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Núria Salas Massó

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Núria Salas Massó

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

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DOCTORAL THESIS 2019

Directed by Prof. Mª Jose Figueras Salvat Department of Basic Health Sciences Microbiology Unit (URV)

by Dra. Dolors Furones Nozal

and by Dr. Karl B. Andree Animal Production Area Aquaculture Programme (IRTA)

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Núria Salas Massó

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STATE that the present study, entitled **"Epidemiology of *Arcobacter*-related spp. in shellfish exposed to marine and brackish water with different levels of fecal pollution**", presented by **Nuria Salas Massó** for the award of the degree of Doctor, has been carried out under our supervision and to confirm this, we sign this letter.

Reus, 22th August 2019

Doctoral Thesis Supervisors

Mª Jose Figueras Salvat **Dolors Furones Nozal** **Karl B. Andree**

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EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

To my daughters.
Always B.E.

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

Do or do not.
There is no try.

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

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UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

Table of contents

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

SUMMARY

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

SUMMARY

The genus *Arcobacter*, which recently has been divided into 7 new genera: *Arcobacter*, *Aliarcobacter*, *Pseudarcobacter*, *Halarcobacter*, *Malacioarcobacter*, *Poseidoniarcobacter*, and candidatus *Arcomarinus* comprises species that have been considered zoonotic agents and emergent pathogens. *Arcobacter*-related species have been recovered with a high positivity from seafood, that together with the fact that are consumed raw or slightly cooked, possess a risk for the consumers. In this thesis, the relationship between these bacteria and shellfish exposed to water with different levels of fecal pollution has been studied. A new approach, for improving the recovery of *Arcobacter*-related genera from marine and brackish environments was developed and consisted on the supplementation of Arcobacter-CAT broth with 2.5% NaCl and posterior culture in marine agar. Results showed that the combination of the conventional and the new approach yielded a 40% more of positive samples. The correlation between the levels of *Escherichia coli* and *Arcobacter* was also examined in shellfish and their surrounding water, showing positive results between the presence of the fecal indicator bacteria and pathogenic species like *A. butzleri* and *A cryaeropilus*. However, when shellfish were harvested from water with a temperature above 26.2ºC, *E. coli* would fail to predict the presence of these pathogens. The distribution of *Arcobacter*-related genera within the tissues of mussels and oysters showed that the intervalval liquid was the compartment with the highest prevalence and diversity of *Arcobacter*. The analysis of the depuration of *E. coli, A. butzleri,* and *M. molluscorum* in mussels and oysters under different bacterial loads and in two seasons (summer and winter) showed that the efficacy of the conventional depuration process may not fully eliminate *Arcobacter* from shellfish and, probably, it is a temperature dependent process. A viable qPCR method for the detection of viable *Arcobacter* spp. cells in different shellfish matrixes was developed with a satisfactory inhibition of DNA amplification from dead cells in 85% of the *Arcobacter* species tested. The comparison of the results from the q-PCR and the v-qPCR in the studied shellfish samples showed that, on average, 1 log of the copy number detected corresponded to dead cells. As a result of all these experiments, and because of the use in parallel of the NaCl enriched and non-enriched approaches, the isolation and description of seven new species belonging to three recently described genera are presented.

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

INTRODUCTION

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

23

Arcobacter

Nuria Salas-Massó, Alba Perez-Cataluña, Luis Collado, Arturo Levican, and Maria José Figueras

CONTENTS

23.1 Introduction

The genus *Arcobacter* embraces gram-negative curved-shaped bacteria, and some of the species are considered emergent enteropathogens to humans and animals [1]. Interest in these bacteria has risen exponentially in recent years, because they are able to colonize a wide diversity of habitats and hosts [1–3 and references therein]. Among the habitats, livestock animals, wastewater, and marine ecosystems (seawater, shellfish, etc.) seem to be the most important reservoirs for these bacteria. Fecal contamination with wastewater could be the way of dissemination to other habitats, like drinking water, irrigation water, ready-to-eat vegetables, and other processed food products [1–5 and references therein]. Food products of animal origin, especially meat products like poultry and pork, show a high prevalence of *Arcobacter* species, and their sources of contamination have been related with the processing procedures in the abattoirs. These bacteria can produce abortions, mastitis, and other disorders in animals, and the species *Arcobacter butzleri* have been considered a serious hazard to human health by the International Commission on Microbiological Specifications for Foods and also a significant zoonotic pathogen [1 and references therein, 6]. In addition, dairy products, such as milk and cheese, as well as vegetables (spinach, lettuce, etc.) have shown contamination by *Arcobacter* species. Therefore, several reviews summarizing and discussing the existing data and the potential role of *Arcobacter* spp. as foodborne pathogens have been published [5,7–10].

Strategies to minimize the impact of these bacteria in the food chain in order to prevent foodborne outbreaks include different procedures to inactivate *Arcobacter*; however, *A. butzleri* shows resistance to several antibiotics and the ability to develop biofilms, and both characteristics may represent a potential hazard for public health [11,12].

23.2 Taxonomic Classification

The genus *Arcobacter* is closely related to the genus *Campylobacter*, because it was proposed in 1991 to accommodate two aerotolerant species previously considered atypical campylobacters since they were able to grow at lower temperatures, that is, 15°C–30°C [13,14]. Both genera are members of the family *Campylobacteraceae* and the order Campylobacterales [1]. The genus *Arcobacter* was enlarged in 1992 to four species, and it increased to six species in 2005, evolving since then very quickly to the 26 species (Figure 23.1) known in 2017 [15–17] and (http://www.bacterio.net/arcobacter.html). As shown in Figure 23.1, the origin of many species is the marine environment or marine shellfish. However, wastewater or sewage seems also to be an important reservoir of

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

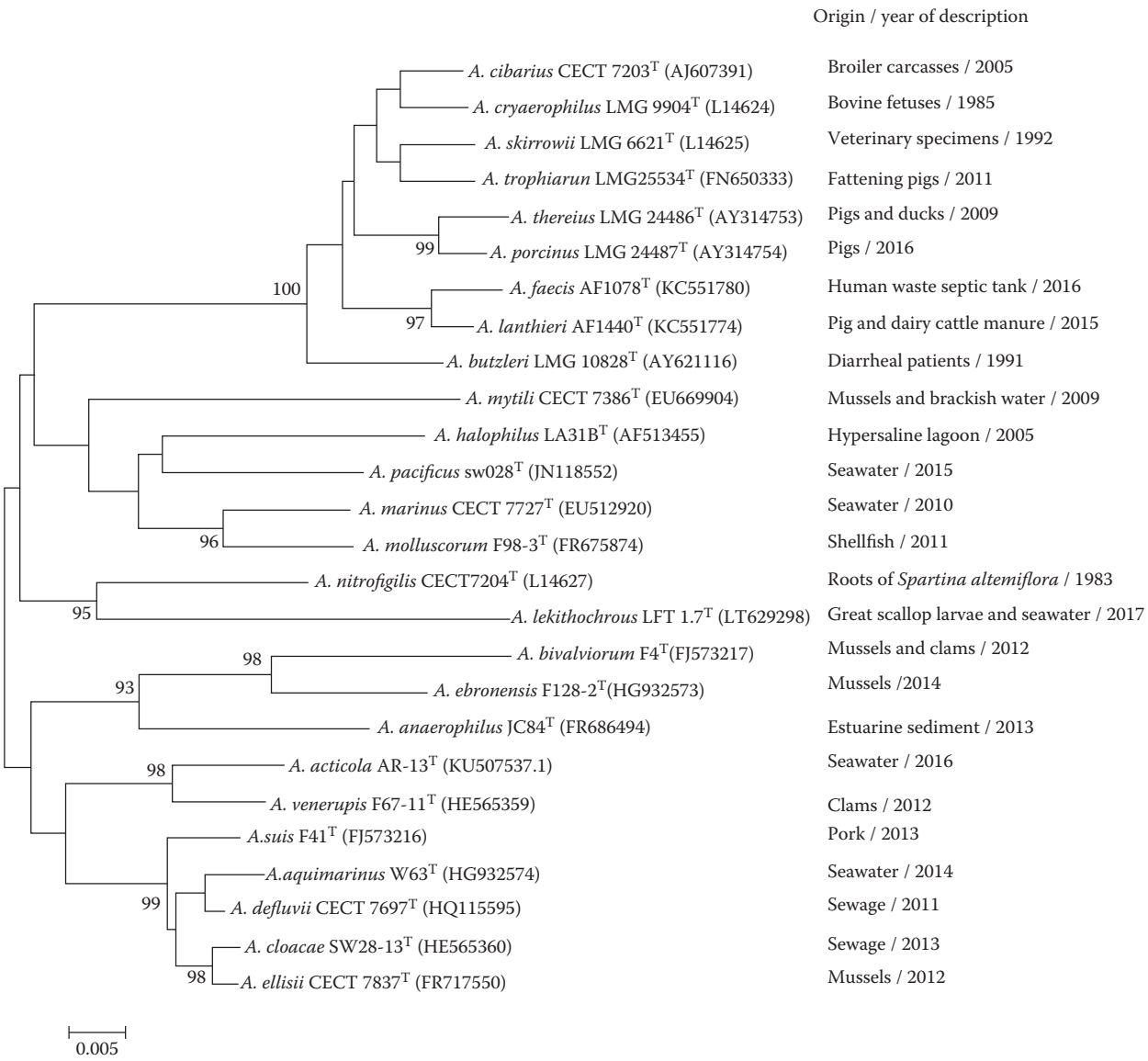

FIGURE 23.1 An unrooted neighbor joining phylogenetic tree constructed with the 16S rRNA sequences (1410 bp) of 26 *Arcobacter* species with their origins and the years of description. Numbers at the nodes depict only support bootstrap values >90% obtained by repeating the analysis 1000 times. The scale bar indicates the number of substitutions per nucleotide position.

species, for instance, *Arcobacter cloacae* and *Arcobacter defluvii* have been discovered from this environment [18 and references therein] as was recently *Arcobacter faecis* and *Arcobacter lanthieri* that have been recovered from a human waste septic tank and from pig and dairy cattle manure, respectively [19,20].

23.2.1 Morphology and Biochemical Characteristics of *Arcobacter* Species

As commented, *Arcobacter* spp. are gram-negative, non-spore-forming curved or helical rod-shaped cells (0.2–0.9 × 1–3 μm) that are motile via a single polar flagellum [14]. This morphology is similar to that of *Campylobacter* spp. However, growth requirements are somewhat less fastidious because most *Arcobacter,* depending upon species and/or strains, display aerotolerance of at least 5% O_2 and grow at a broad range of temperatures (15°C, 25°C, 37°C–42°C). It has been stated that optimal growth can be obtained at 30°C under microaerobic conditions. However, using an incubation temperature of 25°C Van den Abeele et al. [21] detected a similar incidence of *Arcobacter* among patients with diarrhea (1.31%) than previous studies from India that employed 37°C [22 and references therein]. In addition, in a recent study performed by Levican et al. [23], there was no significant difference between the species recovered from wastewater when comparing results obtained after incubation under aerobic or microaerobic conditions. However, recently an obligate anaerobic species, *Arcobacter anaerophilus* has been described [24], but this is so far an atypical characteristic for the genus. An additional atypical member of the genus is an obligate halophile species, that is, *Arcobacter halophilus* that requires the presence of at least 2% NaCl in the culture media to grow [25]. Recently, six isolates of *A. halophilus* and 52 new isolates of *Arcobacter marinus* were recovered from

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

water and shellfish using an enrichment step in *Arcobacter*-CAT (Cefoperazone, Amphotericin B, and Teicoplanin) liquid medium supplemented with 2.5% NaCl (w/v) followed by culturing on marine agar after passive filtration [26]. In the same study, several new species from shellfish and water, pending description, were discovered mainly from the medium containing salt [26].

The majority of *Arcobacter* species can be classified as fastidious bacteria, because they grow and replicate slowly (plates should be incubated between 48 and 72 hours), with the only exception of *A. butzleri* that is the most common species of the genus [1]. This is the least fastidious species able to growth in the presence of 1.5% NaCl, lactose, glucose, and citrate, with some strains showing the ability to reduce nitrate and to be thermotolerant to 42°C [14,18,26]. The fast-growing characteristics of *A. butzleri* in the enrichment culture mask the abundance of other species like *Arcobacter cryaerophilus* [1,23,27]. In the study of Salas-Massó et al. [26], *A. cryaerophilus* was not recovered using medium supplemented with salt (2.5% NaCl), but it was the second most abundant species recovered from sewage-contaminated water using the *Arcobacter*-CAT medium without salt. The dominance of *A. cryaerophilus* is in agreement with its high abundance in the metagenome of wastewater [28].

Even though differential phenotypic characteristics between all accepted species have been defined [14,15,17], unequivocal identification using this approach is impossible. The introduction of molecular identification methods has contributed to the rapid expansion of the genus. The 16S rRNA gene has been classically used as the identification tool for many bacteria species, and in the genus *Arcobacter* this gene shows a wide range of similarity among the type strains of all described species (94.7%–99.6%) [15]. There exist also very closely related species like *Arcobacter suis* and *Arcobacter clocae* with similarities >99%, where this gene is not useful for their separation [15].

The most reliable molecular identification of the species is based on the use of housekeeping genes sequences such as *rpoB* and *gyrB,* performing a Basic Local Alignment Search Tool (BLAST) comparison together with the construction of a phylogenetic tree with representatives of all species (see Section 23.7). As these new methods are more routinely used, a further expansion of the genus is likely to occur. The concatenated sequences of five housekeeping genes (*gyrA*, *atpA*, *rpoB*, *gyrB*, and *hsp60*) named multilocus sequence analysis (MLSA) or multilocus phylogenetic analysis (MLPA) have been used in the description of several new species, because they provide a more robust separation of the species than the 16S rRNA gene [15,17]. In the description of *Arcobacter lekithochrous*, Diéguez et al. [17] stated that a deep revision of the genus should be performed to clarify the taxonomy of *Arcobacter*, because three separate clusters were obtained with the MLPA. In addition, the low similarity of the 16S rRNA gene observed between species (<95%) of these clusters, together with their different ecological characteristics, seems to indicate that they belong to a different genus.

23.3 Biology and Ecology

The capacity of *Arcobacter* to survive and grow in aerobiosis, at ambience temperature, together with their tolerance to NaCl are probably the reasons for the higher environmental persistence of these bacteria than that of *Campylobacter* species [1,3,11]. In fact, the genome and proteome of *A. butzleri* have been described as more similar to *Sulfuromonas denitrificans* and *Wolinella succinogenes*, both members of the *Helicobacteraceae*, and also more similar to the deep-sea vent Epsilonproteobacteria *Sulfurovum* and *Nitratiruptor,* than to the other members of the Campylobacteraceae [29]. Furthermore, a substantial proportion of the *A. butzleri* genome is devoted to growth and survival under diverse environmental conditions, with a large number of proteins associated with respiration, signal transduction, chemotaxis, DNA repair, and adaptation [29].

Although it is easy to think that the abundance of *Arcobacter* (>10^6 MPN/100 mL) in highly polluted waters has its origin in feces from humans and animals [30], the incidence in the latter is not high enough to justify the densities of these bacteria in wastewater and sewage [28,31–33]. These data, together with the information obtained from *Arcobacter* genomes, suggest that arcobacters are free-living bacteria that have the ability to adapt and replicate in diverse environments [28,32,34].

It is thought that *Arcobacter* may play a role as an opportunistic pathogen, as it has been found in feces from both asymptomatic and symptomatic individuals [35–38] as well as from livestock (see Section 23.4.2). So far, many of the studies about *Arcobacter* are focused on food and products of animal origin, showing a high percentage of positivity for these bacteria at farm and retail levels [1,3].

23.4 Epidemiology

Consumption of *Arcobacter*-contaminated water or food is considered the main route of transmission to humans and animals [1,3]. In fact, so far four outbreaks have been attributed to these bacteria, three associated with consumption of fecal-contaminated water and the other one to the consumption of roasted chicken [1,10 and references therein]. *Arcobacter* has been detected not only from raw products (i.e., retail meat, shellfish, vegetables) but also in meals at restaurants in Bangkok, with a higher prevalence than other pathogens like *Salmonella* or *Campylobacter* [1 and references therein]. Considering that *Arcobacter* spp. have been recovered in 66.6%–100% of wastewater samples in different studies [23 and references therein], the use of reclaimed water for irrigation of crops or ready-to-eat vegetables may increase the potential for human infection, especially considering that *Arcobacter* have been found in different types of vegetables [40–43]. Additionally, a study performed in Canada shows that *Arcobacter* signifies a greater threat to human health than *Campylobacter* in irrigation waters because it is present at 2–3 log higher concentrations [44]. The use of manure and sewage effluents in soils for agricultural practices has been proposed as an introduction route of *Arcobacter* in the food chain [45].

Despite the lack of regulated protocols for the isolation or detection of *Arcobacter*, some tools are available for the epidemiological traceability of outbreaks by means of genotyping methods, that is, enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR), randomly amplified polymorphic DNA-PCR (RAPD-PCR), amplified fragment length polymorphism (AFLP), pulsed-field gel electrophoresis (PFGE), and in addition, a multilocus sequence typing (MLST) scheme with its database is available [1]. The latter method allows

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

investigators to deposit gene sequences from their isolates into a database to know if they are already identical to existing already deposited sequences (or alleles) or new ones. Of the sequences available at the database, at the time of writing, 29.7%, come from strains isolated from food and 13.8% from humans. Several of the above mentioned PCR typing methods have been compared in recent studies, showing that all of them have advantages and disadvantages [1,5,46]. So far, ERIC-PCR has been the most widely used technique, due to its reproducibility and easy performance. However, PFGE has been shown to have more discriminatory power than ERIC-PCR, AFLP, or RAPD-PCR [46], but it is a complex technique. The MLST has also been found to be discriminatory; it is free of experimental variation and enables a database to be built for comparison. However, it is costlier and time consuming than the PCR methods, and the still reduced database limits proper analysis of the epidemiological information derived from this technique [5,46]. The use of these typing methods has shown that *Arcobacter* can persist in time and that cross-contamination between food and processing facilities may occur [5 and references therein, 47].

23.4.1 *Arcobacter* in Water

Arcobacter can be found in many different types of water worldwide, including well water, drinking water, rivers, lakes, seawater, wastewater, and in water used during food production as reviewed by Hsu and Lee [3]. The presence of *Arcobacter* in water, as commented before, has been linked to fecal contamination [28,31–33]. However, certain species, that is, *A. marinus* and *A. halophilus*, may be natural residents of marine environments [26]. In addition, it has been demonstrated that *Arcobacter* was effectively removed during disinfection treatments used to produce drinking water [48]. So, the quality of the water used in food processing facilities will have to be monitored for *Arcobacter* to avoid contamination of the food products. These occurred, for instance, during spinach processing where these bacteria were highly abundant in the water from the washing basins, as occurs also for carrot washing [41 and references therein]. The presence of *Arcobacter* in shellfish growing areas is also the source of contamination of shellfish [26 and references therein, 49].

23.4.2 *Arcobacter* in Food

The presence of *Arcobacter* has been described from different food processing facilities in different countries (Table 23.1). Globally, a similar incidence of ca. 40% is found in processing plants of dairy products, which involved mainly production of milk and Italian cheese, than in poultry processing plants. However, the incidence in the latter, depended on the country, ranging from 13.6% in Denmark to 84.6% in Belgium. This is in agreement with the high incidence of *Arcobacter* found in carcasses and feces of poultry (mainly chickens and turkeys) as shown in Table 23.2. The latter table shows that livestock animals are carriers of *Arcobacter*, making therefore easier the contamination of food products derived from them when they are processed at slaughterhouses [1,3,5 and references therein]. The global prevalence of *Arcobacter* in animals (Table 23.2) shows that these bacteria are more abundant in cattle (39.1%), swine (36.4%), poultry (29.9%), and sheep (12.6%). However, the prevalence of *Arcobacter* in the retail meat products in different studies shows the highest incidence from 45.2% in poultry, followed by 32.4% seafood, 30.4% pork, 26.9% dairy, 24.9% lamb, 17.1% beef, and 10% rabbit (Table 23.3).

As *Arcobacter* is closely related to *Campylobacter*, most of the studies have focused on poultry (Tables 23.2 and 23.3) because this is considered the main source of food-related transmission to humans for the latter bacteria [57,85]. Poultry meat has been reported worldwide to be contaminated with *Arcobacter*, showing a prevalence which ranges from 0.8% in studies from South Korea to 96.8% in those of Canada (Table 23.3). This information along with the high prevalence of *Arcobacter* in poultry in different countries makes poultry meat a product of high risk for human health.

The prevalence in pork meat ranged from 0.5% in the Netherlands to 60.9% in Thailand; the average prevalence, 30.4%, is similar to that found in swine, 36.4% (Tables 23.2 and 2.33). *Arcobacter* have been described from pig effluents [45] and pig manure, and from healthy swine feces in Belgium, India, Japan, and the United States [34 and references therein, 62]. Similar to what occurs in poultry, pork meat may get contaminated at slaughterhouse in the process of evisceration. Prevalence of *Arcobacter* in beef meat ranged from 1.5% in the Netherlands to 55.6% in the Czech Republic (Table 23.3). However, in a study

TABLE 23.1

Overview of the Incidence of *Arcobacter* Species in Different Food-Processing Facilities and Countries

Category	Country	Positive Samples/Samples Studied (%)	Species[a]	Identification Method[b]	References
Beef	Malaysia	8/36 (22.2)	*Ab, As*	CI; mPCR[c] [50]	[51]
Dairy	Italy	10/30 (33.3)	*Ab*	CI, mPCR [50]	[52]
73/182		5/6 (83.3)	*Ab*	CI, mPCR [50]	[53]
(40.1%)		58/146 (39.7)[c]	*Ab, Ac*	CI, mPCR [50; 54]	[3]
Poultry	Belgium	44/52 (84.6)	*Ab, Ac*	CI, mPCR [50]	[27]
178/421	Denmark	32/235 (13.6)	*Ab*	DD, CI, mPCR [54]; PCR [55]	[3]
(42.3%)	Iran	94/120 (78.3)	*Ab, Ac, As*	DD, mPCR [50]; [54]	[56]
	The Netherlands	8/14 (57.1)	*Ab, Ac, As*	DD, CI mPCR [50]	[57]
Vegetable	Germany	2/5 (40.0)	*Arcobacter* spp.	DD, CI 23S specific qPCR [41]	[3]

[a] *Ab*: *A. butzleri*; *Ac*: *A. cryaerophilus*; *As*: *A. skirrowii*; *Arcobacter* spp.: when more than three species were detected.

[b] Data from the original paper if not mentioned in the review of Hsu and Lee [3] and include direct detection (DD), colony identification (CI), mPCR: multiplex polymerase chain reaction.

[c] Values represent the sum of data of all studies from the same country shown in Table 23.3 of Hsu and Lee [3].

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

TABLE 23.2

Overview of the Incidence of *Arcobacter* Species in Carcasses,(1) Feces or Rectal Swabs,(2) and Viscera(3) of Food Animals from Different Countries

Category	Country	Positive Samples/ Samples Studied (%)	Species[a]	Identification Method[b]	References
Cattle	Australia(1)	45/130 (34.6)	*Ab, Ac, As*	DD, CI, mPCR [50]	[3]
3489/8939	Belgium(1),(2)	179/574 (31.2)[c]	*Ab, Ac, As*	CI, mPCR [50]	[34]
(39.1%)	Iran(2)	16/200 (8)	*Ab, Ac*	DD, CI, mPCR [50,54]	[58]
	Italy(2)	49/239 (20.5)	*Ab, Ac, As*	CI, mPCR [50]	[52]
		29/30 (96.7)	*Ab, Ac, As*	CI, mPCR [50]	[3]
	Japan(2)	17/393 (4.3)[c]	*Arcobacter* spp.	CI, mPCR [59]	[34]
	Malaysia(2)	12/193 (6.2)	*Ab, As*	CI, mPCR [50]	[51]
	Turkey(2)	19/200 (9,5)	*Ab, Ac, As*	CI, mPCR [50]	[34]
	United States(2)	254/1,889 (13.5)[c]	*Arcobacter* spp.	DD, CI, mPCR [60]	[34]
	United Kingdom(2)	2,869/5,091 (56.3)[c]	*Arcobacter* spp.	CI, mPCR [50]	[3]
Poultry	Belgium(2)	2/20 (10.0)	*Ab, Ac*	CI, mPCR [50]	[34]
1158/3880	Costa Rica(3)	26/150 (17.3)	*Ab, Ac, As*	CI, mPCR [54]	[3]
(29.9%)	Czech Republic(1)	57/420 (13.6)	*Ab, Ac*	DD, CI, mPCR [50]	[3]
	Denmark(2)	39/152 (25.7)[c]	*Ab, Ac*	CI, mPCR [50]	[34]
	Germany(1)	89/305 (29.2)	*Ab, Ac, As*	CI, mPCR [50]	[61]
		87/170 (51.2)	*Ab*	CI, biochemical identification	[34]
	India(2)	11/75 (14.7)	*Ab, Ac, As*	CI, mPCR [50]	[62]
	Iran(1)	138/400 (34.5)	*Ab, Ac, As*	DD, mPCR [50,54]	[56]
	Japan(2)	34/234 (14.5)	*Arcobacter* spp.	CI, PCR [59]	[34]
	Spain(1), (3)	16/36 (44.4)	*Ab*	DD, CI, mPCR [50]	[63]
		6/32 (18.8)	*Ab*	CI, mPCR [50]	[3]
	The Netherlands(1), (2), (3)	202/396 (51.0)	*Ab, Ac, As*	DD, CI, mPCR [50]	[57]
		42/80 (52.5)[c]	*Ab, Ac*	DD, CI, mPCR [50]	[34]
	Turkey(2)	16/90 (17.7)	*Ab, Ac, As*	CI, mPCR [50]	[34]
	United Kingdom(2), (3)	3/70 (4.3)	*Ab, Ac, As, Arcobacter* sp.	CI, biochemical identification	[34]
	United States(1), (2)	390/1250 (31.2)[c]	*Arcobacter* spp.	DD, CI, mPCR [59,60]	[34]
Sheep	Iran(2)	11/108 (10.2)	*Ab, Ac*	DD, CI, mPCR [50,54]	[58]
43/344	United Kingdom(2)	32/236 (13.6)	*Arcobacter* spp.	CI, mPCR [50]	[3]
(12.6%)					
Swine	Belgium(1), (2)	321/545 (58.9)[c]	*Ab, Ac, As*	CI, mPCR [50]	[34]
980/2691	Denmark(3)	23/55 (41.8)	*Ab, Ac, Arcobacter* sp.	CI, biochemical identification	[35]
(36.4%)	India(2)	16/75 (21.3)	*Arcobacter* spp.	CI, mPCR [50]	[62]
	Japan(2)	25/250 (10.0)	*Arcobacter* spp.	CI, mPCR [59]	[34]
	United States(2), (3)	595/1766 (33.7[c]	*Ab, Ac*	DD, CI, DNA probes; 16S sequencing; mPCR [60]	[34]

[a] *Ab*: *A. butzleri*; *Ac*: *A. cryaerophilus*; *As*: *A. skirrowii*; *Arcobacter* sp.: species not specified; *Arcobacter* spp.: when more than three species were detected.

[b] Data from the original paper if not mentioned in the reviews of Hsu and Lee [3] and Wesley and Miller [34], and include direct detection (DD), colony identification (CI), mPCR: multiplex polymerase chain reaction.

[c] Values represent the sum of data of all studies from the same country shown in the reviews [3].

performed in Malaysia, *Arcobacter* occurrence was higher in imported beef than in locally processed beef [51]. In addition, it has been demonstrated that calves and housed cattle are more susceptible to *Arcobacter* than are adult and pastured cattle, respectively [3,10].

The number of studies reporting the presence of *Arcobacter* in seafood has increased in recent years, mainly due to the high incidence reported from shellfish, which ranges from 17.1% in a study performed in India [62] to 90% in a study performed in Thailand [74] (Table 23.3). Mussels are the most studied type of shellfish, followed by clams and oysters [31,37,49,68,81]. A recent study has shown that *A. butzleri* is present in 48% (20/42) of the shellfish samples (mussels and clams) that possessed levels of *Escherichia coli* >230 MPN/100 g, which is the standard established in European legislation for shellfish that will require depuration before consumption [83]. The fact that shellfish are usually consumed raw or slightly cooked may pose a special risk to consumers [26,31,49].

So far, there are no regulations concerning the presence of *Arcobacter* spp. in food products, probably due to the fact that

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

TABLE 23.3

Overview of the Incidence of *Arcobacter* Species in Different Food Products by Country

Category	Country	Positive Samples/ Samples Studied (%)	Species[a]	Identification Method[b]	References
Beef	Australia	7/32 (22.0)	*Ab*	DD, CI, mPCR [50]	[3]
150/879	Belgium	9/100 (9.0)	*Ab, Ac*	CI, mPCR [50]	[64]
(17.1%)	Czech Republic	5/9 (55.6)	*Ab, Arcobacter* sp.	CI, PCR [60]	[34]
		5/13 (38.4)	*Ab, Ac*	DD, CI, mPCR [50]	[3]
	Japan	2/90 (2.2)	*Ab*	CI, mPCR [59]	[31]
	Malaysia	20/81 (24.7)[c]	*Ab, Ac, As*	CI, mPCR [50]	[3]
	Mexico	13/45 (28.8)	*Ab, As*	CI, PCR [65]	[34]
	Poland	14/70 (20.0)	*Ab, Ac*	CI, mPCR [50]	[66]
	Spain	5/16 (31.3)	*Ab, Ac*	DD, CI, 16S rRNA-RFLP [67]	[31]
		1/20 (5.0)	*Ab*	CI, mPCR [50]	[68]
	Thailand	14/50 (28.0)	*Ab*	CI, biochemical identification	[31]
	The Netherlands	1/68 (1.5)	*Arcobacter* spp.	CI, biochemical identification	[31]
	Turkey	5/97 (5.1)	*Ab*	CI, mPCR [50]	[31]
	United Kingdom	49/188 (26.1)[c]	*Ab, Ac, As*	CI, mPCR [50]	[31]
Dairy	Finland	26/117 (22.2)	*Ab, Ac*	CI, mPCR [50]	[69]
185/688	India	1/100 (1.0)	*Ab*	CI, mPCR [50]	[70]
(26.9%)	Italy	7/32 (21.9)	*Ab*	CI, mPCR [50]	[52]
		8/10 (80.0)	*Ab*	CI, mPCR [50,54]	[53]
	Malaysia	5/86 (5.8)	*Ab, Ac*	CI, mPCR [50]	[3]
	Spain	15/35 (42.9)	*Ab, Ac, As*	CI, mPCR [50]	[68]
	Turkey	3/50 (6.0)	*Ab, As*	CI, mPCR [50]	[71]
		73/156 (46.8)[c]	*Arcobacter* spp.	CI, 16S rRNA-RFLP [72]	[3]
	United Kingdom	47/102 (46.1)[c]	*Ab, Arcobacter* sp.	CI, mPCR [50]; biochemical identification	[34]
Lamb	Australia	2/13 (15.0)	*Ab*	DD, CI, mPCR [50]	[3]
13/52 (24.9%)	Czech Republic	11/39 (28.2)	*Ab, Ac, As*	DD, CI, mPCR [50]	[3]
Pork	Australia	6/21 (29.0)	*Ab*	DD, CI, mPCR [50]	[3]
392/1288	Belgium	10/47 (21.3)	*Ab, Ac*	CI, mPCR [50]	[34]
(30.4%)	Czech Republic	1/26 (3.8)	*Ab*	CI, mPCR [50] and MALDI-TOF MS	[73]
	India	12/75 (16.0)	*Arcobacter* spp.	CI, mPCR [50]	[62]
	Japan	7/100 (7.0)	*Ab, Ac, As*	CI, mPCR [59]	[31]
	Mexico	23/45 (51.1)	*Ab, Ac, As*	CI, PCR [65]	[34]
	Poland	11/70 (15.7)	*Ab, Ac*	CI, mPCR [50]	[66]
	Spain	2/20 (10.0)	*Ab*	CI, mPCR [50]	[68]
		9/17 (53.0)	*Ab, As*	DD, CI, 16S rRNA-RFLP [67]	[3]
	Thailand	8/50 (16.0)	*Ab*	CI, biochemical identification	[31]
		14/23 (60.9)	*Ab*	CI, biochemical identification	[74]
	The Netherlands	1/194 (0.5)	*Arcobacter* spp.	CI, biochemical identification	[31]
	United Kingdom	35/101 (35.0)	*Ab, Ac, As*	CI, mPCR [50]	[3]
	United States	231/499 (46.3)[c]	*Arcobacter* spp.	CI, PCR [31], DNA probe [75]	[31]
Poultry	Australia	16/22 (73.0)	*Ab*	DD, CI, mPCR [50]	[3]
1949/4312 (45.2)	Belgium	41/66 (62.1)[c]	*Ab, Ac*	DD, CI, mPCR [50]	[31]
		596/620 (96.1)[c]	*Ab, Ac*	CI, mPCR [50]	[34]
	Canada	121/125 (96.8)	*Ab*	CI, biochemical identification	[34]
	Costa Rica	28/50 (56.0)	*Ab, Ac*	CI, mPCR [50]	[3]
	Czech Republic	25/61 (41.0)	*Ab, Arcobacter* sp.	CI, PCR [60]	[34]
		41/53 (77.4)	*Ab, Ac*	CI, mPCR [50] and MALDI-TOF MS	[73]
	Germany	15/56 (26.8)	*Ab*	CI, mPCR [50]	[76]
	India	46/304 (15.1)	*Ab, Ac*	CI, mPCR [50]	[70]
	Iran	85/540 (15.7)[c]	*Ab, Ac, As*	DD, CI, mPCR [50]	[3]
	Japan	23/100 (23.0)	*Ab, Ac*	CI, mPCR [59]	[31]
		20/41 (48.7)	*Ab, Ac*	CI, mPCR [59]	[34]
	Malaysia	48/123 (39.0)[c]	*Ab*	CI, mPCR [50]	[5]
	Mexico	18/45 (40.0)	*Ab, Ac, As*	CI, PCR [65]	[34]

(*Continued*)

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

TABLE 23.3 (*Continued*)

Overview of the Incidence of *Arcobacter* Species in Different Food Products by Country

Category	Country	Positive Samples/ Samples Studied (%)	Species[a]	Identification Method[b]	References
	Poland	60/70 (85.7)	*Ab, Ac*	CI, mPCR [50]	[66]
	South Korea	76/360 (21.1)	*Ab, Ac*	DD, CI, mPCR [50]	[3]
		4/475 (0.8)	*Arcobacter* spp.	DD, CI, mPCR [50]	[77]
	Spain	36/42 (85.7)	*Ab, Ac*	DD, CI, PCR [78]	[78]
		80/128 (62.5)[c]	*Arcobacter* spp.	DD, CI, mPCR [50]	[34]
		11/20 (55.0)	*Ab*	CI, mPCR [50]	[68]
		44/100 (44.0)[c]	*Arcobacter* spp.	DD, CI, mPCR [50], 16S rRNA-RFLP [67]	[3]
	Thailand	52/210 (24.76)[c]	*Ab, Ac*	CI, mPCR [59], biochemical identification	[34]
		31/40 (77.5)	*Ab*	CI, biochemical identification	[74]
	The Netherlands	53/220 (24.1)	*Arcobacter* spp.	CI, biochemical identification	[31]
	United Kingdom	23/25 (92.0)[c]	*Arcobacter* spp.	CI, biochemical identification; SDS-PAGE	[34]
		58/94 (62.0)	*Ab, Ac, As*	CI, mPCR [50]	[3]
	United States	346/445 (77.8)[c]	*Arcobacter* spp.	CI, PCR [66], mPCR [59]	[34]
Rabbit	Spain	1/10 (10.0)	*Ab*	DD, CI, 16S rRNA-RFLP [67]	[3]
Seafood	Chile	28/80 (35.0)	*Arcobacter* sp.	CI, biochemical identification	[31]
		43/106 (40.5)	*Arcobacter* spp.	CI, 16S rRNA-RFLP [67]	[37]
356/1100	Czech Republic	6/13 (45.2)	*Ab, Ac, As*	CI, mPCR [50] and MALDI-TOF MS	[73]
(32.4%)	Germany	17/50 (34.0)	*Ab, Ac*	CI, mPCR [50]	[76]
	India	13/75 (17.3)	*Arcobacter* spp.	CI, mPCR [50]	[62]
		13/76 (17.1)	*Arcobacter* spp.	DD, CI, mPCR [50]	[79]
	Italy	10/20 (50.0)	*Ab*	CI, 16S sequencing	[80]
		16/70 (22.9)	*Ab, Ac*	CI, 16S rRNA-RFLP [68]	[81]
		12/40 (30.0)	*Ab, Ac*	CI, *rpoB* sequencing	[82]
		41/162 (25.3)	*Arcobacter* spp.	CI, *rpoB* sequencing	[83]
	Spain	12/16 (75.0)	*Ab, Ac*	CI, mPCR [50]	[68]
		84/260 (32.3)[c]	*Arcobacter* spp.	DD, CI, 16S rRNA-RFLP [67]	[3]
		15/22 (68.2)	*Arcobacter* spp.	CI, 16S rRNA-RFLP [72]	[26]
		37/100 (37.0)	*Ab, Ac, Ad*	CI, 16S rRNA-RFLP [72]	[84]
	Thailand	9/10 (90.0)	*Ab*	CI, biochemical identification	[73]
Vegetable	Italy	44/160 (27.5)	*Ab*	DD, 16S rRNA-RFLP [72]	[43]
51/210 (24.3%)	Spain	7/50 (14.0)	*Ab*	DD, CI, 16S rRNA-RFLP [72]	[3]

[a] *Ab*: *A. butzleri*; *Ac*: *A. cryaerophilus*; *Ad*: *A. defluvii*; *As*: *A. skirrowii*; *Arcobacter* sp.: species not specified; *Arcobacter* spp.: when more than three species were detected.

[b] Data from the original paper if not mentioned in the reviews of Hsu and Lee [3], Collado et al. [31], and Wesley and Miller [34] and include direct detection (DD), colony identification (CI), mPCR: multiplex polymerase chain reaction, 16S rRNA-RFLP: 16S rRNA restriction fragment length polymorphism, *rpoB* sequencing: partial sequencing of gene *rpoB*.

[c] Values represent the sum of data of all studies from the same country shown in the reviews[3].

they are poorly known, underestimated pathogens [1]. However, in 2002, the International Commission on Microbiological Specifications for Foods considered *A. butzleri* as a serious hazard to human health [6]. As shown in Table 23.3, the most frequent recovered species is by far *A. butzleri*, followed by *A. cryaerophilus*, and *Arcobacter skirrowii* [1,3,5 and references therein, 34]. However, the detected high incidence of *A. butzleri* is a bias influenced by the use of an enrichment step before culturing, because this species grows faster than the rest [9,24].

Similar to what occurs with shellfish, ready-to-eat vegetables are also consumed raw and the species *A. butzleri* and *A. cryaerophilus* have been recovered from leafy green vegetables such as washed and blanched spinach, and precut (ready-to-eat) lettuce, rocket, and valerian [41,43]. As commented, contamination of vegetables can occur at field level, by the use of contaminated irrigation water but also at retail level [5,40,41,44].

23.4.3 Survival of *Arcobacter*

Due to the prevalence of *Arcobacter* in food matrices, the mechanisms of survival through the whole food production process have been recently reviewed by Ferreira et al. [10]. These authors demonstrated that *Arcobacter* has the capacity to survive several "decontamination" procedures implemented in the food chain, like for instance scalding procedures (52°C, 3 minutes) in poultry slaughterhouses. Therefore, this type of water could become a source of cross-contamination within flocks. In fact, *A. butzleri* has shown to have a higher survival capacity than *A. skirrowii* and *A. cryaerophilus* to the different temperatures tested (7°C, 20°C, 52°C, 56°C, and 60°C) [86]. In dairy facilities, *A. butzleri* and *A. cryaerophilus* survived in raw, ultrahigh-temperature (UHT) and pasteurized milk stored at 4°C–10°C, but *A. butzleri* also showed this capacity at 20°C, surviving the

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

production and storage conditions of buffalo mozzarella cheese [87]. In a longitudinal study performed in Italy by Giacometti et al. [53], strains of *Arcobacter* showing closely related PFGE patterns from teats, milking machines, and milk samples were found, indicating the prevalence of the strains in the different production stages. In the same study, only *A. butzleri* was recovered from the milking system, in contrast with the higher prevalence of *A. cryaerophilus* observed in dairy animal feces, and the authors indicated that this is due to the higher resistance of *A. butzleri* to sanitizing processes than other *Arcobacter* species [53]. Cold storage temperatures may be the first barrier to preserving food against bacteria; however, *Arcobacter* can survive and even form biofilms at 5°C [88]. It has been demonstrated that *Arcobacter* may present cross-protection responses, if they are first stressed by a heat shock, because they can become more resistant to posterior stress situations generated by temperature and pH [89].

Applying vacuum packaging, irradiation, disinfection with chlorine, or using other natural compounds like bearberry, chamomile, cinnamon, rosemary, and sage extracts have shown to reduce the levels of *Arcobacter* found during food processing [10 and references therein]. However, as indicated by Ferreira et al. [10] in their review, the working concentration of chlorine of 0.2%–0.5% used for sanitizing food processing environments is below or very close to 0.5% which is the determined minimum inhibitory concentration of sodium hypochlorite for *Arcobacter* isolates. So, common cleaning practices such as the use of 5% ethanol or 0.2% sodium chloride have no effect in the survival of *A. butzleri* [10 and references therein]. Moreover, even though *Arcobacter* is susceptible to traditional wastewater treatment, exceptions have been noted [42,48,90]. Although appropriate doses of chlorine are highly effective for inactivating *Arcobacter* (i.e., 5 minute exposure to properly chlorinated drinking eliminates culturable *A. butzleri*), recontamination can occur and result in outbreaks [1 and references therein].

23.5 Clinical Features

The main clinical presentation of *Arcobacter* is diarrhea [1,3,10 and references therein, 20,22,38]. However, the role of this bacteria in human infection is still under discussion, because there is a lack of clear etiological association, despite some species having been isolated from feces of patients with diarrhea (Tables 23.4–23.6). In this sense, although the most commonly isolated species by culturing have been *A. butzleri* and *A. cryaerophilus,* some cases of diarrhea by *A. skirrowii* [91] and *Arcobacter thereius* [21] have also been reported.

The diarrhea surveys in which *Arcobacter* species were detected were reviewed by Figueras et al. [22] and updated in the present chapter (Tables 23.4–23.6). The prevalence in studies conducted in stools of patients with diarrhea by culture ranged from 0.1% in Belgium and France to 3.6% in Chile (Table 23.6). Nevertheless, in a recent study conducted in Canada, using an ad hoc molecular PCR detection, the observed incidence of *A. butzleri* among diarrheic patients was 56.7% versus 0.8% found by culture [38]. It has been stated that the high prevalence observed when molecular methods are applied indicates that *Arcobacter* spp. could be underestimated as enteropathogens because of limitations in the current culturing methods [1,22]. In addition, the variability of culturing methods used may also influence the results. To determine the true relevance of these bacteria, molecular methods that can differentiate dead and alive bacteria should be used for screening stool samples concurrently. Webb et al. [38] determined the load of *A. butzleri* DNA by quantitative PCR (qPCR) and observed that although *A. butzleri* DNA loads were low in both diarrheic (1.6 ± 0.59 $\log_{10}$ mg^{-1}) and nondiarrheic individuals (1.3 ± 0.63 $\log_{10}$ copies mg^{-1}), the density was higher in stools from diarrheic people ($P = 0.007$) [37]. The authors concluded that either *A. butzleri* is not a pathogen or its pathogenicity depends on the strain and on the host. In this sense, the link between factors related to strain and host that may influence the development of diarrhea were recently discussed for *Aeromonas* in another study [93].

Regarding the clinical features of the reviewed studies, when symptoms presented for patients are described, the most common characteristic is acute and watery diarrhea with abdominal pain, and in a few cases vomiting and fever, while blood has been rarely observed (Tables 23.4–23.6). In most reviewed population studies, *Arcobacter* was the only pathogen isolated, and the presence of no underlying disease occurred in 13.7%–50% of the patients (Table 23.6). Despite this, when case studies are reviewed (Table 23.4), chronic diarrhea together with abdominal pain are the main clinical presentations. Regarding the extraintestinal presentation, as observed in Table 23.5, *Arcobacter* causes bacteremia mainly in patients with underlying conditions, and recovery occurred after antibiotic treatment (Tables 23.4 and 23.5). In this sense, it has been stated that despite most cases of enteritis caused by *Arcobacter* appearing to be self-limiting and not requiring antimicrobial treatment, the severity or prolongation of symptoms may justify the use of antibiotic treatment as shown in Table 23.4. The absence of other enteropathogens and the remission of the diarrheal symptoms after antibiotic treatment seem to indicate that *Arcobacter* could in fact be considered the etiological agent of the diarrhea process [22].

23.6 Pathogenesis and Virulence

Although the mechanisms that regulate pathogenesis of *Arcobacter* spp. are poorly known, the prevalence of nine putative virulence genes (*cadF*, *cj1349*, *ciaB*, *mviN*, *pldA*, *tlyA*, *hecA*, *hecB,* and *irgA*) are widely studied because they have been found in the genome of *A. butzleri* (ATCC49616^T) and are related with pathogenesis in other microorganisms [1,10 and references therein, 94]. The genes *cadF* and *cj1349* encode two fibronectin binding proteins (CadF and Cj1349); *ciaB* encodes the invasion protein CiaB, while the *mviN* gene codifies an homologue of MniV protein related with peptidoglycan synthesis in *E. coli*; the *pldA* gene corresponds to a phospholipase associated with hemolytic activity; the *tlyA* gene codifies for an hemolysine; *hecB* gene is related with the hemolysis activation; the *hecA* gene encodes for an adhesin of the filamentous hemagglutinin family; and finally, the gene *irgA* codifies the iron-regulated outer membrane protein IrgA. The presence of these virulence genes has been screened for by PCR using primers developed by Douidah et al. [94] on the basis of their sequences retrieved from the genome of *A. butzleri* (ATCC49616^T). In that study, 14.3% of the *A. butzleri* strains tested ($n = 192$) showed the presence of the nine virulence genes simultaneously. However, six of these genes (*cadF*, *cj1349*,

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

TABLE 23.4

Cases of *Arcobacter* Intestinal Infections

Country	Patient Sex/Age	Presentation/Duration	Species	Antimicrobial Susceptibility Testing Method	Recovery in Days (d)/Treatment	Underlying Condition
Australia	M/35 years	Chronic diarrhea and abdominal pain/6 months; coinfection with *Iodamoeba butchlii* and *Entamoeba coli*	*Ac*	NS	NS	Sexually active homosexual
Belgium	M/73 years	Chronic diarrhea/2 months	*As*	NS	10 d/None	Prosthetic aortic heart valve
Chile	1. M/2 years	Chronic mucous diarrhea and vomiting/3 months	*Ab*	Susceptible to ERY, GEN, and CIP; resistant to AMP, CLR, and TET	10 d/ERY	None
	2. F/1 year	Sister of case 1; chronic diarrhea with abdominal cramps and pain/4 months		Susceptible to ERY, GEN, and CIP; resistant to AMP, CLR, and TET; method: E test	10 d/ERY	None
Germany	1. M/48 years	Acute watery diarrhea and abdominal cramps/12 days	*Ab*	Susceptible to ERY, TET, AMG, and QUI; resistant to AMP, MZL, AMXCA, CEPH, and SXT	3 d/OFX	Type 1 diabetes mellitus
	2. F/52 years	Chronic diarrhea and abdominal cramps/3 weeks		Susceptible to ERY and TET; resistant to AMP, MZL, AMXCA, CEPH, and QUI; method: NS	2 d/DXC	Alcohol abuse, hyperuricemia
Italy	4M and 6F 3 years to 7 years	Abdominal pain and occasional vomiting with no diarrhea or fever	*Ab*	NS	5–10 d depending on the intensity of symptoms/none	None
Spain	M/26 years	Persistent bloody and watery diarrhea/3 weeks	*Ac*	Susceptible to AMX/CA, and GEN; resistant to CIP and ERY; method: disk diffusion	8 d/AMX/CA	Acute gastroenteritis 4 months earlier
Turkey	M/30 years	Acute watery diarrhea, abdominal pain, nausea and sweating	*Ab*	Susceptible to AMK, ERY, DXC, PIPT, SXT, CIP, LEV, and NA; resistant to AMP, CFR, and CD; method: disk diffusion	2 d/CIP	None
United States[a]	M/85 years	Chronic persistent diarrhea/4 weeks and bacteremia (Table 23.5)	*Ab*	Not done because microbe did not grow in MicroScan or Muller-Hinton	2 d/VAN and PIPT	Chronic lymphocytic leukemia

Source: Adapted and updated from Figueras MJ et al. *New Microbes New Infect.* 2014;2:31–7 [22], with a new study Arguello E et al. *J Clin Microbiol* 2015;53:1448–51 [92].

Note: NS: Not specified; *Ac*: *A. cryaerophilus*; *Ab*: *A. butzleri*; *As*: *A. skirrowii*; AMG: aminoglycosides; AMK: Amikacin; AMP: ampicillin; AMX: amoxicillin; AMX/CA: amoxicillin/clavulanic acid; CAZ: ceftazidime; CD: clindamycin; CEPH: cephalosporin (any); CFR: cefuroxime; CFZ: cefazolin; CIP: ciprofloxacin; CLR: cloramphenicol; CTR: ceftriaxone; CTZ: ceftizoxime; DXC: doxycycline; ERY: erythromycin; GEN: gentamicin; LEV: levofloxacin; MIN: minocycline; NA: nalidixic acid; OFX: ofloxacin; P: penicillin; PIP: piperacillin; PIPT: piperacillin/tazobactam; QUI: quinolones (any); SXT: trimethoprim-sulfamethoxazole; TET: tetracycline; TIC: ticarcillin/clavulanic acid; TOB: tobramycin; VAN: vancomycin.

[a] Arguello et al. [92], the diarrhea was considered a side effect of Idelalisib therapy, but diarrhea persisted by the time of admission, despite it was interrupted 10 days before. No culture was carried out from feces, but *Ab* (identified by 16S rRNA sequencing) was isolated from blood culture from this patient.

ciaB, *mviN*, *pldA,* and *tlyA*) were present in all tested strains of *A. butzleri* isolated from broiler carcasses ($n = 52$), cattle and sheep fecal samples ($n = 25$), processing water ($n = 15$) and processing line equipment ($n = 21$) of a slaughterhouse, meat ($n = 37$), water ($n = 9$), and humans ($n = 6$) [95,96]. None of the *A. cryaerophilus* or *A. skirrowii* strains tested were positive for all the nine genes in the study of Douidah et al. [94]. The latter authors suggested that this was probably due to the presence of heterogeneity in the sequences of these genes in these two species. The most prevalent genes among *A. cryaerophilus* strains tested (n = 113) in the study of Douidah et al. [94] were *ciaB* (98%) and *mviN* (92%), while genes *irgA* and *hecA* presented the lowest incidence (3% and 4%, respectively). Regarding *A. skirrowii* strains (n = 44), the most prevalent gene was *ciaB* (98%) and the genes *mviN, cadF*, *tlyA*, *pldA, cj1349*, and *hecB* were present with an incidence from 36% to 23%. For this species, the gene *irgA* was not detected. None of the strains of *A. butzleri* (n = 62) isolated from shellfish by Collado et al. [37] or by Mottola et al. [81] showed simultaneously all the five studied genes (*cadF*, *ciaB*, *cj1349*, *irgA*, and *hecA*) that showed a varying prevalence with only *cadF* gene present in all the tested strains. In a recent study using strains recovered from ready-to-eat vegetables, all 40 *A. butzleri* strains tested showed the genes *ciaB*, *cj1349*, *mviN*, *tlyA*, and *pldA*, while genes *cadF*, *hecB*, and *hecA* were detected in lower amounts [43]. However, in the four strains of *A. cryaerophilus* tested, only *cadF* and *mviN* genes were detected. Despite the existence of several studies determining the prevalence of these genes, nothing is known about their expression or if the function is similar to that described by their homologues in *Campylobacter* [1].

Flagellin genes are related with flagellum and, therefore, with the capacity of infection and invasion. In this regard, alterations in the *flaB* gene in one strain of *A. butzleri* did not influence

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

TABLE 23.5

Cases of Arcobacter Extraintestinal Infections

Country	Patient Sex/Age	Presentation/Duration	Species	Antimicrobial Susceptibility Testing Method	Recovery in Days (d)/ Treatment	Underlying Condition
China	F/63 years	Peritonitis with fever and abdominal pain after repositioning a catheter	*A.* sp.	ND. Empiric treatment	15d/prophylactic CFZ and LEV before procedure and then TIC	End-stage renal failure
Hong Kong	F/69 years	Bacteremia with fever and lower quadrant pain	*Ab*	ND. Empiric treatment	3 d/CFR and MET	Gangrenous appendicitis
Taiwan	F/72 years	Bacteremia and hematogenous pneumonia; fever, progressive cough with purulent sputum and frequent loose stool/2 months before admission	*Ac*	Susceptible to AMP, AMX/CA, CFZ, CTX, CTZ, AZT, ERY, CLA, CLR, TOB, and CIP; resistant to CFZ, TET, and MIN, SXT and RIF; method: E-test	14 d/CTZ and TOB	Chronic renal failure
Taiwan	M/60 years	Bacteremia with fever	*Ab*	Susceptible to: AMP, AMX/CA, and CLA; resistant to CPH, CFR, and CTX; method: E-test	4 d/CFR	Chronic hepatitis B and liver cirrhosis
United Kingdom	Neonate	Bacteremia with hypotension, hypothermia, and hypoglycemia	*Ab*	Resistant to AMX, PIP, CFR, CAZ, CTX, AMX/CA, and SXT; method: NS	6 days/P and CTX	Placenta previa, prenatal bleeding, and delivery at 26th week
United States[a]	M/85 years	Chronic persistent diarrhea (Table 23.4), with fever and hypotension	*Ab*	ND. Empiric treatment	3 d/VAN and PIPT	Chronic lymphocytic leukemia (CLL)

Source: Adapted and updated from Figueras MJ et al. *New Microbes New Infect.* 2014;2:31–37, with a new study Arguello E et al. *J Clin Microbiol* 2015;53:1448–51 [92].

Note: ND: Not done; NS: not specified; *A.* sp.: *Arcobacter* sp.; Ab: *A. butzleri*; AMK: amikacin; AMP: ampicillin; AMX: amoxicillin; AMX/CA: amoxicillin/clavulanic acid; CAZ: ceftazidime; CD: clindamycin; CEPH: cephalosporin (any); CFR: cefuroxime; CFZ: cefazolin; CIP: ciprofloxacin; CLR: clor-amphenicol; CTR: ceftriaxone; CTZ: ceftizoxime; DXC: doxycycline; ERY: erythromycin; GEN: gentamicin; LEV: levofloxacin; MET: metronidazole; MIN: minocycline; NA: nalidixic acid; OFX: ofloxacin; P: penicillin; PIP: piperacillin; PIPT: piperacillin/tazobactam; QUI: quinolones (any); SXT: trimethoprim-sulfamethoxazole; TET: tetracycline; TIC: ticarcillin/clavulanic acid; TOB: tobramycin; VAN: vancomycin.

[a] Arguello et al. [92], the immunocompromised status of the patient (CLL) facilitated the severe and chronic diarrhea and bacteremia. Anaerobic blood culture bottle was positive after 23 hours of incubation.

the formation of the flagella, but mutations in the *flaA* gene can produce a loss of flagella and of motility [1 and references therein]. It has been observed that the genome of *A. butzleri* RM4018 carries genes for the flagella structure; however, some of the genes involved in the regulation of flagella transcription typically found in other Epsilonbacteria like *flgM* gene or the sigma factor gene *rpoN*, were not present. However, it has been indicated that the presence of other sigma factor genes present in that genome could compensate for the functions of the missing genes [57].

23.6.1 Adhesion, Invasion, and Cytotoxicity

The capacity of *Arcobacter* spp. to adhere, invade, and produce cytotoxicity has been studied using *in vitro* cell cultures of different cell lines as reviewed by Collado and Figueras [1]. In a recent study, Ferreira et al. [10] adapted and updated the information of the latter review summarizing results from 11 different studies that investigated the adhesion, invasion, and cytotoxicity. Table 23.7 summarizes data from Ferreira et al. [10], adding the most recent study of Bruegge et al. [97]. The reviewed studies showed that adherence and cytotoxicity were the most common interactions, with 71.3% and 88.5% of the tested strains, respectively, showing this behavior while in only 47.3% of the tested strains was invasion observed (Table 23.7). However, the response depended on the strain origin and the type of cell line tested [1]. The most studied species are *A. butzleri* and *A. cryaerophilus*, and the most frequently used cell lines are the human larynx carcinoma (Hep-2), the African green monkey kidney (Vero), and the human colon carcinoma (Caco-2) cells. The Caco-2 cells were used to analyze the activity of 16 *Arcobacter* spp. by Levican et al. [98], and all species tested except *A. bivalviorum* and *A. aquimarinus* (*Arcobacter* sp. strain W63) showed adhesion, and most species (10/16) were invasive. According to Levican et al. [98], the most invasive species were *Arcobacter trophiarum* (100%), *A. skirrowii* (50%), *A. cryaerophilus* (20%), *A. butzleri* (16%), and *A. defluvii* (12.5%), and all the invasive strains were positive for the *ciaB* gene, which as commented, encodes for an invasion protein. Few studies have related the detection and presence of virulence genes with the adhesion and invasion of *Arcobacter* spp. and strains [95, 98]. However, a part of the finding of Levican et al. [98] was that no specific pathotype has been recognized on the basis of the carried genes.

Although *Arcobacter* spp. presented cytotoxic activity against Vero and Chinese hamster ovary (CHO) cells, a cytolethal distending toxin (CDT) similar to that described in *Campylobacter* was not detected; however, it was suggested that another toxin different from CDT could be responsible for the observed toxicity [1 and references therein].

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

TABLE 23.6

Characteristics of the Patients from Different Diarrhea Surveys in which *Arcobacter* spp. Were Detected by Culture or PCR-Based Methods

	Prevalence of *Arcobacter* spp. (%) among Diarrheic Patients					**Type of Diarrhea and Symptoms (%)**											
Country	**Number Patients**	**Age (range or mean)**	**M/F Ratio**	**Culture**	**PCR**	**Acute**	**Chronic**	**Watery**	**Blood**	**Nausea/ Vomiting**	**Abdominal Pain**	**Fever >38°C**	**Monomicrobial Infection (%)**	**Underlying Disease (%)**	**Antimicrobial Treatment (%)**	**Relapse (%)**	**Asymptomatic (%)**
South Africa	19,535	Pediatric (NS)	NS	0.4	ND	100	0	–	–	–	–	–	–	–	–	–	49.7
Belgium	6774	Mean 42 years		1.31	ND	55.1	11.2	–	1.1	–	–	–	–	43.8	–	–	0
Belgium and France	67,599	30 days–90 years	NS	0.11	ND	50.8	16.4	50.8	6.0	27.9	29.5	32.8	82.0	16.4	26.2	6.6	19.7
Canada[a]	1506	0–>65 years	0.69	0.8	57[b]	–	–	–	–	–	–	–	74	–	–	–	0
Chile	140	<5–>50 years	0.96	0.7	1.4	100	0	–	–	–	–	–	50.0	–	–	–	45.3
	83[c]	Pediatric (NS)	NS	3.6	ND	–	–	–	–	–	–	–	–	–	–	–	–
France[d]	2855[e]	54 years	1.44	1.0	ND	59.0	3.4	–	21.1	10.5	57.9	26.3	93.3	15.8	26.3	5.3	–
	345	41.4 years	0.56	0	1.2	100	–	–	–	–	–	–	–	–	–	–	0
India	400[f]	≥18 years (NS)	1.4	1.25	ND	100	0	–	–	–	–	–	–	50.0	–	–	0
South Africa	322	1 month–88 years	0.77	ND	13.0	47.8	–	–	3.1	–	–	–	83.1	13.7	–	–	20.8
New Zealand	1380	49 years	1.0	0.9	ND	100	0	–	–	8.3	–	–	75.0	–	–	–	0
The Netherlands	493	35 years	0.88	0	0.4	100	–	–	–	–	–	–	0.4	–	–	–	–
Turkey	3287	26.6 years	1.25	0.3	ND	100	0	33.3	0	22.2	100	11.1	–	–	–	–	0
United States/ Europe	201[g]	NS	NS	ND	8.0	100	–	–	–	–	–	–	78.6	–	–	–	0

Source: Adapted and updated from Figueras MJ et al. *New Microbes New Infect.* 2014;2:31–7 [22], with new studies Fernández H et al. *Braz J Microbiol* 2015;46:145–7, Van den Abeele AM et al. *Emerg Infect Dis* 2014;20:1731–4, Webb AL et al. *J Clin Microbiol* 2016;54:1082–8 [4,21,38]

Note: NS: Not specified; ND: Not determined.

[a] Webb et al. [38].

[b] Overall cell density of *A. butzleri* in this study was significantly higher in diarrheic than nondiarrheic stools.

[c] Fernandez et al. [4].

[d] Van de Abeele et al. [21].

[e] Surveillance study including only patients with diarrhea due to *Campylobacter*-like microorganisms.

[f] Case control study that included 200 HIV-1 infected patients and 200 noninfected controls.

[g] U.S. or European travelers who suffered acute diarrhea after returning from México, Guatemala, or India.

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

TABLE 23.7

Overview of the Pathogenicity of *Arcobacter* spp. in Different Cell Lines

		Percentage of Positives (Number of Tested Strains)		
Species	**Cell Line**	**Adhesion**	**Invasion**	**Cytotoxicity**
A. cryaerophilus	Hep-2	57.1% (7)	100% (2)	ND
	He-La	ND	ND	100% (3)
	Vero	ND	ND	100% (2)
	INT407	ND	ND	100% (3)
	Caco-2	68.7% (16)	77.7% (9)	ND
	IPI-2I	100% (4)	25% (4)	ND
A. butzleri	Hep-2	88.2% (93)	ND	ND
	He-La	31.4% (35)	0% (18)	100% (7)
	Vero	ND	ND	84.8% (119)
	INT407	5.5% (18)	0% (18)	100% (6)
	CHO	ND	ND	94.4% (18)
	Caco-2	100% (19)	89.5% (19)	ND
	IPI-2I	100% (1)	0% (1)	ND
	HT-29	66.6% (6)	50% (6)	ND
	THP-1[a]	ND	100% (6)	100% (6)
A. skirrowii	Vero	ND	ND	89.5% (19)
	Caco-2	100% (4)	25% (4)	ND
	IPI-2I	100% (2)	0% (2)	ND
A. thereius	Caco-2	100% (5)	80% (5)	ND
A. cibarius	Caco-2	100% (6)	50% (6)	ND
	IPI-2I	100% (1)	0% (1)	ND
Other species[b]	Caco-2	86.7% (30)	60% (30)	ND
Total		71.3% (247)	47.3% (131)	88.5% (183)

Source: Adapted and updated from Ferreira S et al. *Crit Rev Microbiol* 2016 May;42(3):364–83 [10].

Note: ND: Not done.

[a] Data extracted from Bruegge et al. [97].

[b] Nine different species tested by Levican et al. [98].

The mechanism by which *Arcobacter* produces diarrhea was illustrated by Collado and Figueras [1 and references therein] in a scheme that shows the interaction of the bacteria with intestinal epithelial cells. This interaction produces a decreasing expression of Claudin in the tight junctions of the colon intestinal epithelial cells that alter the epithelial barrier, leading to the production of a leak-flux type of diarrhea. A recent *in vitro* study confirmed again the cytotoxicity of strains of *A. butzleri* (isolated from chicken, pork meat, human samples, and water) to the human colon (HT-29/B6) but not to the porcine intestinal (IPEC-J2) epithelial cells [99]. In addition, the latter study ratified that the epithelial barrier alteration observed in the HT-29/B6 cells resulted in a leak-flux type of diarrhea.

23.6.2 Host Immune System

Host immune studies regarding *Arcobacter* spp. interactions are reviewed by Ferreira et al. [10]. Defensins are antimicrobial peptides that can decrease the number of bacteria present during the infection [100]. These peptides are secreted by immune system cells (leukocytes, neutrophils, natural killer cells …) and by epithelial cells. Veldhuizen et al. [100] found that infected epithelial cells of the porcine intestine line (IPI-21), with *A. cryaerophilus* LMG 7535, produce no effect on the expression of β-defensins 1 and 2 secreted by the IPI-21 cells [100]. Other activity of the innate immune response reviewed by Ferreira et al. [10] was the resistance or susceptibility to the complement. Complement helps the immune system to remove damaged cells and microorganisms during the infection process, destroying the pathogen membrane and promoting an inflammation reaction. Complement activity against 10 strains of *A. butzleri* demonstrated that this species is sensitive to the complement, but sensitivity levels are related to the strain origin, because bacteremia strains were more resistant to complement activity than diarrheal strains [10].

Interleukin-8 (IL-8) secreted by macrophages and other epithelial cells during an infection is an important mediator of the innate immune system and the inflammatory reaction [10]. It has been demonstrated that the four tested *Arcobacter* spp. (*A. butzleri*, *A. cryaerophilus*, *A. skirrowii*, and *Arcobacter cibarius*) can enhance the expression of IL-8 both in Caco-2 cells and in IPI-21; however, no changes in the adhesion and invasion levels were observed [1 and references therein].

Toll-like receptor-4 (TLR-4) is a transmembrane receptor of macrophages and dendritic cells that mediate essential signaling pathways involved in the innate and adaptive host immune responses to commensal and pathogenic bacteria. The TLR-4 senses molecules such as lipopolysaccharides (LPS) and lipooligosaccharides (LOS) present in the cell walls of gram-negative bacteria. A German group from the Freie Universität of Berlin has investigated the TLR-4 immune response during

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

Arcobacter infections in gnobiotic or germ-free mice [101–107]. Their research demonstrated that the local and systemic immune response during *A. butzleri* infection is dependent on TLR-4 expression [101]. Furthermore, recognition of LPS and LOS by TLR-4 produces an increase of the inflammatory response during *A. butzleri* infections [102]. Comparative study of the immune reaction in TLR-4/IL-10–deficient mice in relation to deficient mice only for IL-10 shows that the absence of TLR-4 reduced the apoptosis of colonic cells during *Arcobacter* infection [103]. In addition, the number of cells involved in the immune response (i.e., macrophages, monocytes, T and B lymphocytes, etc.) was lower than when experiments were performed in IL-10–deficient mice [104]. Gene expression of inflammatory mediators and matrix-degrading gelatinases has been studied in TLR-4/IL-10– and IL-10–deficient mice after infection with *A. butzleri* [105,106]. It was observed that the infection changes the expression of inflammatory and regulatory immune response genes (i.e., TNF, IFN-γ, and IL-1β), and these changes were related with the presence of TLR-4 [105,106]. Results from the German group with gnobiotic mice models infected with *A. butzleri* were reviewed in a meta-analysis made by Gölz et al. [107].

23.6.3 Animal Models

Reproduction of the *Arcobacter* infections in several animal models has been published in different studies as previously reviewed [1,10]. Recent studies have evaluated other animal models like the zebrafish [108] and the gnobiotic mice [101–107]. The main conclusion is that virulence of the tested *Arcobacter* species is host and species dependent. For instance, *A. butzleri* showed longer bacteria shedding in 1-day-old cesarean-delivered colostrum-deprived piglets orally infected, while *A. cryaerophilus* and A. *skirrowii* did not show this behavior [10 and references therein]. Furthermore, *A. butzleri* was the most invasive species, because it was isolated in almost all sampled tissues of the piglets [10].

In poultry models, the behavior of *A. butzleri* was different depending on the host, because no colonization of the intestine of chickens and turkeys was observed. However, when Beltsville white turkeys were used as a model, *A. butzleri* could colonize and kill the animals [1 and references therein].

Rainbow trout has also been used as an animal model for *A. cryaerophilus* infection [1 and references therein]. After oral infection, the fish showed degeneration of the respiratory system (opercula and gills); hemorrhage in the liver, heart, and kidney; serum accumulation in the inflamed intestine and spleen; alteration of blood parameters; and finally death. Açik et al. [108] used zebrafish to evaluate the pathogenicity of *A. butzleri* infecting the animals by intraperitoneal and immersion assays, but macroscopic lesions or clinical symptoms were not observed. However, microscopic lesions in some organs and tissues were found.

Orally infected albino rats have also been used as diarrhea models for *A. butzleri*, and the rats showed symptoms and lesions like necrosis of the villi and infiltration of leukocytes in the lamina propria [10 and references therein]. The same behavior (diarrhea and histological lesions) was observed when the albino rats were infected with *A. cryaerophilus*. An increase in the inoculum use in albino rats produced an increase of the symptoms [10 and references therein]. However, orally infected gnobiotic IL-10–deficient mice with *A. butzleri* did not show clinical symptoms but showed colonic apoptosis and also accumulation of leukocytes in lamina propria [101]. Furthermore, the inflammatory response was produced in the extraintestinal tissues of infected mice [103,104].

23.7 Microbiological Diagnosis

The isolation methods for *Arcobacter* were reviewed by Collado and Figueras [1], and they indicated that despite different isolation broths and plating media having been described, there is not a standardized method for the recovery of these bacteria. The different enrichment broths available include selective supplements, that is, antibiotics, which can influence and reduce *Arcobacter* species diversity and may favor the prevalence of resistant and fast-growing species [1]. The most used protocol is plating by passive filtration through a 0.45 μm filter onto blood agar combined with the same procedure after an enrichment step in a selective broth concurrently. As mentioned before, the most employed broth is the Arcobacter-CAT that includes cefoperazone, amphotericin B, and teicoplanin. The addition of 2.5% of NaCl to the Arcobacter-CAT broth and inoculation by passive filtration on marine agar had increased the recovery and diversity of *Arcobacter* species found in shellfish, and sea and brackish water [26].

23.7.1 Identification

Identification of *Arcobacter* spp. using phenotypic methods and biochemical assays is unreliable and time consuming, and molecular methods have been developed for this purpose. Some of these methods were reviewed by Levican and Figueras [109] that showed the advantages and disadvantages of each compared method. The most used methods are the multiplex-PCR (m-PCR) developed by Houf et al. [50] and Douidah et al. [54]. However, these methods cannot differentiate between some species. For instance, the m-PCR described by Houf et al. [50] produces the same pattern for *Arcobacter nitrofigilis* and *A. skirrowii* [109] or between *A. butzleri* and *A. lanthieri* (unpublished results). The m-PCR of Douidah et al. [54] combined with the PCR of De Smet et al. [55] cannot differentiate *A. butzleri* from *A. defluvii*, *Arcobacter venerupis*, and *A. suis* [106]. A method that in our hands has been highly useful is the 16S rRNA-RFLP that was designed to produce species-specific patterns for the 17 species described [72]. However, the cost improvement of the sequencing approaches makes the use of sequences of housekeeping genes a more efficient and reliable alternative, as we discuss later in this chapter. The main conclusion of the study of Levican and Figueras [109] was that methods targeting 16S or 23S rRNA genes may be problematic, because many of the new species share a high similarity (>99%) of the 16S rRNA gene, and in addition, the 23S rRNA gene is only available for a few species. Furthermore, the exponential increase in the number of species described in the genus in the last years can make these methods obsolete if they are not validated for possible interference with new species. One unequivocal method to identify the species is the amplification and sequencing of housekeeping genes, that is, *rpoB* [39], *hsp60* [110], and *gyrB* [111]. This is the approach used

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

in our laboratory, which has enabled recognition of *Arcobacter* by *Campylobacter* and many new species [22,26]. Real-time PCR (qPCR) methods targeting 16S rRNA and 23S rRNA genes have been described to quantify *Arcobacter* spp. in different matrices [41,112]. Based also in one of the housekeeping genes mentioned above (*hsp60*), a qPCR method has been developed for the detection of *A. butzleri* from stool samples [113]. So far, no studies have evaluated possible interference with other new species. However, in a study that compared different qPCR methods for the detection of *A. butzleri* and *Campylobacter* spp. from irrigation water, they discover that *Arcobacter* may uncover *Campylobacter* spp. [44]. Another qPCR method for the detection of *A. butzleri* from stool samples is that developed by Webb et al. [38] that targets the gamma subunit of a quinohemoprotein amine dehydrogenase (qhnDH). This method was tested in 130 *A. butzleri* strains, and the developed primers amplified all the tested strains. An alternative, fast, cheap, and reproducible method that has helped to recognize *Arcobacter* in the clinical setting is the use of matrix-assisted laser-detected ionization–time-of-flight (MALDI-TOF) mass spectrometry [15,114,115]. However, the reliability of the method depends on the amount of strains of different species included in the database that in some of the systems, like in the Biotyper of Bruker, is quite limited. Other systems like the one of bioMérieux did not provide any identification for an *A. butzleri* strain isolated from a bacteremia case, and this was attributed to the fact that the specie is not present in the database [92].

23.8 Treatment

There is no official-specific protocol for the treatment of gastrointestinal or systemic infection by *Arcobacter* species [1,10,116]. This is probably due to the fact that this emerging pathogen is not yet included in the routine microbiological diagnosis of gastroenteritis and/or bacteremia. Therefore, *Arcobacter* species are still very poorly known by physicians, and the clinical data available come from the few microbiological investigations or clinical cases described so far [22,38,92].

Given the taxonomic and clinical similarities with *Campylobacter* spp., and as done with many enteropathogens, fluid therapy should be the first step in the management of gastrointestinal infections produced by *Arcobacter* spp. Antimicrobial treatment, as commented, would only be necessary in patients with severe, prolonged, and relapsing illness or in patients with underlying diseases [1]. However, opposite to this assumption, the scarce available clinical data have shown that antimicrobial treatment is relatively common in cases of enteritis, probably due to the chronic characteristics of the *Arcobacter* diarrhea (Table 23.4), and mandatory in the cases of bacteremia (Table 23.5). The most common antimicrobial agents used in gastrointestinal infections produced by arcobacters have been macrolides, such as erythromycin, and fluoroquinolones, such as ciprofloxacin or ofloxacin (Table 23.4). To a lesser extent, tetracycline (doxycycline) has also been used as an alternative choice in the treatment. A recent case that occurred in a 26-year-old male in Spain showed that amoxicillin/clavulanic acid was also effective in the treatment of persistent bloody and watery diarrhea of 3 weeks' duration [22]. Unlike intestinal infections, most cases of bacteremia have been treated with β-lactam antibiotics, such as second- and third-generation cephalosporins (Table 23.5). The use of β-lactamase inhibitors combined with β-lactam antibiotics (i.e., ticarcillin/clavulanic acid or piperacillin/tazobactam) has also apparently been an effective treatment (Table 23.5). Nevertheless, it is important to note that several of these clinical cases have been treated empirically. This means that no antibiotic resistance tests were analyzed before implementing these treatments (Tables 23.4 and 23.5).

23.8.1 Antibiotic Resistance

A recent review performed by Ferreira et al. [10] about the *in vitro* antimicrobial susceptibility testing (AST) of strains of *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii*, mainly isolated from food like poultry, livestock, and shellfish, show patterns of antimicrobial resistance for these species that can guide the election of the treatment in cases of intestinal and extraintestinal infections. This accumulative evidence shows some interesting trends such as a remarkable resistance to ampicillin (β-lactam), nalidixic acid (quinolone), ciprofloxacin (fluoroquinolone), erythromycin (macrolide), and gentamicin (aminoglycoside), with resistance rates ranging between 0% and 100%. In contrast, a low resistance was encountered for tetracycline (0%–9% of the strains tested). These observations are in agreement with the results of a Belgian study, performed by Van den Abeele et al. [116], where the antimicrobial resistance of 106 strains isolated from human enteritis (63 *A. butzleri* and 43 *A. cryaerophilus*), was analyzed. The authors indicate that fluoroquinolones and macrolides should not be used as empirical treatment in cases of *Arcobacter*-related gastrointestinal infections, suggesting instead the use of tetracycline. However, comparisons between the *Arcobacter* antibiotic susceptibility results should be made with caution, since these studies have been developed using different methodologies to evaluate the antimicrobial susceptibility, such as disc diffusion, agar dilution, E-test, or broth microdilution, and using different interpretation guidelines [10]. A guide or recommendation on the treatment of invasive *Arcobacter* infections is needed, taking into account that, in practice, the most used drugs are β-lactam antibiotics (Table 23.5), which are at the same time the antimicrobials against which most strains show resistance [10,116].

In relation with molecular mechanisms of antibiotic resistance, two independent studies (carried out in France and Belgium) have assessed the presence of potential mutations in the quinolone resistance determining region (QRDR) of *gyrA* gene in ciprofloxacin-resistant clinical strains [116,117]. Both studies found similar results, in the French study two *A. butzleri* and one *A. cryaerophilus* strains harbored a cytosine-to-thymine transition in position 254 of the *gyrA* gene, resulting in the amino acid substitution Thr-85-Ile in the GyrA protein [117], while in the Belgian study 10 *Arcobacter* strains showed the same point mutation [116]. So far, there are no other studies investigating the resistance mechanisms in *Arcobacter*. However, the genome of *A. butzleri* (RM 4018 strain) has some putative resistance genes, such as (1) the *cat* gene related to chloramphenicol resistance because it encodes a chloramphenicol *O*-acetyltransferase; (2) three putative β-lactamase genes (AB0578, AB1306, and AB1486); and (3) the *lrgAB* operon, which is associated with

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

β-lactam resistance [29]. However, the *upp* gene, encoding uracil phosphoribosyltransferase, associated with 5-fluorouracil resistance, was not detected in the *A. butzleri* genome. The genomic analysis of an *A. cryaerophilus* strain detected in sewage was published [118], and the authors highlight the capacity of this bacterium to accumulate a large number of antibiotic resistance genes (ARGs). It was observed that 5% of open reading frames (ORFs) encoded ARGs belonging to 25 categories, being macrolides, fluoroquinolones, aminocoumarin, and vancomycin resistance genes the most abundant groups [118]. Considering the availability of at least 46 draft genomes of *Arcobacter* spp., it would be interesting to screen them for the presence of those and other potential resistance genes in the near future.

Several important questions about antibiotic resistance in *Arcobacter* are still pending to be answered. For example, it is still unknown whether *Arcobacter* is suffering an increase in antimicrobial resistance due to the use of antibiotics, either as therapeutic agents or as growth promoters in the agricultural and aquaculture industries [37]. Most molecular mechanisms of antibiotic resistance and the ways by which they can be transmitted, that is, through horizontal gene transfer between *Arcobacter* and related species, are still unknown. Therefore, in addition to having an adequate treatment for *Arcobacter* infections, it is also necessary to understand the mechanisms and routes of acquisition and transmission of the antibiotic resistance either among strains and species, or between them and well-known pathogenic campylobacteria, and vice versa.

23.9 Discussion and Future Perspectives

As commented, the interest in the genus *Arcobacter* has increased significantly in recent years, due to its implication in human and animal disease. This was also influenced by its common isolation from food products of animal origin, and also more recently from irrigation water, vegetables, and shellfish. Among the most striking findings of the last decade is the discovery of many new species that have quadruplicate the number of species from 6 in 2006 to 24 in 2016. Moreover, this has also highlighted that the genus embraces currently three main clusters of species that could each correspond to separate genera. The definition of these new genera is one of the things that can be expected to happen in the near future. Despite global interest in the genus, many questions remain, for instance, its true incidence in different types of samples. Results from culturing show discordance with the high detection reported using molecular methods, including conventional mPCR, qPCR, or even metagenomic studies. To which extent the latter is due to false positives, to the existence of a VNC state, or to the culture methods used requires full clarification. In fact, the ideal culture method for these microbes does not yet exist. Furthermore, we now know that the introduction of a pre-enrichment step, despite being necessary for increasing the number of positive samples, favors the growth of *A. butzleri* overestimating the importance of this species. The use of several culture approaches in parallel, together with the characterization of an elevated number of colonies (more than 8 or 10) has been the most effective way of discovering the great diversity of species associated with shellfish and marine environments. In fact, the last approach has led to the discovery of the high number of species described during the last years. Therefore, the use of a similar approach in other types of samples is necessary, along with the characterization of the species by sequencing a housekeeping gene (*rpoB*, *cpn60*, *gyrB*, etc.).

Metagenomic studies made the genus popular, because it was in many cases unexpectedly discovered as an abundant one in many environments like wastewater and coastal marine ecosystems. In fact, it has been indicated that *Arcobacter* can grow in the sewerage system, and this would explain the abundance in sewage and wastewater ($>10^5$/100 mL). These data ratified the previous association of *Arcobacter* and fecal pollution. Comparative studies on irrigation water using different qPCR methods with primers considered specific for *Campylobacter* have revealed that *Arcobacter* could be responsible for generating many false positives. Similarly, *Campylobacter* strains recovered from clinical cases may uncover *Arcobacter* as revealed by MALDI-TOF and other molecular methods. The extended use of MALDI-TOF at the clinical microbiological laboratories will probably help to recognize more clinical cases. There is an urgent need for defining guidelines for interpretation of the antimicrobial susceptibility testing of these bacteria.

Considerable research has been carried out on *Arcobacter*'s virulence using cell cultures and animal models as well as detecting putative virulence genes. However, further research is necessary to determine the role of the virulence genes described in this genus, generating mutants that could pinpoint their true association with virulence. It is expected that the genome analysis of *Arcobacter* spp. will provide more light on their virulence and common metabolic pathways associated to this genus.

Probably the eradication and elimination of foodborne infections and diseases produced by *Arcobacter* can be achieved by good sanitation practices preventing contamination of the water and food products with feces or wastewater that, as we commented, are the main sources of these bacteria [119].

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UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

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UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

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INTEREST AND OBJETIVES

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

INTEREST AND OBJECTIVES

As it has been outlined in the introduction, the "Sanitary importance of *Arcobacter*" was reviewed by Arturo Levican in his Ph.D. thesis (Levican, 2013) and some of his conclusions became the starting point of the present Ph.D. thesis, and these are presented below.

The high prevalence and diversity of *Arcobacter* species found by Levican in sewage contaminated waters and in shellfish were attributed to the parallel use of a direct culture plating medium and an enrichment step in combination with the incubation of the media under aerobic and microaerobic conditions. Considering such results and that shellfish grow in marine and brackish environments, the use of culture media that better mimics the conditions of these habitats (possessing a higher concentration of salts) could favor still further the isolation of *Arcobacter* species. This became the first hypothesis of this Ph.D., and therefore, the design and evaluation of media that contained an augmented salt concentration was one of the first objectives. In addition, Levican and other researchers consider that the exposure of the shellfish to sewage contaminated waters is the reason that made them reservoirs and hosts of *Arcobacter* species, including those considered human pathogens, like *A. butzleri* and *A. cryaerophilus.* However, experiments that demonstrated the extent of the shellfish contamination, and colonization of the different organs after exposure to sewage polluted waters, were missing and this became another one of the aims of this thesis.

It is well known that bivalves have a filter feeding behavior which make them vehicles of concentration, accumulation, and dissemination of a diverse number of microorganisms. Thus, together with the fact that bivalve mollusks are usually consumed raw or slightly cooked, their consumption possess an extra risk for the safety of the consumers. Spain is the main producer of marine mollusks in Europe and ensures the sanitary quality of marketable shellfish following the European legislation EU 854/2004. This regulation assesses if bivalve mollusks are safe for human consumption, and it is based on the specific load of the fecal indicator bacteria *E. coli* that bivalves carry. The *E. coli* concentration is used to determine if shellfish can be directly consumed or if they will require a depuration process. However, some reports showed that *E. coli* is not universally suitable for predicting the presence of some pathogens, like viruses of human origin or marine-borne *Vibrio* spp. Despite the demonstration that the presence of *E. coli* can predict the presence of *Arcobacter* in fecally contaminated waters, there is only one study, performed in mussels, which correlated the concentration of *E. coli* with the presence of *A. butzleri*. Thus, evaluating the potential correlation between the concentrations

of *E. coli* and *Arcobacter* spp. in both marine water and shellfish, would be useful to determine to which extent the EU legislation can protect the consumers from being exposed to *Arcobacter* contaminated shellfish. Additionally, new strategies for quantifying *Arcobacter* in shellfish were also required to enable its comparison with the concentrations of *E. coli*. Furthermore, the lack of studies of depuration strategies for *Arcobacter*, make necessary an assessment of the conventional depuration times established for *E. coli* to confirm if they are sufficient to remove *Arcobacter* from shellfish before they are placed in the market.

The contamination of food products with *Arcobacter*, has mainly been associated with cross-contamination with fecally polluted waters. However, recent studies have demonstrated that *Arcobacter* species are part of the shellfish microbiota, nevertheless, they can be concomitant pathogens of oysters in cases of coinfections caused by *Vibrio* spp. Therefore, studies to address this aspect are of paramount importance to guide epidemiological and surveillance studies with special focus on the distribution within shellfish tissues. The latter will enable differentiation of those species of *Arcobacter* found within shellfish that are autochthonous from the allochthonous. Moreover, the relationship between the load of *Arcobacter* species present in shellfish and the time required to remove, or reduce this bacteriological load during the depuration process has never been explored and requires investigation.

One of the main problems in the research of *Arcobacter* is that their isolation and identification by culture is fastidious and time consuming, requiring on average 4 days. Additionally, as previously shown in Levican's Ph.D. thesis, the current PCR identification methods are unable to unequivocally characterize all the known *Arcobacter* species. Furthermore, the conventional PCR procedures have the handicap that they cannot differentiate between living and dead cells. Therefore, developing a method able to discriminate viable from non viable cells, like the ones that use propidium monoazide (PMA) in combination with qPCR, would represent a step forward in the recognition of this fastidious bacteria for the agro-alimentary industry. These more specific diagnostic methods can also help to prevent illness and to reduce product recalls avoiding economic losses.

Furthermore, the Ph.D. thesis "Epidemiology and taxogenomics of the genus *Arcobacter*" by Alba Pérez-Cataluña (2018) has recently demonstrated that the diversity of *Arcobacter* is greater than previously considered. In fact, the thesis studies of this genus uncovered at least seven different genera, for which the names *Arcobacter*, *Aliarcobacter* gen.

nov., *Pseudarcobacter* gen. nov., *Halarcobacter* gen. nov., *Malacioarcobacter* gen. nov., *Poseidoniarcobacter* gen. nov., and candidatus *Arcomarinus* gen. nov., have been proposed. The present Ph.D. thesis will retain mainly the old taxonomy of *Arcobacter,* because many results were written down before the taxonomic study of Alba Pérez-Cataluña was accepted (**Studies 3.1, 3.2, 3.5, and 3.6**).

In that work the genomic analysis showed the existence of at least 7 potential new species belonging to the new *Arcobacter*-related genera. However, a complete polyphasic characterization of these 7 potential new species, was not performed and it is required for their accurate description and validation, and this is going to be done in the present thesis.

The general objective of the present thesis is to provide new insights on the relationship between *Arcobacter* and shellfish, and on whether shellfish could be considered a potential source of human infections by these bacteria. To achieve this, the following specific objectives have been defined:

1. To develop new methods for culturing, detection, and quantification of *Arcobacter* spp. from water and shellfish samples. **Study 3.1**
2. To evaluate if the fecal indicator *E. coli* predicts the presence of *Arcobacter* in water and shellfish samples, and to study the diversity of *Arcobacter* species in shellfish and in their surrounding water. **Study 3.2**
3. To determine the distribution and prevalence of *Arcobacter* related spp. in the different compartments and shellfish tissues in order to characterize the dynamics of colonization by these bacteria. **Study 3.3**
4. To study if the depuration time-periods established by the EU shellfish legislation for reducing the load of *E. coli*, would ensure the parallel reduction of *Arcobacter*- related spp. in mussels and oysters. **Study 3.4**
5. To improve available molecular tools for discriminating among viable and non-viable *Arcobacter* cells in shellfish samples. **Study 3.5**
6. To assess with a polyphasic taxonomic approach, including genomic information, if strains that seem to correspond to potential new phylogenetic lines represent new species of the recently described new *Arcobacter*-related genera. **Studies 3.6 and 3.7.**

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

RESULTS AND DISCUSSION

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

3.1 Enhanced recovery of *Arcobacter* spp. using NaCl in culture media and re-assessment of the traits of *Arcobacter marinus* and *Arcobacter halophilus* isolated from marine water and shellfish Salas-Massó N, Andree KB, Furones MD, Figueras MJ. *Science of the total Environment*, 2016; 566-567: 1355–1361.

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
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Enhanced recovery of *Arcobacter* spp. using NaCl in culture media and re-assessment of the traits of *Arcobacter marinus* and *Arcobacter halophilus* isolated from marine water and shellfish

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HIGHLIGHTS

- A new culture approach for the improved recovery of *Arcobacter* from marine samples
- The addition of NaCl to the growth media enhances the number of positive samples.
- The new culture strategy allowed the isolation of high number of *Arcobacter* spp.
- First report of *A. marinus* and *A. halophilus* from shellfish samples

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ABSTRACT

The genus *Arcobacter* is a relatively poorly known group of bacteria, and the number of new species and sequences from non-culturable strains has increased considerably in recent years. This study investigates whether using media that contain NaCl might help to improve the recovery of *Arcobacter* spp. from marine environments. To this aim, 62 water and shellfish samples were analysed in parallel, with both a commonly used culture method (enrichment in *Arcobacter*-CAT broth followed by culture on Blood Agar) and a new one that supplements the *Arcobacter*-CAT enrichment broth with 2.5% NaCl (w/v) followed by culturing on Marine Agar. The new method yielded ca. 40% more positive samples and provided a higher diversity of known (11 vs. 7) and unknown (7 vs. 2) *Arcobacter* species. Among the 11 known species recovered, *Arcobacter marinus* and *Arcobacter halophilus* were isolated only by this new method. No more strains of these species have been isolated since their original descriptions, both of which were based only on a single strain. In view of that, the phenotypic characteristics of these species are re-evaluated in the present study, using the new strains. Strains of *A. halophilus* had the same phenotypic profile as the type strain. However, some strains of *A. marinus* differed from the type strain in that they did not hydrolyse indoxyl-acetate, becoming, therefore, the first *Arcobacter* species to show a varying ability to hydrolyse indoxyl-acetate. This study shows to what extent a simple variation to the culture media can have a big influence on positive samples and on the community of species recovered.

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1. Introduction

The genus *Arcobacter* (within the Epsilonbacteria) includes both pathogenic and free-living species (Collado and Figueras, 2011; Hsu and Lee, 2015). The species of clinical relevance are *Arcobacter butzleri*, *Arcobacter cryaerophilus* and *Arcobacter skirrowii*, all of which have been linked to gastrointestinal diseases and bacteraemia in humans (Collado and Figueras, 2011; Figueras et al., 2014; Van den Abeele et al., 2014) and also to abortions, mastitis and diarrhoea in animals (Van Driessche and Houf, 2007; Collado and Figueras, 2011). Although many isolates have been recovered from sewage and from faecally contaminated water samples, marine ecosystems seem to be a primary source for new *Arcobacter* species (Fera et al., 2004; Wesley and Miller, 2010; Collado and Figueras, 2011). Ten of the 23 species that make up the genus were isolated from marine water and/or seafood, six of which (*Arcobacter mytili*, *Arcobacter molluscorum*, *Arcobacter ellisii*, *Arcobacter bivalviorum*, *Arcobacter venerupis* and *Arcobacter ebronensis*) were isolated from shellfish (Collado et al., 2009a; Collado and

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UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

Nomenclature

AB	Alfacs Bay
BA	Blood Agar
CAT	cefoperazone, amphotericin B, and teicoplanin
MA	Marine Agar
PNC	Poble Nou Channel

Figueras, 2011; Figueras et al., 2011a,b; Levican et al., 2012; Levican et al., 2015). The other four species were identified as *Arcobacter halophilus*, which was isolated from an hypersaline lagoon in Hawaii (Donachie et al., 2005), *Arcobacter marinus* recovered from a sample containing seaweed, starfish and seawater in Korea (Kim et al., 2010), *Arcobacter aquimarinus* from a seawater sample at the Garraf beach on the north-eastern coast of Spain (Levican et al., 2015) and *Arcobacter pacificus* from seawater in the South Pacific Gyre (Zhang et al., 2015).

Culture-independent studies of the bacteria present in seawater, sediments, shellfish and hydrothermal vents have recovered many 16S rRNA genes whose sequences show the highest similarity with species of the genus *Arcobacter* (Vandamme et al., 1991; Teske et al., 1996; Naganuma et al., 1997; Egas et al., 2012; Fernández-Piquer et al., 2012; King et al., 2012). A phylogenetic analysis of some of those sequences reveals that most of them might be new *Arcobacter* spp. (Miller et al., 2007; Wesley and Miller, 2010; Collado and Figueras, 2011). This highlights the importance of improving the methodology for isolating bacteria of this genus from these environments. Furthermore, finding multiple isolates of known species is essential if we are to understand better their intra-specific diversity, particularly *A. halophilus* and *A. marinus* that were originally described on the basis of only a single strain (Figueras et al., 2011c).

Few studies have focused on the diversity of *Arcobacter* in shellfish, with *A. butzleri* and *A. cryaerophilus* being the most frequently detected species (Collado et al., 2009b; Nieva-Echevarria et al., 2013; Levican et al., 2014; Collado et al., 2014; Mottola et al., 2016). Other, less-prevalent, species (*A. skirrowii*, *Arcobacter defluvii*, *Arcobacter mytili*, and *Arcobacter nitrofigilis*) have also been found (Collado et al., 2009b, 2014). However, Levican et al. (2014, 2015) reported the highest incidence of different *Arcobacter* species from any type of food sample studied to date, with 11 *Arcobacter* species isolated from mussels, oysters and clams, including all the species named above and, *A. molluscorum*, *A. ellisii*, *A. bivalviorum*, *A. thereius* and *A. ebronensis*. Determining the prevalence of *Arcobacter* spp. in shellfish and in the water of their harvesting area, using two culturing methods in parallel, is the subject of a large ongoing project in which we have isolated what we believe are new *Arcobacter* species together with several strains of *A. halophilus* and *A. marinus*, the first isolates of these two species that have been obtained since their description. The aims of the present study, therefore, were to show how the new culturing method is able to recover more and new *Arcobacter* species than the previously used one and to re-evaluate the phenotypic characteristics described for *A. halophilus* and *A. marinus*.

2. Material and methods

2.1. Location and sampling

Shellfish and water samples were collected over 7 months (March–August, and November–December, 2013) from Alfacs Bay (designated AB) (40° 34′ 22.43″N, 0° 39′ 12.96″ E; Ebro River Delta, Tarragona, Spain). The processed bivalves were provided by fishermen and consisted of 1.5–2 kg of mussels (*Mytilus galloprovincialis*), 20–25 individual oysters (*Crassostrea gigas*), 0.5 kg of clams (*Ruditapes philippinarum*) and 0.5 kg of cockles (*Cerastoderma edule*). Half of the shellfish samples (1 kg of mussels, 10–15 oysters, 250 g of clams and cockles) were directly analysed for the presence of *Arcobacter* as described below. The other half were exposed to water from a channel of untreated sewage that drains from the town of Poble Nou (designated PNC) (40° 38′ 30.8″N; 0°41′ 37.2″E). The shellfish were left in a cage immersed in the channel for 4 days to ensure natural contamination. When the bivalves were removed, 2 l of water were also taken from the same sites for analysis. We analysed a total of 37 shellfish samples (22 from AB and 15 from PNC) i.e. 13 samples of mussels (8 from AB, 5 from PNC), 20 samples of oysters (11 from AB and 9 from PNC), 3 samples of clams (2 from AB and 1 from PNC), 1 sample of cockles (AB) and 25 samples of water (11 from AB and 14 from PNC). Not all the shellfish exposed to the PNC were available for analysis because they died. The samples were immediately stored in cool boxes and processed within 4 h of their collection. Shellfish samples consisting of 10 g of homogenated flesh and intervalval liquid from several individuals were processed for the isolation of *Arcobacter* spp. according to the protocol mentioned in the following section. The temperature (°C) and salinity (‰) of every water sample were measured by means of a portable multiparameter probe (YSI professional, Xylem Inc., Ohio, USA).

2.2. Isolation and detection

The optimum NaCl concentration that was added to the media for the recovery of *Arcobacter* species from marine water and shellfish was determined after a preliminary screening using different concentrations. The Arcobacter broth with cefoperazone, amphotericin B, and teicoplanin (Arcobacter-CAT broth; Oxoid, Basingstoke, UK) was supplemented with 1, 1.5, 2, and 2.5% (w/v) of NaCl and the media were used for culturing the type strains of 19 *Arcobacter* species for 48 h and then the $OD_{450\ nm}$ was measured. The highest OD was observed at 1% (w/v) NaCl for 9 species, including *A. butzleri*, *A. cryaerophillus* and *A. skirrowii*. However, environmental species (10 in total), such as *A. halophilus*, *A. marinus* and *A. molluscorum* showed little growth at 1% (w/v) NaCl $OD_{450\ nm} \leq 0.1$, but growth was enhanced at 2.5% NaCl. Therefore the latter was the concentration chosen for the present study.

For the isolation of *Arcobacter* species from water, we followed the procedure described by Collado et al. (2008). Briefly, this consisted of filtering 200 ml of water through a 0.45 μm nitrocellulose membrane filter (Millipore, Darmstadt, Germany). Then, those filters were rolled up and put into two tubes, one containing 9 ml of Arcobacter-CAT broth, and the other containing 9 ml of the same broth but supplemented with 2.5% (w/v) NaCl. Both tubes were incubated for 48 h at 30 °C, which is the optimal time and temperature described for *Arcobacter* (Vandamme et al., 1991). In the case of shellfish, duplicate samples of 10 g of flesh and intervalval liquid were homogenized with 90 ml of the Arcobacter-CAT broth. One of the duplicates was supplemented with NaCl (2.5%; w/v) and incubated for 48 h at 30 °C. After the enrichment period, the protocol for both the shellfish and the water samples was the same, i.e. 200 μl of the post-enrichment broth without the NaCl supplement was inoculated by passive filtration through 0.45 μm membrane onto Blood Agar (BA) plates (Tryptone Soy Agar, (TSA) supplemented with 5% sheep blood (BD Difco, Le Pont de Claix, France), as described by Atabay and Corry (1997). The same inoculation by passive filtration was done onto Marine Agar (MA, Scharlab, Barcelona, Spain) from the enrichment tubes supplemented with NaCl. Plates of BA and MA were incubated (48 h at 30 °C) under aerobic and microaerobic atmosphere. The latter was generated using the Gas Pak EZ Campy container sachets™ (oxygen, 6% to 16%; carbon dioxide, 2% to 10%; and nitrogen, 80%; Becton Dickinson, Sparks, MD, USA). From each positive culture, eight presumed *Arcobacter* colonies (non-swarming, small, beige to off-white, translucent, circular with entire margins) from BA were selected and sub-cultured on the same media for further identification, including Gram staining reaction to recognise their typical curved rod morphology (Collado et al., 2008). The same was done by sub-culturing on new MA plates the colonies selected from MA that

were similar in morphology to those of BA, but in this media their colour was from pale yellow to orange.

2.3. Genotyping and identification

The DNA from each isolate was extracted using InstaGene™ DNA Purification Matrix (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions. Isolates were first genotyped with Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR) as described by Houf et al. (2002) in order to eliminate clonal redundant isolates, and then the first isolate found was chosen as the representative of each genotype. Next, the representative of each genotype was identified to species level by comparing its 16S rRNA gene Restriction Fragment Length Polymorphism (16S rRNA-RFLP) patterns with those that have been described for the different species of the genus following the previously described protocols (Figueras et al., 2008, 2012; Levican et al., 2015). In case of doubt, the 16S rRNA-RFLP was repeated using the type strain of the presumed species in parallel and identification was confirmed, when necessary, using the partial sequences of the *rpoB* (621 bp) gene. The *rpoB* amplification-sequencing primers rpoB-Arc15F (5′-TCTCAATTTATGGAYCAAAC-3′) and rpoB-Arc24R (5′-AGTTATATCCATTCCATGGCAT-3′) and conditions were those described previously (Levican, 2013). Each reaction was performed in a final volume of 50 μl containing 1 μl genomic DNA (20–40 ng), 0.2 μM of each dNTP, 0.2 μM of each primer, 2 mM $MgCl_2$, 1 U Taq DNA polymerase (Invitrogen, Fisher Scientific, Madrid, Spain) and the buffer that was supplied with the enzyme. The PCR conditions applied were 3 min at 94 °C followed by 35 cycles of 15 s at 94 °C, 30s at 50 °C and 45 s at 72 °C, with a final extension step of 5 min at 72 °C. The sequences were analysed and assembled using BioEdit software. Alignment was made using Clustal W (Larkin et al., 2007). A neighbour-joining tree was built with a bootstrap analysis with 1000 replicates (MEGA6; Tamura et al., 2013).

2.4. Phenotypic assays

A total of 32 phenotypic tests were carried out in triplicate for all the strains of *A. halophilus* and *A. marinus*, and for the type strains used as controls (strains LA31B^T and CECT 7727^T, respectively). The methods used were those from their original description (Donachie et al., 2005; Kim et al., 2010). Positive and negative controls were included in parallel for each specific test. The tests were repeated again in triplicate for strains that did not yield the expected results.

To evaluate motility, the bacteria were first grown in a 0.5% increasing concentration of NaCl (from 0.5 to 4% NaCl w/v) in Tryptone Soy Broth (TSB, BD Difco) at 30 °C for 48 h. From there, wet mounts were prepared and observed through phase-contrast microscopy.

Growth was evaluated in duplicate at two different temperatures, 30 and 37 °C, in 3 culture media: Saline Blood Agar (SBA; per 500 ml distilled water: 20 g BBL™ Blood Agar Base, 25 ml defibrinated sheep blood, 15 g NaCl), Tryptone Soy Agar (TSA, BD Difco) and Marine Agar. Additionally, the effect of the NaCl concentration added to TSA (from 0.5 to 4% NaCl w/v) was evaluated in duplicate at room temperature (22 °C) and at 30 °C under aerobic conditions. Catalase, oxidase, hydrolysis of urea and indoxyl acetate hydrolysis testing was carried out according to Levican et al. (2015).

2.5. Statistical analyses

The chi-square test of independence was performed using the Statistical Package for Social Sciences (version 15.0; SPSS Inc., Chicago, IL) to determine if there were any significant differences between the species recovered using culture media with and without NaCl. The Spearman correlation coefficient was used to test the influence of water temperature and salinity of the original sample in the recovery of *A. halophilus* and *A. marinus*. Statistical significance was assessed at $P < 0.05$.

3. Results and discussion

3.1. Isolation and identification of Arcobacter species depending upon the culturing method

From the 62 studied samples of water and shellfish, 83.9% (52/62) were positive for *Arcobacter* (Table 1). Of those, 51.9% (27/52) were positive with both culturing methods, 38.5% were only positive when the Arcobacter-CAT broth was supplemented with 2.5% NaCl followed by growth on Marine Agar, whereas 9.6% were positive exclusively in Arcobacter-CAT broth followed by growth on Blood Agar. The latter is the culture approach used for these bacteria in several studies (Collado et al., 2010; Nieva-Echevarria et al., 2013; Çelik and Ünver, 2015; Levican et al., 2015). All 15 shellfish samples that were left in the PNC were positive for the presence of *Arcobacter* while 15 (68.18%) of the 22 samples studied from Alfacs Bay were positive. This is a higher incidence than the one reported by Hsu and Lee (2015) in their review, which indicated that the average prevalence of *Arcobacter* in seafood was 32.3%. In conclusion, the use of the media containing NaCl increased significantly the number of positive samples found in water and shellfish (51.6% vs. 75.8%; $P = 0.023$).

From the positive samples, 643 *Arcobacter* isolates were recovered (Table 1). Of those, 235 (36.5%) were isolated with the previously used culture media (Arcobacter-CAT followed by culturing on BA) and the remaining 408 (63.5%) isolates were recovered using the new method (supplementing the Arcobacter-CAT broth with 2.5% NaCl followed by growth on MA). After genotyping with ERIC-PCR, the 643 isolates corresponded to 291 (45.3%) different genotypes (strains), of which 160 (55.0%) were found with the new method and 135 (46.4%) with the previous one and 4 genotypes were common and accounted to both methods (Table 1). Among those 135 strains, seven known species (*A. bivalviorum*, *A. butzleri*, *A. cryaerophilus*, *A. defluvii*, *A. ellisii*, *A. molluscorum* and *A. mytili*) were recognised by the RFLP patterns described for those species (Figueras et al., 2008, 2012; Levican et al., 2015). However, two strains had unknown RFLP patterns and were classified as *Arcobacter* sp. 2 and sp. 4 (Table 1). The 160 strains obtained using the new culture media with NaCl showed RFLP patterns that corresponded to 11 known *Arcobacter* species. Six of those species were common to those found by the previous method but five were exclusively found by the new method (*A. cloacae*, *A. halophilus*, *A. marinus*, *A. nitrofigilis* and *A. skirowii*) (Table 1). Six strains had the same unknown RFLP pattern found before and named *Arcobacter* sp. 2 and 37 strains also had different patterns to those described and were referred to as *Arcobacter* sp. 1, 3, 5, 6, 7 and 8 (Table 1).

The potentially new species were confirmed as novel phylogenetic lines within the genus on the basis of the *rpo*B and the 16S rRNA genes sequences, and their description is underway on the basis of a polyphasic study that included whole genome sequencing (data not shown). Table 1 shows that the species *A. bivalviorum*, *A. molluscorum*, and *A. mytili* were recovered more often ($P < 0.05$) using the new method. The potentially new *Arcobacter* species (8 in total) were also significantly more often isolated using the culture media with NaCl (Table 1). In addition, the species *A. halophilus* and *A. marinus* were only isolated using the new method. The species *A. cryaerophilus* on the other hand, which was the second most abundant using the previously known method, was not recovered using the media with NaCl. According to D'Sa and Harrison (2005) optimum growth of *A. cryaerophilus* is achieved with 0.5 to 1% NaCl, but they reported that this species can tolerate 5% of NaCl for up to 2 days. Our data seems to indicate that *A. cryaerophilus* is intolerant to a concentration of 2.5% NaCl, because the observed $OD_{450\ nm}$ was ≤0.05, indicating no growth.

Enrichment on Arcobacter-CAT broth followed by culturing on BA, reflects what it is already thought about *Arcobacter*, that *A. butzleri* is

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

Table 1
Positive samples and species found in 62 samples of water and shellfish depending upon the employed culture approach.

Species	Total no. of positive samples	Water		Shellfish				Total strains/isolates	Strains (%)[a]	
		AB	CPN	Mussel	Oyster	Clam	Cockle		Arcobacter CAT broth + BA	Arcobacter CAT-NaCl broth + MA
A. bivalviorum	17	1	4	3	6	2	1	33/73	2 (6.06)	31 (93.94)[b]
A. butzleri	26	1	9	6	8	1	1	99[c]/171	92 (92.93)[b]	9 (9.09)
A. cloacae	2	1				1		2/2	ND	2 (100)[d]
A. cryaerophilus	6		6					27/31	27 (100)[d]	ND
A. defluvii	2		2					7[c]/12	4 (57.14)	4 (57.14)
A. ellisii	1					1		1[c]/18	1(100)	1 (100)
A. halophilus	2	1			1			2/6	ND	2 (100)[d]
A. marinus	8	1	2	1	3	1		10/52	ND	10 (100)[d]
A. molluscorum	23	5	3	7	6	1	1	38/128	1 (2.63)	37 (97.37)[b]
A. mytili	14	4		5	5			23/49	6 (26.09)	17 (73.91)[b]
A. nitrofigilis	2		1		1			3/3	ND	3 (100)[d]
A. skirrowii	1		1					1/2	ND	1 (100)[d]
Arcobacter sp.[e]	21		6	5	7	2	1	45/96	2 (3.64) sp 2–sp 4	43 (95.56)[b] sp1–sp3; sp5–sp8
Total	52	8	14	10	16	3	1	291/643[f]	135 (46.4)	160 (55.0)

Strain: isolate/s showing a unique ERIC-PCR pattern (genotype). ND: no detection.
[a] Percentage of total number of strain/s recovered from each individual species.
[b] Significantly higher recovery ($P \leq 0.05$).
[c] Identical ERIC-genotype/s was/were found with both approaches (in total $n = 4$).
[d] Statistical analyses could not be performed because of non-detection of the species in one method.
[e] The new candidate *Arcobacter* species recovered from each culture approach were referred to as sp1 to sp8; each of them had a different number of genotypes (strains) i.e. sp 1, $n = 16$; sp 2, n (Arcobacter CAT broth + BA) = 1, n (Arcobacter CAT-NaCl broth + MA) = 6; sp. 3, $n = 4$; sp. 4, $n = 1$; sp. 5, $n = 1$; sp. 6, $n = 10$; sp. 7, $n = 4$ and sp. 8, $n = 2$.
[f] 235 (36.54%) isolates were recovered using the Arcobacter CAT broth + BA and 408 (63.46%) isolates were recovered using the Arcobacter CAT-NaCl broth + MA.

the most prevalent species in water and shellfish samples (68.1%, 92/135, in the present study), usually followed by *A. cryaerophilus* (Maugeri et al., 2000; Fernández et al., 2001; Collado et al., 2009b, 2014; Levican et al., 2014; Mottola et al., 2016). In this sense, *A. cryaerophilus* was only detected in 6/14 (42.8%) water samples from the PNC (that showed a mean salinity of 2.07%). The most prevalent *Arcobacter* spp. recovered with the new culturing method were *A. molluscorum*, *A. bivalviorum* and *A. mytili* (Table 1). These three species embraced 53% of strains, giving further evidence of their common association with bivalves (Levican et al., 2014). Combining the results from the two methods offers a completely different view of the prevailing species in these environments and shows they are dominated by *A. butzleri*, *A. molluscorum*, *A. bivalviorum*, *A. cryaerophilus* and *A. mytili*, all together being 75% of the strains recovered.

3.2. A. halophilus and A. marinus

The species *A. halophilus* and *A. marinus* were recovered from 2 and 8 samples, respectively (Table 1). In the case of *A. halophilus*, 6 isolates were recovered from water and oysters and belonged to 2 ERIC genotypes or strains (Table 2; Fig. S2) that showed the expected RFLP pattern (on 3.5% agarose gel) described for *A. halophilus* (551, 141 and 138 bp, after digestion with *Mnl*I enzyme, Figueras et al., 2008). The 52 isolates that corresponded to 10 different strains (ERIC genotypes; Tables 1 and 2; Fig. S2) and that showed (on agarose gel) the typical RFLP pattern of *A. marinus* (440, 126, 106, 87 and 59 bp bands when digested with *Mse*I enzyme, Figueras et al., 2008, 2012) were found in 3 water and 5 shellfish samples (Tables 1 and 2). None of the ERIC-genotypes of *A. halophilus* and *A. marinus* were found simultaneously among isolates recovered from water and shellfish. However, one genotype of *A. marinus* was found at the same time in oyster and mussel samples collected in PNC (Table 2). Globally the species *A. halophilus* and *A. marinus* were present in 4% (1/25) and 12% (3/25), respectively, of all water samples and in 3% (1/37) and 13.5% (5/37), respectively, of the shellfish samples.

To fully confirm the 16S rRNA-RFLP results of *A. halophilus* and *A. marinus*, the *rpo*B gene was partially sequenced (621 bp). The derived neighbour-joining tree (Fig S1) demonstrates that sequences from strains that had the RFLP pattern of *A. marinus* clustered with the type strain of this species (*A. marinus* CECT 7727^T), as occurred with those that showed the pattern of *A. halophilus*. A 100% bootstrap was observed in both cases, indicating that these groups were well supported. The percentage of similarity between the partial sequences of the *rpo*B gene obtained from these new isolates of each species (deposited on GenBank with the accession numbers LT160080–LT160092) and their type strains ranged from a 99.68 to 99.84% for *A. halophilus* and 99.52% to 100% for *A. marinus*. These strains are therefore extremely similar even though they were recovered from different samples and from different geographical origins such as Spain–USA and Spain–Korea, respectively. These two species might therefore be found to have a worldwide distribution if a suitable isolation method is employed.

The congruent results found between the 16S rRNA-RFLP identification and the *rpo*B gene confirms, once more, that this is a reliable method for identifying *Arcobacter* spp. as indicated in other studies (Levican and Figueras, 2013; González et al., 2014; Giacometti et al., 2015; Whiteduck-Léveillée et al., 2016; Mottola et al., 2016).

Table 2
Number of isolates, genotypes and origin of the *A. marinus* and *A. halophilus* isolates studied.

Species	Number of isolates	Strain/genotype	Sample	Location	Month
A. halophilus	$n = 6$				
	1	W119-41/AhI	Water	AB	July
	5	F166-37/AhII	Oysters	PNC	December
A. marinus	$n = 52$				
	1	W110-35/AmI	Water	PNC	March
	2	W110-43/AmII	Water	PNC	March
	7	F139-34/AmIII	Oysters	PNC	March
	2	F139-44/AmIV	Oysters, mussels	PNC	March
	8	F140-37/AmV	Clams	AB	April
	1	W112-47/AmVI	Water	PNC	April
	2	F162-33/AmVII	Oysters	AB	November
	15	W130-33/AmVIII	Water	AB	December
	10	F165-33/AmIX	Oysters	AB	December
	4	F165-36/AmX	Oysters	AB	December

When analysing whether the presence of *A. halophilus* and *A. marinus* in shellfish and water samples correlates with the temperature and/or salinity of the water samples at the two sampling sites, no significant correlation could be established between the temperature of the water from the PNC (Fig. 1), or with salinity at the two sampling points (AB and PNC) for *A. marinus*. However, a significant negative correlation (−0.595; *p*-value = 0.04) was found between the recovery of *A. marinus* and temperature of the water in AB. As shown in Fig. 2, a high proportion of the *A. marinus* isolates came from samples (shellfish and water) collected in the coldest months (April, November and December) when the temperature ranged from 10–16 °C. This preference for lower water temperatures has been reported previously for other *Arcobacter* species, such as *A. cryaerophilus*, *A. nitrofigilis*, and *A. skirrowii* (Fisher et al., 2014; Levican et al., 2014). A smaller number of isolates of *A. marinus* were found in the PNC than in AB. This might be related to the water salinity (‰) in the channel, which varied from 6 to 35‰, with a mean of 19.2‰ (brackish water). This is much lower than in AB, which ranged from 33 to 36‰ with a mean salinity of 34.7‰. It should be noted, too, that the highest salinity values recorded annually in the PNC were in March (31.3‰) and April (35.1‰) when the temperatures were between 12 and 15 °C and it was the only period in which *A. marinus* was recovered from the PNC water (Fig. 1). This data suggests that environments with salinity of at least 30‰ together with cold temperatures (≤15 °C) might be potential niches for *A. marinus*. The detection of *A. halophilus* did not correlate with either of the two environmental parameters recorded, due probably to the small number of strains recovered from this species.

As mentioned above, the two species *A. halophilus* and *A. marinus* were recovered only when the Arcobacter-CAT broth, used as the enrichment medium, was supplemented with NaCl and then sub-cultured onto MA. Isolating colonies of these bacteria took only 4 days, much quicker than when these species were originally described (Kim et al., 2010; Donachie et al., 2005). Kim et al. (2010) used Maltose-Yeast medium incubated for 2 weeks at 30 °C, followed by growth as pure cultures on MA to recover *A. marinus*. Similarly *A. halophilus* was recovered after incubating the water sample for 12 days at 25 °C in a medium containing aspartic acid, after which the colonies were transferred to MA (Donachie et al., 2005).

In a recent study on Italian salami, *A. marinus* was detected by PCR-DGGE because, according to Pisacane et al. (2015), the alignment of the sequences showed a similarity of 98% with a sequence (accession number in GenBank U34386) that they considered to be this species. However, after checking the sequence (U34386) in our laboratory we noticed that it was annotated as *A. butzleri*. To verify whether it was wrongly annotated and whether it truly corresponded to an *A. marinus* sequence, we included this sequence in a neighbour-joining tree (data not shown) together with representatives of all the species. The U34386 sequence clustered with *A. butzleri*, a finding that agreed with previous studies in which *A. butzleri* was found to be abundant in chicken, turkey, pork meat and beef (Pejchalová et al., 2008; Collado et al., 2009b; González et al., 2014).

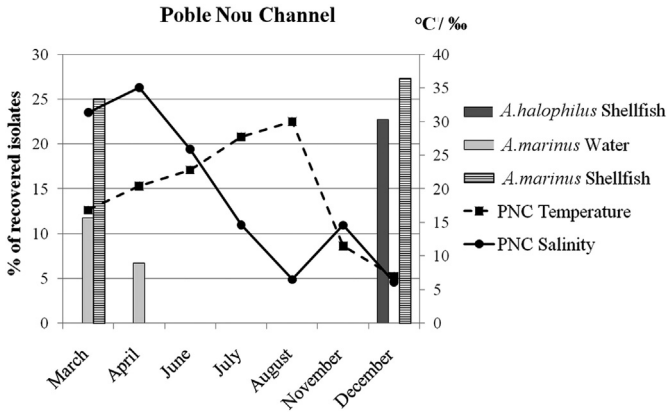

Fig. 1. Relationship between the water temperature and salinity of the Poble Nou Channel (PNC) and the prevalence of *A. marinus* and *A. halophilus* in the water and in the exposed shellfish samples.

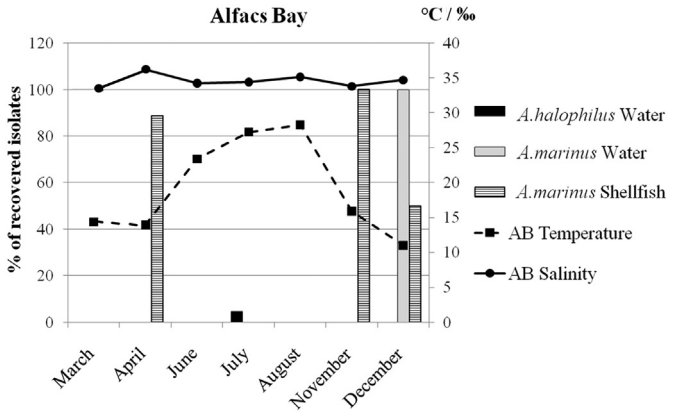

Fig. 2. Relationship between the water temperature and salinity of the Alfacs Bay (AB) and the prevalence of *A. marinus* and *A. halophilus* in the water and in the harvested shellfish samples.

3.2.1. Phenotypic characterization

The two new strains of *A. halophilus* showed colonies with morphology (shape, diameter and sticky consistency) similar to that of the type strain (Donachie et al., 2005). Similarly, all the strains in the present study had phenotypical and biochemical traits similar to those of the original description of the type strain (Table S1). Regarding *A. marinus*, its colony morphology on Saline Blood Agar (SBA; 3.5% NaCl w/v) was larger in diameter (4 mm vs 1 mm) and greyer in colour than when it grew on MA (2% NaCl w/v plus other salts) although both features agree with the original description by Kim et al. (2010). Different phenotypic traits were shown by 72.7% of the *A. marinus* strains when compared with the type strain (Table S1). As reported above, water temperatures significantly influenced the ability to detect *A. marinus*, therefore, growth was tested in increasing concentrations of salt (from 0.5 to 4% NaCl) in TSA at 30 °C and at room temperature (22 °C). The strains that did not grow in 2% NaCl at 30 °C did grow at room temperature and 70% of the *A. marinus* strains were able to tolerate a NaCl concentration of 1.5% at room temperature while no growth was observed at 30 °C.

In agreement with the genus description, all the strains of *A. marinus* were negative for the production of catalase and urease and positive for oxidase. However, there was some variation because only 60% of the strains produced hydrolysis of the indoxyl-acetate under microaerobic conditions, as the type strain of *A. marinus* CECT 7727^T did. The present study, therefore, shows that *A. marinus* is the first species of the genus to present a variable response to the hydrolysis of indoxyl-acetate.

The *A. marinus* strains were not motile and growth was almost absent when they were cultured in TSB in a concentration of less than 2.5% NaCl. On the other hand, growth was enhanced in concentrations of 3.5% and 4% NaCl, agreeing with the original description of this species in which the optimal growth is described to occur at NaCl concentrations between 3 and 5% (Kim et al., 2010). These results suggest that NaCl is a requirement not only for the growth of *A. marinus*, but also directly affects motility. Previous studies on *Campylobacter jejuni* have shown that motility is affected by the concentration of NaCl in the media. By adding more than 1.5% NaCl to the media leads to morphological changes in these bacteria and a complete loss of motility (Cameron et al., 2012). Data on *Arcobacter* indicates that the bacteria of this genus are more tolerant to

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

environmental changes than *Campylobacter* spp., including their ability to grow in higher concentrations of NaCl (D'Sa and Harrison, 2005). That might suggest, then, that the relationship between salinity and motility is physiologically regulated in a different way in these two genera (D'Sa and Harrison, 2005).

4. Conclusion

Isolation of the rare species *A. marinus* and *A. halophilus* has only been achieved after supplementing the enrichment media (Arcobacter-CAT broth) with NaCl and culturing on Marine Agar. This new approach has enabled to recover 7 potentially new *Arcobacter* species and enhanced the number of positive samples found from water and shellfish. Furthermore, we have been able to isolate *A. marinus* and *A. halophilus* much more quickly, in only 4 days rather than in 12 days reported in the original descriptions. We strongly recommend, therefore, that when analysing *Arcobacter* from marine or brackish water samples, this new protocol should be followed in parallel with other commonly used methods. This would also avoid any possible underestimation of the number and/or the presence of species of this genus.

This study shows that a simple modification to the media (such as the addition of salt) can have a big influence on the community of species recovered. We believe this is especially relevant now in this era of metagenomics if we are to elucidate to what extent discordances found between culturing methods and metagenomics are influenced by the specific culture media and conditions used in testing.

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Authors and contributors

NSM carried out the experiments and literature review and drafted the manuscript, being the principal author; KBA and MDF evaluated results, drafted the manuscript and supervised; and MJF designed the research project evaluated results, drafted the manuscript and supervised. All the authors read and approved the final manuscript.

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Supplementary material

Table S1 Phenotypic characteristics of *A. marinus* (n=11) and *A. halophilus*(n=4) strains; including the type strains. Shaded in grey are the results from the original description of *A. marinus* (Kim et al., 2010), and *A. halophilus* (Donachie et al., 2005). The specific responses for type strains coincided with the ones of the original description. Unless otherwise indicated: +, ≥95% strains positive; −, ≤11% strains positive; V, 12–94% strains positive.

Characteristics	***A.marinus* CECT7727^T (Kim *et al.*, 2010)**	***A. marinus***	***A.halophilus* LA31B^T (Donachie *et al.*, 2005)**	***A. halophilus***
Aerobiosis 37ºC				
MA	+	V	+	+
SBA	+	+	+	+
TSA 2%	+	V	+	+
TSA 3%	+	V	+	+
TSA 4%	+	+	+	+
Microaerophilia 37ºC				
MA	+	V	+	+
SBA	+	V	+	+
TSA 3%	+	V	+	+
Aerobiosis 30°C				
TSA 2%		V		+
TSA 3%		+		+
TSA 4%		+		+
Microaerophilia 30°C				
MA		+		+
SBA		+		+
TSA 2%		V		+
TSA 3%		+		+
TSA 4%		+		+
Indoxyl acetate hydrolisis[a]	+[b]	V[b]	+	+

[a] Test performed with cultures grown under aerobic and microaerophilic conditions.

[b] This species was only positive with cultures grown under microaerophilic conditions.

Figure S1 Neighbour-joining tree based on the sequences of *rpo*B gene (442bp) showing the phylogenetic position of the new strains of *A. marinus* and *A. halophilus* within the genus *Arcobacter*. Bootstrap values (>70%) based on 1000 replicates are shown at the nodes of the tree. Scale Bar = 2 nucleotide substitutions per 100 nt.*Only the type strain is available.

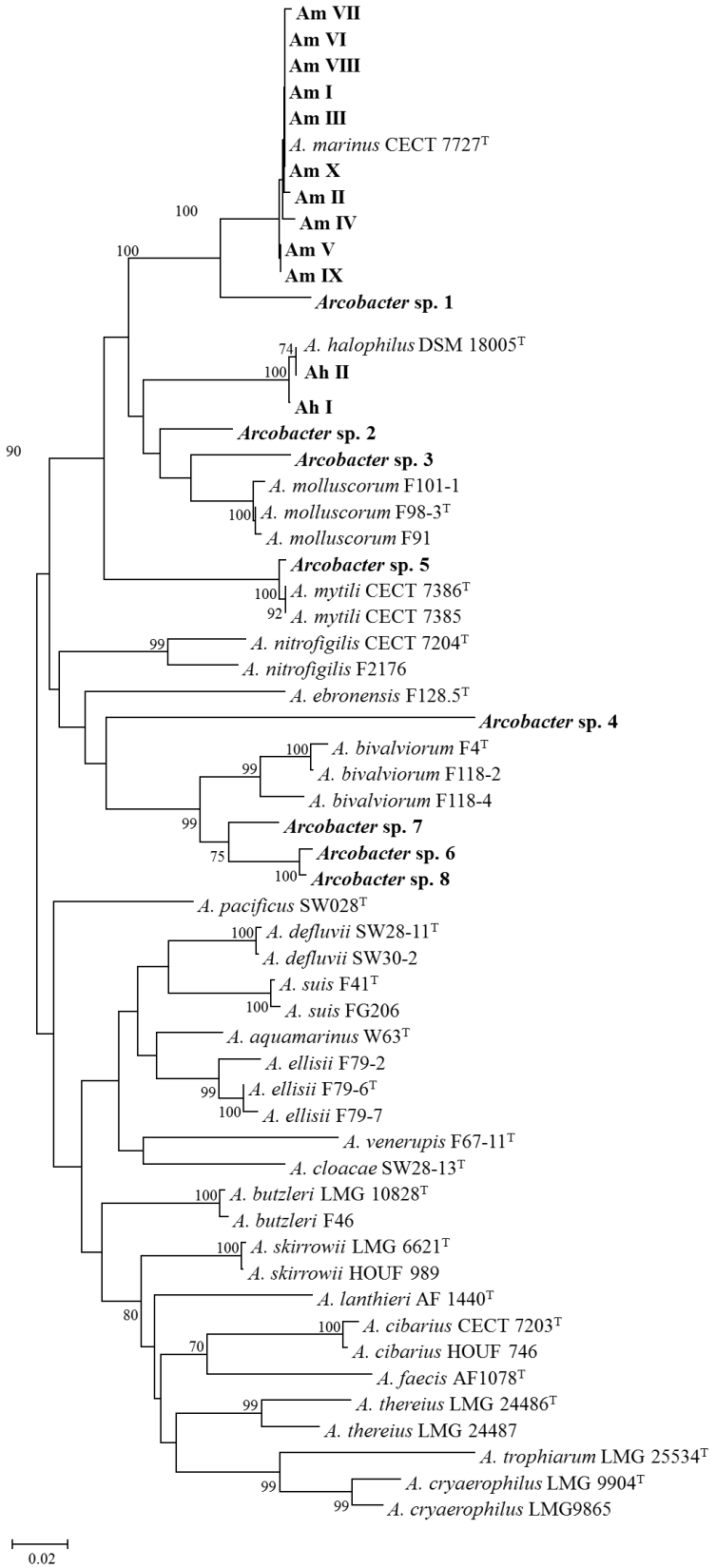
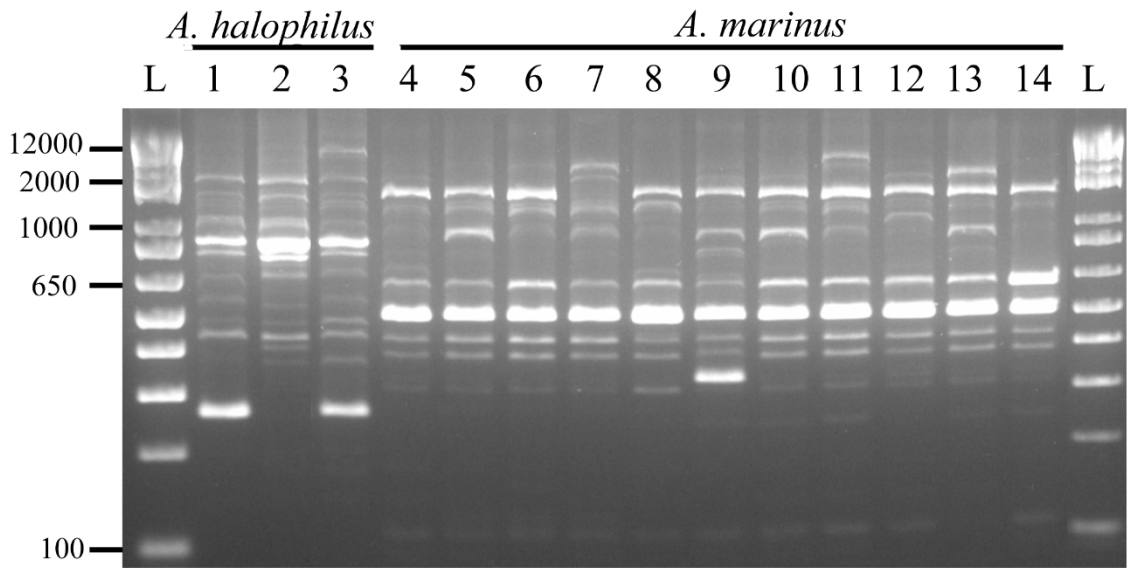


Figure S2 Agarose gel showing the different ERIC patterns of isolates of *A. halophilus* and *A. marinus.* Lanes: **L**, ladder 1Kb plus Invitrogen; **1**, *A. halophilus* LA31B^{T};**2**, AhI; **3**, AhII; **4**, *A. marinus* CECT7727^{T}; **5**, AmI; **6**, AmII; **7**, AmIII; **8**, AmIV; **9**, AmV; **10**, AmVI; **11**, AmVII; **12**, Am VIII; **13**, AmIX; **14**, AmX.



3.2 Do the *Escherichia coli* European Union shellfish safety standards predict the presence of *Arcobacter* spp., a potential zoonotic pathogen? Salas-Massó N, Figueras MJ, Andree KB, Furones MD. *Science of the total Environment*, 2018; 624: 1171–1179.

UNIVERSITAT ROVIRA I VIRGILI
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Do the *Escherichia coli* European Union shellfish safety standards predict the presence of *Arcobacter* spp., a potential zoonotic pathogen?

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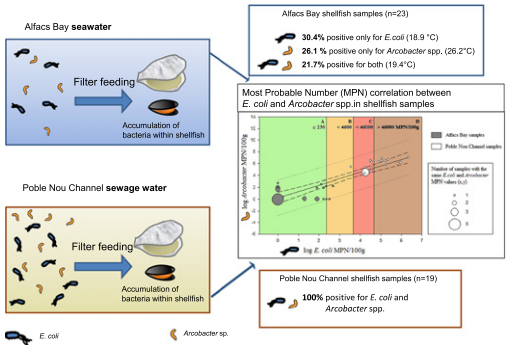
HIGHLIGHTS

- *E. coli* does not predict *Arcobacter* occurrence in shellfish from warm waters (>28 °C).
- In shellfish, the presence of *E. coli* correlates with *A. butzleri* and *A. cryaerophilus*.
- Independently of the *Arcobacter* load in water, latter predicts its incidence in shellfish.

GRAPHICAL ABSTRACT



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ABSTRACT

The genus *Arcobacter* comprises *Campylobacter*-related species, considered zoonotic emergent pathogens, the presence of which in water has been associated with fecal pollution. Discharges of fecal polluted water into the sea have been considered as one of the main reasons for the presence of *Arcobacter* in shellfish, and this may represent a risk for public health. In this study, the European Union shellfish food safety criteria based on levels of *Escherichia coli* were studied in relation to their capacity to predict the presence of *Arcobacter* species. In addition, the accumulation factor (AF) that measures the concentration ratio between the microbes present in the shellfish and in the water, was also studied for both bacteria. The results show that the presence of *E. coli* correlated with the presence of the potentially pathogenic species *A. butzleri* and *A. cryaerophilus*. However, in 26.1% of the shellfish samples (corresponding to those taken during summer months) *E. coli* failed to predict the presence of, for instance *A. butzleri* and *A. skirrowii*, among other species. In the rest of the samples a significant correlation between the concentration of *E. coli* and *Arcobacter* spp. (mussels and oyster; $R^2 = 0.744$) was found. This study indicates that the presence of *E. coli* can predict the presence of pathogenic *Arcobacter* species in shellfish samples harvested from water with temperatures lower than 26.2 °C. Consumption of shellfish collected at higher temperatures which may not be permissive to the growth of *E. coli* but does allow growth of *Arcobacter* spp., may represent a risk for consumers.

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1. Introduction

The genus *Arcobacter* includes species that are capable of causing diarrhoea and bacteremia in humans (Collado and Figueras, 2011; Van

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Nomenclature

EU	European Union
MPN	Most Probable Number
AF	accumulation factor
AB	Alfacs Bay
PNC	Poble Nou Channel
CAT	Cefoperazone, Amphotericin B, and Teicoplanin
GM	geometric mean

den Abeele et al., 2014; Hsu and Lee, 2015) and, more specifically, *Arcobacter butzleri* has been considered a zoonotic agent and an emergent pathogen by the International Commission on Microbiological Specifications for Foods (ICMSF, 2002). These microorganisms can be transmitted to humans and animals through the consumption of water and food products contaminated with sewage (Ho et al., 2006; Fong et al., 2007; Miller et al., 2009; Collado and Figueras, 2011; Hsu and Lee, 2015; Ferreira et al., 2016). In fact, *Arcobacter* spp. have been associated with the fecal contamination of water samples and are persistently found in wastewater because they are considered to be able to grow in this environment (Collado et al., 2008; McLellan et al., 2010; Fisher et al., 2014). Several studies have demonstrated a high worldwide prevalence of *Arcobacter* in shellfish ranging from a 14.7% found in India, to a 73.3% found in Spain (Fernández et al., 2001; Collado et al., 2009, 2014; Nieva-Echevarria et al., 2013; Levican et al., 2014; Mottola et al., 2016; Laishram et al., 2016; Salas-Massó et al., 2016; Leoni et al., 2017). These differences in prevalence may depend on the methods used for the detection and isolation of these microbes and also on the different environmental conditions of the water in relation to the degree of fecal contamination (Collado et al., 2008; Collado and Figueras, 2011; Levican et al., 2016; Salas-Massó et al., 2016; Leoni et al., 2017). Many studies consider shellfish as reservoirs for *Arcobacter* species and, in fact, 8 of the 27 species that are included in the genus *Arcobacter* have been described from shellfish (Collado et al., 2009; Figueras et al., 2011a,b, 2017; Levican et al., 2012, 2014; Diéguez et al., 2017; Tanaka et al., 2017). The high prevalence of *Arcobacter* in shellfish may pose a potential health risk for consumers as they are usually consumed raw or lightly cooked (Collado et al., 2009).

Food safety regulations governing the production and sale of shellfish have been developed throughout the world. Within the European Union (EU), the shellfish harvesting areas are classified into four categories (A, B, C and D) following the 2004 EU regulation (Anon, 2004) updated in 2015 (Anon, 2015). These categories designate increasing concentrations of the fecal indicator bacteria *Escherichia coli* that should predict the presence of pathogenic microbes in flesh and intervalval liquid. In category A, shellfish do not require depuration before placing them on the market. This is because at least 80% of the samples, collected as part of a regular monitoring program, do not exceed 230 Most Probable Number (MPN) *E. coli*/100 g and the remaining 20% do not exceed 700 MPN *E. coli*/100 g. The other categories (B–D) that have equal requirements in the updated and earlier version of this regulation involve higher concentrations of *E. coli* and therefore, shellfish require depuration to reach the values of category A before consumption (Anon, 2004, 2015). For category B, 90% of samples must have ≤4600 MPN *E. coli*/100 g and the remaining 10% should not exceed 46,000 MPN *E. coli*/100 g; category C, all samples are ≤46,000 MPN *E. coli*/100 g; and category D 100% of the samples show values ≥ 46,000 MPN *E. coli*/100 g. As indicated shellfish obtained from categories B-D cannot be placed directly on the market. Thus, samples of B category require 24 h of depuration, while samples of category C must be maintained in a clean water area for at least one month to reach category A, and harvesting of shellfish is prohibited for category D (Anon, 2004, 2015).

It has been proven that *E. coli* is not suitable for predicting the presence of some additional pathogens such as species of *Vibrio* which naturally occur in marine environments and are not related to fecal pollution (Roque et al., 2009; Oliveira et al., 2011). Regarding this problem the National Shellfish Sanitation Program (NSSP, USA) has included among others, the evaluation of levels of *V. parahaemolyticus* and *V. vulnificus* in their standards for harvesting shellfish (NSSP, 2013). Human viruses (mainly enteroviruses, Noroviruses and Hepatitis A viruses) can persist after being released into seawater for longer periods than *E. coli* (from weeks to months), thus the latter is neither a suitable proxy for the presence of viruses (Formiga-Cruz et al., 2002, DePaola et al., 2010; Manso and Romalde, 2013; Brake et al., 2014; Rodríguez-Manzano et al., 2012).

The capacity of *E. coli* to predict the presence of *Arcobacter* in water has been demonstrated in some studies (Collado et al., 2008, 2010). However, the information about this relationship in shellfish derives from only one very recent study which demonstrated that concentrations of *E. coli* >230 MPN/100 g in the shellfish were associated with a higher number of positive samples for *A. butzleri* (Leoni et al., 2017). Nevertheless, the latter study did not investigate the concentration of *Arcobacter* in shellfish or in the surrounding ambient water. Therefore, the objective of the present study was to quantify *Arcobacter* in shellfish and their surrounding water by means of the MPN and to correlate these values with those of *E. coli* in two scenarios with different levels of fecal pollution. Thus, the primary objective is to evaluate if the presence of *E. coli* is able to predict the presence of *Arcobacter* in water and shellfish. In addition, the accumulation factor (AF), which is the ratio between the MPN of the bacteria in the shellfish and in the water (Shieh et al., 2003; Martins et al., 2006; Derolez et al., 2013), was also evaluated for *E. coli* and *Arcobacter*.

2. Experimental procedures

2.1. Location and sampling

Sampling was performed at two sampling sites once a month between March 2013 and June 2014, except in July and August 2013 when the samples were collected fortnightly. The two sampling sites were Alfacs Bay (AB) which is a shellfish harvesting area situated at the Ebro River Delta, Spain (40° 34′ 22.43″ N, 0° 39′ 12.96″ E), and classified as B category according to the Annex II criteria of EU Regulation 854/2004 (Order APA/3228/2005), and a channel that receives untreated sewage from the village of Poble Nou (40° 38.515N′; 00° 41.617′E), designated as PNC in this study. In each sampling occasion the bivalve mollusks taken from AB consisted of 1.5–2 kg of mussels (*Mytilus galloprovincialis*) and 20–25 individual oysters (*Crassostrea gigas*), to provide a minimum weight of 100 g of flesh, with the exception of November 2013 and December 2013 when mussels did not have the recommended commercial size and only oysters were collected. In addition, 2 L of the surrounding water were also sampled each time. Half of the amount of the collected shellfish and all the water samples were directly studied for the presence of *E. coli* and *Arcobacter* spp. The remaining half of the shellfish, i.e. approximately 1 kg of mussels and 10–15 oysters, were placed in a cage in the PNC to be exposed to its fecal contaminated water. Three exposure times were preliminarily tested 24, 48 and 72 h, but no differences were observed in the MPN of *E. coli* and *Arcobacter* found in the oysters and mussels (data not shown). Most of the samples were exposed for 72 h with the exception of the samples of July and August that were exposed for shorter periods of 24 and 48 h because a more extended exposure to the high water temperatures of summer could affect the survival of the shellfish. After that, the mussels and oysters were removed, along with 2 L of the PNC water, to perform the same analyses as that from the AB samples. A total of 75 samples were analyzed i.e. 33 from water (21 from AB and 12 from PNC) and 42 from shellfish (11 mussel samples from AB and 8 from the PNC; 12 oyster samples from AB and 11 from the

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

PNC). Four samples of shellfish exposed to the PNC were not available for analysis because in two of them, the shellfish died and the other two were lost. Water temperature (°C) and salinity (parts per thousand, ‰) were recorded at each site during sampling by means of a portable multi- parameter probe (YSI professional, Ohio, US).

2.2. Analyses of E. coli and Arcobacter spp.

2.2.1. Quantification of E. coli and Arcobacter spp.

Quantification of *E. coli* from water and shellfish was performed using the two step MPN method involving a presumptive and a confirmatory step, according to ISO/TS 16649-3:2005. Briefly, 100 mL of water or 100 g of shellfish flesh and intervalval liquid were mixed thoroughly and homogenized in a stomacher (Lab·Blender 400, West Sussex, UK), respectively, with peptone water. The homogenate was used for preparing 3 dilutions (i.e. 1, 0.1 and 0.01 mL or g of the original sample) that were each inoculated into 5 tubes containing Glutamate broth (OXOID, Basingstoke, UK) that were incubated for 24 h at 37 °C (±1 °C). Tubes in which the color of the media changed from purple to yellow indicated the presence of coliforms and were then confirmed for the presence of *E. coli*. The confirmation was performed by subculturing cells from the yellow Glutamate broth tubes in Tryptone Bile X-glucuronide Agar medium (TBX, OXOID, Basingstoke, UK) at 44 °C, ±1 °C, for 24 h. Colonies showing the typical greenish-blue color were considered to belong to *E. coli*. The number of positive confirmed tubes per dilution were counted and used to derive the MPN results of *E. coli* (per 100 mL or 100 g) using the CEFAS MPN tables (Appendix 2 CEFAS issue No. 11, 2015).

The same original dilutions prepared in peptone water were used for investigating the MPN of *Arcobacter* as described by Collado et al. (2008). However, for comparison purposes with *E. coli*, the volume of the initial sample used in our study was 100 mL or 100 g instead of the 10 g used in Collado's protocol. Dilutions were performed in Arcobacter broth supplemented with Cefoperazone, Amphotericin B and Teicoplanin, i.e. Arcobacter-CAT broth (OXOID, Basingstoke, UK); and incubation was performed at 30 °C for 48 h. Confirmation of the presence of *Arcobacter*, in tubes which presented turbidity, was done as described by Collado et al. (2008). The confirmation consisted on the detection of the typical small, beige to off-white, translucent and convex colonies obtained after having inoculated and cultured at 30 °C for 48 h under aerobic conditions 100 μL of the enrichment tubes by passive filtration (0.45 μm nitrocellulose filters; Millipore) on Blood Agar (BA) plates (Tryptone Soy Agar supplemented with 5% sheep blood BD Difco, Le Pont de Claix, France). Presumptive colonies were confirmed as *Arcobacter* spp. by Gram staining. The MPN final values from 100 mL or 100 g were obtained using the software MPN Build 23 (Mike Curiale software; http://i2workout.com/mcuriale/mpn/index.html). When processing samples of the PNC, up to 6 dilutions were performed because higher bacterial counts were expected.

2.2.2. Detection of Arcobacter spp.

Additionally, all the samples were analyzed for the presence of *Arcobacter* species using two methods. The conventional one, described in previous studies (Collado et al., 2008; Levican et al., 2014; Salas-Massó et al., 2016), involved the use of a pre-enrichment in Arcobacter-CAT broth followed by subculturing by passive filtration on BA. The second method included enrichment in Arcobacter-CAT broth supplemented with 2.5% NaCl (w/v) and subculturing was done on Marine Agar (MA, Scharlab, Barcelona, Spain) where the presumed *Arcobacter* showed pale yellow to orange colonies. When present, eight presumptive colonies were obtained from each media that were first genotyped with Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR) in order to eliminate clonal redundant isolates. The different ERIC genotypes or strains were identified to species level by the 16S rRNA gene Restriction Fragment Length Polymorphism (16S rRNA-RFLP) method described by Figueras et al. (2012). When necessary identification was confirmed using the partial sequences of the *rpoB* (621bp) gene using primers and PCR conditions described by Salas-Massó et al. (2016).

2.3. Data analysis

The geometric mean (GM) and standard deviation of the MPN results were used for the statistical analyses. Counts <10 *E. coli* and <20 *Arcobacter* MPN/100 mL or 100 g, which were the limits of detection of the method, were assigned a value of 1 to allow log transformation. All the statistical analyses were performed with the IBM SPSS Statistics 22.0. Normality distribution of the data was assessed using the Shapiro-Wilk and Kolmogorv-Smirnov test. For data that did not follow a normal distribution the non-parametric Spearman's rho (correlation coefficient) tests was used for the analyses. To calculate the regression between the concentration of *E. coli* and *Arcobacter*, a linear regression model (SigmaPlot 9.0 software) was used with log-transformed data and statistical significance was established at $P < 0.05$. A *t*-test for equality of means was performed to evaluate significant differences between the MPN geometric means of *E. coli* and *Arcobacter* found in water and shellfish samples from both the AB and PNC origins.

The AF of each microorganism (*E. coli* and *Arcobacter*) within the shellfish was calculated by dividing the GM of the MPN obtained from the shellfish by the one obtained from the water (GM MPN shellfish/GM MPN water) as described by Burkhardt and Calci (2000). We also analyzed during the study period the AF data of *E. coli* and *Arcobacter* to determine if a hyperaccumulation occurred. The latter have been defined by Burkhardt and Calci (2000) as the accumulation factor of a particular organism greater than the mean for the entire data + 1 standard deviation ($\overline{X}$ + 1SD).

3. Results

3.1. Presence of E. coli and Arcobacter in water and shellfish samples

In AB, 6/21 water samples (28.6%) were exclusively positive for *E. coli*, 4/21 (19.0%) for *Arcobacter* and 1 (4.8%) sample was simultaneously positive for both microbes (Table 1). Of the 23 shellfish samples, only 12 (43.5%) were positive for *E. coli* (7 alone and 5 in combination with *Arcobacter*, Table 1). The shellfish samples presented the same GM (1.2×10^2) for *E. coli* and *Arcobacter*, while in the water the density of *Arcobacter* was higher (Table 1).

The Alfacs Bay samples (water and shellfish) that were positive for *Arcobacter* presented a statistically higher ($P = 0.001$) mean water temperature (26.2 °C) than those that were only positive for *E. coli* (18.9 °C) and those positive for both microbes (19.4 °C; Table 1). As shown in Table 1, a similar number of positive samples for *E. coli* and *Arcobacter* were obtained by the MPN from water (i.e. 7/21 and 5/21, respectively) and shellfish (i.e. 12/23 and 11/23, respectively). In addition, the same GM value ($1.2 \times 10^2 \pm 2$) was obtained from the shellfish for both microbes, while in the water the values were slightly different i.e. $5.6 \times 10^1 \pm 2.2$ for *E. coli* and $1.0 \times 10^2 \pm 3.1$ for *Arcobacter* (Table 1). The higher number of positive samples for *Arcobacter*, 81% (17/21) in water and 69.6% (16/23) in shellfish, were obtained with the culture approach that used enrichment in Arcobacter-CAT broth supplemented with salt followed by isolation on Marine Agar (Table 1). In contrast, the enrichment in Arcobacter-CAT followed by isolation on Blood Agar yielded a low number of positive samples i.e. 19% (4/21) from water and 26.1% (6/23) from shellfish (Table 1).

From PNC all the samples of water and shellfish were positive for both bacteria with the MPN method, while with both culture approaches the positive samples for *Arcobacter* ranged between 66.7% and 78.9% (Table 2). The densities of *E. coli* (GM = $6.6 \times 10^4 \pm 5.1$) and *Arcobacter* spp. (GM = $5.4 \times 10^5 \pm 7.8$) in the shellfish exposed

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

Table 1
Positive samples for *E. coli* and *Arcobacter* spp. from the water and shellfish of Alfacs Bay (AB).

Sample	N of positives (%)	No (%) positive samples by MPN for *E. coli* and *Arcobacter* spp. Mean temperature (°C)			Geometric mean ± SD[a]		No (%) positive samples for *Arcobacter* spp. by culture[b]	
		Only *E. coli*	Only *Arcobacter*	*E. coli* + *Arcobacter*	*E. coli*	*Arcobacter*	Arcobacter CAT broth + BA[c]	Arcobacter CAT-NaCl broth + MA[d]
Water n = 21	11 (52.4)	6 (28.6)	4 (19.0)	1 (4.8)	$5.6 \times 10^1 \pm 2.2$	$1.0 \times 10^2 \pm 3.1$	4[e] (19.0)	17[f] (81.0)
Shellfish n = 23	18 (78.3)	7 (30.4)	6 (26.1)	5 (21.7)	$1.2 \times 10^2 \pm 2.7$	$1.2 \times 10^2 \pm 2.2$	6[g] (26.1)	16[h] (69.6)
Total n = 44	29 (65.9)	13 (29.5) 18.9 °C	10 (22.7) 26.2 °C[i]	6 (13.6) 19.4 °C	$8.9 \times 10^1 \pm 2.7$	$1.1 \times 10^2 \pm 2.4$	10 (22.7)	33 (75.0)

[a] Geometric mean of the MPN/results obtained from 100 mL of water or 100 g of shellfish.
[b] Enrichment was performed in Arcobacter CAT (Cefoperazone, Amphotericin B, and Teicoplanin) broth.
[c] Enrichment followed by culturing on Blood Agar (BA) after passive filtration.
[d] Enrichment broth supplemented with 2.5% NaCl (w/v) and followed by culturing on Marine Agar (MA) after passive filtration.
[e] Species recovered: *A. butzleri*, *A. molluscorum*, and *A. mytili*.
[f] Species recovered: *A. bivalviorum*, *A. butzleri*, *A. cloacae*, *A. ebronensis*, *A. halophilus*, *A. marinus*, *A. molluscorum*, *A. mytili*, *A. skirrowii* and *Arcobacter* sp.
[g] Species recovered: *A. butzleri*, *A. mytili* and *Arcobacter* sp.
[h] Species recovered: *A. bivalviorum*, *A. butzleri*, *A. marinus*, *A. molluscorum*, *A. mytili*, and *Arcobacter* sp.
[i] Mean temperature of the water samples positive only for *Arcobacter* was higher (P = 0.001) than that of samples only positive for *E. coli* and higher (P = 0.005) than those positive for *E. coli* and *Arcobacter*. Species recovered: *A. bivalviorum*, *A. butzleri*, *A. cloacae*, *A. halohilus*, *A. molluscorum*, *A. mytili*, *A. skirrowii* and *Arcobacter* sp.

for 72 h to the PNC contaminated water were slightly higher than the densities of these bacteria found in water (Table 2). When comparing the *Arcobacter* and the *E. coli* MPN values obtained from both water and shellfish in the PNC, the former had significantly higher MPN than the latter in both matrices (Table 2).

3.2. Correlation of E. coli and Arcobacter

A significant positive correlation between the detection of *E. coli* and *Arcobacter* was found when comparing the concentrations of both bacteria in shellfish ($R^2 = 0.744$, $P < 0.05$) and in water ($R^2 = 0.791$, $P < 0.05$), (Figs. 1 and 2, respectively). As shown in Table 1, the lower densities of both bacteria were found in the water and shellfish samples from AB. The majority of the MPN results obtained from AB corresponded to A category (<230 *E. coli*/100 g) and only a few to B category, while the higher concentrations corresponded to PNC samples (Figs. 1 and 2).

In addition, a significant positive correlation (Table S1) was obtained when considering data from AB and PNC together, not only between microorganisms, but also when comparing separately the detection of one microorganism (*E. coli* or *Arcobacter* spp.) in water versus its detection in both types of shellfish (mussel/s or oyster/s).

When the data from AB and PNC were analyzed separately, it was shown that in AB the presence of *E. coli* and *Arcobacter* in water predicted ($P < 0.05$) their presence in shellfish (Table S1). However, in PNC it was observed that the presence of *E. coli*, both in water and shellfish, correlated with the presence of *Arcobacter* in both matrices. Also, the presence of *Arcobacter* in water was positively correlated with its presence in shellfish (Table S1).

When investigating if levels of *E. coli,* classified according to the categories (A–D) of the EU legislation, found in the shellfish samples could predict the presence or absence of *Arcobacter* spp. in these samples (Table S2), we observed that at the lowest level of *E. coli* (<230 MPN/100 g) oysters were more positive for *Arcobacter* than mussels (83 vs. 44%). When examining the species of *Arcobacter* identified in those samples (Table S2), *A. molluscorum* was the most recovered species among mussels and *A. marinus* in oysters. Shellfish from the PNC showed higher concentrations of *E. coli* and corresponded to classes C (between 4600 and 46,000 MPN/100 g) and D (>46,000 MPN/100 g) and presented a higher diversity of *Arcobacter* species like *A. cloacae*, *A. cryaerophilus*, *A. defluvii*, *A. ellisii* and *A. halophilus* (Table S2). However, the dominating species in both mussels and oysters was *A. butzleri*. Similar diversity of species was also observed in water (Table S3). Regarding the distribution of species depending on the matrix (shellfish vs. water), *A. aquimarinus* and *A. ellisii* were found in shellfish, but not in water (Table S2). On the contrary, *A. ebronensis*, *A. nitrofigilis* and *A. skirrowii* were isolated from water, but not from shellfish (Tables S2 and S3).

3.3. Accumulation factor of E. coli and Arcobacter in shellfish

The mean AF for *E. coli* and *Arcobacter* in mussels from Alfacs Bay in the period studied were 72.61 ± 122.89 and 38.84 ± 112.94

Table 2
Positive samples for *E. coli* and *Arcobacter* spp. from the water of the Poble Nou Chanel (PNC) and from shellfish exposed to this water for 3 days.

Sample	N	Geometric $\overline{X}$ ± SD[a] Mean temperature (°C)			No (%) positive samples for *Arcobacter* spp. by culture[b]	
		Both	*E. coli*	*Arcobacter*	Arcobacter CAT broth + BA[c]	Arcobacter CAT-NaCl broth + MA[d]
Water	12	12 (100)	$4.1 \times 10^4 \pm 3.6$	$4.5 \times 10^5 \pm 9.3$[e]	9 (75.0)[f]	8 (66.7)[g]
Shellfish	19	19 (100)	$6.6 \times 10^4 \pm 5.1$	$5.4 \times 10^5 \pm 7.8$[e]	15 (78.9)[h]	15 (78.9)[i]
Total	31	31 (100) 18.3 °C	$5.6 \times 10^4 \pm 4.5$	$5.0 \times 10^5 \pm 8.1$	24 (77.4)	23 (74.2)

[a] Geometric mean of the MPN/results obtained from 100 mL of water or 100 g of shellfish.
[b] Enrichment was performed in Arcobacter CAT (Cefoperazone, Amphotericin B, and Teicoplanin) broth.
[c] Enrichment followed by culturing on Blood Agar (BA) after passive filtration.
[d] Enrichment broth supplemented with 2.5% NaCl (w/v) and followed by culturing on Marine Agar (MA) after passive filtration.
[e] The *Arcobacter* MPN values obtained from PNC water (P = 0.004) and shellfish (P = 0.002) samples were significantly higher than those of *E. coli* from the same samples.
[f] Species recovered: *A. butzleri*, *A. molluscorum*, and *A. mytili*.
[g] Species recovered: *A. bivalviorum*, *A. butzleri*, *A. cloacae*, *A. ebronensis*, *A. halophilus*, *A. marinus*, *A. molluscorum*, *A. mytili*, *A. skirrowii* and *Arcobacter* sp.
[h] Species recovered: *A. aquimarinus*, *A. bivalviorum*, *A. butzleri*, *A. cloacae*, *A. cryaerophilus*, *A. defluvii*, *A. ellisii* and *Arcobacter* sp.
[i] Species recovered: *A. bivalviorum*, *A. butzleri*, *A. cloacae*, *A. cryaerophilus A*, *halophilus*, *A. marinus*, *A. molluscorum*, *A. mytili*, and *Arcobacter* sp.

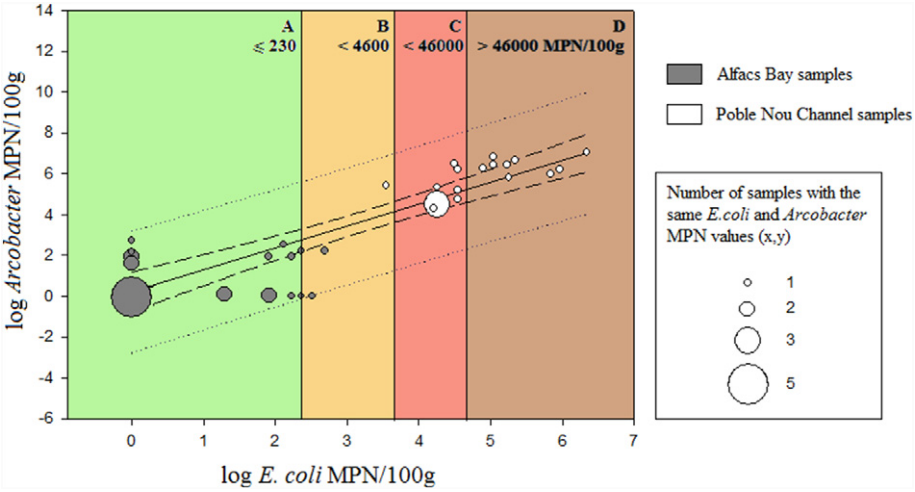

Fig. 1. Linear regression showing the correlation between the MPN concentration of *E. coli* and *Arcobacter* sp. in the AB (grey) and in the PNC (white) for 100 g of shellfish (rho = 0.873, P = 0.000). The different colors indicate the standards of the four categories (A, B, C and D) established by the European Union for the shellfish harvesting areas on the basis of the MPN results of *E. coli*/100 g (Anon, 2004, 2015): class A (green), shellfish do not require depuration and can go direct to the market; class B (orange), 24 h of depuration is needed; class C (red), shellfish have to be placed in a clean water for at least one month and class D (brown), these shellfish are prohibited for consumption. The size of the circles represents how many samples presented the same MPN values for *E. coli* and *Arcobacter*. Line = linear regression; lines — = 95% confidence interval; lines ··· = 95% predictive concentration interval. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

respectively (Fig. 3A); and for oysters 39.31 ± 80.78 and 35.16 ± 54.28, respectively (Fig. 3B). The mussels from AB presented 2 hyperaccumulation (mean + 1SD) moments for *E. coli* and both occurred in June (2013 and 2014), and only one for *Arcobacter* that occurred in May 2014 (Fig. 3B). The oysters from AB also presented 2 hyperaccumulation moments for *E. coli*, one in December 2014 and one in June 2014. Hyperaccumulation of *Arcobacter* within oysters took place on 3 occasions, two in June (2013 and 2014) and one in December 2013.

In PNC samples, we observed that for mussels two hyperaccumulation peaks for *E. coli* occurred (in May and June 2014), whereas there were 3 episodes of hyperaccumulation for *Arcobacter* in August 2013, March and June 2014 (Fig. 3C). The oysters from PNC showed 3 hyperaccumulation peaks for *E. coli* (December 2013, May and June 2014) and 3 for *Arcobacter* (February, May and June 2014; Fig. 3D).

4. Discussion

In our study the relationship between presence and abundance of species from the emergent pathogen genus *Arcobacter* and the fecal indicator *E. coli* were evaluated to determine if the fecal indicator bacteria could predict the presence of *Arcobacter* spp. This relationship was determined analyzing the concentration of both microbes in the shellfish and their surrounding harvesting waters. In order to increase the knowledge about the ecology of both bacteria, the relationship was studied in two completely different scenarios: a shellfish harvesting area (Alfacs Bay) and a heavily fecal polluted channel.

Alfacs Bay represents a commercial shellfishery officially classified as a B harvesting area, where during our study 91% (21/23) of the shellfish samples obtained from there were below the 230 *E. coli* MPN threshold that EU Regulation establishes as the limit for harvesting areas of A category (Anon, 2004, 2015). In fact, *E. coli* was not detected in 48% of those

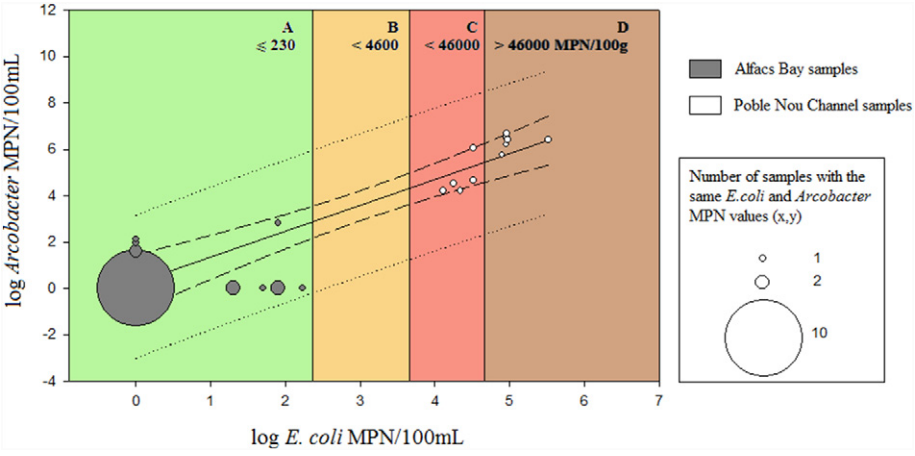

Fig. 2. Linear regression showing the correlation between the MPN concentration of *E. coli* and *Arcobacter* sp. in the AB (grey) and in the PNC (white) for 100 mL of water (rho = 0,791, P = 0.000). The different colors indicate the standards of the four categories (A, B, C and D) established by the European Union for the shellfish harvesting areas on the basis of the MPN results of *E. coli*/100 g (Anon, 2004, 2015): class A (green), shellfish do not require depuration and can go direct to the market; class B (orange), 24 h of depuration is needed; class C (red), shellfish have to be placed in a clean water for at least one month and class D (brown), these shellfish are prohibited for consumption. The size of the circles represents how many samples presented the same MPN values for *E. coli* and *Arcobacter*. Line = linear regression; lines — = 95% confidence interval; lines ··· = 95% predictive concentration interval. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

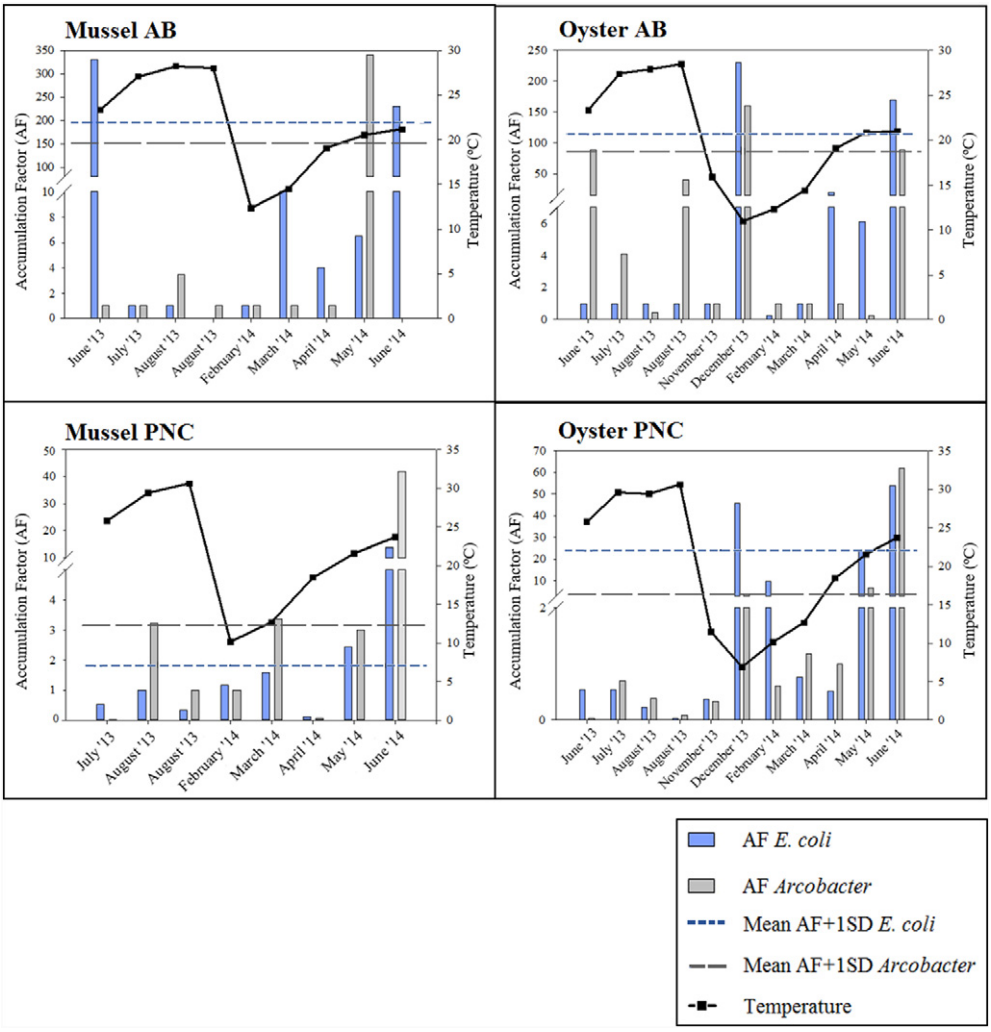

Fig. 3. Accumulation factor (AF; GM MPN shellfish/GM MPN water) of *E. coli* and *Arcobacter* in the shellfish from Alfacs Bay (AB) and Poble Nou Channel (PNC) in relation to the sampling months and temperature.

samples. Moreover, the percentage of samples with *E. coli* values higher than 230 MPN, but not exceeding 700 MPN was 9% (n = 2). Our data indicates that although AB is a harvesting zone classified as B, it is close to the criteria of a category A zone.

As expected, a higher prevalence of positive samples for both bacteria was found in shellfish (18/23; 78.3%) than in the water (11/21; 52.4%) due to the shellfish accumulation capacity. This is to our knowledge the first study that investigates simultaneously the presence of *Arcobacter* and *E. coli* both in the harvesting waters and in the shellfish.

The AB shellfish samples that were only positive for *Arcobacter* MPN (26.1%) were the ones collected during the summer months (July and August) when the water temperature was above 26.2 °C, while those exclusively positive for *E. coli* (30.4%) showed a mean temperature of 18.9 °C (Table 1). These results would support previous findings that indicate that fecal indicator bacteria decrease when the temperature of the water increases (Burkhardt III et al., 2000; Chigbu et al., 2005; Leight et al., 2016). The no detection of *E. coli* in these summer samples suggests that this fecal indicator would fail to predict the presence of *A. butzleri* and *A. skirrowii* among other species found at 26.2 °C (Table 1). Levican et al. (2014), in a study performed in the same area, showed that the levels of *Arcobacter* tend to decrease in colder temperatures. However, this seasonality may depend on the species, i.e. *A. cryaerophilus* and *A. skirrowii* are more prevalent at colder temperatures (9.8–19.8 °C) than in warmer ones (20–29.5 °C), where *A. butzleri* prevail (Fisher et al., 2014; Levican et al., 2014). Recently, Leoni et al. (2017) found that *A. butzleri* is most frequently recovered from Italian shellfish in the winter-spring season, attributing this difference to geographical and climatic features and to different inputs of fecal contamination.

In general, no correlation between *E. coli* and the *Arcobacter* spp. was observed in the Alfacs Bay samples. However, after a deeper analysis taking into account the different *Arcobacter* species recovered from all the water and shellfish samples with different levels of *E. coli* (Tables S2 and S3) correlations with concrete species were observed. The lack of significant correlation observed between the MPN of *E. coli* and *Arcobacter* in any type of samples from the AB (Table S1) was probably due to the low levels of fecal pollution found in the water of the Bay, because only 33% (7/21) of the samples were positive for *E. coli* with a GM of 56 MPN/100 mL and maximum values of 170 MPN/100 mL. In fact, a previous study has demonstrated that inputs of fecal pollution of 4.9×10^3 CFU/100 mL of *E. coli* entering the seawater were not detected at 200 m distance from the discharge point, as a consequence of an important dilution effect (Collado et al., 2008). The deeper analysis showed that in agreement with results of Leoni et al. (2017) the presence of *E. coli* in shellfish was associated with the presence of the dominating species *A. butzleri*, and *A. cryaerophilus* (Tables S2 and S3). These two species have been recovered from patients with intestinal illnesses (Figueras et al., 2014). However, species recovered from shellfish and seawater like *A. molluscorum* and *A. mytili* showed an inverse relationship with *E. coli* (Tables S2 and S3). When the concentration of *E. coli*

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

in water and shellfish was low, indicating low levels of fecal contamination, the prevalence of the mentioned marine species increased. A possible explanation for this behavior is that these species are indigenous of marine environments and as such could be adapted to survive better in seawater than *E. coli* (D'Sa and Harrison, 2005). However, other species such as *A. butzleri* and *A. cryaerophilus* are introduced in the seawater with the fecal pollution (Maugeri et al., 2000; Wirsen et al., 2002; Fera et al., 2004; Collado et al., 2009; Salas-Massó et al., 2016).

The methodology of the MPN for *Arcobacter* uses Arcobacter-CAT broth followed by subculturing on Blood Agar plates for confirmation, and this combination of media has shown to cause a bias in the detection of environmental species (Table 1). For instance, species like *A. bivalviorum*, *A. marinus*, *A. ebronensis* and *A. mytili*, previously related to shellfish and new potential *Arcobacter* species that were only recovered with the method supplemented with NaCl (Salas-Massó et al., 2016), would not be detected with the MPN method. The pathogenicity of these *Arcobacter* species to humans remains unknown. However, when analyzing marine samples, culture media with at least 2.5% NaCl should be used in order to ensure enhanced recovery results (Salas-Massó et al., 2016). A bias in relation to the species detected and caused by the enrichment step has also been described in other studies (Ho et al., 2008; Levican et al., 2016). It was demonstrated that when analyzing samples directly, *A. cryaerophilus* may be the predominant species, but after the enrichment step, *A. butzleri* becomes the most prevalent one due to its faster growth capacity (Ho et al., 2008; Levican et al., 2016).

Although Alfacs Bay is a good representative of the western Mediterranean shellfish growing areas, its low fecal contamination levels did not provide a wide range of conditions to generate multiple scenarios where the performance of the correlation of *E. coli* and *Arcobacter* spp. could be compared. As a second scenario for the study, the Poble Nou Channel was chosen as the water harbored high levels of fecal pollution (geometric mean of *E. coli* 4.1×10^4 MPN/100 mL). In this water, the concentration of *Arcobacter* spp. (4.5×10^5 MPN/100 mL) was one log higher ($P = 0.05$) than that of *E. coli*, which agrees with the concentrations described by Collado et al. (2008) in contaminated freshwater that impacted a seawater bathing area (3.7×10^5 MPN/100 mL for *Arcobacter* spp. vs. 4.9×10^3 CFU/100 mL for *E. coli*). This difference in the concentration of both bacteria has also been observed in a recent study that investigated the efficiency of a tertiary treatment by lagooning, which is a natural (biological) process of purifying wastewater by storing it in open air lagoons and where the wastewater to be treated showed concentration of *Arcobacter* (7.51×10^6 MPN/100 mL) higher than those of *E. coli* (7.23×10^4 MPN/100 mL) (Fernandez-Cassi et al., 2016). Some authors have indicated that the high prevalence of *Arcobacter* spp. found in sewage could be associated to contamination from human feces (Moreno et al., 2003; Collado et al., 2008; Merga et al., 2014). However, the prevalence found in human feces does not support this statement and therefore other studies indicate that this high abundance is related to the capacity of *Arcobacter* to multiply in the sewage system (McLellan et al., 2010; Vandewalle et al., 2012; Fisher et al., 2014). Interestingly, we found that all the shellfish samples exposed during 24, 48 and 72 h to the PNC tested positive for both *E. coli* and *Arcobacter* and their concentrations increased 3 and 4 logs respectively from their original concentration in AB (Tables 1 and 2). The MPN of PNC water (10^4 *E. coli* and 10^5 *Arcobacter*) were on the same log rank as the values reached in the shellfish for both microbes, respectively (Table 2). This similarity of concentrations inside the shellfish with respect to water may be related to what was suggested by Jozić et al. (2012) that bioaccumulation via filtering reaches a plateau of the maximum concentration of particles that the shellfish body can support. Moreover, the low salinity of the PNC can be stressful for the shellfish and could also contribute to a lowering of the filtration rates (Gosling, 2003). However, when considering only the PNC results, a statistically positive correlation between the presence of *E. coli* and *Arcobacter* within the shellfish was found. In this case, only *Arcobacter* showed a positive correlation between its concentration in the water column and the shellfish (Table S1).

As mentioned above, this study corroborates the results obtained from water by Collado et al. (2008) and Leoni et al. (2017) that demonstrated that the presence of *Arcobacter* is related to the fecal contamination. However, in those studies the correlation between the two microbes (*E. coli* and *Arcobacter*) was not quantified as has been done in the present study for the first time. The correlation values (Spearman's rho) obtained between the MPN values of *Arcobacter* and *E. coli* found in water (rho = 0.791) and those found in shellfish (rho = 0.873) (Figs. 1 and 2) seem to indicate that detection of *Arcobacter* in water may predict its presence in shellfish, independently of the concentration of the bacteria in water, as the correlation coefficients obtained in both AB (0.527) and PNC (0.472) were statistically significant.

In addition to the enumeration of both bacteria in water and within shellfish, we established for the first time the AF for *E. coli* and *Arcobacter* in mussels and oysters. It was observed that in June 2014, mussels and oysters from AB and PNC presented AF for *E. coli* and *Arcobacter* higher than the threshold established as their hyperaccumulation. This is something that could be expected because a positive correlation between the temperature and the filtration rates of bivalves has been described (Gosling, 2003; Anestis et al., 2010; Galimany et al., 2011). In fact, in June 2014, the temperature of the water was 21 °C at AB and 23.7 °C at PNC, warmer than the rest of the sampling period (mean temperatures of 19.13 °C for AB and 18.01 °C for PNC). Iwamoto et al. (2010) showed that seafood associated infections caused by bacteria occurred with a higher prevalence in warm months (from June to August). The hyperaccumulation of *E. coli* and *Arcobacter* that primarily occurred in June, may be considered as an extra risk for the consumer, as previously reported by Burkhardt and Calci (2000). These authors found a relationship between the hyperaccumulation events of F^+ coliphages and the illness caused by Norwalk-like virus. The generally accepted mathematical models that explain the filtration rates in oysters indicate that this rate has a positive correlation with the temperature (Ehrich and Harris, 2015). However, there is another mathematical model supporting oyster's higher filtration rates in winter (Powell et al., 1992). The latter model applied to oysters is based on the size of the bivalve (i.e. juvenile and market sized). During the winter months oysters reach their adult size thus their filtration rate would increase despite the lower temperatures. This explanation would support the hyperaccumulation event observed in December 2013 for oysters in our study.

The fact that the concentration of the bacteria (*E. coli* and *Arcobacter*) found in the water and in the shellfish, was very similar in the PNC, lowered the AF in this site in relation with what we observed for the AB site. Additionally, the exposure of the shellfish to the PNC water allowed us to see how the two types of shellfish studied behaved regarding the accumulation of both bacteria (Fig. 3). In our experiment, both, oysters and mussels were equally exposed to the PNC water, while in AB we had no record when the shellfish were introduced in the system and for how long they were exposed to the surrounding water. Interestingly, mussels and oysters from the PNC did not present their hyperaccumulation episodes at the same time (Fig. 3). This can be attributed to how different bivalves control their accumulation rates. Nowadays, there exist two theories, one that indicates that the filtration rate is physiologically controlled (Bayne, 1998; Hawkins et al., 1998) and the other that postulates that this depends on the capacity of the pump and the concentration of food particles in the water (Jørgensen, 1996). The most accepted is that the pumping rate and retention efficiency is a function of the nutritional needs or gut satiation of the individual bivalves (Gosling, 2003). However, given that the environmental conditions were the same, as occurred with the shellfish exposed to PNC water, the filtration rate may be controlled by the gills and also be dependent on body size (Powell et al., 1992; Gosling, 2003). In this sense the gill's structure is different in both types of shellfish

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

(Pechenik, 1991), and the body size of oysters is bigger than for mussels. In addition to that the accumulation rates can also be affected by the different susceptibility of the mussels and the oysters to the physicochemical characteristics of the surrounding water (Gosling, 2003 and references therein).

5. Conclusions

This study is the first to provide comparative data of the concentration of *E. coli* and *Arcobacter* spp. from shellfish and from their surrounding water, including information about the accumulation rate of both bacteria in two different scenarios: low and high fecal pollution and in two types of shellfish (mussels and oysters).

The genus *Arcobacter* comprises species that are emergent pathogens like *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* (Figueras et al., 2014; Van den Abeele et al., 2014). As shown by several studies *A. butzleri* is not only the most frequent species recovered from human samples (Figueras et al., 2014; Van den Abeele et al., 2014), but also from shellfish samples (Levican et al., 2014; Salas-Massó et al., 2016; Leoni et al., 2017). Although this prevalence may be overestimated due to the common use of a pre-enrichment step in the recovery of *Arcobacter* species (Ho et al., 2008; Levican et al., 2016). Our results show that the presence of *E. coli* correlates with the presence of two of these potentially pathogenic species, *A. butzleri* and *A. cryaerophilus*. However, *E. coli* would fail to predict the presence of *A. butzleri* and *A. skirrowii* among other species in 26.1% of the shellfish samples harvested from Alfacs Bay during the warmer months (>26.2 °C) and this may have significant public health implications. The presence of *Arcobacter* in shellfish when *E. coli* was not detected would mean that this shellfish would be classified as class A which can be directly consumed without depuration (Anon, 2004, 2015). Thus, the presence of potential pathogenic *Arcobacter* species in shellfish may pose a risk to consumers. More studies need to be performed to know if the depuration process established by the European Regulation for *E. coli*, would also be enough to eliminate the burden of *Arcobacter* spp. from shellfish samples.

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Authors and contributors

NSM carried out the experiments and literature review and drafted the manuscript, being the principal author; KBA, MDF evaluated results, drafted the manuscript and supervised; and MJF designed the research project evaluated results, drafted the manuscript and supervised. All the authors read and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2017.12.178.

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Supplementary material

Table S1.- Spearman's correlation coefficient (rho) between the MPN values of *E. coli* and *Arcobacter* found in water and shellfish samples obtained from Alfacs Bay (AB) and the Poble Nou Channel (PNC).

Comparison between		AB + PNC		AB		PNC	
		rho	N	rho	N	rho	N
E. coli water	*E. coli* shellfish	0.835**	40	0.450*	21	0.094	19
	E. coli mussel	0.807**	17	0.411	9	-0.072	8
	E. coli oyster	0.863**	23	0.543	12	0.180	11
	Arcobacter water	0.791**	33	-0.083	21	0.718**	12
E. coli shellfish	*Arcobacter* shellfish	0.873**	42	0.078	23	0.697**	19
Arcobacter water	*Arcobacter* shellfish	0.893**	40	0.527*	21	0.472*	19
	Arcobacter mussel	0.885**	17	0.283	9	0.492	8
	Arcobacter oyster	0.911**	23	0.658*	12	0.544	11

* Correlation is significant at the 0.05 level.

** Correlation is significant at the 0.01 level.

Table S2. Presence/absence of *Arcobacter* spp. in 42 shellfish samples classified according to their levels of *E. coli* as determined by the EU legislation for bivalve mollusks.

		No. of samples positive for/No. of samples analysed (%)[b]												
Type of shellfish	*E. coli* MPN/100 g[a]	*Arcobacter* spp.	*A. aqu*	*A. biv*	*A. but*	*A. clo*	*A. cry*	*A. def*	*A. ell*	*A. hal*	*A. mar*	*A. mol*	*A. myt*	*Arcobacter* sp.
Mussels	≤ 230	4/9 (44)	-	1/9 (11)	1/9 (11)	-	-	-	-	-	-	4/9 (44)	4/9 (44)	3/9(33)
	230 < X <4600	1/1 (100)	-	-	-	-	-	-	-	-	-	1/1 (100)	-	-
	4600<X<46000	4/4 (100)	-	2/4 (50)	3/4 (75)	-	2/4 (50)	-	-	-	2/4 (50)	2/4 (50)	-	2/4 (50)
	> 46000	4/4 (100)	1/4 (25)	2/4 (50)	4/4 (100)	1/4 (25)	3/4 (75)	-	1/4 (25)	-	1/4 (25)	1/4 (25)	-	2/4 (50)
Oysters	≤ 230	10/12 (83)	-	-	3/12 (25)	-	-	-	-	-	6/12 (50)	4/12 (33)	3/12 (25)	4/12 (33)
	230 < X < 4600	2/2 (100)	-	1/2 (50)	1/2 (50)	-	-	-	-	-	1/2 (50)	1/2 (50)	-	1/2 (50)
	4600<X< 46000	5/5 (100)	-	3/5 (60)	2/5 (40)	-	1/5 (20)	1/5 (20)	-	-	1/5 (20)	2/5 (40)	2/5 (40)	5/5 (100)
	> 46000	5/5 (100)	-	3/5 (60)	4/5 (80)	1/5 (20)	3/5 (60)	-	-	1/5 (20)	2/5 (40)	-	1/5 (20)	5/5 (100)
Total	≤ 230	14/21 (67)	-	1/21 (5)	4/21 (19)	-	-	-	-	-	6/21 (29)	8/21 (38)	7/21 (33)	7/21 (33)
	230 < X < 4600	3/3 (100)	-	1/3 (33)	1/3 (33)	-	-	-	-	-	1/3 (33)	2/3 (66)	-	1/3 (33)
	4600<X< 46000	9/9 (100)	-	5/9 (56)	5/9 (56)	-	3/9 (33)	1/9 (11)	-	-	3/9 (33)	4/9 (44)	2/9 (22)	9/9 (100)
	> 46000	9/9 (100)	1/9 (11)	5/9 (56)	8/9 (89)	2/9 (22)	6/9 (67)	-	1/9 (11)	1/9 (11)	3/9 (33)	1/9 (11)	1/9 (11)	7/9 (78)

[a] EU regulation (Anon 2004; 2015) category A: ≤ 230 MPN/100 g; category B: between 230 and 4600 MPN/100 g; category C: between 4600 and 46000 MPN/100 g; and catergory D: >46000.

[b] *A. aqu*: *A. aquimarinus*; *A. biv*: *A. bivalviorum*; *A. but*: *A. butzleri*; *A. clo*: *A. cloacae*; *A. cry*: *A. cryaerophilus*; *A. def*: *A. defluvii*; *A. ell: A. ellisii; A. hal*: *A. halophilus*; *A. mar*: *A. marinus*; *A. mol*: *A. molluscorum*; *A. myt*: *A. mytili*. The slash means no detection of that *Arcobacter* species.

Table S3. Presence/absence of *Arcobacter* spp. in 33 water samples classified according to their levels of *E. coli* as determined by the EU legislation for bivalve mollusks.

		No. of samples positive for/No. of samples analysed (%) [b]													
	E. coli MPN/100 mL[a]	*Arcobacter* spp.	*A. biv*	*A. but*	*A. clo*	*A. cry*	*A. def*	*A. ebr*	*A. hal*	*A. mar*	*A. mol*	*A. myt*	*A. nit*	*A. ski*	*Arcobacter* sp.
Water	≤ 230	17/21 (81)	1/21 (5)	3/21(14)	1/21 (5)	-	-	3/21 (14)	1/21 (5)	5/21 (24)	6/21 (29)	2/21 (10)	1/21 (5)	1/21 (5)	2/21 (10)
	230 < X <4600	2/2 (100)	1/2 (50)	1/2 (50)	-	-	1/2 (50)	-	-	-	-	-	-	-	1/2 (50)
	4600<X<46000	4/4 (100)	1/4 (25)	3/4 (75)	-	1/4 (25)	-	1/4 (25)	-	1/4 (25)	2/4 (50)	-	-	-	2/4 (50)
	>46000	6/6 (100)	1/6 (17)	5/6 (83)	-	4/6 (67)	-	1/6 (17)	-	1/6 (17)	1/6 (17)	-	-	1/6 (17)	4/6 (67)

[a] EU regulation (Anon 2004; 2015) category A: ≤ 230 MPN/100 g; category B: between 230 and 4600 MPN/100 g; category C: between 4600 and 46000 MPN/100 g; and catergory D: >46000.

[b] *A. biv*: *A. bivalviorum*; *A. but*: *A. butzleri*; *A. clo*: *A. cloacae*; *A. cry*: *A. cryaerophilus*; *A. def*: *A. defluvii*; *A. ebr: A. ebronensis; A. hal*: *A. halophilus*; *A. mar*: *A. marinus*; *A. mol*: *A. molluscorum*; *A. myt*: *A. mytili*; *A. nit*: *A. nitrofigilis*; *A. ski*: *A. skirrowii.* The slash means no detection of that *Arcobacter* species.

3.3 Tissue distribution of *Arcobacter* spp in mussels and oysters. Salas-Massó N, Figueras MJ, Andree KB, Furones MD. (In preparation)

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EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

Tissue distribution of *Arcobacter*-related spp in mussels and oysters

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Abstract

Shellfish are one of the important vehicles for the transfer of pathogen of both humans and animals. During the process of filter feeding, bivalves can concentrate and accumulate bacteria and viruses. *Arcobacter*-like genus' species are frequently recovered from shellfish all around the world, with different prevalence. This relative new family comprises species pathogens for humans, and recently, the role of *Arcobacter* as potential opportunistic pathogens of moribund oysters have been revealed. The tissue distribution of *Arcobacter* was studied in oysters (*Crassostea gigas*) and mussels (*Mytilus galloprovincialis*) in two different scenarios with different levels of fecal pollution. In Alfacs Bay (AB), the interval liquid was the compartment which showed the highest positivity (65.4% for mussels and 84.6% for oysters). The detection of *Arcobacter* in the different tissues was independent of the methodology used, but the origin of the samples (lead to a better performance of NaCl supplemented media methodologies in marine samples (AB), than those which were not supplemented. The diversity of AB (H_{max}= 1.95) was lower than that of the Poble Nou Channel (H_{max} 2.56). Two species were identified as autochthonous species of shellfish microbial diversity, i) *A. butzleri* which due to its pathogenic potential represents a risk for the shellfish consumers; and ii) *M. canalis* which may represent a candidate for opportunistic pathogens of shellfish.

Keywords: Diversity, *Arcobacter*, sodium chloride, opportunistic pathogen, shellfish, marine water.

1 Introduction.

Nowadays the genus *Arcobacter* has been split into 7 genera comprising 28 species (Pérez-Cataluña et al., 2018a) of which 8 species (28.6%): *Halarcobacter bivalviorum, Halarcobacter ebronensis, Malaciobacter canalis, Malaciobacter mytili*, *Malaciobacter molluscorum, Poseidonibacter lekithochrous, Pseudarcobacter ellisii* and *Pseudarcobacter venerupis*; have been described from isolates recovered from molluscs (mussel, oyster, clams, abalone and scallop). Since 2002, *Arcobacter butzleri* (comb. nov. *Aliarcobacter butzleri*; Pérez-Cataluña et al., 2018a) was considered a zoonotic and emergent pathogen by the International Commission on Microbiological Specifications for Foods (ICMSF, 2002). In fact, so far 4 outbreaks have been attributed to these bacteria, 3 associated with consumption of fecal contaminated water and the other one to the consumption of roasted chicken among attendees of a wedding (Vandamme et al., 1991; Rice et al., 1999; Fong et al., 2007; Lappi et al., 2013). Additionally, *A. butzleri* has been recovered from bivalves both in higher prevalence than any other *Arcobacter*-like species (Collado et al., 2009; Levican et al., 2014, Salas-Massó et al., 2016; 2018; Leoni et al., 2017).

Bivalves concentrate and accumulate microorganism during filtration undertaken as part of their feeding process, including human pathogenic bacteria and viruses, especially where they grow in seawater-polluted water areas (Doré and Lees, 1995; Grodzki et al., 2014). During filter-feeding, the uptake of bacteria by bivalves have shown to have some specificity leading to distinguish between two kinds of microbiota i) the allochthonous, which are microorganism that simply pass through with the water and food and ii) the autochthonous that are relatively permanent and intimately associated with shellfish tissues i.e. vibrios and pseudomonas (Olafsen et al., 1993; Romero et al., 2002). The different distribution of a specific bacteria within the molluscs tissues may influence its elimination rate (Grodzki et al., 2014). Those microorganisms that are in transit in the different tissues during the digestive process, are usually eliminated during depuration (Grodzki et al., 2014).

Reducing the levels of bacterial load (including pathogens) through the depuration of shellfish, is used as the system to ensure its safety for the consumer since they are usually consumed raw or slightly cooked (Potasman et al., 2002; Polo et al., 2014; Taylor et al., 2018). The depuration time applied to shellfish, prior to be placed in the market depends on the classification level of the area from where they were harvested. In the European Union (EU) such classification is based on a regular monitoring of the *Escherichia coli* levels present in the

flesh and intervalval liquid, used as the indicator according to the EU regulation 854/2004 (Anon, 2015; Salas-Massó et al., 2018). Usually, shellfish are harvested from A and B classified areas. The first ones can go directly to consumption, and those from B areas require minimum of 24h of depuration (Anon, 2015; Salas-Massó et al., 2018).

Determining the distribution of *Arcobacter*-like genera within shellfish, would help to elucidate whether the depuration times stablished by the EU regulation could be effective eliminating these bacteria from the shellfish. However, little information on this is currenty available, Romero et al. (2002) demonstrated that *Arcobacter* spp. are autochthonous bacteria of Chilean oysters and Ottaviani et al. (2013) performed a study of *A. butzleri* accumulation in mussels, they examined the digestive tissues separately from the rest of the body including the intervalval liquid, where no differences between both type of samples were found, suggesting that, for *A. butzleri*, the digestive tissues were not an elective tissue for bioaccumulation. Additionally, as far as we are concern, there are no studies reporting whether the *Arcobacter* genotypes found in the surrounding harvesting water are also found in shellfish and their tissues distribution. Levican et al., (2014) however, demonstrated that the same genotypes of *A. butzleri* (n=1) and *A. molluscorum* (comb. nov. *Malaciobacter molluscorum*; Pérez-Cataluña et al., 2018a) (n=5) were recovered from different samples taken on equal or different months and/or years, indicating bivalves may have persistent genotypes.

Based on these findings, the aims of the present work are i) to elucidate how the different *Arcobacter*-genera like species are distributed within the tissues of mussels and oyster under two different scenarios: seawater and sewage water and ii) to study whether the different genotypes found in the surrounding water are also found in the shellfish tissues. Thus, to provide relevant information on shellfish safety knowing that *E. coli*, as indicator, cannot correctly predict the presence of the potentially pathogenic bacteria of this family (Salas-Massó et al., 2018)

2. Material and methods.

2.1 Sample collection.

Mussels (*Mytilus galloprovincialis*) and oysters (*Crassostrea gigas*) were collected monthly from a harvesting area in Alfacs bay (AB; Ebro Delta River, Spain , 40° 34′ 22.43″ N, 0° 39′ 12.96″ E) between January and June (2014) matching a commercial production cycle. Samples consisted on 1kg of mussels and 50-60 pieces of oysters. Additionally, batches of these samples were artificially contaminated in a channel that receives sewage from the village of Poble Nou (PNC; 40° 38.515N'; 00° 41.617'E), as previously described (Salas-Massó et al., 2016). Each time shellfish samples were collected, 2 L of the surrounding water (seawater or PNC water) were also sampled. A total of 42 samples were analyzed i.e. 18 from water (12 from AB and 6 from PNC) and 24 from shellfish (6 mussel samples from AB and 6 from the PNC; 6 oyster samples from AB and 6 from the PNC).

2.2 Sample dissection.

From each different sample set, 14 individuals were opened in sterile conditions. The intervalval liquid (IL) was collected and different tissues were dissected: the gills (G), digestive glands (DG; stomach, gut and digestive diverticula) and residual tissues (RT; mantle and adductor muscle). The tissues from 4 individuals were analyzed independently, while the tissues from the remaining 10 animals were pooled together (Fig S1). Each tissue was weighted and an equal volume of sterile saline 0.9%NaCl (SS) was used to homogenize the sample. Homogenized were used for culture and molecular biology purposes.

In total, both for intervalval liquid (IL) and for each different tissue (G, DG, RT) 24 pooled samples were analyzed (12 from Alfacs Bay, 6 mussels and 6 oysters, and 12 from PNC (6 mussels and 6 oysters) and 88 individual samples: 20 from mussels and 20 from oysters in AB, and 24 from mussels and 24 from oysters in CPN. In total for each single compartment we had 112 samples. (Table S1).

2.3 Detection of *Arcobacter*-like species.

2.3.1 Culture methods and identification.

Isolation of *Arcobacter*-like spp. was performed as described by Salas-Massó et al., (2016). Briefly, tubes containing 9ml of Arcobacter-CAT broth (Arcobacter broth supplemented with Cefoperazone, Amphotericin B and Teicoplanin; OXOID, Basingstoke, UK) in parallel with and without a supplement of 2.5% of NaCl were inoculated with 1ml of the homogenized tissue or intervalval liquid and incubated for 48h at 30ºC. 200µl of the pre-enrichment were put on 0.45µm nitrocellulose membrane filter (Millipore, Darmstadt, Germany) and were passive filtrated during 30 minutes onto Marine agar (MA, Scharlab, Barcelona, Spain) or Blood Agar (BA; Tryptone Soy Agar supplemented with 5% sheep blood, BD Difco, Le Pont de Claix, France) respectively for 48h at 30ºC.

Identification of the isolates was performed according to Levican (2013). Presumptive *Arcobacter*-like colonies (confirmed by Gram staining) were picked and pure cultures were obtained for DNA extraction. Enterobacterial Repetitive Intergenic Consensus Sequences -PCR (ERIC-PCR, Houf et al., 2002) was performed with two aims, first to avoid working with redundant clones, and secondly to evaluate the epidemiology of the species and the presence of a strain along the different tissues of the molluscs. To identify the isolates to species level Restriction Fragment Length Polymorphism – PCR (RFLP-PCR) was performed using the conditions described by Figueras et al. (2012). When necessary, the partial *rpoB* sequence gene was obtained in order to stablish the correct taxonomic position of the strain (Levican et al., 2013; Salas-Massó et al., 2016).

2.3.2 Molecular Biology methods.

DNA was extracted at different stages during the samples processing, to evaluate the presence of *Arcobacter*-like species by means of a multiplex PCR (Houf et al., 2000; m-PCR). Extraction of the DNA was performed from the raw homogenized tissues, and from both the pre-enrichment broth with and without NaCl.

2.4 Quantification of *E. coli*.

As part of a broader study (Salas-Massó et al., 2018) all the samples used in the present study were also used for the quantification by means of the Most Probable Number (MPN) of *E. coli* (ISO/TS 16649-3:2005) and was performed as described in the mentioned work.

2.5 Statistical analysis

For evaluation of species diversity, the ecological indexes Shannon–Wiener (H) and Evenness (equitability; E) were calculated using the equations from Krebs (Krebs., 1989). The former was used as a quantitative measure for diversity of species in a specific sample and the latter to evaluate the similarity of frequencies of the different *Arcobacter*-like species making up a sample. Additionally, richness (S) was estimated as described by Oguntoyinbo (2011), to infer the different number of species in a given sample. The potential maximum diversity for our samples in both origins was calculated as H_{max}=lnS (Lloyd and Ghelardi, 1964). The differences on the prevalence of *Arcobacter*-like species among tissues was calculated using the non-parametric test, Kruskal-Wallis. The estimation of the effect of the methodology used on the positivity of a sample was calculated using Pearson's Chi-Square test. To evaluate the relationship between environmental parameters such as temperature and salinity, but also to evaluate how species would relate between them, a binary logistic regression and estimation of odd ratios (OR) were used. All the analyses were performed using Software SPSS Statistical (IBM Analytics).

Furthermore, ERIC-PCR patterns were analyzed by means of BioNumerics 6.5 software (Applied Math, Ghent, Belgium). A dendrogram per species was constructed using the Dice similarity coefficient and the cluster analysis of similarity matrices was calculated with the unweighted pair group method with arithmetic averages (UPGMA).

3. Results

3.1 Detection and prevalence of *Arcobacter*-like spp.

A total of 90.5% (38/42) of the studied water and shellfish samples were positive for the isolation of *Arcobacter*-like spp. Only 1 batch of oyster and 3 of mussel samples from Alfacs Bay were negative (4/12; 33.3%) for the recovery of *Arcobacter*-like strains. A total of 1281 isolates (581 genotypes) were recovered i.e. 1153 isolates (526 genotypes) from shellfish and 129 isolates (62 genotypes) from water, comprising a total of 16 *Arcobacter*-like spp. belonging to 4 different genera (13 and 8 different species from shellfish and water, Table 1). Additionally, two of those 16 species, are new and are being described, "*Halarcobacter mediterraneus*" and "*Malaciobacter ostreae*".

3.1.1 Detection of *Arcobacter*-like spp. in shellfish tissues: methodology effect.

As stated in material and methods, up to five different methodologies were used to detect *Arcobacter*-like spp. in the tissue samples i) m-PCR from DNA obtained directly from the raw homogenized tissues (m-PCR-D), ii) m-PCR from DNA obtained from the pre-enrichment broth in Arcobacter-CAT broth (m-PCR-PE1), iii) m-PCR from DNA obtained from the pre-enrichment broth in Arcobacter-CAT broth supplemented with NaCl (m-PCR-PE2), iv) positive isolation on blood agar (BA-C), and v) positive isolation on Marine agar (MA-C).

When evaluating the positivity of the tissues (a sample was considered positive when at least one of the methodologies mentioned above yielded a positive results), it was observed that all the pool samples from both origins were positive. The results given in Table 2 correspond to the sum of pooled and individual samples. When analyzed separately those tissue samples that are statistically more positive than other tissues for the pooled samples, were also positive for their individuals' equivalent sample (data not shown). The intervalval liquid was significatively ($P< 0.05$) the most positive sample for both type of shellfish in Alfacs Bay (65.4% for mussels and 84.6% for oysters; Table 2). Whereas in the PNC, none of the tissues were significative more positive than the others (Table 2).

When the methodologies for the detection of *Arcobacter* are evaluated independently, it could be observed that the intervalval liquid was significatively the most positive compartment of the shellfish when using the m-PCR-D ($P< 0.05$; Table 2). The post enrichment m-PCR (m-PCR-PE1 and m-PCR-PE2) were the methodologies that yielded a higher number

of positives samples (Table 2). When focusing in shellfish from AB, only the digestive gland (DG) and gills (G) from mussels (3.8% in both cases) and rest of tissues (RT) from oyster (7.7% %) were the ones positive for culture on blood agar (Table 2). In the PNC, all the tissues presented a higher percentage of positivity for culture on blood agar than for culture on marine agar (Table 2). The total counts show that the origin influenced the methodology. When samples from Alfacs Bay were analyzed, higher number of positive samples were obtained with those methodologies that have a step of supplementing the media with NaCl (i.e m-PCR-2 and MA-C). However, in the Poble Nou channel the effect is the opposite (Table 2) being the conventional methods, those that did not included the NaCl supplement, for the isolation of *Arcobacter*-like more effective.

3.2 Distribution of *Arcobacter* species within tissues.

The bacterial diversity was determined by the origin of the samples rather than by the type of shellfish. In Alfacs Bay only 4 different species were isolated (Figure 1), this value determines the maximum diversity indices. Therefore, the potential Shannon–Wiener maximum index (H_{max}) in AB was 1.95. The DG collected in January and the IL collected in May, both from mussels, presented the highest H index (0.69). Additionally, both samples shared values of Evenness (E) and richness (S) indexes. Richness was in most cases reduced to a single species (Table 3).

However, the highest positivity in the Poble Nou Channel offers a different picture. As shown in Figure 2, different species were isolated. The interval liquid was the tissue that presented highest values for the Shannon–Wiener and richness indexes, for mussels occurred in February (H= 1.37 and S=6) and for oysters in June (H=1.17 and S=5), being in PNC the H_{max}=2.56. In February the richness that was shown between the 4 sections of both type of shellfish was similar (Table 3). On the other hand, bacterial diversities in richness showed little difference between the digestive gland and the rest of tissues at different months, showing mussels and oysters the same trend (Table 3).

On average, the interval liquid showed that the number of species recovered, in terms of richness, in this particular compartment compared with the other was significatively higher ($P < 0.001$). Additionally, May was statistically the month when a lower number of positive samples for *Arcobacter*-like genera was recovered ($P<0.001$; Table 3).

Figure1 and 2 show how *Arcobacter*-like genera species are distributed within the tissues of shellfish. In AB only 4 species were recovered in total from both types of shellfish: *A. butzleri*, *M. canalis*, *M. marinus* and *M. mytili* (Figure 1). However, in the PNC up to 13 different species were recovered. Some species were recovered exclusively from one tissue i.e: *P. aquimarinus* from the RT of mussels; *P. ellisii* and *P. defluvii* and from the IL of mussels and oysters, respectively; and *A. lacus* from the DG of oysters (Figure 2). However, *M. canalis* and *M. marinus* were present in all the tissues, but were significatively more prevalent in the IL of both shellfish ($P_{M.canalis}$=0.013 and $P_{M.\ marinus}$=0.000, respectively; Figure 1 and Figure 2). Among the different species recovered from water samples, only *A. butzleri*, *A. cryaerophilus*, *M. canalis* and *M. marinus* were also recovered from shellfish tissues (Figure 1 and 2). Table 4 shows the simultaneous prevalence of four species in different shellfish's tissues and their surrounding water. Only *M. marinus* was found at the same time in Alfacs Bay water samples and its correspondent shellfish sample, with no differences of presence among tissues. Regarding the PNC, when *A. butzleri* and *A. cryaerophilus* were found in the water, they were also equally found in the 4 different compartments (Table 4). *M. marinus* was also found in the 4 compartments when it was present in water, but in this case intervalval liquid was significative more positive than the other 3 tissues (Table 4). However, *M. canalis* was only found in the intervalval liquid and the rest of tissues (Table 4).

Figure S1 shows how the MPN of *E. coli* correlates with the presence of *Arcobacter* in different tissues. When the levels of *E. coli* were i) of category A, only four species were recovered *A. butzleri*, *M. canalis* (only present in the IL), *M. marinus* and *M. mytili*, and none of the gills were positive, (in concordance with Figure 1), all these samples corresponded to Alfacs Bay samples. The samples from PNC showed levels of *E. coli* corresponding to the 3 remaining categories. ii) In samples of category B, only *M. molluscorum* was present in IL and *A. marinus* in the DG; iii) in those of category C, there was a predominance of *A. butzleri* in the four tissues. *M. marinus* was the second species present in the tissues, but a trend could be observed, and its presence decreased as it followed the digestive process i.e. IL = 73.3%, G= 33.33%; DG =13.33% and no detection in the RT. iv) In category D the tissues had their highest diversity of species (Figure S1).

3.3 Effect of abiotic and biotic parameters in the presence of *Arcobacter* spp.

Salinity and temperature of the surrounding water influenced the presence of some *Arcobacter* species as shown in Figure 3. Among all the different species recovered in this study, only the presence of *M. marinus* was slightly positive influenced by salinity (OR=1.075), whereas *A. butzleri*, A. *cryaerophilus* and *M. mytili* showed a negative relationship with this parameter (Figure 3A). A negative relationship between temperature and the presence of *A. butzleri*, *A. cryaerophilus* and *M. marinus*, was significative; on the other hand, *M. molluscorum* showed to have a positive correlation with this parameter (OR=1.81; Figure 3B).

The influence of the presence of one *Arcobacter* species on the presence of other species was also evaluated. Figure 4 shows that *A. butzleri*, *A. cryaerophilus*, *H. bivalviorum*, *M. canalis*, *M. marinus*, *M. molluscorum*, *M. mytili* and "*M. ostreae*" were significative more or less present in the environment depending on the species recovered at the same time. For instance, when *A. butzleri* was present the probability of isolation of *A. cryaerophilus* or *M. marinus* was lower than when *A. butzleri* was not recovered (Figure 4).

3.4 Persistence of *Arcobacter* genotypes.

The bioinformatic analysis of the ERIC-PCR patterns, revealed that among the total genotypes of *M. molluscorum*, and "*M. ostreae*" only one of them was found in the tissues of the pool and individuals from the same batch of samples (Table S2 and Figure S1). In the case of *M. mytili* the same genotype was found in different tissues from the same sample (Table S2 and Figure S1). *A. butzleri* and *M. marinus* were the species with a higher number of genotypes distributed among different types of samples (n=25 and n=8 respectively). In both cases we could find that a genotype was found in water and shellfish samples corresponding to the same origin and collected in the same month. Although the most common trend was that the same genotype was found between individuals and pool from the same sample batch. One of the *A. butzleri* genotypes was recovered in 4 different months always in shellfish samples collected from the PNC (Table S2). In the case of *M. canalis* the same genotype was recovered from PNC water samples taken in February and March; additionally, another genotype was recovered from the RT of a single mussel collected in February and the IL of an oyster collected in June, both from PNC. *H. bivalviorum* and *A. cryaerophilus* also showed two genotypes with isolates recovered from different samples (Table S2 and Figure S2).

4. Discussion

This study was undertaken to investigate the distribution of *Arcobacter*-like genera within the tissues of mussels and oysters, but also in water. The use of two scenarios, one with low fecal pollution from which shellfish were harvested and the other one, with high levels of fecal pollution where the shellfish were exposed to natural contamination, were necessary in order to evaluate how fecal pollution could (or not) affect to distribution of these microorganisms within the tissues, along with the hypothesis that some strains could be autochthonous from bivalves .

The detection of *Arcobacter*-like genera in the studied samples was high. In the Poble Nou channel all the samples, including water and shellfish, were positive. However, in Alfacs Bay 66.7% of the shellfish samples were positive for the isolation of *Arcobacter*-like genera isolates, which is, still a high percentage, results from other studies in the same area have been previously reported with a range of positivity between 29.9% and 68.2% (Collado et al.,2009; Levican et al., 2014: Salas-Massó et al., 2016;2018).

The two m-PCR performed with the DNA obtained from the two enrichment broths were the methodologies that resulted in higher number of positive tissue samples. This result could be expected, as DNA was obtained from a pre-enrichment step, meaning that we may have more bacteria than in a direct sample. Also, DNA from dead or viable but non culturable bacteria (VBNC) could been detected in the pre-enrichment broth, but when culturing approaches are used, a lower result is obtained. In concordance with previous studies performed by our group, the methodologies comprising a step in which salt is added, i.e: pre-enrichment in Arcobacter CAT-broth +2.5%NaCl and culture on marine agar, resulted in a higher number of tissues that were positive in Alfacs Bay compared to those methodologies that did not include salt (Salas-Massó, et al., 2016; 2018). Although, it was observed that m-PCR-PE2 in oysters' IL from Alfacs Bay had a higher performance than for the rest of the tissues, the use of m-PCR-PE1 in mussels and oysters and the m-PCR-PE2 in mussels, from AB, did not yield any difference between tissues. Therefore, these methodologies were not influenced by the studied tissue.

When evaluating pools versus individuals, all the pool samples were positive for *Arcobacter*-like species. This is just according to sampling methodologies a way to ensure the detection of this bacteria. In surveillance studies, especially in studies of antimicrobial

resistance or detection of bacteria in fecal samples, it has been demonstrated that pooling samples have the pro to improve time and cost efficiency of sample processing. On the contrary pooling samples reduce the methodological sensitivity at low occurrences resulting in misclassification. Pooling samples at the level of the epidemiological unit makes sense if the loss of methodological sensitivity is limited and if detection only is the primary aim (Wells, et al., 2003; Muñoz-Zanzi et al., 2006; Schmidt et al., 2015) Therefore, pooling samples at the current level of descriptive epidemiology where the aim was giving an overall picture of the distribution of *Arcobacter*, would make sense.

Regarding the distribution of the *Arcobacter*-like genera among the tissues, it was observed that the origin had an effect in the tissue positivity. The intervalval liquid, was the compartment which presented a higher percentage of positivity 65.4% in mussels and 84.6% in oysters, becoming the potential main source of *Arcobacter*-like species in shellfish, at least in harvesting areas of class B as Alfacs Bay. However, positive results were also obtained from marine agar cultures from the DG and the RT of both types of shellfish, meaning that *Arcobacter*-like genera are internalized by bivalves. Due to its legal classification (Order APA/3228/2005), the shellfish from Alfacs Bay, require 24h depuration before consumption (Anon 2015; Salas-Massó et al., 2018). For that, the conventional depuration processes may be enough for the removal of *Arcobacter*-like genera from the intervalval liquid, but the removal from the rest of the tissues should be assayed, as there are no studies in the bibliography which have addressed this issue and our study demonstrates the presence of different *Arcobacter*-like species in the tissues of shellfish, some of them have appeared exclusively in tissue samples, i.e, *M. mytili*. In our study none of the AB gills samples were positive for any of the methodologies. Previous results by Wang and col. (2008; 2010;2014) have shown the opposite. They observed that gills and digestive glands of oysters were the main tissues which bioaccumulated bacteria and viruses. Chen et al., (2019) found that *Arcobacter* was present in gills of fresh pacific oysters. This difference among our results may be attributed to environmental, habitat season and specimen factors. Wang et al., suggest that all the allochthonous species would be in the gills and not in other tissues. The allochthonous bacteria in shellfish have been defined as those that would pass through with the water (Romero et al., 2002) and the autochthonous bacteria may supply nutrient factors to keep shellfish alive (Pujalte et al.,1999; Zurel et al., 2011; Wang et al., 2014). Taking into account these considerations, we could propose that *Arcobacter*-like genera species have both, autochthonous and allochthonous representatives, and autochthonous species. i.e. *A. butzleri*, *M. marinus* and *M. canalis,* have

been found in both, oysters (Romero et al., 2002), and mussels without apparent discrimination among them. Recent studies have shown that *Arcobacter* species may be part of the microbiota of oyster's hemolymph (Lokmer and Wegner, 2015) and mussel's gut (Li et al., 2018), acting as opportunistic pathogens of bivalves, being the main taxon recovered in microbiota studies, when the shellfish are in a moribund state caused by other infections or increases in the water temperature. After comparing the similarity of the 16S sequences of the *Arcobacter*'s OTU from the study of Lokmer and Wegner, (2015), 20/101 of the OTUs were related to already known *Arcobacter* species. Interestingly, all these clones, clustered with environmental species described from isolates recovered from marine environments, among them *M. canalis*, which together with our results, could be considered an autochthonous bacterium of oysters with an opportunistic pathogenic capacity in bivalves (Salas-Massó in preparation). However, further studies are needed to study this pathogenic capacity in bivalves. Additionally, Chen et al., (2019) has recently observed that in spoiled oysters' gills, *Arcobacter* was the dominated bacteria, corroborating their opportunistic pathogenic potential for shellfish.

In the PNC samples it was observed that the RT was slightly more positive than the IL, (RT_{mussel}=86.7% vs. IL_{mussel}= 83.3% and RT_{oyster}=73.3% vs. IL_{oyster}= 66.7%). The reason for this small difference could be attributed to the conjunction of two factors, first the filter-feeding behavior of shellfish that lead to an accumulation of microbiota within them and secondly, because of the high levels of bacteria that have been recorded in the PNC water, i.e. levels of 4.1×10^4 *E.coli* MPN/100ml and 4.5×10^5 *Arcobacter* MPN/100ml (Salas-Massó, et al., 2018) which may saturate the bivalves.

The distribution of species within the shellfish' tissues was clearly segmented by the origin from which the animals were collected as well as by the type of shellfish that was analyzed. The results in Table 3, showed that no seasonality was observed in terms of diversity indexes in none of the origins. In Alfacs Bay, in most cases, only one species was recovered at a time in each sample. On the contrary, in the PNC, up to 6 different species were isolated from a same sample. The higher diversity on the PNC could be consequence of the different effluent that the PNC received, i.e. waste water from the village without treatment, irrigation water, seawater from the Alfacs Bay. This higher diversity was previously reported by Salas-massó et al. (2018).

As seen in Figure 1, despite both shellfish were in the same environment, Alfacs Bay, and that only 4 species were recovered from both type of bivalves; the distribution of the species

was different. For example, *A. butzleri* was detected for both shellfish in the IL however, it was present in the digestive gland of mussels, but in the rest of tissues of oysters. Ottaviani et al. (2013) observed that the DT was not a specific tissue for bioaccumulation of *A. butzleri,* because they did not found differences between the counts in DT and the rest of the animal including the intervalval liquid. Probably they did not found differences because they put together the rest of the mussel's body and the intervalval liquid, instead of considering as individual tissues as presented in this study. In the case of *M. mytili* and *M. canalis* were not detected in the oysters' RT, but they were in mussels' RT. These results may indicate a different retention of these species by the two shellfish as have been previously pointed out by Salas-Massó et al. (2018), and the main reason for this, would be their different gill structures. However, *M. marinus* was present in the DG and RT of both bivalves, and in the IL of oysters. This later result could be explained by the capability of bacteria to survive the immune system defense of shellfish. There are few studies investigating this phenomenon. It has been demonstrated that some bacterial surface domains, like mannose-sensitive ligands (type 1 fimbriae in the case of *E. coli* and MSHA pilus in *Vibrio* species) are involved in binding to and killing by hemocytes mussels (*M. galloprovincialis* Lam.) haemocytes (Pruzzo et al., 2005; Canesi et al., 2016). However, this relationship between the immune system of bivalves and *Arcobacter*-like genera has not yet been studied, which opens new investigation lines that will help to address better strategies for their depuration.

In the PNC samples, the distribution of species offers a different picture probably due to the high numbers of bacteria in the surrounding water. For instance, *M. marinus* is present in all the mussels' compartments and in 3 of oysters' with the exception of the DG. The species *A. butzleri* and *A. cryaerophilus* are present in all the tissues of both, mussels and oyster. Salas-Massó and col. (2018) demonstrated that these two species were especially abundant, when compared with the rest, in harvesting areas of class C and D; and PNC would be classified as a D area. For other species like *P. cloacae*, *P. ellisii*, *P. defluvii*, or *A. lacus* only one isolate was recovered, thus no preference for any of the tissues could be stablished. As mentioned above the microbial quality and overall bacterial load of the water had an impact on the distribution of the species within the shellfish's tissues. When the distribution of species is analyzed on the bases of the category to which the shellfish belong according to the EU regulation 854/2004, could be observed that in class C shellfish, *A. butzleri* and *M. marinus* reached their maximum percentages of positivity. However, in class D the percentage for these two species lowered, especially for *M. marinus*, which can be attributed to higher levels of pollution, thus fecal

associated species like *A. cryaerophilus, P. cloacae, P. defluvii* may prevail above environmental species like *M. marinus*.

Interestingly, only 4 species (*M. marinus* in both origins and *A. butzleri*, *M. canalis* and *A. cryaerophilus* in the PNC samples) were found at the same time in water and in the bivalves' tissues, among them the pathogenic nature of *A. butzleri* has been demonstrated as it has caused different episodes of cramps and diarrhea in humans (Salas-Massó et al., 2018b and references therein). *A. butzleri* showed no significative difference between its presence in the different tissues. These results seem to indicate that this pathogen can be easily incorporated to the shellfish tissues. However, Ottaviani et al. (2013) observed that in water and in mussels, *A. butzleri* decrease approximately 1 log every 24 h from the contamination, concluding that this species would not effectively grow in seawater and would not bioaccumulate in mussels. A recent study performed by the same authors (Ottaviani et al., 2016), in which they observed that *A. butzleri* type strain induced the phagocytic answer of mussels' hemocytes, would reinforce their hypothesis of the rapid removal of *A. butzleri* from the host tissues. On the contrary, *M. marinus* showed a preferential distribution for the IL in the PNC compared to the rest of the compartments, probably because of lower salinities of the PNC which may not favor the permanence of this species within the shellfish tissues.

The effect of salinity and temperature on *Arcobacter* species has been have previously reported (D'Sa and Harrison,2005; Fisher et al., 2014; Levican et al., 2014; Salas-Massó et al., 2018). Our results showed that *M. marinus* is favored by higher salinities which is in concordance with the fact that *M. marinus* has only been recovered when the media was supplemented with 2.5% (w/v) NaCl and Marine Agar were used (Salas-Massó et al., 2016; 2018). Regarding temperature, *A. cryaerophilus* would be favored by lower temperatures, which is in concordance with previous studies showing that is recovered at lower temperatures than other species like *A. butzleri* or *A. molluscorum*. However, in this study *A. butzleri* seem to have the same tendency as *A. cryaerophilus*. Leoni et al. (2017) in a study performed in Italy, showed that *A. butzleri* was more prevalent in the winter-spring season, which was not in concordance with the results of Levican et al. (2014), ascribing this difference to geographical and climatic features. Levican et al. (2014) reported that *A. butzleri* species was predominant in shellfish samples recovered from Alfacs Bay from June to October when the water temperature was between 23°C and 27°C. The reason that our results are contradictory with those previous findings could be due to our limited number of samples that were taken in the

winter-spring season (January to June) when the temperature range was 9.14-23.70ºC, therefore summer samples are not included in the analysis, leading to this bias.

As far as we are concern, this is the first study that stablished a relationship between the *Arcobacter*-like genera species. According to the odd ratio results, it could be observed that species belonging to the genera *Halarcobacter* and *Malaciobacter* would favor the presence of other species of these same genera. Additionally, when *H. bivalviorum* was isolated, it was very likely that *A. butzleri* was also recovered, and *vice versa*. The opposite relationship between *A. butzleri* and *M. marinus* was observed. Interestingly, the presence of *A. butzleri* was barely affected by the presence of *A. cryaerophilus*, however, the fact that *A. butzleri* was present in a sample diminish the probability of finding *A. cryaerophilus*. Several authors (Ho et al., 2008; Fisher et al., 2014; Levican et al.,2014) have reported similar findings, and reached the conclusion that the enrichment step used favors the growth of *A. butzleri* masking the rest, specially *A. cryaerophilus*.

The genetic diversity of *Arcobacter*-like genera species was 45.8%, which agreed with results obtained by Collado et al. (2014) that obtained a 68.5% of variability in shellfish from Chile. Levican et al. (2014), reported a lower variability (24.8%) in Spanish shellfish. Despite the variation in the percentage of variability, we also observed that strains were in their majority different between months, with some exceptions i.e a genotype of *M. canalis* recovered in February and March, which is in concordance with the results presented by Levican et al. (2014). However, as far as we know this is the first study analyzing ERIC patterns in both water and shellfish, and we could find that for *A. butzleri* (genotype 13 in Table S2) and *M. marinus* (genotype 7 in Table S2), the same genotypes were found in water and within the shellfish, demonstrating that these species are taken by the bivalves from water and then incorporated to the tissues. In the case of *A. butzleri*, the genotype was in the PNC water and was incorporated to both, mussels and oysters exposed to that water, additionally this genotype was also found in other months, indicating that this genotype could be a concurrent inhabitant of the PNC.

5. Conclusion

This is the first study in which the distribution of *Arcobacter*-like genera species is carefully examined within the tissues of shellfish. Choosing shellfish production areas (A, B) and heavily fecal contaminated waters (C, D) has allowed us to get a comparative profile of the *Arcobacter*-like distribution in the different compartments. This was very relevant both, to provide data on the *Arcobacter*-like species, which posed a risk for consumption (zoonotics), and those which could be of marine indigenous origin, which could play a different role, even as shellfish pathogens. Our finding showed that the marine environment was less diverse, and some species (*M. mytili*) are found only associated with shellfish. *A. butzleri* was ubiquitous and found in all compartments associated with fecal polluted waters. However, in marine environments, *A. butzleri* behaved as an autochthonous species of shellfish microbial diversity, posing a risk for the shellfish consumers due to its pathogenic potential. This work advance on this issue, opening very challenging questions for the monitoring basis of *Arcobacter*-like species in marine environments, but also in depuration strategies. The results determined that the intervalval liquid was the compartment that presented a higher percentage of positivity, thus the conventional depuration process stablished by the legislation should be enough, for the removal of this group of bacteria. However, further studies are needed to confirm this.

6. Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7. Author Contributions Statement

NSM, KA, MJF and DF designed the work. NSM, performed all the assays the compartmentalization protocol for PMA. NS-M, KA, MJF and DF wrote the paper.

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11. Figures and tables

Table 1. Positive samples and species found in 42 samples of water and shellfish. The genetic diversity is indicated as the % between strains and isolates for each species.

		Water		Shellfish		
Species	**Total nº of positive samples**	**AB**	**PNC**	**Mussel**	**Oyster**	**TOTAL Strains/Isolates (%)**
Aliarcobacter butzleri	20	3	4	7	6	327/734 (44.6)
Aliarcobacter cryaerophilus	14		3	6	5	54/79 (68.4)
Aliarcobacter lacus	1				1	1/1 (100)
Arcobacter nitrofigilis	1	1				1/1 (100)
Halarcobacter bivalviorum	4			2	2	66/66 (100)
Halarcobacter ebronensis	3	2	1			8/12 (66.7)
"Halarcobacter mediterraneus"	1		1			1/1 (100)
Malaciobacter canalis	13	2	3	3	5	26/64 (40.6)
Malaciobacter marinus	20	6	2	4	8	81/254 (31.9)
Malaciobacter molluscorum	3	1		2		4/14 (7.1)
Malaciobacter mytili	4			2	2	9/34 (26.5)
"Malaciobacter ostreae"	1			1		4/11 (28.6)
Pseudarcobacter aquimarinus	1			1		1/1 (100)
Pseudarcobacter cloacae	2			1	1	2/7 (28.6)
Pseudarcobacter defluvii	1				1	1/1 (100)
Pseudarcobacter ellisii	1			1		1/1 (100)
Total						587/1281 (45.8)

Table 2. Relationship between the type of shellfish from both origins Alfacs Bay with the positivity of the different tissues for the different methodologies. The table shows the results of pooled plus individual samples. m-PCR-D: m-PCR from DNA obtained directly from the raw homogenized tissues; m-PCR-PE1: m-PCR from DNA obtained from the pre-enrichment broth in Arcobacter-CAT broth; m-PCR-PE2: m-PCR from DNA obtained from the pre-enrichment broth in Arcobacter-CAT broth supplemented with 2.5% (w/v) NaCl; BA-C : positive isolation on blood agar; and MA-C: positive isolation on Marine agar; IL: intervalval liquid; G: gills; DG: digestive gland; RT: rest of tissues.

			Tissue nº positive simples (%)				
Origin	**Shellfish**	**Methodology**	**IL**	**G**	**DG**	**RT**	**Total**
Alfacs Bay	**Mussel**	m-PCR D	6 (23.1) *	-	-	-	6/104 (5.8)
		m-PCR-PE1	9 (34.6)	5 (19.2)	3 (11.5)	4 (15.4)	21/104 (20.2)
		m-PCR-PE2	9 (34.6)	3 (11.5)	6 (23.1)	6 (23.1)	24/104 (23.1)
		BA-C	-	1 (3.8)	1 (3.8)	-	2/104 (1.9)
		MA-C	4 (15.4) *	-	2 (7.7)	2 (7.7)	8/104 (7.7)
		Total	**17/26 (65.4)**	**8/26 (30.8)**	**8/26 (30.8)**	**8/26 (30.8)**	**41/104 (39.4)**
	Oyster	m-PCR D	9 (34.6) *	ND	3 (11.5)	ND	12/104 (11.5)
		m-PCR-PE1	10 (38.5)	5 (19.2)	5 (19.2)	10 (38.5)	30/104 (28.8)
		m-PCR-PE2	19 (73.1) *	5 (19.2) **	9 (34.6)	11 (42.3)	44/104 (42.3)
		BA-C	ND	ND	ND	2 (7.7)	2/104 (1.9)
		MA-C	12 (46.2) *	ND	4 (15.4)	2 (7.7)	18/104 (17.3)
		Total	**22/26 (84.6)**	**9/26 (34.6)**	**13/26 (50.0)**	**16/26 (61.5)**	**60/104 (57.7)**
Poble Nou Channel	**Mussel**	m-PCR D	16 (53.3) *	5 (16.7)	1 (3.3) **	5 (16.7)	27/120 (22.5)
		m-PCR-PE1	25 (83.3)	25 (83.3)	25 (83.3)	25 (83.3)	100/120 (83.3)
		m-PCR-PE2	25 (83.3)	22 (73.3)	19 (63.3)	20 (66.7)	86/120 (71.7)
		BA-C	24 (80.0)	18 (60.0)	21 (70.0)	18 (60.0)	81/120 (67.5)
		MA-C	20 (66.7) *	4 (13.3) **	9 (30.0)	10 (33.3)	43/120 (35.8)
		Total	**25/30 (83.3)**	**25/30 (83.3)**	**25/30 (83.3)**	**26/30 (86.7)**	**101/120 (84.2)**
	Oyster	m-PCR D	15 (50.0) *	3 (10.0)	1 (3.3) **	2 (6.7)	21/120 (17.5)
		m-PCR-PE1	20 (66.7)	20 (66.7)	20 (66.7)	20 (66.7)	80/120 (66.7)
		m-PCR-PE2	18 (60.0)	20 (66.7)	15 (50.0)	17 (56.7)	70/120 (58.3)
		BA-C	20 (66.7)	18 (60.0)	18 (60.0)	19 (63.3)	75/120 (62.5)
		MA-C	16 (53.3) *	10 (33.3)	6 (20.0)	9 (30.0)	41/120 (34.2)
		Total	**20/30 (66.7)**	**20/30 (66.7)**	**20/30 (66.7)**	**22/30 (73.3)**	**82/120 (68.3)**

ND: no detection

* The number of samples positive for a specific methodology in a specific tissue was higher ($P< 0.05$) than in other tissues.

** The number of samples positive for a specific methodology in a specific tissue was lower ($P< 0.05$) than in other tissues.

Table 3. Data on positive sample by culture and relative species diversity (H), evenness (E), and richness (S) estimates from analysis of the isolates recovered from mussels and oyster from Alfacs Bay (AB) and Poble Nou Channel (PNC). IL: intervalval liquid; G: gills; DG: digestive gland; RT: rest of tissues.

			Mussels			**Oysters**		
Origin	**Month**	**Tissue**	**Shannon–Wiener Index (H)**	**Evenness (E)**	**Richness (S)**	**Shannon–Wiener Index (H)**	**Evenness (E)**	**Richness (S)**
Alfacs Bay	January	IL	0	1	1	-	-	-
		G	-	-	-	-	-	-
		DG	0.69	1	2	-	-	-
		RT	0	1	1	-	-	-
	February	IL	-	-	-	0.34	0.46	2
		G	-	-	-	-	-	-
		DG	-	-	-	-	-	-
		RT	-	-	-	0	1	1
	March	IL	-	-	-	0.64	0.92	2
		G	-	-	-	-	-	-
		DG	-	-	-	-	-	-
		RT	-	-	-	-	-	-
	April	IL	0	1	1	0.68	0.99	2
		G	-	-	-	-	-	-
		DG	0	1	1	-	-	-
		RT	-	-	-	0	1	1
	May	IL	0.69	1	2	-	-	-
		G	-	-	-	-	-	-
		DG	-	-	-	0	1	1
		RT	0	1	1	-	-	-
	June	IL	-	-	-	0	1	1
		G	-	-	-	-	-	-
		DG	-	-	-	0	1	1
		RT	-	-	-	0	1	1

Table 3. Continuation

Origin	Month	Tissue	Mussels			Oysters		
			Shannon–Wiener Index (H)	Evenness (E)	Richness (S)	Shannon–Wiener Index (H)	Evenness (E)	Richness (S)
Poble Nou Channel	January	IL	0.60	0.43	3	0.33	0.44	2
		G	0.47	0.67	2	0	1	1
		DG	0.76	0.63	3	0	1	1
		RT	0	1	1	0.64	0.92	2
	February	IL	1.37	0.37	6	0.87	0.40	4
		G	1.01	0.39	4	0.74	0.53	3
		DG	0.68	0.98	2	0.59	0.46	3
		RT	1.16	0.69	4	1.03	0.91	3
	March	IL	0.69	0.99	2	0.95	0.33	4
		G	0	1	1	0.69	1	2
		DG	0.49	0.33	3	0.24	0.33	2
		RT	0.24	0.33	2	0	1	1
	April	IL	1.05	0.45	4	-	-	-
		G	0.58	0.46	3	-	-	-
		DG	0.83	0.70	3	-	-	-
		RT	0.68	0.37	3	0.45	0.63	2
	May	IL	-	-	-	-	-	-
		G	-	-	-	-	-	-
		DG	-	-	-	-	-	-
		RT	1.05	0.93	3	0.56	0.81	2
	June	IL	0.93	0.52	4	1.17	0.62	5
		G	-	-	-	0.17	0.24	2
		DG	1.12	0.67	4	0.49	0.70	2
		RT	0.92	0.42	4	0.18	0.25	2

Table 4. Positive samples at the same time for water and tissues for different species in Alfacs Bay (AB) and the Poble Nou Channel (PNC).

		Present in positive tissue sample for each species/ Total positive water samples for each species (%)			
Origen	Species	IL	G	DG	RT
AB	*M. marinus*	6/22 (27.3)	-	3/22 (13.6)	2/22 (9.1)
PNC	*A. butzleri*	32/40 (80.0)	29/40 (72.5)	30/40 (75.0)	26/40 (65.0)
	A. cryaerophilus	2/30 (6.7)	5/30 (16.7)	3/30(10.0)	4/30 (13.3)
	M. canalis	4/30 (10.0)	-	-	3/30 (7.5)
	M. marinus	13/20 (65.0)*	5/20 (25.0)	1/20 (5.0)	4/20 (20.0)

* The number of samples positive in a specific tissue was higher ($P < 0.05$) than in other tissues.

Figure 1. Bacterial diversity in the tissues of mussels, oysters and water from Alfacs Bay (AB). The total number of isolates for each species isolated from a specific tissue is shown in brackets. The species marked in red in the water box correspond to species also found within the shellfish. ND: no detection.

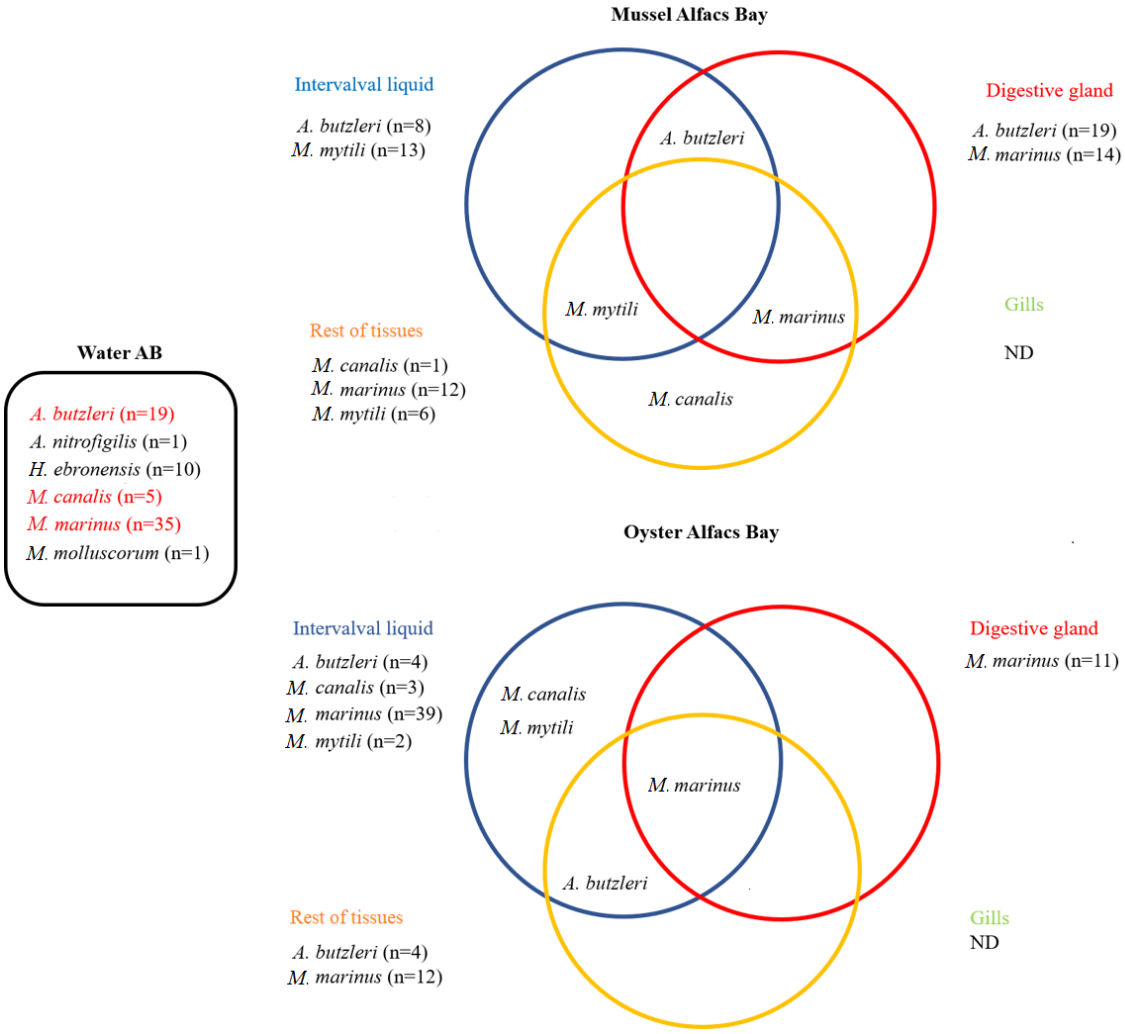


Figure 2. Bacterial diversity in the tissues of mussels, oysters and water from Poble Nou Channel (PNC). The total number of isolates for each species isolated from a specific tissue is shown in brackets. The species marked in red in the water box correspond to species also found within the shellfish.

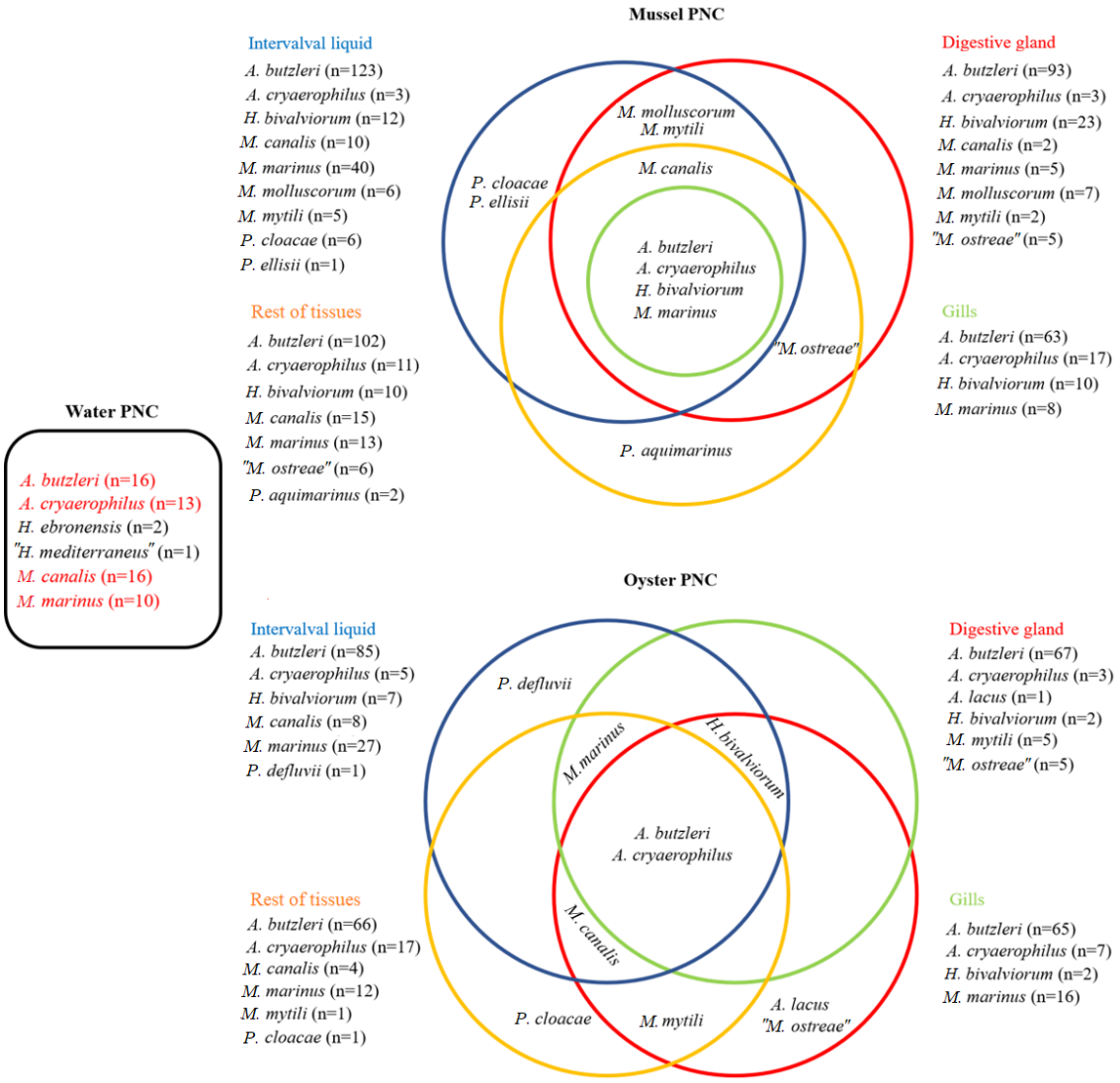
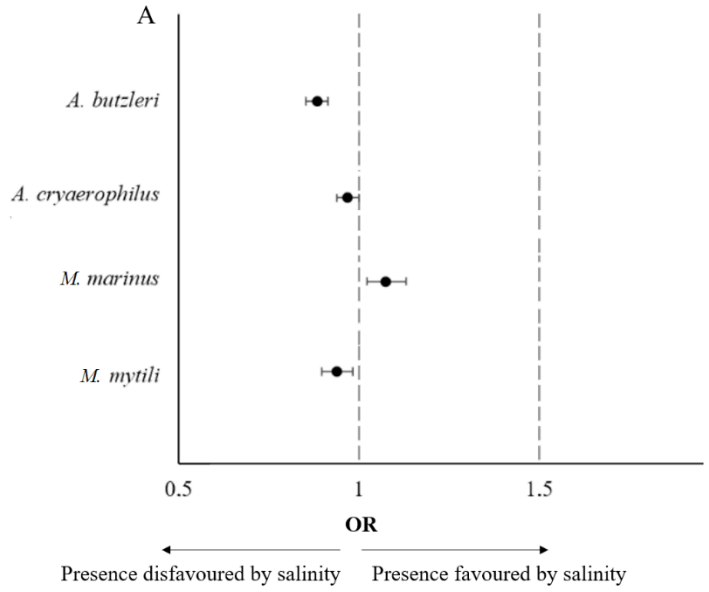


Figure 3. Odd ratios to show the relationship between *Arcobacter*-like genera and abiotic parameters like A) Salinity and B) Temperature



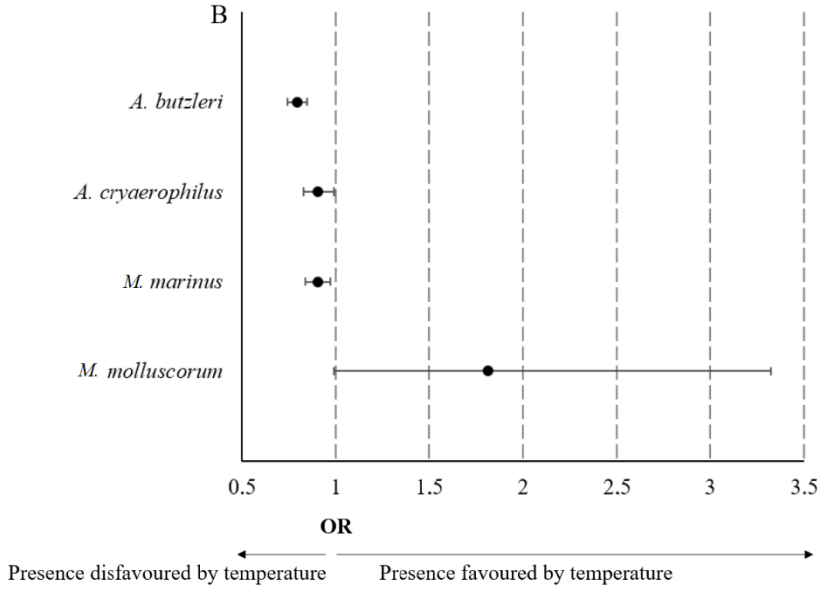
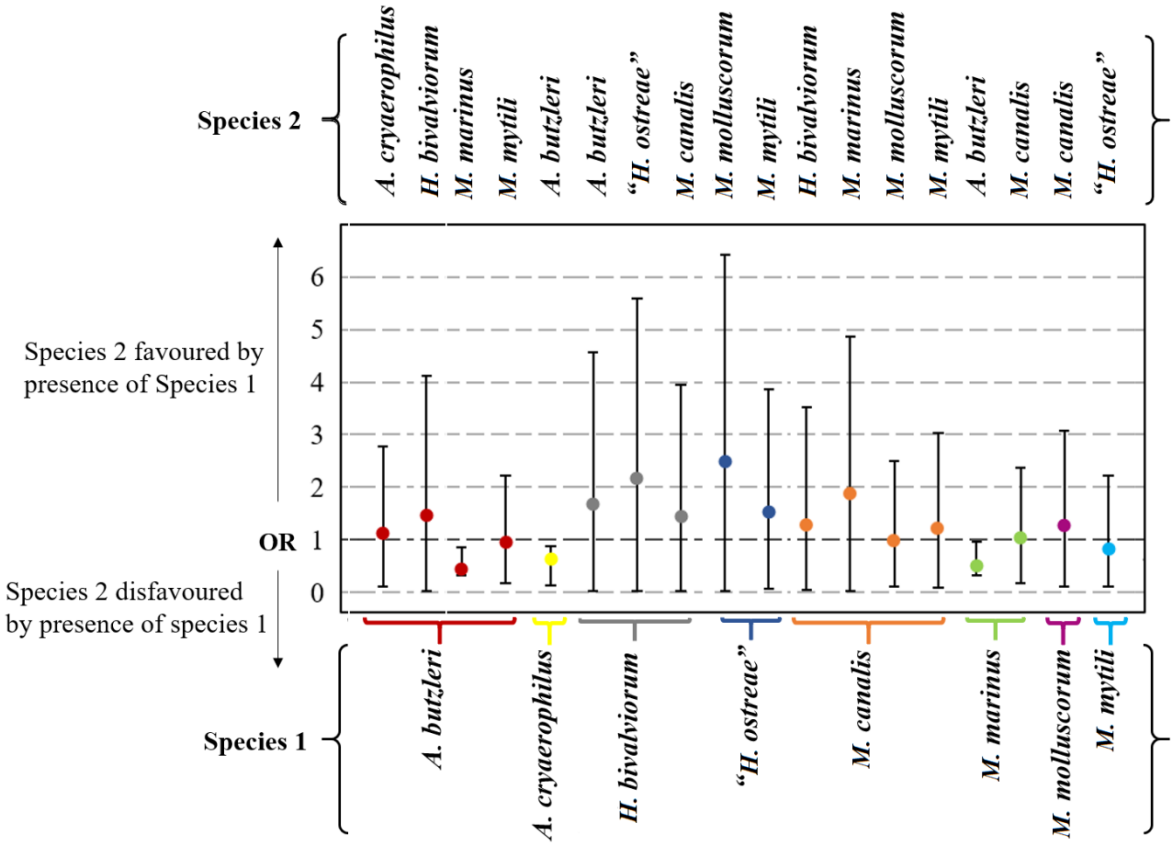


Figure 4. Odd ratios to show the relationship between the different species within the *Arcobacter*-like genera



Supplementary Material

Table S1. Total analyzed samples from mussels and oysters collected from Alfacs Bay (AB) and Poble Nou Channel (PNC).

Origin	Shellfish	Pool samples	Individual samples	
AB	Mussel	6	20	
	Oyster	6	20	
PNC	Mussel	6	24	
	Oyster	6	24	
Total		24	88	112*

* The total number of samples is 448 (112x4), because 4 different compartments were dissected and analyzed from each sample independently if it was a pool or a individual.

Table S2. Distribution of the same genotypes among different samples according to type of samples, origin, tissue and month and it is based on the bioinformatic analysis with BioNumerics.

Species	Genotype	Samples	Pool/ Individual code	Origin	Tissues	Month
A. butzleri	1	Mussel	F169-3**	PNC	IL	January
		Mussel	F169-3**	PNC	DG	January
		Mussel	F169-3**	PNC	RT	January
		Mussel	F169-4**	PNC	IL	January
		Oyster	F170*	PNC	RT	January
	2	Oyster	F170-4**	PNC	IL	January
		Oyster	F170-4**	PNC	DG	January
	3	Mussel	F169-2**	PNC	DG	January
		Mussel	F169-2**	PNC	G	January
	4	Mussel	F173-3**	PNC	IL	February
		Mussel	F173-4**	PNC	IL	February
		Oyster	F174-2**	PNC	DG	February
		Oyster	F174-3**	PNC	DG	February
	5	Mussel	F189-3**	PNC	RT	June
		Oyster	F190-1**	PNC	G	June
	6	Mussel	F177-3**	PNC	IL	March
		Mussel	F177-4**	PNC	IL	March
	7	Oyster	F170-1**	PNC	DG	January
		Oyster	F170-1**	PNC	RT	January
		Oyster	F174-1**	PNC	DG	February
		Mussel	F181*	PNC	IL	April
		Mussel	F189-4**	PNC	F	June
	8	Mussel	F189-4**	PNC	IL	June
		Oyster	F190-1**	PNC	G	June
		Oyster	F190-1**	PNC	DG	June
		Oyster	F190-2**	PNC	F	June
		Oyster	F190-3**	PNC	IL	June
	9	Mussel	F189-1**	PNC	F	June
		Oyster	F190-1**	PNC	DG	June
	10	Mussel	F189-1**	PNC	IL	June
		Oyster	F190*	PNC	DG	June
		Oyster	F190-2**	PNC	DG	June
		Oyster	F190-4**	PNC	IL	June
		Oyster	F190-4**	PNC	G	June
		Oyster	F190-4**	PNC	DG	June
	11	Mussel	F177*	PNC	G	March
		Oyster	F178-1**	PNC	F	March
		Oyster	F178-1**	PNC	IL	March
		Oyster	F178-2**	PNC	IL	March
		Mussel	F189-1**	PNC	F	June

Table S2. Continuation

Species	Genotype	Samples	Pool/ Individual code	Origin	Tissues	Month
	12	Mussel	F173-3**	PNC	IL	February
		Mussel	F173-3**	PNC	DG	February
	13	Mussel	F169*	PNC	G	January
		Mussel	F169-2**	PNC	IL	January
		Oyster	F170*	PNC	G	January
		Oyster	F170-3**	PNC	DG	January
		Oyster	F170-3**	PNC	RT	January
		Mussel	F173-3**	PNC	RT	February
		Mussel	F177-1**	PNC	IL	March
		Oyster	F178*	PNC	RT	March
		Oyster	F178*	PNC	RT	March
		Mussel	F189*	PNC	RT	June
		Oyster	F190-2**	PNC	RT	June
		Water	W134	PNC	-	January
	14	Mussel	F177*	PNC	IL	March
		Mussel	F177-3**	PNC	RT	March
		Mussel	F177-4**	PNC	IL	March
	15	Oyster	F190-3**	PNC	DG	June
		Oyster	F190-3**	PNC	G	June
	16	Mussel	F181*	PNC	RT	April
		Mussel	F181*	PNC	G	April
	17	Mussel	F177-3**	PNC	DG	March
		Mussel	F177-4**	PNC	IL	March
	18	Mussel	F173*	PNC	G	February
		Mussel	F173*	PNC	DG	February
		Mussel	F181*	PNC	IL	April
		Mussel	F189-1**	PNC	IL	June
	19	Oyster	F178-1**	PNC	IL	March
		Oyster	F178-3**	PNC	G	March
	20	Mussel	F177*	PNC	IL	March
		Mussel	F177*	PNC	F	March
		Mussel	F177-1**	PNC	DG	March
		Mussel	F177-1**	PNC	G	March
	21	Oyster	F174*	PNC	RT	February
		Oyster	F174-1**	PNC	G	February
	22	Mussel	F181-1**	PNC	G	April
		Mussel	F181-1**	PNC	IL	April
	23	Mussel	F169-2**	PNC	DG	January
		Mussel	F169-2**	PNC	RT	January
		Mussel	F169-3**	PNC	IL	January
	24	Mussel	F169-2**	PNC	G	January
		Mussel	F169-2**	PNC	IL	January

Table S2. Continuation

Species	Genotype	Samples	Pool/ Individual code	Origin	Tissues	Month
	25	Oyster	F170-1**	PNC	G	January
		Oyster	F170-1**	PNC	IL	January
A. cryaerophilus	1	Mussel	F181-3**	PNC	F	April
		Mussel	F181-3**	PNC	G	April
	2	Oyster	F170-1**	PNC	F	January
		Oyster	F170-2**	PNC	IL	January
H. bivalviorum	1	Mussel	F173*	PNC	RT	February
		Mussel	F173-3**	PNC	DG	February
		Mussel	F173-4**	PNC	IL	February
		Mussel	F173-4**	PNC	DG	February
		Mussel	F173-4**	PNC	G	February
		Mussel	F189-3**	PNC	DG	June
	2	Mussel	F173*	PNC	G	February
		Mussel	F173*	PNC	DG	February
		Mussel	F173*	PNC	RT	February
		Mussel	F173-1**	PNC	DG	February
		Oyster	F174-3**	PNC	DG	February
"H. ostreae"	1	Mussel	F189*	PNC	RT	June
		Mussel	F189-3**	PNC	DG	June
M. canalis	1	Water	W137	PNC	-	February
		Water	W140	PNC	-	March
	2	Mussel	F190-1**	PNC	RT	February
		Oyster	F173-3**	PNC	IL	June
M. marinus	1	Oyster	F180-2**	Bay	IL	April
		Mussel	F181-1**	PNC	IL	April
	2	Oyster	F172*	Bay	IL	February
		Oyster	F172- 3**	Bay	IL	February
		Oyster	F174*	PNC	IL	February
		Oyster	F188*	Bay	DG	June
	3	Oyster	F174*	PNC	IL	February
		Water	W133	Bay	-	January
	4	PNC	F173-1**	Mussel	RT	February
		PNC	F173-1**	Mussel	IL	February
		PNC	F174-4**	Oyster	IL	February
	5	Oyster	F172- 3**	Bay	IL	February
		Oyster	F172-4**	Bay	IL	February
	6	Oyster	F172-3**	Bay	IL	February
		Oyster	F172-4**	Bay	IL	February
		Mussel	F173*	PNC	IL	February
		Oyster	F176*	Bay	IL	March

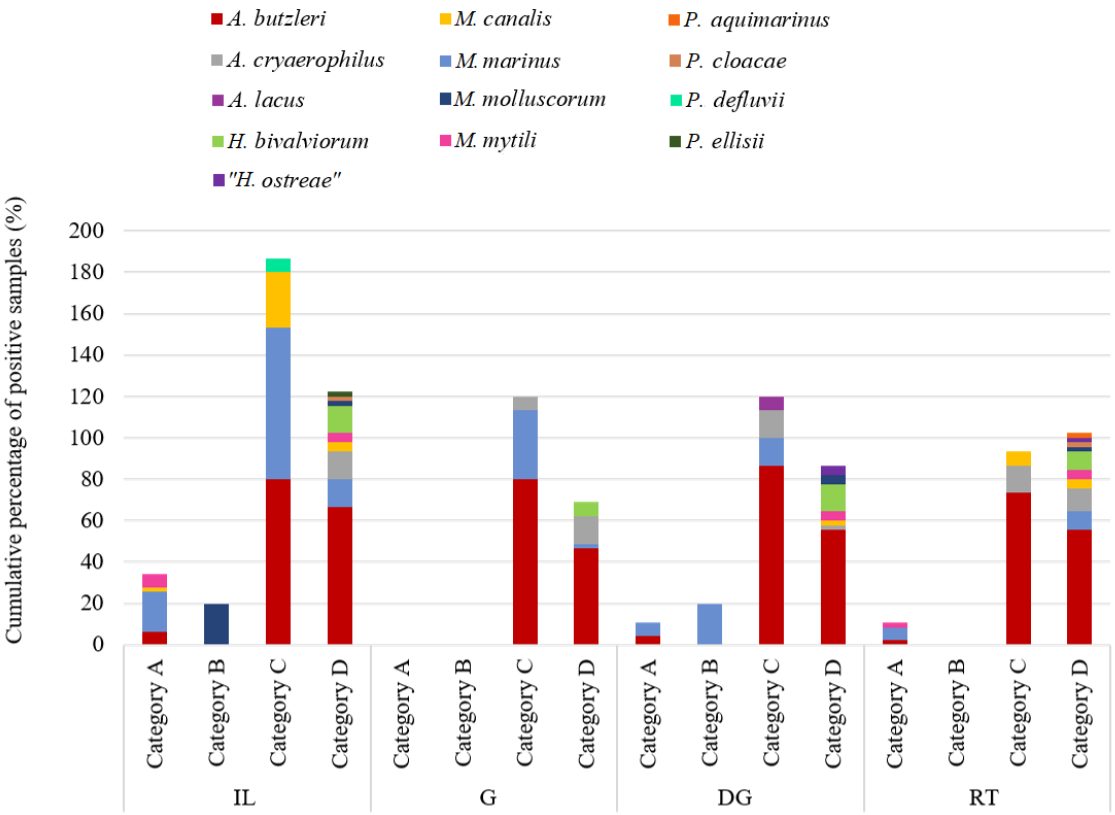
Table S2. Continuation

Species	Genotype	Samples	Pool/ Individual code	Origin	Tissues	Month
	7	Mussel	F167*	Bay	DG	January
		Water	W133	Bay	-	January
	8	Oyster	F188-1**	Bay	IL	June
		Oyster	F188-4**	Bay	IL	June
M. molluscorum	1	Mussel	F189*	PNC	DG	June
		Mussel	F189-2**	PNC	IL	June
		Mussel	F189-3**	PNC	DG	June
M. mytili	1	Mussel	F167*	Bay	IL	January
		Mussel	F169*	PNC	DG	January
		Mussel	F169*	PNC	IL	January

*Pool sample

**Individual sample

Figure S1. Percentage of samples positive for each tissue and for each species depending upon the classification of shellfish harvesting areas (Anon, 2015). Category A (n=188): at least 80% of the samples, collected do not exceed 230 MPN *E. coli*/100 g and the remaining 20% do not exceed 700 MPN E. coli/100 g; category B (n=20): 90% of samples must have ≤4600 MPN *E. coli*/100 g and the remaining 10% should not exceed 46,000 MPN *E. coli*/100 g; category C (n=60): all samples are ≤46,000 MPN *E. coli*/100 g; and category D (n=180): 100% of the samples show values ≥ 46,000MPN *E. coli*/100 g.



UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

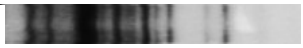
Figure S2. Dendrogram of the ERIC-PCR patterns of the different species isolated in the study.

ERIC fKBplus

A. aquimarinus

F185 IL-171	Mussel	PNC	May

A. defluvii



F178-1 IL 20	Oyster	PNC	March

A. ellisii

F173-2 IL 18	Mussel	PNC	February

A. lacus

F178-4 DG 21	Oyster	PNC	March

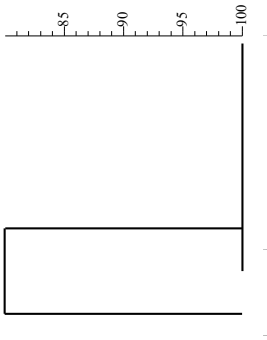
"A. mediterraneus"

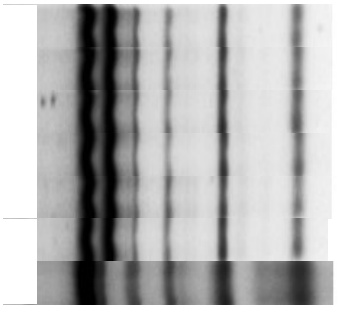
W143-33	Water	PNC	April

A. nitrofigilis

W138-33	Water	Bay	March

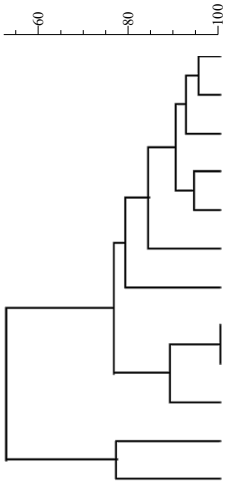
A. cloacae

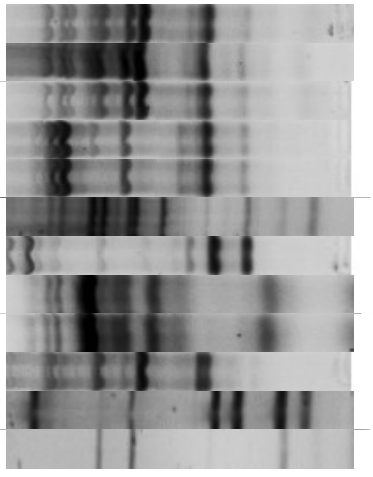




F173-2 IL 33	Mussel	PNC	February
F173-2 IL 34	Mussel	PNC	February
F173-2 IL 35	Mussel	PNC	February
F173-2 IL 36	Mussel	PNC	February
F173-2 IL 37	Mussel	PNC	February
F173-2 IL 38	Mussel	PNC	February
F186 IL 201	Oyster	PNC	August

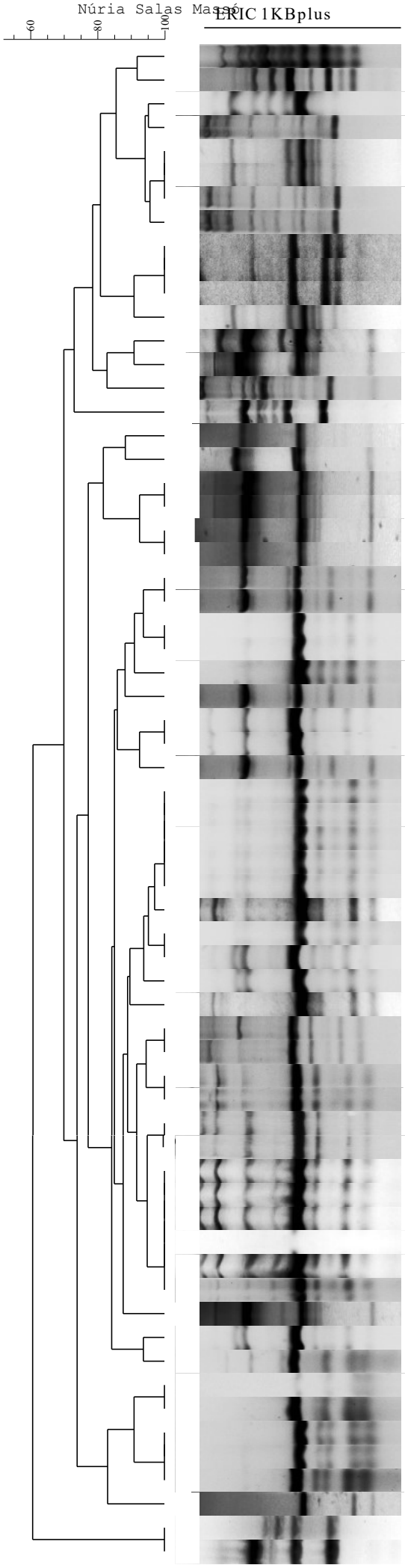
A. ebronensis



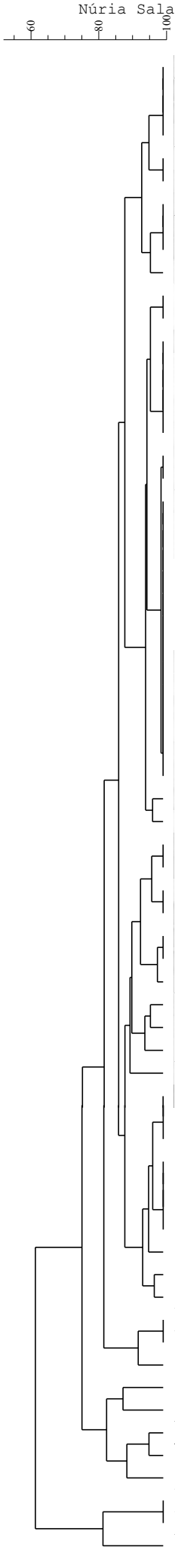


W145-381	Water	Bay	May
W145-34	Water	Bay	May
W145-361	Water	Bay	May
W145-341	Water	Bay	May
W145-351	Water	Bay	May
W136-34	Water	Bay	February
W145-331	Water	Bay	May
W140-331	Water	PNC	March
W140-371	Water	PNC	March
W145-371	Water	Bay	May
W135-38	Water	Bay	February
W136-36	Water	Bay	February

A. canalis

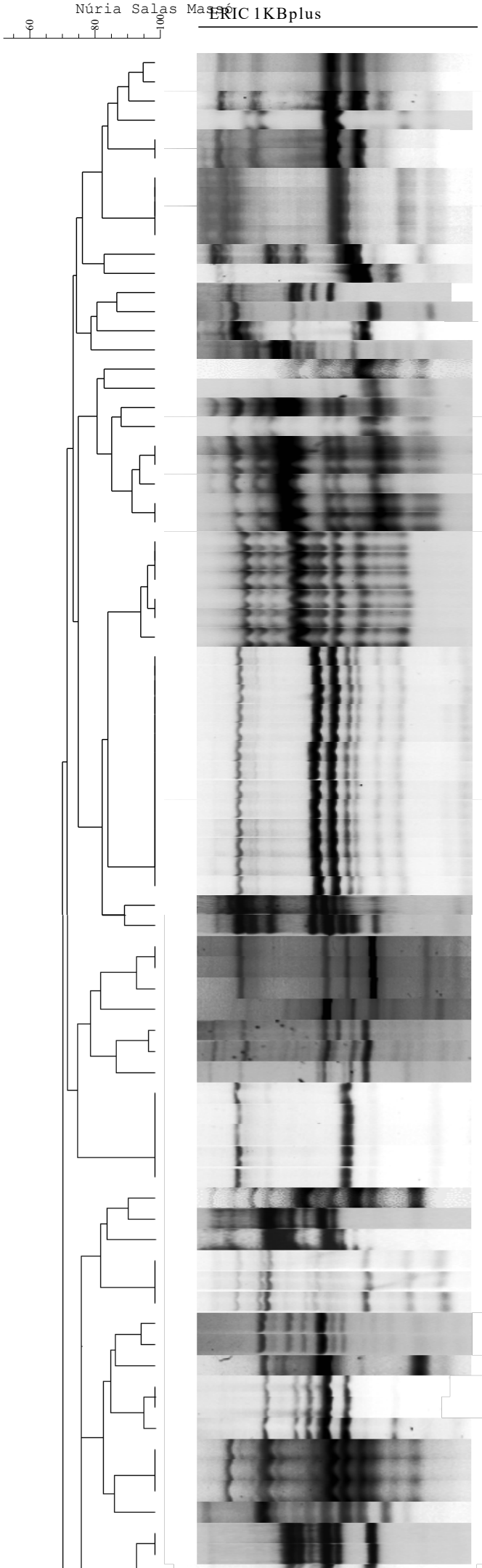


F190-1 IL 33	Oyster	PNC	June
W143-34	Water	PNC	April
F181-2 IL 34	Mussel	PNC	April
W144-35	Water	Bay	May
F173 F 33	Mussel	PNC	February
F173 F 34	Mussel	PNC	February
W144-38	Water	Bay	May
W144-36	Water	Bay	May
F176-1 IL 33	Oyster	Bay	March
F176-1 IL 34	Oyster	Bay	March
F176-1 IL 35	Oyster	Bay	March
F173-1 IL 33	Mussel	PNC	February
F174 IL 381	Oyster	PNC	February
W140-38	Water	PNC	March
W144-34	Water	Bay	May
F167 F 33	Mussel	Bay	January
W140-36	Water	PNC	March
W140-37	Water	PNC	March
W140-33	Water	PNC	March
W140-35	Water	PNC	March
W137-33	Water	PNC	February
W140-34	Water	PNC	March
F190-2 IL 33	Oyster	PNC	June
F190-2 IL 34	Oyster	PNC	June
F181-1 IL 34	Mussel	PNC	April
F181-1 IL 35	Mussel	PNC	April
F181-1 IL 38	Mussel	PNC	April
F190-2 IL 35	Oyster	PNC	June
F181-3 IL 34	Mussel	PNC	April
F181-3 IL 37	Mussel	PNC	April
F190-2 IL 37	Oyster	PNC	June
F181-1 F 34	Mussel	PNC	April
F181-1 F 35	Mussel	PNC	April
F181-1 F 36	Mussel	PNC	April
F181-1 F 37	Mussel	PNC	April
F181-1 F 38	Mussel	PNC	April
F174 IL 351	Oyster	PNC	February
F181-1 F 33	Mussel	PNC	April
F181-3 IL 33	Mussel	PNC	April
F181-2 IL 35	Mussel	PNC	April
F174 IL 361	Oyster	PNC	February
W143-35	Water	PNC	April
W143-36	Water	PNC	April
W143-341	Water	PNC	April
W143-351	Water	PNC	April
F178-1 IL 33	Oyster	PNC	March
F178-1 IL 34	Oyster	PNC	March
F173-3 F 33	Mussel	PNC	February
F173-3 F 34	Mussel	PNC	February
F173-3 F 35	Mussel	PNC	February
F173-3 F 36	Mussel	PNC	February
F173-3 F 37	Mussel	PNC	February
F190-1 IL 36	Oyster	PNC	June
W137-35	Water	PNC	February
F181-3 IL 36	Mussel	PNC	April
W142-341	Water	Bay	April
F182 IL 331	Oyster	PNC	April
W140-381	Water	PNC	March
F181 IL 331	Mussel	PNC	April
F181 IL 341	Mussel	PNC	April
W143-331	Water	PNC	April
W137-38	Water	PNC	February
F169 DG 35	Mussel	PNC	January
F169 DG 36	Mussel	PNC	January



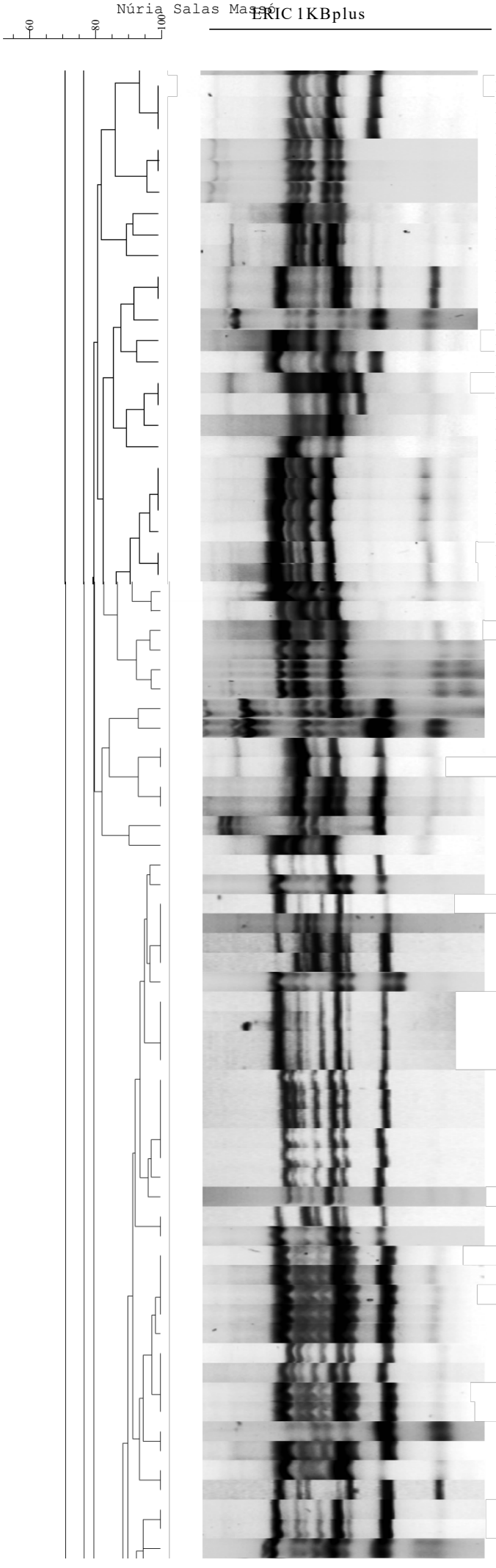
F174 IL 35	Oyster	PNC	February
F189-1 IL 37	Mussel	PNC	June
F189-1 IL 38	Mussel	PNC	June
F189-2 F 34	Mussel	PNC	June
F174 IL 33	Oyster	PNC	February
F174 IL 37	Oyster	PNC	February
F189-1 IL 34	Mussel	PNC	June
F189-1 IL 35	Mussel	PNC	June
F189-1 IL 36	Mussel	PNC	June
F173 DG 35	Mussel	PNC	February
F173-4 IL 33	Mussel	PNC	February
F173-4 IL 34	Mussel	PNC	February
F173- 4 G 33	Mussel	PNC	February
F173-4 IL 35	Mussel	PNC	February
F173-4 IL 36	Mussel	PNC	February
F173-4 IL 37	Mussel	PNC	February
F173-4 IL 38	Mussel	PNC	February
F173-3 DG 35	Mussel	PNC	February
F173-3 DG 37	Mussel	PNC	February
F173- 4 G 36	Mussel	PNC	February
F173- 4 G 37	Mussel	PNC	February
F173- 4 G 38	Mussel	PNC	February
F173-3 DG 33	Mussel	PNC	February
F173-3 DG 34	Mussel	PNC	February
F173-3 DG 36	Mussel	PNC	February
F173-3 DG 38	Mussel	PNC	February
F173-4 DG 33	Mussel	PNC	February
F173-4 DG 34	Mussel	PNC	February
F173-4 DG 35	Mussel	PNC	February
F173-4 DG 36	Mussel	PNC	February
F173-4 DG 37	Mussel	PNC	February
F173-4 DG 38	Mussel	PNC	February
F173- 4 G 34	Mussel	PNC	February
F173- 4 G 35	Mussel	PNC	February
F173 F 37	Mussel	PNC	February
F189-3 DG 35	Mussel	PNC	June
F173 DG 33	Mussel	PNC	February
F189-3 DG 33	Mussel	PNC	June
F189-3 F 34	Mussel	PNC	June
F189-3 F 36	Mussel	PNC	June
F173 IL 331	Mussel	PNC	February
F173-3 F 38	Mussel	PNC	February
F189-1 IL 33	Mussel	PNC	June
F189-2 F 36	Mussel	PNC	June
F174-2 IL 35	Oyster	PNC	February
F173 G 35	Mussel	PNC	February
F173 G 36	Mussel	PNC	February
F173 G 37	Mussel	PNC	February
F173 DG 34	Mussel	PNC	February
F173 F 35	Mussel	PNC	February
F173-1 DG 35	Mussel	PNC	February
F173-1 DG 37	Mussel	PNC	February
F173 F 36	Mussel	PNC	February
F173-1 DG 33	Mussel	PNC	February
F173-1 DG 34	Mussel	PNC	February
F174-3 DG 33	Oyster	PNC	February
F174-3 DG 34	Oyster	PNC	February
F173 DG 36	Mussel	PNC	February
F173-1 DG 36	Mussel	PNC	February
F189-3 F 33	Mussel	PNC	June
F173 G 34	Mussel	PNC	February
F190-2 IL 36	Oyster	PNC	June
F190-2 IL 38	Oyster	PNC	June
F174 G 17	Oyster	PNC	February
F174 G 21	Oyster	PNC	February
F190-1 IL 35	Oyster	PNC	June
F173 G 36	Mussel	PNC	February
F173 G 37	Mussel	PNC	February
F173 DG 34	Mussel	PNC	February

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Masó



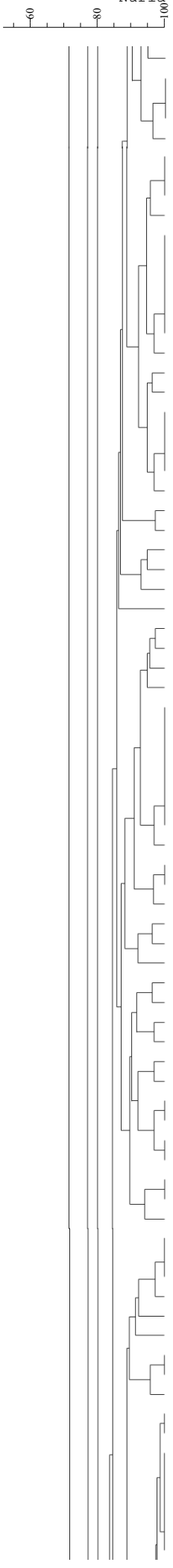
F170-3 G 19	Oyster	PNC	January
F170-3 IL 18	Oyster	PNC	January
F169-1 IL 18	Mussel	PNC	January
F181-3 DG 17	Mussel	PNC	April
F170 DG 17	Oyster	PNC	January
F170 DG 18	Oyster	PNC	January
F170 F 18	Oyster	PNC	January
F170 F 19	Oyster	PNC	January
F170 F 20	Oyster	PNC	January
F170-1 IL 17	Oyster	PNC	January
F169-1 F 18	Mussel	PNC	January
F169-1 IL 19	Mussel	PNC	January
F178-4 IL 17	Oyster	PNC	March
F190-3 DG 18	Oyster	PNC	June
F169 IL 17	Mussel	PNC	January
W145-33	Water	Bay	May
F174 F 22	Oyster	PNC	February
F179-3 IL 33	Mussel	Bay	April
F179-1 DG 37	Mussel	Bay	April
F179-3 IL 34	Mussel	Bay	April
F179-1 DG 33	Mussel	Bay	April
F179-1 DG 34	Mussel	Bay	April
F179-1 DG 38	Mussel	Bay	April
F179-1 DG 35	Mussel	Bay	April
F179-1 DG 36	Mussel	Bay	April
F183-2 IL 35	Mussel	Bay	May
F183-2 IL 36	Mussel	Bay	May
F183-2 IL 37	Mussel	Bay	May
F183-2 IL 33	Mussel	Bay	May
F183-2 IL 34	Mussel	Bay	May
F183-2 IL 38	Mussel	Bay	May
F169-3 DG 17	Mussel	PNC	January
F169-3 DG 18	Mussel	PNC	January
F169-3 F 33	Mussel	PNC	January
F169-3 F 34	Mussel	PNC	January
F169-3 F 35	Mussel	PNC	January
F169-3 IL 17	Mussel	PNC	January
F169-3 IL 18	Mussel	PNC	January
F169-4 IL 17	Mussel	PNC	January
F169-4 IL 18	Mussel	PNC	January
F169-4 IL 19	Mussel	PNC	January
F169-4 IL 33	Mussel	PNC	January
F169-4 IL 34	Mussel	PNC	January
F169-4 IL 35	Mussel	PNC	January
F178-4 G 20	Oyster	PNC	March
F178-4 IL 22	Oyster	PNC	March
F170-4 IL 18	Oyster	PNC	January
F170-4 IL 19	Oyster	PNC	January
F170-4 DG 17	Oyster	PNC	January
F170-4 IL 20	Oyster	PNC	January
W135-36	Water	Bay	February
W135-37	Water	Bay	February
W135-35	Water	Bay	February
F169-2 DG 17	Mussel	PNC	January
F169-2 G 18	Mussel	PNC	January
F169-2 G 19	Mussel	PNC	January
F169-2 G 33	Mussel	PNC	January
F169-2 G 34	Mussel	PNC	January
F174-1 IL 34	Oyster	PNC	February
F178-1 DG 18	Oyster	PNC	March
F177 DG 21	Mussel	PNC	March
F169-3 IL 19	Mussel	PNC	January
F169-3 IL 20	Mussel	PNC	January
F169-3 IL 33	Mussel	PNC	January
F190-2 IL 17	Oyster	PNC	June
F190-2 IL 18	Oyster	PNC	June
F173-3 G 21	Mussel	PNC	February
W134-18	Water	PNC	January
W134-19	Water	PNC	January
W134-17	Water	PNC	January
F174-2 IL 20	Oyster	PNC	February
F174-2 IL 34	Oyster	PNC	February
F174-2 IL 36	Oyster	PNC	February
F169-4 DG 19	Mussel	PNC	January
F178-2 IL 18	Oyster	PNC	March
F178-2 IL 19	Oyster	PNC	March

A. butzleri continuation



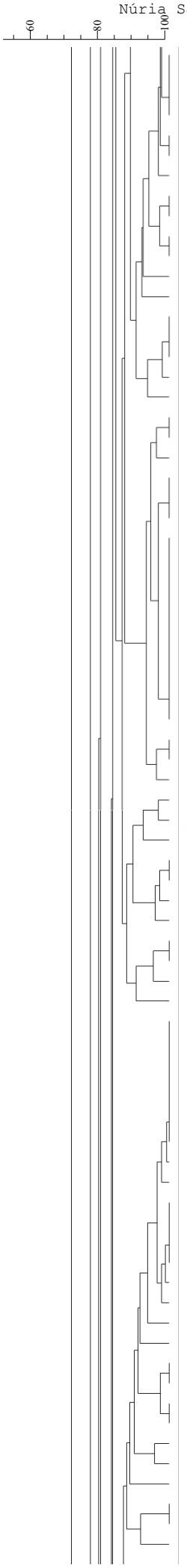
F174-2 DG 34	Oyster	PNC	February
F174-2 DG 35	Oyster	PNC	February
F174-2 DG 36	Oyster	PNC	February
F181-F 19	Mussel	PNC	April
F181-F 22	Mussel	PNC	April
F181-F 17	Mussel	PNC	April
F174 G 18	Oyster	PNC	February
F174-3 F 22	Oyster	PNC	February
F174-3 F 19	Oyster	PNC	February
F173-1 DG 21	Mussel	PNC	February
F173-1 DG 22	Mussel	PNC	February
F190-1 DG 22	Oyster	PNC	June
F169-4 DG 20	Mussel	PNC	January
F174 IL 21	Oyster	PNC	February
F173 IL 22	Mussel	PNC	February
F173-1 F 19	Mussel	PNC	February
F169-4 F 17	Mussel	PNC	January
F174 G 19	Oyster	PNC	February
F174-2 DG 19	Oyster	PNC	February
F174-2 DG 20	Oyster	PNC	February
F174-2 DG 21	Oyster	PNC	February
F173-3 IL 17	Mussel	PNC	February
F174-3 DG 18	Oyster	PNC	February
F174-3 DG 19	Oyster	PNC	February
F173-4 IL 20	Mussel	PNC	February
F174-2 F 21	Oyster	PNC	February
F169-4 DG 17	Mussel	PNC	January
F189-3 F 18	Mussel	PNC	June
F190-1 G 18	Oyster	PNC	June
F190-1 G 20	Oyster	PNC	June
F190-1 G 17	Oyster	PNC	June
F190-2 DG 19	Oyster	PNC	June
F174-3 IL 17	Oyster	PNC	February
F174-3 IL 19	Oyster	PNC	February
F173 G 18	Mussel	PNC	February
F173 G 22	Mussel	PNC	February
F169 IL 19	Mussel	PNC	January
F174 IL 19	Oyster	PNC	February
F177-3 F 17	Mussel	PNC	March
F181-G 21	Mussel	PNC	April
F178 IL 19	Oyster	PNC	March
W137-21	Water	PNC	February
F177 IL 171	Mussel	PNC	March
F177 IL 191	Mussel	PNC	March
F181-IL 20	Mussel	PNC	April
F178 G 17	Oyster	PNC	March
F178 G 18	Oyster	PNC	March
F178 G 20	Oyster	PNC	March
F178 G 21	Oyster	PNC	March
F177-3 IL 18	Mussel	PNC	March
F177-3 IL 19	Mussel	PNC	March
F177-3 IL 20	Mussel	PNC	March
F177-4 IL 19	Mussel	PNC	March
F177-4 IL 22	Mussel	PNC	March
F177-4 IL 18	Mussel	PNC	March
F190-2 F 21	Oyster	PNC	June
F177-3 DG 21	Mussel	PNC	March
F181-G 17	Mussel	PNC	April
W133-25	Water	Bay	January
W133-32	Water	Bay	January
W133-28	Water	Bay	January
W133-29	Water	Bay	January
W133-30	Water	Bay	January
F169-4 IL 17	Mussel	PNC	January
F173-2 G 17	Mussel	PNC	February
W133-26	Water	Bay	January
W133-27	Water	Bay	January
F190-4 DG 22	Oyster	PNC	June
W133-31	Water	Bay	January
F173-2 F 21	Mussel	PNC	February
F177-4 DG 19	Mussel	PNC	March
F174-4 G 35	Oyster	PNC	February
F174-4 G 36	Oyster	PNC	February
W140-171	Water	PNC	March

A. butzleri continuation



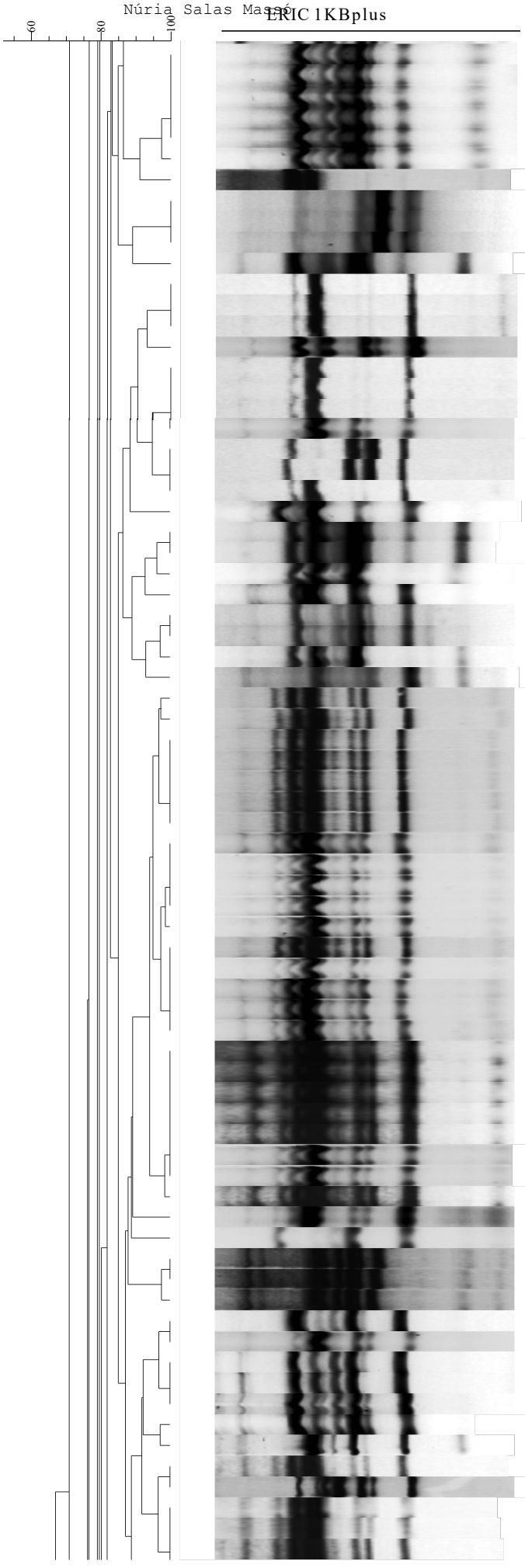
F190-F 20	Oyster	PNC	June
F177-4 DG 17	Mussel	PNC	March
F177-4 DG 18	Mussel	PNC	March
F189-4 F20	Mussel	PNC	June
F189-IL 19	Mussel	PNC	June
W133-21	Water	Bay	January
W133-22	Water	Bay	January
W133-23	Water	Bay	January
F181 IL 181	Mussel	PNC	April
F170-1 DG 17	Oyster	PNC	January
F170-1 F 17	Oyster	PNC	January
F174-1 DG 18	Oyster	PNC	February
F174-1 DG 20	Oyster	PNC	February
F189-4 F22	Mussel	PNC	June
F181 IL 171	Mussel	PNC	April
F178-4 IL 20	Oyster	PNC	March
F173-2 IL 20	Mussel	PNC	February
W133-20	Water	Bay	January
F174-3 IL 22	Oyster	PNC	February
W133-17	Water	Bay	January
W133-18	Water	Bay	January
W133-19	Water	Bay	January
F174-4 G 17	Oyster	PNC	February
F173-2 IL 19	Mussel	PNC	February
F177-3 DG 22	Mussel	PNC	March
F173-1 G 22	Mussel	PNC	February
F190-3 G 21	Oyster	PNC	June
F174-4 G 20	Oyster	PNC	February
W137-17	Water	PNC	February
F190-4 G 20	Oyster	PNC	June
F190-F 17	Oyster	PNC	June
F190-F 21	Oyster	PNC	June
F190-F 19	Oyster	PNC	June
F173-2 IL 22	Mussel	PNC	February
F174-3 F 21	Oyster	PNC	February
F181-4 G 18	Mussel	PNC	April
F181-4 G 19	Mussel	PNC	April
F181-4 G 20	Mussel	PNC	April
F181-4 G 21	Mussel	PNC	April
F181-4 G 22	Mussel	PNC	April
F178-2 DG 18	Oyster	PNC	March
F174-4 G 33	Oyster	PNC	February
F189-DG 17	Mussel	PNC	June
F177 F 20	Mussel	PNC	March
F173-2 F 18	Mussel	PNC	February
F174 IL 17	Oyster	PNC	February
F178-1 IL 21	Oyster	PNC	March
F173-3 IL 20	Mussel	PNC	February
F174 G 20	Oyster	PNC	February
F174 IL 22	Oyster	PNC	February
F177 IL 20	Mussel	PNC	March
F169-4 IL 19	Mussel	PNC	January
F178-2 DG 17	Oyster	PNC	March
F178-2 G 17	Oyster	PNC	March
F178-4 DG 17	Oyster	PNC	March
F173-2 F 17	Mussel	PNC	February
F173-2 F 20	Mussel	PNC	February
F174-2 F 17	Oyster	PNC	February
F189-3 F 17	Mussel	PNC	June
F174 IL 18	Oyster	PNC	February
F173-2 G 19	Mussel	PNC	February
F173-2 G 20	Mussel	PNC	February
F173-2 G 22	Mussel	PNC	February
F177-2 G 17	Mussel	PNC	March
F174-2 IL 17	Oyster	PNC	February
F174-1 DG 17	Oyster	PNC	February
F173-3 F 19	Mussel	PNC	February
F173-3 F 20	Mussel	PNC	February
F174-4 IL 17	Oyster	PNC	February
F190-1 DG 19	Oyster	PNC	June
F190-2 F 19	Oyster	PNC	June
F189-4 IL 17	Mussel	PNC	June
F190-2 F 20	Oyster	PNC	June
F189-4 IL 20	Mussel	PNC	June
F190-1 DG 20	Oyster	PNC	June
F190-1 DG 21	Oyster	PNC	June
F190-2 F 18	Oyster	PNC	June

A. butzleri continuation



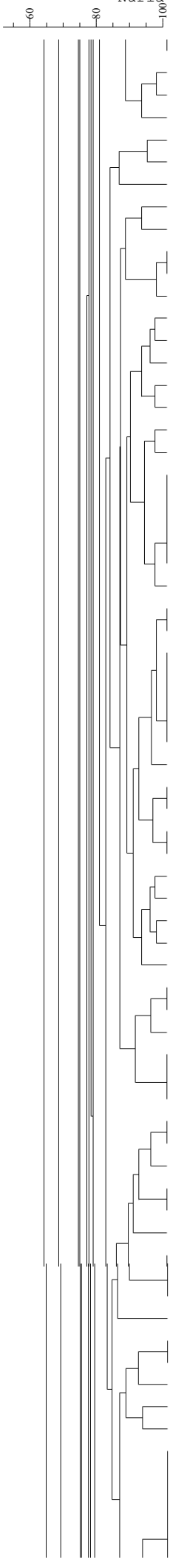
F189-4 IL 18	Mussel	PNC	June
F189-4 IL 19	Mussel	PNC	June
F189-4 IL 21	Mussel	PNC	June
F189-4 IL 22	Mussel	PNC	June
F190-3 IL 17	Oyster	PNC	June
F190-3 IL 18	Oyster	PNC	June
F190-1 G 19	Oyster	PNC	June
F169-4 IL 18	Mussel	PNC	January
F190-1 G 21	Oyster	PNC	June
F169-4 G 17	Mussel	PNC	January
F174-4 G 19	Oyster	PNC	February
F190-2 DG 18	Oyster	PNC	June
F190-3 IL 20	Oyster	PNC	June
F189-1 F 19	Mussel	PNC	June
F189-1 F 21	Mussel	PNC	June
F190-1 DG 18	Oyster	PNC	June
F190-3 F 17	Oyster	PNC	June
F169-4 F 18	Mussel	PNC	January
F190-4 DG 21	Oyster	PNC	June
F190-4 G 22	Oyster	PNC	June
F190-4 G 21	Oyster	PNC	June
F190-4 DG 18	Oyster	PNC	June
F190-4 DG 20	Oyster	PNC	June
F190-4 DG 19	Oyster	PNC	June
F189-1 IL 18	Mussel	PNC	June
F189-1 IL 20	Mussel	PNC	June
F189-1 IL 21	Mussel	PNC	June
F189-1 IL 22	Mussel	PNC	June
F190-4 IL 17	Oyster	PNC	June
F190-4 IL 18	Oyster	PNC	June
F190-4 IL 19	Oyster	PNC	June
F190-4 IL 20	Oyster	PNC	June
F190-4 IL 21	Oyster	PNC	June
F190-4 IL 22	Oyster	PNC	June
F190-4 G 19	Oyster	PNC	June
F190-DG 20	Oyster	PNC	June
F190-2 DG 17	Oyster	PNC	June
F189-DG 21	Mussel	PNC	June
F190-3 G 18	Oyster	PNC	June
F190-3 G 20	Oyster	PNC	June
F173-2 F 19	Mussel	PNC	February
F190-3 G 22	Oyster	PNC	June
F174-3 IL 21	Oyster	PNC	February
F189-IL 20	Mussel	PNC	June
F173-3 F 17	Mussel	PNC	February
F173-3 F 18	Mussel	PNC	February
F190-2 G 17	Oyster	PNC	June
F189-3 IL 18	Mussel	PNC	June
F173 F 17	Mussel	PNC	February
F173 F 19	Mussel	PNC	February
F173 F 22	Mussel	PNC	February
F173-1 DG 17	Mussel	PNC	February
F173-1 DG 18	Mussel	PNC	February
F173-1 DG 19	Mussel	PNC	February
F173-1 DG 20	Mussel	PNC	February
F173-4 DG 19	Mussel	PNC	February
F173-1 IL 20	Mussel	PNC	February
F174-4 IL 18	Oyster	PNC	February
F174-4 IL 19	Oyster	PNC	February
F174-4 IL 20	Oyster	PNC	February
F174-4 IL 22	Oyster	PNC	February
F174-4 IL 21	Oyster	PNC	February
F174-3 DG 17	Oyster	PNC	February
F178-1 IL 17	Oyster	PNC	March
F190-3 IL 19	Oyster	PNC	June
F189-DG 18	Mussel	PNC	June
F189-DG 20	Mussel	PNC	June
F173-1 IL 17	Mussel	PNC	February
F173-1 IL 18	Mussel	PNC	February
F174-3 F 18	Oyster	PNC	February
F174-3 F 20	Oyster	PNC	February
F177 IL 17	Mussel	PNC	March
F173-4 DG 21	Mussel	PNC	February
F173-4 DG 22	Mussel	PNC	February
F173-1 G 21	Mussel	PNC	February

A. butzleri continuation



F173- 4 G 18	Mussel	PNC	February
F173- 4 G 19	Mussel	PNC	February
F173- 4 G 20	Mussel	PNC	February
F173- 4 G 21	Mussel	PNC	February
F173- 4 G 22	Mussel	PNC	February
F173- 4 G 17	Mussel	PNC	February
F178-3 F 17	Oyster	PNC	March
F170-3 DG 17	Oyster	PNC	January
F170-3 DG 18	Oyster	PNC	January
F170-3 DG 19	Oyster	PNC	January
F173 IL 18	Mussel	PNC	February
F177-4 G 20	Mussel	PNC	March
F177-4 G 21	Mussel	PNC	March
F177-4 G 22	Mussel	PNC	March
F190-1 IL 22	Oyster	PNC	June
F177-4 G 17	Mussel	PNC	March
F177-4 G 18	Mussel	PNC	March
F177-4 G 19	Mussel	PNC	March
F181-IL 19	Mussel	PNC	April
F177-2 DG 20	Mussel	PNC	March
F177-2 DG 22	Mussel	PNC	March
F177-4 IL 20	Mussel	PNC	March
F173-4 DG 17	Mussel	PNC	February
F173 IL 19	Mussel	PNC	February
F173 IL 21	Mussel	PNC	February
F173-4 IL 21	Mussel	PNC	February
F173-3 DG 22	Mussel	PNC	February
F173 F 20	Mussel	PNC	February
F173 F 21	Mussel	PNC	February
F173-3 IL 21	Mussel	PNC	February
F169-4 DG 18	Mussel	PNC	January
F178-1 F 21	Oyster	PNC	March
F178-2 IL 20	Oyster	PNC	March
F178-1 F 17	Oyster	PNC	March
F178-1 F 18	Oyster	PNC	March
F178-1 F 19	Oyster	PNC	March
F178-1 F 22	Oyster	PNC	March
F178-2 IL 17	Oyster	PNC	March
F189-1 F 18	Mussel	PNC	June
F189-2 IL 20	Mussel	PNC	June
F189-2 IL 18	Mussel	PNC	June
F189-2 IL 19	Mussel	PNC	June
F189-2 IL 21	Mussel	PNC	June
F178-1 IL 22	Oyster	PNC	March
F189-2 IL 17	Mussel	PNC	June
F189-1 F 17	Mussel	PNC	June
F189-1 F 20	Mussel	PNC	June
F189-1 F 22	Mussel	PNC	June
F177 G 17	Mussel	PNC	March
F177 G 18	Mussel	PNC	March
F177 G 19	Mussel	PNC	March
F177 G 20	Mussel	PNC	March
F177 G 21	Mussel	PNC	March
F189-4 F17	Mussel	PNC	June
F189-4 F21	Mussel	PNC	June
F177 G 22	Mussel	PNC	March
W140-181	Water	PNC	March
F181-1 G 19	Mussel	PNC	April
F178-4 G 17	Oyster	PNC	March
F178-4 G 18	Oyster	PNC	March
F178-4 G 19	Oyster	PNC	March
F174-2 DG 18	Oyster	PNC	February
F181-IL 22	Mussel	PNC	April
F173-3 IL 18	Mussel	PNC	February
F173-3 IL 19	Mussel	PNC	February
F173-3 IL 22	Mussel	PNC	February
F173-3 DG 21	Mussel	PNC	February
F174-3 IL 20	Oyster	PNC	February
F177-2 IL 22	Mussel	PNC	March
F178-2 G 18	Oyster	PNC	March
F177-2 IL 17	Mussel	PNC	March
F177-2 IL 18	Mussel	PNC	March
F177-2 IL 19	Mussel	PNC	March

A. butzleri continuation



F177-2 IL 20	Mussel	PNC	March
F173-1 G 18	Mussel	PNC	February
F173-1 G 19	Mussel	PNC	February
F174-1 DG 19	Oyster	PNC	February
F178-3 DG 17	Oyster	PNC	March
F178-4 DG 20	Oyster	PNC	March
F174 F 19	Oyster	PNC	February
F173-2 G 21	Mussel	PNC	February
F174-2 DG 22	Oyster	PNC	February
F177 F 17	Mussel	PNC	March
F177 F 18	Mussel	PNC	March
F177 F 19	Mussel	PNC	March
F173-2 DG 17	Mussel	PNC	February
F173-2 DG 19	Mussel	PNC	February
F173-2 IL 17	Mussel	PNC	February
F173-2 DG 21	Mussel	PNC	February
F173-2 F 22	Mussel	PNC	February
F169 G 17	Mussel	PNC	January
F177-1 IL 18	Mussel	PNC	March
F169 G 18	Mussel	PNC	January
F169 G 19	Mussel	PNC	January
F170-3 DG 20	Oyster	PNC	January
W134-21	Water	PNC	January
W134-22	Water	PNC	January
W134-33	Water	PNC	January
F178 F 18	Oyster	PNC	March
F178 F 19	Oyster	PNC	March
F169-2 IL 18	Mussel	PNC	January
F173-3 F 21	Mussel	PNC	February
F173-3 F 22	Mussel	PNC	February
F178 F 17	Oyster	PNC	March
F178 F 20	Oyster	PNC	March
F170-3 F 18	Oyster	PNC	January
F189-F 20	Mussel	PNC	June
F190-2 F 17	Oyster	PNC	June
F170 G 17	Oyster	PNC	January
F178-4 IL 18	Oyster	PNC	March
F189-3 F 21	Mussel	PNC	June
F189-3 F 22	Mussel	PNC	June
F169 DG 34	Mussel	PNC	January
F189-3 F 19	Mussel	PNC	June
F178 IL 191	Oyster	PNC	March
F170-1 G18	Oyster	PNC	January
F177-2 G 18	Mussel	PNC	March
F170-4 IL 19	Oyster	PNC	January
F177-2 G 20	Mussel	PNC	March
F177-2 G 21	Mussel	PNC	March
F177-2 G 22	Mussel	PNC	March
F173-2 DG 18	Mussel	PNC	February
F173-2 DG 20	Mussel	PNC	February
F173-2 DG 22	Mussel	PNC	February
F178-4 F 18	Oyster	PNC	March
F178-4 F 21	Oyster	PNC	March
F177-2 DG 17	Mussel	PNC	March
F181-2 F 20	Mussel	PNC	April
F181-2 F 21	Mussel	PNC	April
F181-2 F 22	Mussel	PNC	April
F178 G 19	Oyster	PNC	March
F189-IL 21	Mussel	PNC	June
F190-3 F 20	Oyster	PNC	June
F190-IL 22	Oyster	PNC	June
F181-IL 18	Mussel	PNC	April
F189-3 F 20	Mussel	PNC	June
F177-3 F 18	Mussel	PNC	March
F177-3 F 19	Mussel	PNC	March
F177-3 F 20	Mussel	PNC	March
F177-3 F 21	Mussel	PNC	March
F177-4 IL 17	Mussel	PNC	March

A. butzleri continuation

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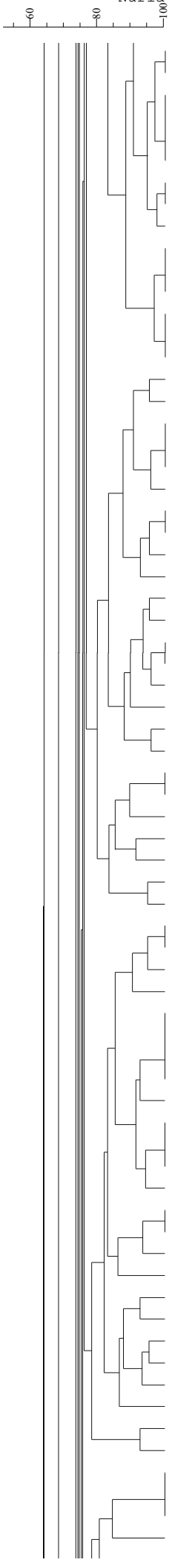
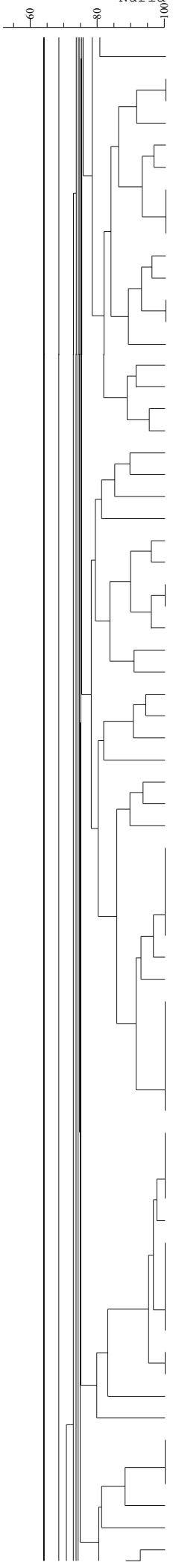
F177 IL 181	Mussel	PNC	March
F177 F 21	Mussel	PNC	March
F190-3 F 18	Oyster	PNC	June
F190-3 F 19	Oyster	PNC	June
F190-3 F 21	Oyster	PNC	June
F190-3 F 22	Oyster	PNC	June
F190-3 DG 21	Oyster	PNC	June
F190-3 DG 22	Oyster	PNC	June
F190-3 DG 17	Oyster	PNC	June
F190-3 DG 19	Oyster	PNC	June
F190-3 DG 20	Oyster	PNC	June
F190-3 G 19	Oyster	PNC	June
F178-4 IL 21	Oyster	PNC	March
F178-4 IL 19	Oyster	PNC	March
F189-3 IL 21	Mussel	PNC	June
F174-1 IL 17	Oyster	PNC	February
F174-1 IL 33	Oyster	PNC	February
F169 DG 33	Mussel	PNC	January
F190-IL 17	Oyster	PNC	June
F190-IL 18	Oyster	PNC	June
F190-IL 19	Oyster	PNC	June
F190-IL 20	Oyster	PNC	June
F190-IL 21	Oyster	PNC	June
W143-181	Water	PNC	April
W143-191	Water	PNC	April
F174-3 G 17	Oyster	PNC	February
F190-4 F 17	Oyster	PNC	June
F190-4 G 18	Oyster	PNC	June
F181-3 DG 20	Mussel	PNC	April
F181-F 20	Mussel	PNC	April
F181-F 21	Mussel	PNC	April
F181-G 18	Mussel	PNC	April
F181-G 19	Mussel	PNC	April
F178-4 F 20	Oyster	PNC	March
F178-4 F 22	Oyster	PNC	March
F181-DG 20	Mussel	PNC	April
F190-3 G 17	Oyster	PNC	June
F181-DG 17	Mussel	PNC	April
F181-DG 19	Mussel	PNC	April
F177-3 DG 19	Mussel	PNC	March
F177-3 DG 20	Mussel	PNC	March
F177-4 IL 21	Mussel	PNC	March
F177-3 DG 17	Mussel	PNC	March
F177-3 DG 18	Mussel	PNC	March
F189-DG 22	Mussel	PNC	June
F173 G 19	Mussel	PNC	February
F173 G 20	Mussel	PNC	February
F173 DG 17	Mussel	PNC	February
F173 DG 18	Mussel	PNC	February
F173 DG 19	Mussel	PNC	February
F173 DG 20	Mussel	PNC	February
F173 G 17	Mussel	PNC	February
F173 DG 21	Mussel	PNC	February
F189-1 IL 17	Mussel	PNC	June
F181 IL 201	Mussel	PNC	April
F181 IL 211	Mussel	PNC	April
F181 IL 191	Mussel	PNC	April
F186 IL 171	Oyster	PNC	May
F186 IL 191	Oyster	PNC	Jul
F186 IL 181	Oyster	PNC	May
F178-3 DG 18	Oyster	PNC	March
F190-1 IL 20	Oyster	PNC	June
F178-3 G 17	Oyster	PNC	March
F178-3 G 18	Oyster	PNC	March
F178-3 G 19	Oyster	PNC	March
F178-3 G 20	Oyster	PNC	March
F178-3 G 21	Oyster	PNC	March
F178-1 IL 18	Oyster	PNC	March
F178-1 IL 19	Oyster	PNC	March

A. butzleri continuation



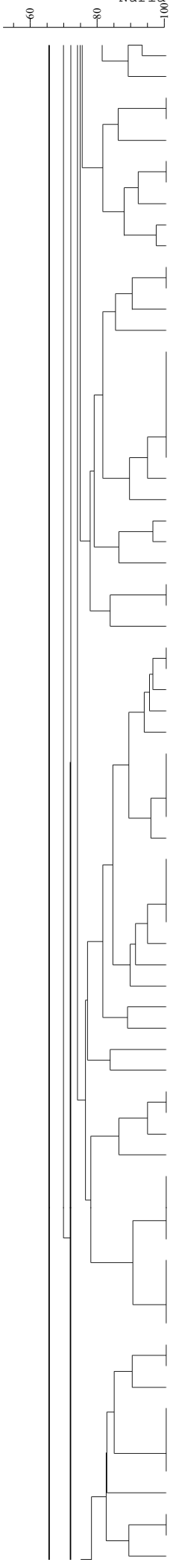
F177-1 DG 18	Mussel	PNC	March
F177-1 DG 22	Mussel	PNC	March
F177-1 DG 17	Mussel	PNC	March
F177-1 DG 19	Mussel	PNC	March
F177-1 DG 20	Mussel	PNC	March
F177-1 DG 21	Mussel	PNC	March
F177 F 22	Mussel	PNC	March
F177-1 G 17	Mussel	PNC	March
F177 IL 19	Mussel	PNC	March
F174-4 DG 19	Oyster	PNC	February
F174-4 DG 20	Oyster	PNC	February
F174-4 DG 22	Oyster	PNC	February
F174-4 DG 17	Oyster	PNC	February
F174-4 DG 18	Oyster	PNC	February
F174-4 DG 21	Oyster	PNC	February
F190-4 F 19	Oyster	PNC	June
F190-F 18	Oyster	PNC	June
F169-1 IL 17	Mussel	PNC	January
F181-G 22	Mussel	PNC	April
F190-4 F 18	Oyster	PNC	June
F190-4 F 22	Oyster	PNC	June
F181-G 20	Mussel	PNC	April
F189-1 IL 19	Mussel	PNC	June
F177 IL 201	Mussel	PNC	March
F181-3 F 21	Mussel	PNC	April
F189-F 17	Mussel	PNC	June
F189-F 22	Mussel	PNC	June
F170 G 18	Oyster	PNC	January
F170 G 19	Oyster	PNC	January
F174-1 IL 18	Oyster	PNC	February
F190-4 F 20	Oyster	PNC	June
F169 DG 19	Mussel	PNC	January
F177-3 IL 21	Mussel	PNC	March
F189-F 19	Mussel	PNC	June
F189-F 21	Mussel	PNC	June
F174-3 G 21	Oyster	PNC	February
F174-2 G 17	Oyster	PNC	February
F177-1 IL 17	Mussel	PNC	March
F178-4 DG 18	Oyster	PNC	March
F178-4 DG 19	Oyster	PNC	March
F170-2 IL 18	Oyster	PNC	January
F181-1 F 17	Mussel	PNC	April
F181-1 F 18	Mussel	PNC	April
F181-1 F 19	Mussel	PNC	April
F181-4 DG 19	Mussel	PNC	April
F181-4 DG 22	Mussel	PNC	April
F181-4 DG 20	Mussel	PNC	April
F181-4 DG 21	Mussel	PNC	April
F178-4 F 17	Oyster	PNC	March
F174 F 36	Oyster	PNC	February
F174-1 G 38	Oyster	PNC	February
F181-2 F 19	Mussel	PNC	April
F170-2 IL 17	Oyster	PNC	January
F169-2 IL 19	Mussel	PNC	January
F170 F 17	Oyster	PNC	January
F177-2 G 19	Mussel	PNC	March
F174-3 IL 18	Oyster	PNC	February
F178 DG 19	Oyster	PNC	March
W137-19	Water	PNC	February
F173-3 G 17	Mussel	PNC	February
F178 DG 17	Oyster	PNC	March
F178 DG 18	Oyster	PNC	March
F190-4 G 17	Oyster	PNC	June
F174 G 22	Oyster	PNC	February
F178 DG 20	Oyster	PNC	March
F174-2 G 19	Oyster	PNC	February
F174-2 G 20	Oyster	PNC	February
F174-2 G 21	Oyster	PNC	February
F174-2 IL 18	Oyster	PNC	February

A. butzleri continuation



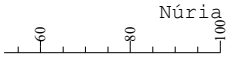
F189-IL 17	Mussel	PNC	June
F189-4 DG 20	Mussel	PNC	June
F189-4 DG 21	Mussel	PNC	June
F189-4 DG 22	Mussel	PNC	June
F173-4 DG 20	Mussel	PNC	February
F178-2 IL 21	Oyster	PNC	March
F189-4 DG 17	Mussel	PNC	June
F189-4 DG 18	Mussel	PNC	June
F189-4 DG 19	Mussel	PNC	June
F189-3 IL 17	Mussel	PNC	June
F189-3 IL 19	Mussel	PNC	June
F189-3 IL 20	Mussel	PNC	June
F189-3 IL 22	Mussel	PNC	June
F177-3 IL 17	Mussel	PNC	March
F173-4 DG 18	Mussel	PNC	February
F181-IL 17	Mussel	PNC	April
F170-3 G 17	Oyster	PNC	January
F170-3 G 18	Oyster	PNC	January
F169-1 IL 20	Mussel	PNC	January
F174-4 G 21	Oyster	PNC	February
F169-1 IL 18	Mussel	PNC	January
F173-4 IL 22	Mussel	PNC	February
F169-1 IL 17	Mussel	PNC	January
F185 IL 201	Mussel	PNC	May
F177-1 IL 19	Mussel	PNC	March
F177-1 IL 20	Mussel	PNC	March
F185 IL 211	Mussel	PNC	May
F170-4 DG 18	Oyster	PNC	January
F173-1 G 17	Mussel	PNC	February
F169-1 F 20	Mussel	PNC	January
F170-2 G17	Oyster	PNC	January
F169-4 F 20	Mussel	PNC	January
W137-20	Water	PNC	February
F167 DG 22	Mussel	Bay	January
F174-1 DG 21	Oyster	PNC	February
F173 DG 22	Mussel	PNC	February
F173 IL 17	Mussel	PNC	February
F173-1 F 17	Mussel	PNC	February
F173-1 F 18	Mussel	PNC	February
F173-1 F 20	Mussel	PNC	February
F173-1 F 21	Mussel	PNC	February
F189-DG 19	Mussel	PNC	June
F173-1 F 22	Mussel	PNC	February
F189-2 DG 17	Mussel	PNC	June
F189-2 DG 18	Mussel	PNC	June
F189-2 DG 19	Mussel	PNC	June
F189-2 DG 20	Mussel	PNC	June
F189-2 DG 21	Mussel	PNC	June
F189-2 DG 22	Mussel	PNC	June
F167 DG 26	Mussel	Bay	January
F167 DG 28	Mussel	Bay	January
F167 DG 29	Mussel	Bay	January
F167 DG 30	Mussel	Bay	January
F167 DG 25	Mussel	Bay	January
F167 DG 19	Mussel	Bay	January
F167 DG 20	Mussel	Bay	January
F167 DG 21	Mussel	Bay	January
F167 DG 23	Mussel	Bay	January
F167 DG 27	Mussel	Bay	January
F167 DG 17	Mussel	Bay	January
F167 DG 18	Mussel	Bay	January
F177 IL 18	Mussel	PNC	March
F170-4 IL 17	Oyster	PNC	January
F178-3 F 18	Oyster	PNC	March
F178-3 F 19	Oyster	PNC	March
F178-3 F 20	Oyster	PNC	March
F169-1 IL 19	Mussel	PNC	January
F189-F 18	Mussel	PNC	June
F174-1 DG 22	Oyster	PNC	February

A. butzleri continuation



F178-1 DG 17	Oyster	PNC	March
F189-IL 22	Mussel	PNC	June
F177 DG 18	Mussel	PNC	March
F177 DG 19	Mussel	PNC	March
F174 IL 211	Oyster	PNC	February
F177-2 DG 18	Mussel	PNC	March
F177-2 DG 19	Mussel	PNC	March
F190-1 IL 19	Oyster	PNC	June
F174-1 IL 21	Oyster	PNC	February
F174-3 F 17	Oyster	PNC	February
F189-4 F18	Mussel	PNC	June
F189-4 F19	Mussel	PNC	June
F181-IL 21	Mussel	PNC	April
F189-IL 18	Mussel	PNC	June
F181-1 G 21	Mussel	PNC	April
F181-1 IL 18	Mussel	PNC	April
F181-1 IL 19	Mussel	PNC	April
F181-1 IL 20	Mussel	PNC	April
F181-1 IL 21	Mussel	PNC	April
F181-1 IL 22	Mussel	PNC	April
F181-1 IL 17	Mussel	PNC	April
F181-1 F 20	Mussel	PNC	April
F173 IL 20	Mussel	PNC	February
F174-4 G 22	Oyster	PNC	February
F173-3 G 20	Mussel	PNC	February
F177-2 F 18	Mussel	PNC	March
F177-2 F 19	Mussel	PNC	March
F177-2 F 17	Mussel	PNC	March
F181-2 F 17	Mussel	PNC	April
F181-2 F 18	Mussel	PNC	April
F169-1 F 19	Mussel	PNC	January
F181 IL 221	Mussel	PNC	April
F173-3 G 19	Mussel	PNC	February
F169-2 DG 18	Mussel	PNC	January
F169-2 F 17	Mussel	PNC	January
F169-3 IL 17	Mussel	PNC	January
F169-3 IL 18	Mussel	PNC	January
F177-2 DG 21	Mussel	PNC	March
F169-2 G 17	Mussel	PNC	January
F169-2 IL 17	Mussel	PNC	January
F169-2 IL 18	Mussel	PNC	January
F169-2 IL 19	Mussel	PNC	January
F169-1 F 17	Mussel	PNC	January
F170-4 F 18	Oyster	PNC	January
F174-1 G 18	Oyster	PNC	February
F190-4 F 21	Oyster	PNC	June
W143-201	Water	PNC	April
F170-4 F 17	Oyster	PNC	January
F181-3 DG 19	Mussel	PNC	April
F169-4 F 19	Mussel	PNC	January
F170-3 IL 17	Oyster	PNC	January
F170 IL 17	Oyster	PNC	January
F190-1 G 22	Oyster	PNC	June
F170-4 IL 17	Oyster	PNC	January
F170-4 IL 18	Oyster	PNC	January
F170-4 IL 20	Oyster	PNC	January
F170-4 IL 33	Oyster	PNC	January
F170-1 G 17	Oyster	PNC	January
F170-1 IL 18	Oyster	PNC	January
F170-1 IL 17	Oyster	PNC	January
F170-1 IL 18	Oyster	PNC	January
F181-3 IL 17	Mussel	PNC	April
F181-3 IL 18	Mussel	PNC	April
F174-1 G 20	Oyster	PNC	February
F181-3 IL 19	Mussel	PNC	April
F181-3 IL 20	Mussel	PNC	April
F181-3 IL 21	Mussel	PNC	April
F181-3 IL 22	Mussel	PNC	April
F180-3 IL 36	Oyster	Bay	April
F180-3 IL 33	Oyster	Bay	April
F180-3 IL 34	Oyster	Bay	April
F180-3 IL 37	Oyster	Bay	April

A. butzleri continuation



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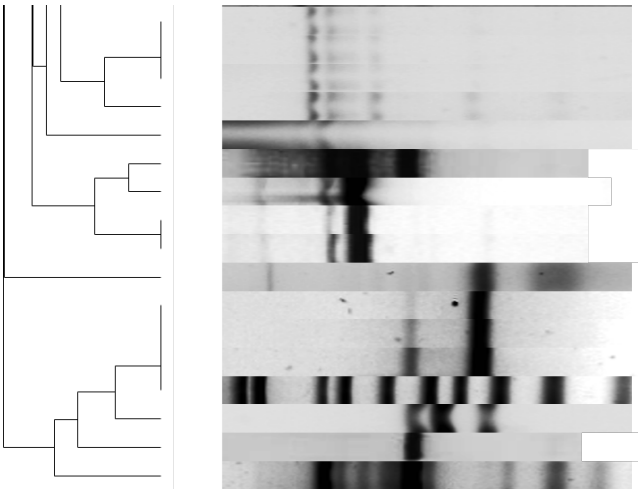
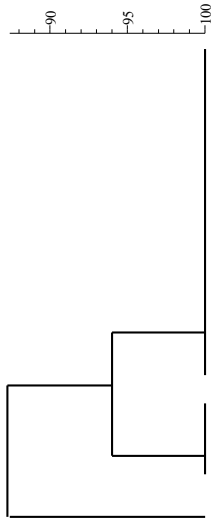


F180-2 F 18	Oyster	Bay	April
F180-2 F 19	Oyster	Bay	April
F180-2 F 20	Oyster	Bay	April
F180-2 F 17	Oyster	Bay	April
F181-1 G 20	Mussel	PNC	April
F178-4 F 19	Oyster	PNC	March
W134-20	Water	PNC	January
F178 IL 17	Oyster	PNC	March
F178 IL 18	Oyster	PNC	March
F190-4 DG 17	Oyster	PNC	June
F173-3 DG 17	Mussel	PNC	February
F173-3 DG 18	Mussel	PNC	February
F173-3 DG 19	Mussel	PNC	February
F173-3 DG 20	Mussel	PNC	February
F181-3 F 18	Mussel	PNC	April
F178 IL 181	Oyster	PNC	March
F173-3 G 22	Mussel	PNC	February

A. molluscorum



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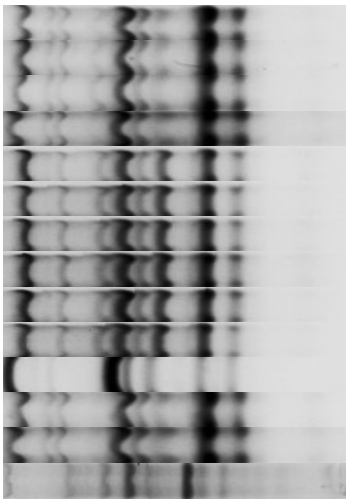
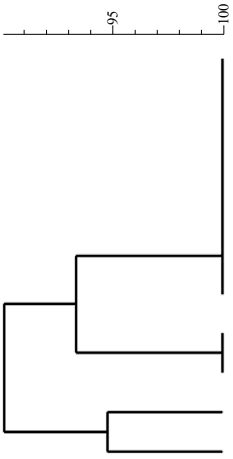


F189-2 IL 34	Mussel	PNC	June
F189-2 IL 35	Mussel	PNC	June
F189-2 IL 36	Mussel	PNC	June
F189-3 DG 34	Mussel	PNC	June
F189-DG 33	Mussel	PNC	June
F189-DG 34	Mussel	PNC	June
F189-DG 35	Mussel	PNC	June
F189-DG 36	Mussel	PNC	June
F189-DG 37	Mussel	PNC	June
F189-DG 38	Mussel	PNC	June
F181-3 IL 35	Mussel	PNC	April
F189-2 IL 37	Mussel	PNC	June
F189-2 IL 38	Mussel	PNC	June
W147-171	Water	Bay	June

"A. ostreae"



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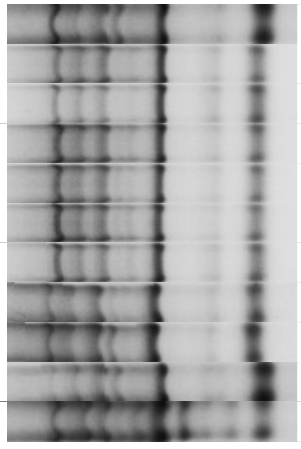

F189-3 DG 36	Mussel	PNC	June
F189-F 33	Mussel	PNC	June
F189-F 34	Mussel	PNC	June
F189-F 35	Mussel	PNC	June
F189-F 36	Mussel	PNC	June
F189-F 37	Mussel	PNC	June
F189-F 38	Mussel	PNC	June
F189-1 DG 33	Mussel	PNC	June
F189-1 DG 34	Mussel	PNC	June
F189-3 DG 37	Mussel	PNC	June
F189-3 DG 38	Mussel	PNC	June

UNIVERSITAT ROVIRA I VIRGILI
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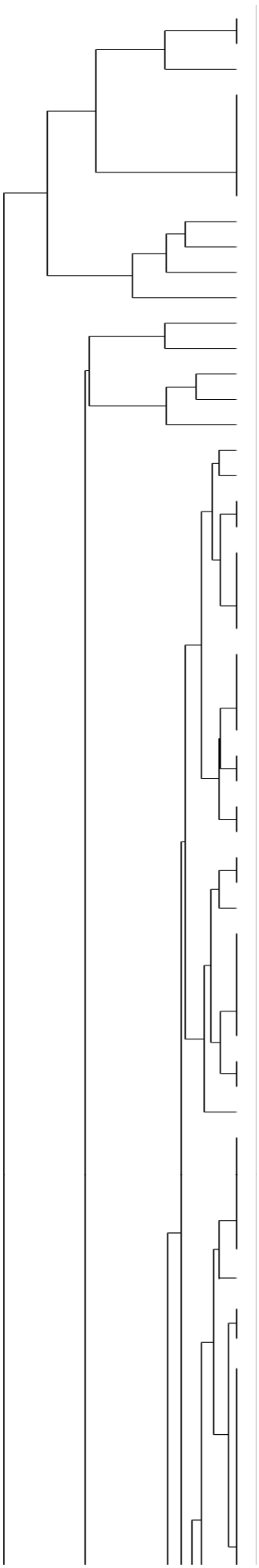
Núria Salas Massó

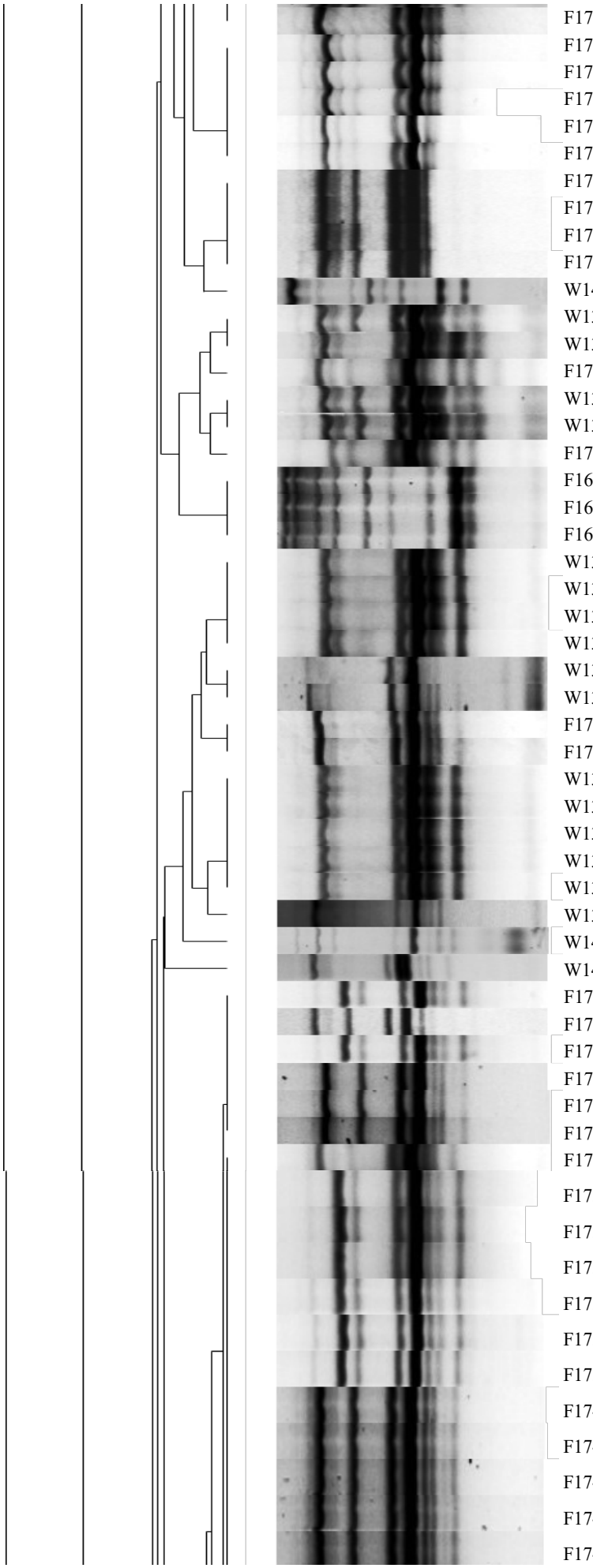
A. marinus

ERIC 1KBplus



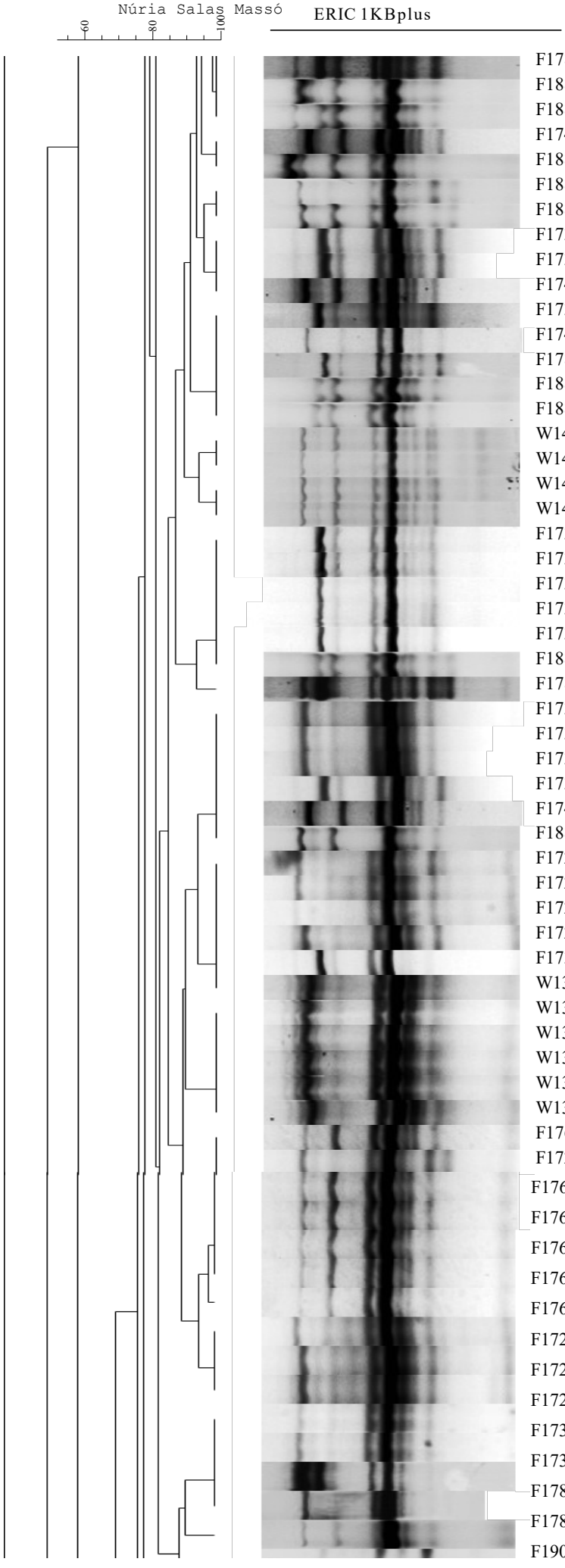
F177-2 IL 36	Mussel	PNC	March
F180-2 IL 33	Oyster	Bay	April
F181-1 IL 36	Mussel	PNC	April
F173-3 G 34	Mussel	PNC	February
F180-2 IL 34	Oyster	Bay	April
F180-2 IL 35	Oyster	Bay	April
F181-1 IL 33	Mussel	PNC	April
F181-1 IL 37	Mussel	PNC	April
F173-3 G 38	Mussel	PNC	February
W137-37	Water	PNC	February
W137-331	Water	PNC	February
F173-3 G 33	Mussel	PNC	February
F177-1 DG 35	Mussel	PNC	March
F185 IL 171	Mussel	PNC	May
F181-1 DG 18	Mussel	PNC	April
F181-1 DG 19	Mussel	PNC	April
F190-1 IL 37	Oyster	PNC	June
F172-4 IL 33	Oyster	Bay	February
W132-46	Water	Bay	January
F178-3 G 33	Oyster	PNC	March
F178-3 G 34	Oyster	PNC	March
F177 IL 33	Mussel	PNC	March
F177 IL 34	Mussel	PNC	March
F177 IL 35	Mussel	PNC	March
F177 IL 36	Mussel	PNC	March
F178-4 G 33	Oyster	PNC	March
F178-4 G 37	Oyster	PNC	March
F178-4 G 35	Oyster	PNC	March
F178-4 G 36	Oyster	PNC	March
F172-4 IL 34	Oyster	Bay	February
F178-4 G 34	Oyster	PNC	March
F188-IL 34	Oyster	Bay	June
F188-IL 36	Oyster	Bay	June
F177 IL 38	Mussel	PNC	March
F188-1 IL 34	Oyster	Bay	June
F178-4 IL 34	Oyster	PNC	March
F178-3 IL 33	Oyster	PNC	March
F178-3 IL 34	Oyster	PNC	March
F178-3 IL 35	Oyster	PNC	March
F178-3 IL 36	Oyster	PNC	March
F178-3 IL 37	Oyster	PNC	March
F177 IL 37	Mussel	PNC	March
F188-3 IL 33	Oyster	Bay	June
F188-DG 35	Oyster	Bay	June
F172- 3 IL 34	Oyster	Bay	February
F172- 3 IL 35	Oyster	Bay	February
F172 IL 341	Oyster	Bay	February
F172 IL 351	Oyster	Bay	February
F172 IL 371	Oyster	Bay	February
F188-DG 34	Oyster	Bay	June
F178-4 IL 36	Oyster	PNC	March
F188-F 33	Oyster	Bay	June
F178-1 G 36	Oyster	PNC	March
F178-1 G 38	Oyster	PNC	March
F188-F 34	Oyster	Bay	June
F188-F 35	Oyster	Bay	June
F188-F 36	Oyster	Bay	June
F188-F 37	Oyster	Bay	June
F188-F 38	Oyster	Bay	June

A. marinus continuation



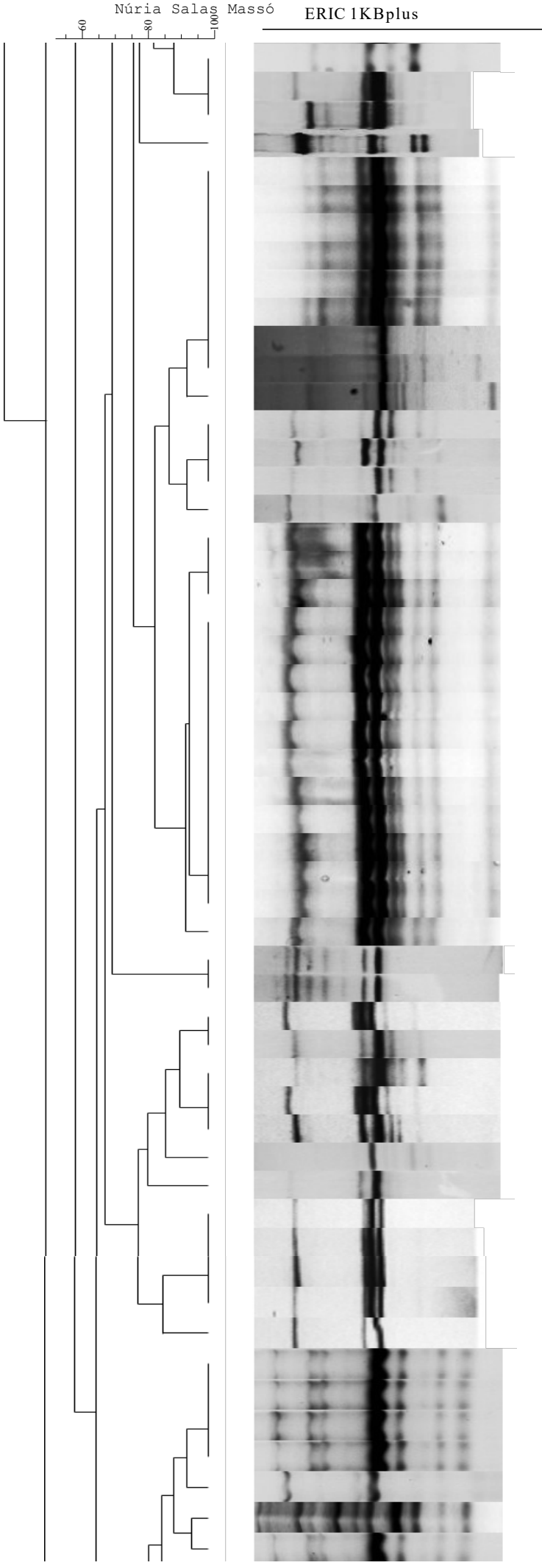
F174 IL 371	Oyster	PNC	February
F173-2 F 33	Mussel	PNC	February
F173-2 F 34	Mussel	PNC	February
F173-2 F 35	Mussel	PNC	February
F173-2 F 36	Mussel	PNC	February
F173-2 F 37	Mussel	PNC	February
F177-2 IL 33	Mussel	PNC	March
F177-2 IL 34	Mussel	PNC	March
F177-2 IL 35	Mussel	PNC	March
F177-2 IL 37	Mussel	PNC	March
W148-331	Water	Bay	June
W132-34	Water	Bay	January
W132-43	Water	Bay	January
F172 IL 331	Oyster	Bay	February
W132-42	Water	Bay	January
W132-47	Water	Bay	January
F172-4 F 33	Oyster	Bay	February
F167 F 37	Mussel	Bay	January
F167 F 38	Mussel	Bay	January
F167 F 39	Mussel	Bay	January
W133-43	Water	Bay	January
W133-44	Water	Bay	January
W133-45	Water	Bay	January
W133-46	Water	Bay	January
W137-361	Water	PNC	February
W137-371	Water	PNC	February
F174 IL 331	Oyster	PNC	February
F174 IL 341	Oyster	PNC	February
W133-33	Water	Bay	January
W133-35	Water	Bay	January
W133-36	Water	Bay	January
W133-41	Water	Bay	January
W133-42	Water	Bay	January
W139-36	Water	Bay	March
W148-341	Water	Bay	June
W142-33	Water	Bay	April
F173-1 IL 35	Mussel	PNC	February
F177-3 IL 34	Mussel	PNC	March
F173-1 IL 36	Mussel	PNC	February
F174-4 F 34	Oyster	PNC	February
F174-4 F 35	Oyster	PNC	February
F174-4 F 37	Oyster	PNC	February
F173 IL 34	Mussel	PNC	February
F173-1 F 34	Mussel	PNC	February
F173-1 F 36	Mussel	PNC	February
F173-1 F 37	Mussel	PNC	February
F173-1 IL 34	Mussel	PNC	February
F173-1 IL 37	Mussel	PNC	February
F173-1 IL 38	Mussel	PNC	February
F174-4 IL 33	Oyster	PNC	February
F174-4 IL 34	Oyster	PNC	February
F174-4 IL 35	Oyster	PNC	February
F174-4 IL 36	Oyster	PNC	February
F174-4 IL 38	Oyster	PNC	February

A. marinus continuation



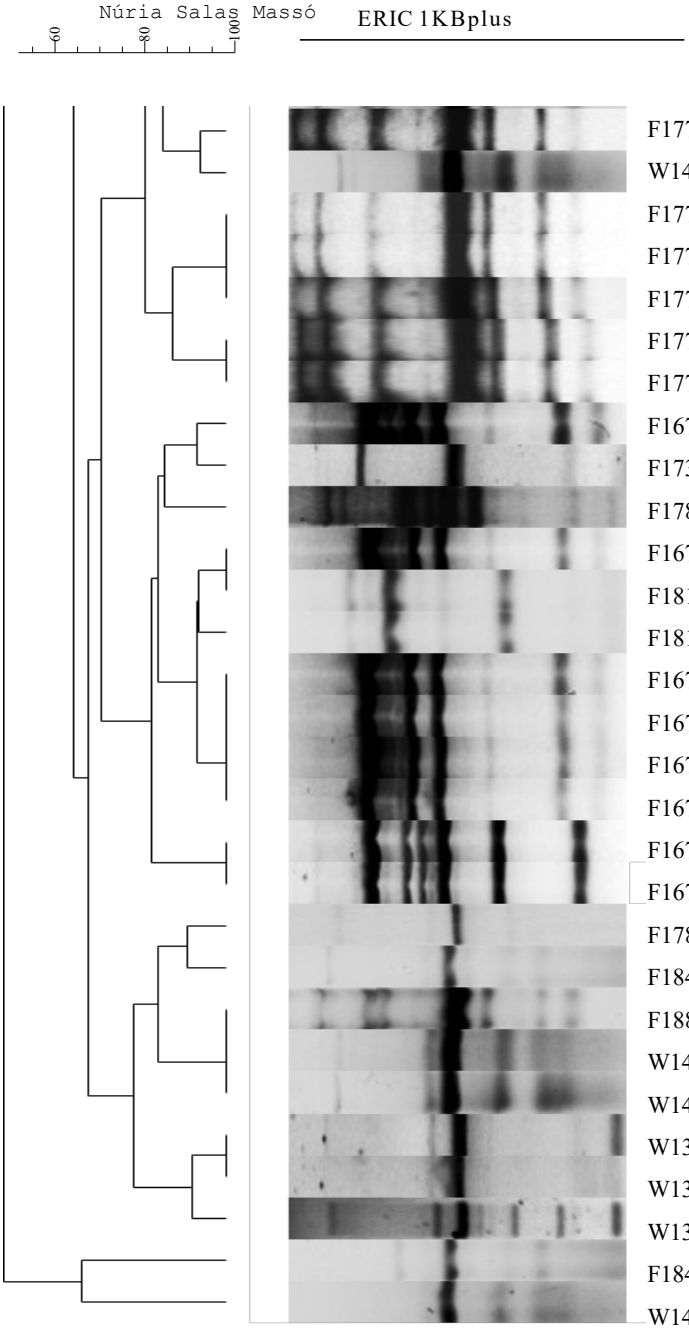
F178-4 IL 37	Oyster	PNC	March
F188-DG 36	Oyster	Bay	June
F188-DG 37	Oyster	Bay	June
F174-4 F 38	Oyster	PNC	February
F188-IL 33	Oyster	Bay	June
F188-4 DG 34	Oyster	Bay	June
F188-DG 33	Oyster	Bay	June
F173-1 F 33	Mussel	PNC	February
F173-1 F 38	Mussel	PNC	February
F174-4 IL 37	Oyster	PNC	February
F173-3 IL 38	Mussel	PNC	February
F174-4 F 33	Oyster	PNC	February
F178-2 G 33	Oyster	PNC	March
F188-DG 38	Oyster	Bay	June
F188-IL 37	Oyster	Bay	June
W148-371	Water	Bay	June
W148-381	Water	Bay	June
W148-351	Water	Bay	June
W148-361	Water	Bay	June
F173-3 IL 33	Mussel	PNC	February
F173-3 IL 34	Mussel	PNC	February
F173-3 IL 35	Mussel	PNC	February
F173-3 IL 36	Mussel	PNC	February
F173-3 IL 37	Mussel	PNC	February
F188-IL 38	Oyster	Bay	June
F178-4 IL 33	Oyster	PNC	March
F173 IL 33	Mussel	PNC	February
F173 IL 35	Mussel	PNC	February
F173 IL 36	Mussel	PNC	February
F173-1 F 35	Mussel	PNC	February
F174-4 F 36	Oyster	PNC	February
F188-4 DG 33	Oyster	Bay	June
F172- 3 IL 33	Oyster	Bay	February
F172- 3 IL 36	Oyster	Bay	February
F172- 3 IL 37	Oyster	Bay	February
F172-4 IL 35	Oyster	Bay	February
F173-2 F 38	Mussel	PNC	February
W132-44	Water	Bay	January
W132-33	Water	Bay	January
W132-35	Water	Bay	January
W132-36	Water	Bay	January
W132-41	Water	Bay	January
W132-45	Water	Bay	January
F176 IL 33	Oyster	Bay	March
F172 IL 361	Oyster	Bay	February
F176 IL 34	Oyster	Bay	March
F176 IL 35	Oyster	Bay	March
F176 IL 36	Oyster	Bay	March
F176 IL 37	Oyster	Bay	March
F176 IL 38	Oyster	Bay	March
F172-3 IL 38	Oyster	Bay	February
F172-4 IL 36	Oyster	Bay	February
F172-4 IL 37	Oyster	Bay	February
F173 IL 37	Mussel	PNC	February
F173 IL 38	Mussel	PNC	February
F178-1 IL 36	Oyster	PNC	March
F178 IL 331	Oyster	PNC	March
F190-1 IL 34	Oyster	PNC	June

A. marinus continuation



F181-2 IL 33	Mussel	PNC	April
F178 IL 351	Oyster	PNC	March
F178 IL 341	Oyster	PNC	March
F178-4 G 38	Oyster	PNC	March
F172 IL 33	Oyster	Bay	February
F172 IL 34	Oyster	Bay	February
F172 IL 35	Oyster	Bay	February
F172 IL 36	Oyster	Bay	February
F172 IL 37	Oyster	Bay	February
F172 IL 38	Oyster	Bay	February
W139-33	Water	Bay	March
W139-34	Water	Bay	March
W139-35	Water	Bay	March
F178-1 G 33	Oyster	PNC	March
F178-2 IL 34	Oyster	PNC	March
F178-1 G 34	Oyster	PNC	March
W141-33	Water	Bay	April
F167 DG 39	Mussel	Bay	January
F167 DG 40	Mussel	Bay	January
F167 DG 45	Mussel	Bay	January
F167 DG 33	Mussel	Bay	January
F167 DG 34	Mussel	Bay	January
F167 DG 35	Mussel	Bay	January
F167 DG 36	Mussel	Bay	January
F167 DG 37	Mussel	Bay	January
F167 DG 38	Mussel	Bay	January
F167 DG 42	Mussel	Bay	January
F167 DG 43	Mussel	Bay	January
F167 DG 44	Mussel	Bay	January
F167 DG 46	Mussel	Bay	January
F167 DG 47	Mussel	Bay	January
W133-34	Water	Bay	January
F178-3 G 35	Oyster	PNC	March
F178-3 G 36	Oyster	PNC	March
F177-3 IL 35	Mussel	PNC	March
F178-2 IL 33	Oyster	PNC	March
F177-1 IL 38	Mussel	PNC	March
F177-3 IL 33	Mussel	PNC	March
F177 IL 331	Mussel	PNC	March
W141-34	Water	Bay	April
F178-1 IL 35	Oyster	PNC	March
F178 IL 33	Oyster	PNC	March
F178 IL 35	Oyster	PNC	March
F178 IL 36	Oyster	PNC	March
F178 IL 37	Oyster	PNC	March
F178 IL 34	Oyster	PNC	March
F188-1 IL 35	Oyster	Bay	June
F188-4 IL 33	Oyster	Bay	June
F188-4 IL 34	Oyster	Bay	June
F188-4 IL 35	Oyster	Bay	June
F184-4 DG 33	Oyster	Bay	May
F167 F 43	Mussel	Bay	January
F188-1 IL 33	Oyster	Bay	June

A. marinus continuation



F177-1 IL 35	Mussel	PNC	March
W140-361	Water	PNC	March
F177-1 IL 33	Mussel	PNC	March
F177-1 IL 34	Mussel	PNC	March
F177-1 IL 37	Mussel	PNC	March
F177-1 DG 33	Mussel	PNC	March
F177-1 DG 34	Mussel	PNC	March
F167 F 35	Mussel	Bay	January
F173-3 G 37	Mussel	PNC	February
F178-4 IL 38	Oyster	PNC	March
F167 F 41	Mussel	Bay	January
F181-1 G 18	Mussel	PNC	April
F181-1 G 17	Mussel	PNC	April
F167 F 34	Mussel	Bay	January
F167 F 36	Mussel	Bay	January
F167 F 40	Mussel	Bay	January
F167 F 42	Mussel	Bay	January
F167 F 44	Mussel	Bay	January
F167 F 45	Mussel	Bay	January
F178-1 G 35	Oyster	PNC	March
F184-4 DG 35	Oyster	Bay	May
F188-4 IL 36	Oyster	Bay	June
W140-341	Water	PNC	March
W142-331	Water	Bay	April
W137-341	Water	PNC	February
W137-351	Water	PNC	February
W137-34	Water	PNC	February
F184-4 DG 34	Oyster	Bay	May
W140-351	Water	PNC	March

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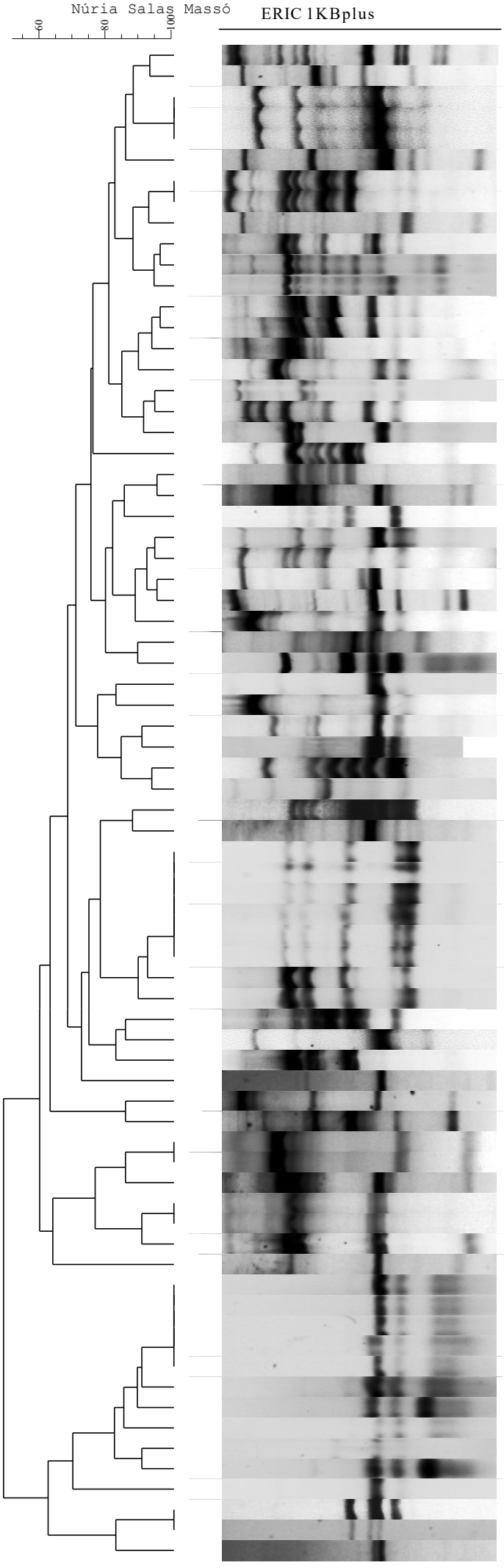
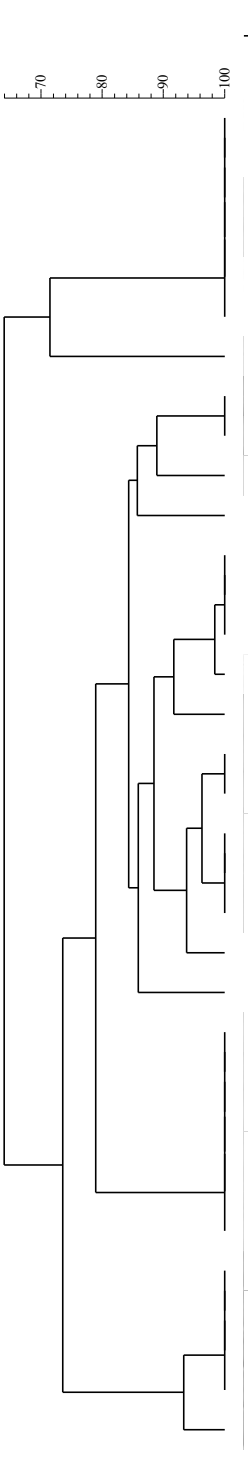


F174-3 G 18	Oyster	PNC	February
F174 IL 201	Oyster	PNC	February
F174 DG 35	Oyster	PNC	February
F174 DG 36	Oyster	PNC	February
F174 DG 37	Oyster	PNC	February
F174 IL 181	Oyster	PNC	February
F174-2 G 22	Oyster	PNC	February
F174-2 G 33	Oyster	PNC	February
F174 IL 171	Oyster	PNC	February
F173-2 G 18	Mussel	PNC	February
F190-1 IL 17	Oyster	PNC	June
F190-1 IL 18	Oyster	PNC	June
F174-2 F 18	Oyster	PNC	February
F174-2 F 19	Oyster	PNC	February
F174-2 F 20	Oyster	PNC	February
F173 IL 191	Mussel	PNC	February
F181-F 18	Mussel	PNC	April
F173 IL 211	Mussel	PNC	February
F185 IL 191	Mussel	PNC	May
F174-2 G 18	Oyster	PNC	February
F170-2 IL 18	Oyster	PNC	January
W137-181	Water	PNC	February
F169-2 IL 20	Mussel	PNC	January
F189-G 17	Mussel	PNC	June
F173 IL 171	Mussel	PNC	February
F174-2 F 22	Oyster	PNC	February
F174-3 G 19	Oyster	PNC	February
F173 IL 181	Mussel	PNC	February
F170-3 F 17	Oyster	PNC	January
W143-221	Water	PNC	April
F181-3 DG 21	Mussel	PNC	April
F173 IL 221	Mussel	PNC	February
F173-2 IL 21	Mussel	PNC	February
F178 IL 171	Oyster	PNC	March
F174-2 IL 19	Oyster	PNC	February
F181-4 G 17	Mussel	PNC	April
F177 DG 17	Mussel	PNC	March
W137-201	Water	PNC	February
F181-3 F 17	Mussel	PNC	April
F181-3 F 20	Mussel	PNC	April
F181-3 G 18	Mussel	PNC	April
F181-3 G 19	Mussel	PNC	April
F181-3 G 20	Mussel	PNC	April
F181-3 G 21	Mussel	PNC	April
F181-3 G 22	Mussel	PNC	April
F181-3 F 22	Mussel	PNC	April
F169-2 IL 17	Mussel	PNC	January
F174 G 36	Oyster	PNC	February
F169-1 G 18	Mussel	PNC	January
W140-18	Water	PNC	March
W137-171	Water	PNC	February
W137-191	Water	PNC	February
F170-3 F 19	Oyster	PNC	January
F170-3 F 20	Oyster	PNC	January
W137-221	Water	PNC	February
F170-1 F 18	Oyster	PNC	January
F170-2 IL 17	Oyster	PNC	January
F169-1 G 17	Mussel	PNC	January
W137-211	Water	PNC	February
F182 IL 171	Oyster	PNC	April
F182 IL 181	Oyster	PNC	April
F182 IL 201	Oyster	PNC	April
F182 IL 211	Oyster	PNC	April
F182 IL 221	Oyster	PNC	April
W140-191	Water	PNC	March
W143-211	Water	PNC	April
F190-G 17	Oyster	PNC	June
F189-G 20	Mussel	PNC	June
W143-171	Water	PNC	April
F181-3 DG 22	Mussel	PNC	April
F177-3 F 22	Mussel	PNC	March
W137-22	Water	PNC	February
W140-17	Water	PNC	March

A. mytili



F169 DG 17	Mussel	PNC	January
F169 DG 18	Mussel	PNC	January
F169 IL 18	Mussel	PNC	January
F169 IL 19	Mussel	PNC	January
F169 IL 17	Mussel	PNC	January
F169 IL 18	Mussel	PNC	January
F167 IL 43	Mussel	Bay	January
F172-1 IL 34	Oyster	Bay	February
F172-1 IL 35	Oyster	Bay	February
F190-F 34	Oyster	PNC	June
F167 IL 38	Mussel	Bay	January
F167 IL 34	Mussel	Bay	January
F167 IL 35	Mussel	Bay	January
F167 IL 36	Mussel	Bay	January
F169-3 IL 34	Mussel	PNC	January
F183 F 38	Mussel	Bay	May
F183 F 36	Mussel	Bay	May
F183 F 37	Mussel	Bay	May
F183 F 33	Mussel	Bay	May
F183 F 34	Mussel	Bay	May
F183 F 35	Mussel	Bay	May
F167 IL 39	Mussel	Bay	January
F167 IL 41	Mussel	Bay	January
F183-1 IL 33	Mussel	Bay	May
F183-1 IL 34	Mussel	Bay	May
F183-1 IL 35	Mussel	Bay	May
F183-1 IL 36	Mussel	Bay	May
F183-1 IL 37	Mussel	Bay	May
F183-1 IL 38	Mussel	Bay	May
F190-2 DG 33	Oyster	PNC	June
F190-2 DG 34	Oyster	PNC	June
F190-2 DG 35	Oyster	PNC	June
F190-2 DG 37	Oyster	PNC	June
F190-2 DG 36	Oyster	PNC	June

3.4 Depuration of *Aliarcobacter butzleri* and *Malaciobacter molluscorum* in comparison with *Escherichia coli* in mussels (*Mytilus galloprovincialis*) and oysters (*Crassostrea gigas*). Salas-Massó N, Fernández-Bravo A, Bertomeu E, Andree KB, Figueras MJ, Furones MD. (In preparation)

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Núria Salas Massó

Depuration of *Aliarcobacter butzleri* and *Malaciobacter molluscorum* in comparison with *Escherichia coli* in Mussels (*Mytilus galloprovincialis*) and Oysters (*Crassostrea gigas*).

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Abstract

The efficiency of commercial depuration for decontaminating *Arcobacter*-related genera (and specifically *Aliarcobacter butzleri* and *Malaciobacter molluscorum*) in comparison with the indicator *Escherichia coli,* from mussels and oysters from the Ebro River Delta was investigated. Four different conditions were assed: i) a control tank with commercially depurated bivalves; ii) a tank with bivalves highly contaminated in sewage water; and two tanks in which the contamination was performed under controlled conditions, containing iii) *A. butzleri* and *E. coli* and iv) *M. molluscorum* and *E. coli.* Results showed that commercial depuration was not effective removing *Arcobacter*-related species from both bivalves. When evaluating the reduction of bacteria load in the shellfish contaminated in raw sewage from Poble Nou Channel (PNC), versus to the challenge infection in the laboratory with strains previously recovered from shellfish we observed a higher reduction of bacterial loads in the shellfish contaminated in the PNC, which may be attributed to strains less adapted to the conditions of high salinity (34‰) in which the depuration process was performed. Although temperature did not statistically make a difference in depuration, at 20ºC higher elimination than at 14ºC of both bacteria was recorded. Therefore, new procedures and strategies should be developed to assure the safety of commercial bivalves in regard to the *Arcobacter*-related species content.

Keywords: fecal pollution, depuration, shellfish, *A. butzleri, E. coli*

1. Introduction

Global aquaculture production in 2016 included 80.0 million tonnes of food fish, among which 17.1 million tons were molluscs (FAO 2018). According to the report published by the FAO (2018) Spain is the main producer of marine mollusks in Europe, where the most common farmed or harvest species are mussel, oysters, clams, cockles, scallop and razors. The filter feeding behavior of bivalves make them vehicles of concentration, accumulation and, dissemination of a diverse number of microorganisms, which are present in the surrounding harvesting waters (Bosch, 1994; Lees, 2000), Among those microorganisms, including bacteria, virus and protozoa, there are several pathogens which either have their origin in fecal contamination (i.e. *Salmonella, Shigella, Arcobacter*, Hepatitis A virus, Norovirus, etc.) or are indigenous from the marine environment, like *Vibrio* species (Lees, 2000; Baker-Austin et al., 2017; FAO 2018). This accumulation together with the fact that bivalves molluscs are usually consumed raw or slightly cooked pose a risk for the consumer (Collado et al., 2009; Polo et al., 2014; Salas-Massó et al., 2016).

The sanitary quality of marketable shellfish is routinely monitored by analyzing their levels of fecal coliforms. In the European Union (EU) this is mandatory to stablish a classification of the shellfish harvesting areas, based on the levels of the *E. coli* as indicator in the shellfish matrix (Anon, 2015). Briefly, in category A areas the shellfish must not exceed 230 Most Probable Number (MPN) of *E. coli*/100 g, and they do not require depuration before placing them on the market. The other categories (B–D) involve increasingly higher concentrations of *E. coli* and therefore, shellfish require depuration (Category B areas), or relaying in clean waters (Category C areas), until they reach the values of category A before consumption (Anon, 2004, 2015); shellfish from Category D areas is prohibited for consumption. Usually shellfish are harvest from categories A or B areas, for the latest (Category B areas) depuration is mandatory for a period between 24 and 42 h (Lees et al., 2010; McMenemy et al., 2018), until its *E. coli* level fall into Category A.

Depuration is a process for reducing microbial contaminants by resubmerging shellfish in clean seawater over time, under controlled conditions (Lopez-Joven et al., 2011; McMenemy et al., 2018; Shen et al., 2019). This process is dependent on the type of shellfish, as well as on the species and/or their bacterial content. Additionally, environmental factors such as temperature, salinity and dissolved oxygen, are also important for determining the efficacy of the depuration process (Lee and Younger, 2002; Lopez-Joven et al., 2011). However, it has

been demonstrated that *E. coli*, may not be a suitable indicator for the presence of pathogens like indigenous *Vibrio* or viruses (Salas-Massó et al., 2018a and references therein), mainly because the depuration rate of these pathogens takes longer in time than for *E. coli* This is mainly caused by the ability of this pathogens to colonize shellfish and to survive in shellfish hemocytes (Polo et al., 2014; Shen et al., 2019). Therefore, environmental parameters in depuration such as temperature and salinity are being studied to modify depuration processes in order to minimize the loads of these microorganism (Shen et al., 2019). Recently, Salas-Massó et al. (2018a) presented that the predictive capacity of *E. coli* could even be seasonality-dependent, as *Arcobacter*-related species were detected in shellfish samples when the temperature of the water was higher than 26.2ºC, but the same samples were negative for the detection of *E. coli*.

The genus *Arcobacter* has been isolated from shellfish worldwide, showing a high prevalence in this type of food, which range from a 14.7% to 73.3% and were summarized by Salas-Massó et al. (2018b). The more frequently recovered species from shellfish have been, in first place *Arcobacter butzleri*, which is capable of causing diarrhea and bacteremia in humans and its presence in shellfish may have a fecal origin (Collado et al., 2009; Levican et al., 2014; Salas-Massó et al., 2016; 2018a), and in second place, *A. molluscorum*, which naturally occurs in marine bivalves. Recently, the genus has been divided into seven different genera: *Arcobacter*, *Aliarcobacter*, *Pseudarcobacter*, *Halarcobacter*, *Malaciobacter*, *Poseidonibacter*, and Candidate "*Arcomarinus*" gen. nov.; according to an exhaustive taxonomic study performed by Pérez-Cataluña et al. (2018). Therefore, *A. butzleri* has become a member of the genus *Aliarcobacter*, which comprises other pathogenic species like *Aliarcobacter cryaerophilus* and *Aliarcobacter skirrowii*, and *A. molluscorum* is now a member of the genus *Malaciobacter*, which comprises species mainly recovered from bivalves and marine environments (Pérez-Cataluña et al., 2018).The roles of these new genus i.e *Malaciobacter*, *Haliarcobacter* or *Poseidonibacter*, which seemed to be associated with shellfish microbiota, are still unclear, but recent hypothesis seem to indicate that may be opportunistic pathogens of these animals (Lokmer and Wegner, 2015; Li et al., 2018) The high incidence of this genus in shellfish, along with the fact that includes pathogenic species like *A. butzleri*, has drawn attention to study how this particular species of bacteria is transmitted and incorporated into the shellfish tissues (Ottaviani et al., 2017). The bioaccumulation capacity of *A. butzleri* in mussels was studied by Ottaviani et al. (2013). The authors found out that the type strain of *A. butzleri* was not capable to survive in seawater for longer than 48 h, thus starting from an inoculum of

10^6 CFU/ml, *A. butzleri* accumulate within mussels' tissues in 24h at 2 logs lower than the inoculum. Therefore, work on the shellfish depuration parameters and on the survival capacity of *Arcobacter*-related species in the marine environment, are paramount to generate the needed information to set the criteria to guarantee the safety of shellfish in regards of these bacteria.

Being the Ebro Delta River region a major producer of mollusks in Spain, cultivating mainly Mediterranean mussel (*Mytilus galloprovincialis)* and pacific oyster (*Crassotrea gigas*) together with the lack of studies involving depuration strategies for *Arcobacter*, the objectives of this study are i) to analyze how *Arcobacter*-related genera species, in comparison to *E. coli*, are depurated in mussels and oysters from this harvesting area , at two different season (summer and winter and ii) to stablished if the pathogenic (*A. butzleri*) and the environmental (*M. molluscorum*) nature of the species would make any differences in their depuration rate. This work has been done taking into account the EU regulation 854/2004 for shellfish harvesting areas.

2. Materials and methods

2.1 Sample collection.

A total of 10kg of local commercial depurated mussels (*Mytilus galloprovincialis*) and 40kg of oysters (*Crassostrea gigas*), were bought at the same establishment in July and December of 2015. The mussels and oysters were analyzed for the quantification of *E. coli* and *Arcobacter*-related spp. by means of Most Probable Number (MPN; see section 2.4), in order to stablish the background load of the bacteria load prior to the trial (tbg).

2.2 Bacterial culture preparation.

One strain of *Aliarcobacter butzleri* (F146-25) and another one of *Malaciobacter molluscorum* (F146-34), both isolated from *M. galloprovincialis* from the Ebro Delta bays, were used for the co-contamination experiment with *E. coli* CECT 434. Each strain was grown separately in Blood Agar (BA; TSA supplemented with 5% sheep blood BD Difco, Le Pont de Claix, France) plates at 30ºC for 48 h in aerobic conditions. For *E. coli*, the culture was prepared in Tryptone Soy Agar (TSA; Oxoid, Basingstoke, UK) plates and incubated at 37ºC for 24h. After incubation, a single colony of each species was individually grown in 100 ml of *Arcobacter* broth supplemented with cefoperazone, amphotericin B and teicoplanin (Arcobacter-CAT broth; Oxoid, Basingstoke, UK) at 30ºC for 48h, in the case of *Arcobacter*-

like genera. *E. coli* was grown in Tryptone Soy Broth (TSBOxoid Basingstoke, UK) and incubated at the same conditions mentioned above. The pure cultures of the three species were transferred to sterile centrifuge tubes and centrifuged at 3,000 g for 15 min. Pelleted cells for each, culture were washed with 50 ml of saline sterile solution (0.9% NaCl w/v; SSE) twice. The suspensions that would serve as inoculums for the co-contaminations assays were prepared individually for each species, *A. butzleri*, *M. molluscorum* and *E. coli*, and consisted of bottles with 0.8L of SSE all with final $OD_{550\,nm}$ of 0.8, which we have predetermine that corresponded with a bacterial load of 10^6-10^7 CFU/ml for *A. butzleri* and *M. molluscorum*. For *E. coli* a bacterial suspension of 10^5-10^6 CFU/ml was achieved by means of a 1 McFarland standard (bioMérieux, Marcy l'Etoile, France).

2.3 Experimental design.

Two assays were performed, in summer (July) and winter (December), under the same conditions. The shellfish, (mussels and oysters) previously cleaned, were placed in different fiberglass tanks (90 x 45 cm), with a total volume of 40L. The water supply consisted of a flow thought system, fed with seawater from Alfacs Bay, which had been previously disinfected going through a device equipped with filtration (5µm pore diameter) and ultraviolet light, to ensure sterile conditions of the water. The temperature and salinity of the depuration tanks was registered in the IRTA facilities given a temperature of 20 and 14ºC in summer and winter respectively, the salinity for both periods was of 34.5‰. The inlet water was microbiologically analyzed to corroborate the efficacy of the disinfection process. The water renewal rate was 0.7L / min. To avoid recontamination with the feces of the shellfish, a perforated plastic grill with 6 legs of 9 cm was placed at the bottom of all the tanks. Shellfish were not fed during the depuration process. In total, 6 different tanks were used, 3 for mussels, which each tank contained c.a.120 mussels, and 3 for oysters, each tank containing c.a.75 oysters, and 4 different treatments prior depuration were given to the shellfish. Afterwards, depuration occurred for 5 days, since extending the trials for more than 120 h, probably would induce weight loss of the animals and organoleptically changes valued by the consumer may occur (Figure 1):

i) PNC tanks: here the shellfish were previously naturally contaminated by direct immersion, during 24h, in a channel that receives untreated sewage from the village of Poble Nou (Tarragona, Spain), which in a previous study (Salas-Massó et al., 2018a), showed levels of *E.coli* corresponding to a harvesting are of class D, and equivalent high loads of *Arcobacter* spp. In summer and winter the temperature of the PNC water was 26.8 and 9.7 ºC, respectively; and presented a salinity of 10.7 and 16.8‰, respectively. After that naturally bioaccumulation process, the shellfish were washed with tap water to remove mud on the shells and were placed

on their depuration tanks. The purpose was to have shellfish highly polluted with natural occurring bacteria.

ii) *A. butzleri* (Ab): Experimental tanks and iii) *M. molluscorum* (Mm) Experimental tanks: Prior to the experimental depuration, the commercial shellfish were inoculated with co-cultures of *E.coli* and *A. butzleri* (Ab) and *E. coli* and with *M. molluscorum* (Mm) respectively, prepared as mentioned in section 2.2. Shellfish were maintained for 24 h in a closed tank in which air was pumped to keep the dissolved oxygen level favorable for the animals and phytoplankton (10^5 cells/mL of axenic *Isochrysis galbana* in the tanks) was added to stimulate uptake of the bacteria (Gentry et al., 2009). Corroboration of the axenic conditions of the phytoplankton was performed for *E. coli* and *Arcobacter*-like spp. by means of the MPN(see section 2.4) and the values obtained wee below the limit of detection of the method, After the artificial contamination, the animals were washed and transferred to the depuration tanks, where they were kept under the same condition of i and ii.

2.4 Microbiological analysis.

The commercial shellfish were initially analyzed to know the bacterial load of *E. coli* and *Arcobacter*-related spp. Additionally, this value corresponded to tbackground After the different bioaccumulation trials (i.e., i) 24h in the PNC, and 24h in the IRTA controlled infection tanks with iii) Ab+ *E. coli* and iv) Mm + *E. coli*), and before the animals were placed in the experimental depuration system at IRTA, the initial bacterial load (t0) for each trial was quantified by means of the MPN. Once the animals were placed in the tanks, microbiological analyses were performed at times 24, 48, 96 and 120 h Briefly, flesh and intervalval liquid from 20 mussel and 10 oysters were mixed thoroughly and homogenized in a stomacher (Lab·Blender 400, West Sussex,UK), respectively, with peptone water. Then 100g of the homogenate were used for preparing the 3 dilutions (i.e. 1, 0.1 and 0.01mL or g of the original sample) that were used in the two step MPN for *E. coli* and *Arcobacter-like* spp. For *E.coli*, the quantification was performed according to previous studies (ISO/TS 16649-3:2005, Salas-Massó et al., 2018a) , five tubes, in triplicates containing Glutamate broth (Oxoid, Basingstoke, UK) were inoculated with the dilutions of the homogenates and incubated for 24h at 37°C. Confirmation of *E.coli* was performed from those tubes changing color from purple to yellow , by subculturing a loop of cells from the Glutamate broth tubes onto Tryptone Bile X-glucuronide Agar medium (TBX) (Oxoid, Basingstoke, UK) at 44°C for 24h.Thus, growth of

typical greenish-blue color colonies confirmed the presence of *E. coli*. The derived MPN results was calculated using the CEFAS MPN tables (Appendix 2 CEFAS issue No. 11, 2015).

An *Arcobacter* two-steps MPN was performed, according to Salas-Massó et al. (2018a). Briefly, 5 tubes in triplicates containing Arcobacter-CAT broth were inoculated with the previously made dilutions and were incubated at 30°C for 48 h, under aerobic conditions. Confirmation of the presence of *Arcobacter*-related spp. consisted on culturing at 30 °C for 48 h under aerobic conditions 100µl of the above enrichment tubes by passive filtration (0.45µm nitrocellulose filters; Millipore) on Blood Agar plates. The MPN final values were obtained using the software MPN Build 23 (Mike Curiale software; http://i2workout.com/mcuriale/mpn/index.html).

To corroborate that the quantification of *A. butzleri* and *M. molluscorum* corresponded to the strains used in the bioaccumulation process, 8 colonies obtained when the MPN tubes were plated for confirming the presence of *Arcobacter*, were subcultured on BA and incubated at 30ºC for 48hr. DNA was extracted and the isolates were genotyped with Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR). DNA from strains F146-25 and F146-34 were used for comparison and verification of the colonies isolated.

2.6 Statistical analysis.

The results of the MPN were transformed into log values, and statistical analyses were performed with Software SPSS Statistical 22.0 (IBM Analytics). Normality distribution of the data was assessed using the Shapiro-Wilk and Kolmogorv-Smirnov test. Bacterial populations in oysters and mussels at different sampling times and at different temperatures were analyzed with the non-parametric Mann Whitney test for non-normal distributed data and significant differences between means of treatments were established at a P value of 0.05.

3. Results

The depurated shellfish bought locally showed that *E. coli* and *Arcobacter*-related spp., at time 0, occurred in mussels at both sampling periods (winter and summer) and in oysters during the winter. The levels of *E. coli* in mussels (2200 MPN/100g in summer and 1700MPN/100g in winter) after the commercial depuration corresponded to a class B area levels (between 230 and 4600 MPN/100g) not being within the legally established bacteriological limit of *E. coli* (<230 MPN/100g), however, for oysters the levels of *E. coli* corresponded to a class A (<18 and 20 MPN/100g, in summer and winter, respectively).

In the tanks which contained shellfish naturally contaminated in the PNC, the levels of *E. coli* in both seasons at t0 were within category C and D. A decrease along time was observed for both type of shellfish, reaching or close to levels of *E. coli* of class A after 48 h (Figures 2A, 2B, 3A and 3B). *E. coli* was removed after 72 h when the temperature of the water was 20ºC, as well as for *Arcobacter*-related spp. in mussels (Figure 2A), but in oysters *Arcobacter*-related spp. persisted until the end of the trial (Figure 3A). During the winter experiment, *E. coli* decreased 2.61 log and 3.8 log in mussels and shellfish, respectively and *Arcobacter*-related spp. showed also a decrease in both, mussels and oysters, of 3.96 log and 2.97 log respectively. In both shellfish, *Arcobacter*-related spp. was not detected at t72, despite being detected the two following days (Figure 2B and 3B).

The bacterial loads of *A. butzleri* and *E. coli* in mussels artificially contaminated, showed no decrease, with the exception of the depuration of *A. butzleri* at 20ºC, in which at t120 no detection of this bacterium occurred (Figure 2C). In the case of oysters, during the first 48 after contamination, the bacterial loads of both bacteria remained within the same category C level, except for *A. butzleri* at 20ºC, which fell into B category level at 48 hr. After that time, 48 hrs., both bacteria started decreasing their load up to 1.3 and 1.78 logs in the case of *E. coli* (for 14ºC and 20ºC respectively) and 1.60 log for *Arcobacter*-related spp. at 14ºC (Figure 3D). As well as happened in mussels, *Arcobacter*-related spp. at 20ºC was not detected anymore in oysters after 96 h of depuration (Figure 3C).

In relation to the shellfish contaminated with *M. molluscorum* and *E. coli*, the dynamics of *E. coli* at both temperatures were similar to those observed in the experimental tank with the co-infection of *A. butzleri* and *E. coli*. *E. coli* barely decreased in mussels (Figures 2E and 2F). However, in oysters achieved reduction loads corresponding to class B areas. Although the MPN values of *E. coli* at 20ºC, at the end of the experiment were very close (280 MPN/100g) to the limit of class A (230 MPN/100g; Figure 3E). During the experiment that took place at 20ºC, the initial loads (t0) of *M. molluscorum* in shellfish were lower than expected, and after 24 h this species was totally removed from the animals (Figures 2E and 3E). However, at 14ºC the levels of *M. molluscorum* in mussels increased 0.46 log during the first 24h to start decreasing during the next 48 h, achieving a final reduction of 2.44 logs which was maintained at times t72, t96 and t120 (Figure 2F). In the case of oysters, the reduction on the levels of *M. molluscorum* was lower (1.02 logs) and, after 72 h the bacterium concentration was stable during the trial (Figure 3F).

4. Discussion

The current study investigated the depuration of *E. coli*, *A. butzleri* and *M. molluscorum* in mussels and oysters in the Ebro River Delta area. Depuration, under regulated criteria, is the most common practice worldwide for bacterial elimination from commercial bivalves going to the market for human consumption (Lopez-Joven et al., 2011; Oliveira et al., 2011; Wang et al., 2010; Shen et al., 2019). Our first results showed that the commercial shellfish , used as control, should have been depurated to assure *E.coli* levels below the limit stablished for category A (≤230 MPN/100g), but, in fact, had *E.coli* levels above that limit in mussels. Thus, mussels presented levels of *E. coli*, in both seasons, above the 230 MPN/100g threshold to place the shellfish in market. However, the levels of *E. coli* in oysters in both seasons, at tbg, were under the legal limits to be sold. It has been reported that depuration is not being 100% efficient in the elimination of pathogenic microorganisms, i.e. *E. coli* and *Vibrio* spp. from bivalve tissues and these may persist in bivalves after depuration (FAO, 2008; Martínez et al., 2009; Rong et al., 2014; Pereira et al., 2017). *Arcobacter*-related genera could be included to this list. Despite the low levels of *E. coli* at 14 ºC, the levels of *Arcobacter*-related species were 2 logs higher than those of *E. coli* posing a potentially serious risk for the consumer.

The performance of the depuration of *E. coli* and *Arcobacter*-related spp. by shellfish contaminated by immersion in the PNC water, was better than those from bivalves artificially contaminated under controlled conditions. Thus, higher logs reductions were achieved in the PNC tanks than in the experimentally contaminated shellfish with pure cultures mixes of *E. coli* and any of the *Arcobacter*-related species tested which could be attributed to the different origin of the strains involved in the trials. Thus, in the PNC, the predominant species are those closely related to fecal pollution, like *A. butzleri*, *A. cloacae* or *A. cryaerophilus* (Salas-Massó et al., 2018a), and when were introduced in the depuration system, and therefore in a more NaCl concentrated environment, their persistence and survival could have been affected, favoring its depuration, because their physiological status might have been compromised . The relationship between the environmental conditions, especially salinity, have been pointed out in previous studies performed by this group (Salas-Massó et al., 2016; 2018a). In the controlled experimental conditions, however, the *A. butzleri* and *M. molluscorum* strains used were originally isolated from resident bivalves harvested in Alfacs Bay, and it is, therefore, highly probable that these strains may have mechanisms that make them persist in high salinity environments, like shellfish tissues. It should be noticed that the concentration values obtained

for *E.coli* in the PNC, corresponded to levels of areas of class C or even D, which require either laying the mollusks in cleaner waters for long periods of times in the case of class C, or prohibition to go into the market, in the case of class D (EU regulation 854/2004), Interestingly, in this work, we observed that, after 120 hrs. in clear water, the PNC *E. coli* load, reached the level of class A. However, in the tanks in which *E. coli* was artificially contaminated, its levels, despite the initial load being lower than those of the PNC, barely change through time or decreased from levels corresponding to a class C area to a B area. Again, this may be due to an effect of the strain used, which belongs to a culture collection and long-term accumulation of mutations make that this isolate behaves totally different from their environmental equivalents (Palková, 2004).

The depuration experiments were performed during two different seasons, summer and winter to understand whether the temperature could influence the depuration rates of the bacteria. Although the statistical test did not prove any significant difference between depuration at 14ºC or 20ºC, it was observed that at 20ºC a higher number of tanks achieved total reduction of bacterial loads than at 14ºC. Some authors have reported that temperature influences depuration rates, Shen et al. (2019) found that depuration of *V. parahaemolyticus* in oysters was enhanced at temperatures between 5 and 15ºC, rather than at ambient temperatures like 22ºC. For F+ coliphages, instead, depuration was more effective at 18ºC than at 9ºC (Lee and Younger, 2002); higher temperatures also favored the depuration of noroviruses (Doré et al., 2010). Therefore, to achieve a consensus on the temperature to set the depuration process, the behavior of the main pathogens to be treated needs to be documented. However physicochemical parameters are limited, as to ensure the animals' welfare is necessary to avoid stress to shellfish by setting these parameters (i.e. temperature and salinity) close to their harvesting areas (McMenemy et al., 2018).

There have been few reports of foodborne illness caused by *Arcobacter*, usually associated with consumption of fecally contaminated water or consumption of chicken and caused by *A. butzleri* or *A. cryaerophilus* (Salas-Massó et al. 2018b). However, the first bacteremia cause by *M. mytili* and acquire by contact with seafood has been recently reported (Vasiljevic et al., 2019). A 65-years old man with several underlaying illnesses, acquired *M. mytili* causing febrile symptoms. The acquisition of the bacteria was hypothesis to have arrived at the blood through wounds in his hands after handling Maryland crab. The high prevalence of *Arcobacter*-related genera in commercial seafood worldwide (Salas-Massó et al., 2018b),

should drive attention to better strategies for purification, as well as better bacterial indicators that ensure safety for the consumer.

5. Conclusion

The commercial depurated shellfish of this study presented loads of *E. coli* above the limits stablished for the European legislation to be sold, but also high numbers of *Arcobacter*, indicating that this process do not ensure the safety of this food. Future studies should test the best depuration system for ensuring the removal of *Arcobacter*-related genera from shellfish, considering economical and animal welfare aspects.

This study provides preliminary information to help to improve depuration standards and protocols of commercial mussel and oysters, focusing in Arcobacter-related bacteria as emergent pathogens for this sector. We based our work frame on the EU standards, which are set for mainly for *E. coli*. Up today, very little is known about *Arcobacter*-related ecological niches, survival capacity in the very variable environment where they are found. This work forms part of the group efforts to increase our information on these issues, aiming to increase food safety standards, which at present, do not seem to protect the consumer against *Arcobacter* threat.

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9. Figures

Figure 1. Scheme of the different experimental conditions that were used in this study.

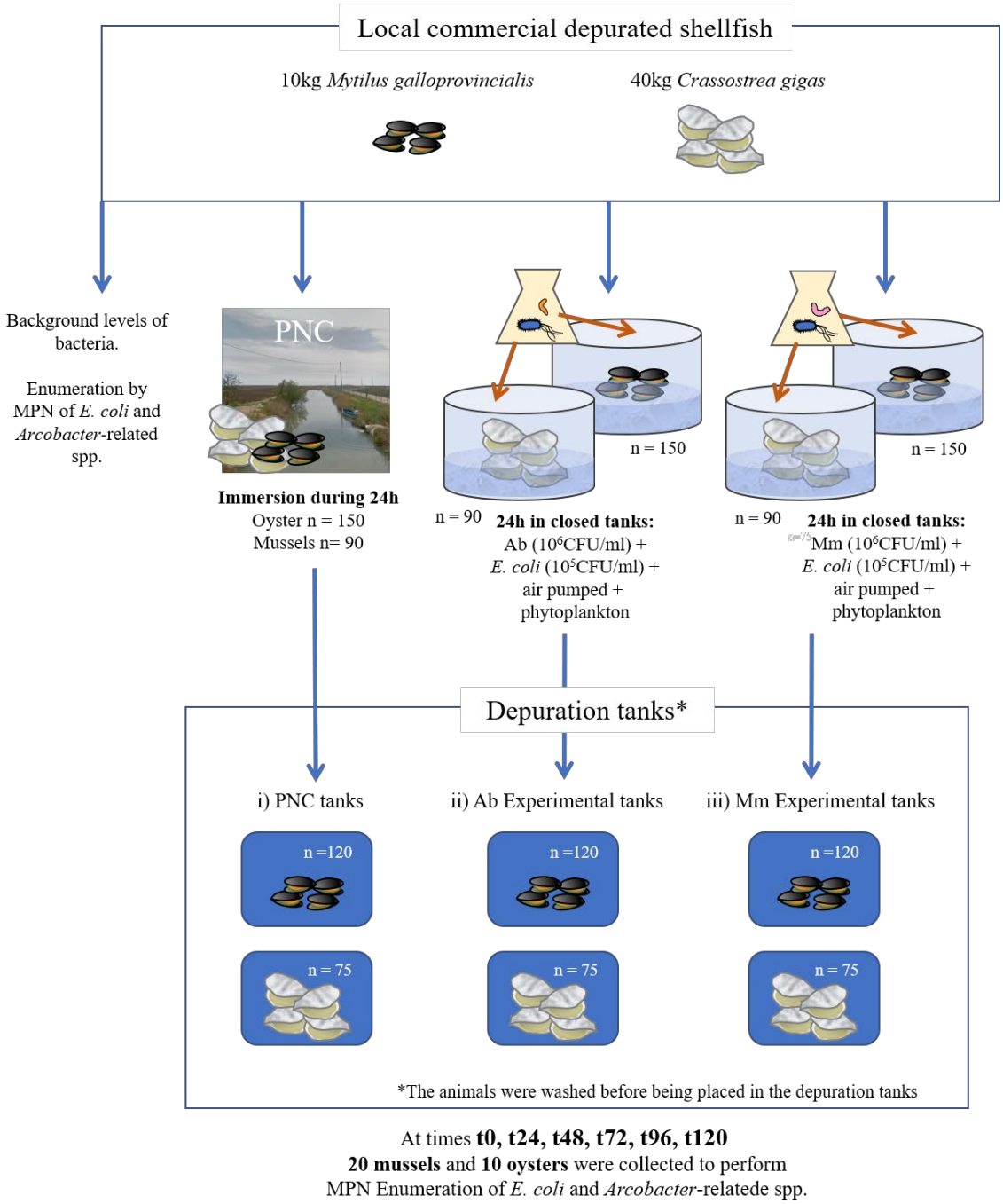


Figure 2. Values of log MPN of *E. coli* and *Arcobacter*-related species in the different trial tanks (A-F) containing mussels along time. The different colors indicate the standards of the four categories (A, B, C and D) established by the European Union for the shellfish harvesting areas on the basis of the MPN results of *E. coli*/100 g (Anon, 2004, 2015) class A (green), ≤230 MPN/100g, shellfish do not require depuration; class B (orange), < 4600 MPN/100g, 24 h of depuration is needed; class C (red), < 46000 MPN/100g, shellfish have to be placed in a clean water for at least one month and class D (brown), ≥ 46000 MPN/100g, these shellfish are prohibited for consumption.

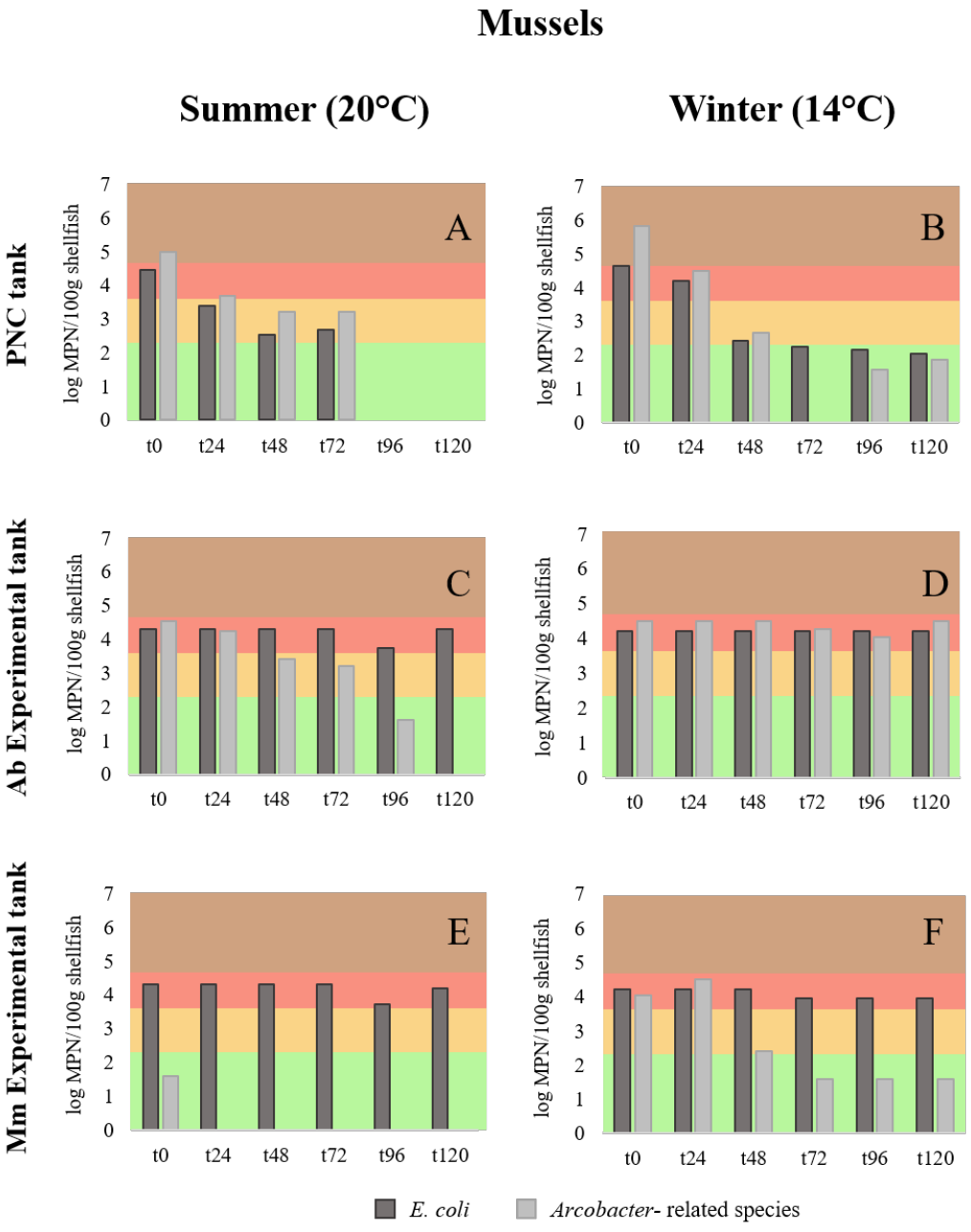
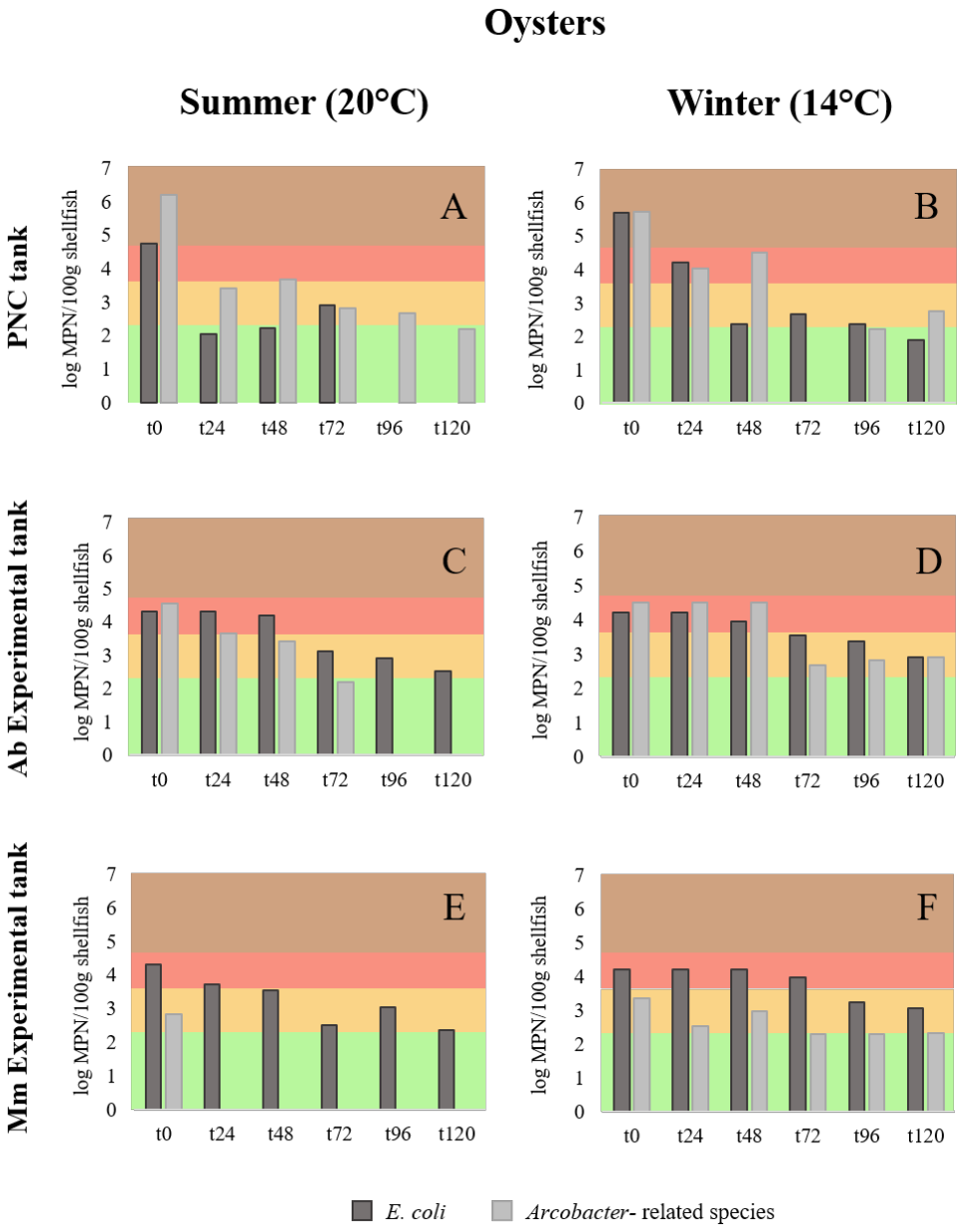


Figure 3. Values of log MPN of *E. coli* and *Arcobacter*-related species in the different trial tanks (A-F) containing oysters along time. The different colors indicate the standards of the four categories (A, B, C and D) established by the European Union for the shellfish harvesting areas on the basis of the MPN results of *E. coli*/100 g (Anon, 2004, 2015) class A (green), ≤230 MPN/100g, shellfish do not require depuration; class B (orange), < 4600 MPN/100g, 24 h of depuration is needed; class C (red), < 46000 MPN/100g, shellfish have to be placed in a clean water for at least one month and class D (brown), ≥ 46000 MPN/100g, these shellfish are prohibited for consumption.



3.5 The use of a DNA-intercalating dye for quantitative detection of viable *Arcobacter* spp. cells (v-qPCR) in shellfish Salas-Massó N, Linh QT, Chin WH, Wolff A, Andree KB, Furones MD, Figueras MJ, Bang DD. *Frontiers in Microbiology,* 2019; 28;10:368.

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
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UNIVERSITAT ROVIRA I VIRGILI
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The Use of a DNA-Intercalating Dye for Quantitative Detection of Viable *Arcobacter* spp. Cells (v-qPCR) in Shellfish

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The genus *Arcobacter* (Vandamme et al., 1991), comprised of *Campylobacter*-related species, are considered zoonotic emergent pathogens. The presence of *Arcobacter* in food products like shellfish, has an elevated incidence worldwide. In this study, we developed a specific viable quantitative PCR (v-qPCR), using the dye propidium monoazide (PMA), for quantification of the viable *Arcobacter* spp. cells in raw oysters and mussels. The high selectivity of primers was demonstrated by using purified DNA from 38 different species, 20 of them from the genus *Arcobacter*. The optimization of PMA concentration showed that 20 μM was considered as an optimal concentration that inhibits the signal from dead cells at different concentrations (OD_{550} from 0.2 to 0.8) and at different ratios of live: dead cells (50:50 and 90:10). The v-qPCR results from shellfish samples were compared with those obtained in parallel using several culture isolation approaches (i.e., direct plating on marine and blood agar and by post-enrichment culturing in both media). The enrichment was performed in parallel in Arcobacter-CAT broth with and without adding NaCl. Additionally, the v-qPCR results were compared to those obtained with traditional quantitative (qPCR). The v-qPCR and the qPCR resulted in c.a. 94% of positive detection of *Arcobacter* vs. 41% obtained by culture approaches. When examining the reduction effect resulting from the use of v-qPCR, samples pre-enriched in Arcobacter-CAT broth supplemented with 2.5% NaCl showed a higher reduction (3.27 log copies) than that of samples obtained directly and those pre-enriched in Arcobacter-CAT broth isolation (1.05 and 1.04). When the v-qPCR was applied to detect arcobacter from real shellfish samples, 15/17 samples tested positive for viable *Arcobacter* with 3.41 to 8.70 log copies $1g^{-1}$. This study offers a new tool for *Arcobacter* surveillance in seafood.

Keywords: PMA, qPCR, viable cells, *Arcobacter*, shellfish

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Nuria Salas Massó

INTRODUCTION

Foodborne disease outbreaks are of public health concern (Zeng et al., 2016 and references therein). In 2015, a total of 4,362 foodborne disease outbreaks, including waterborne disease outbreaks were reported in the European Union (EU). Overall, these outbreaks caused 45,874 cases of illness, 3,892 hospitalizations and 17 deaths (EFSA and ECDC, 2016). Most of the outbreaks reported in 2015 were caused by bacterial agents (33.7% of all outbreaks). The most frequent human foodborne illnesses in order of prevalence were campylobacteriosis, salmonellosis, yersiniosis, shiga toxin-producing *Escherichia coli* infections and listeriosis (EFSA and ECDC, 2016). To avoid the occurrence of disease outbreaks, food is monitored following specific microbiological criteria, which may vary according to culture, climate and economic status of the country (Zhang et al., 2016). In these regulated monitoring programs, the most commonly used methods are based on bacterial isolation in synthetic media, which are time consuming, laborious and cannot detect viable-but-non-culturable bacteria (VBNC) (Barbau-Piednoir et al., 2014).

Molecular methods have been progressively introduced as they are fast, sensitive and specific. Among such methods, PCR is the most widely used. By using PCR, the presence of a pathogen of interest in a sample can be detected rapidly. However, the method is not able to give us a clear picture of the status of the bacterial population, since the method amplifies the DNA from both living and dead cells (Nocker et al., 2006; Fittipaldi et al., 2012; Elizaquível et al., 2014; Zhang et al., 2016; Reyneke et al., 2017). In a food safety context, it is important to know whether the bacteria are still alive in the food, to avoid unnecessary product recalls and economic losses (Zhang et al., 2016). Therefore, RNA-based methodologies are recommended for detecting and determining the number of viable bacterial cells that are metabolically active in the sample. The problem of RNA-based methodologies is that the RNA molecules are easily degraded, and the RNA degradation can easily occur while handling the samples (Fittipaldi et al., 2012; Barbau-Piednoir et al., 2014).

Nocker et al. (2006) were the first to use propidium monoazide (PMA) for examining the suitability of this membrane-impermeant dye to intercalate to genomic DNA from cells with compromised cell membranes as an alternative tool to discriminate between viable and dead cells. The basic ideas of the use of this dye are that (i) the dye is able to covalently bind to DNA after photoactivation using light with 450 nm wavelength, and (ii) the dye is usually not permeable to intact cell membranes, so the dye only can enter into membrane-compromised cells (i.e., dead or damaged cells). Once the PMA is inside the cells it intercalates into the DNA and, after photoactivation it is crosslinked to the DNA. This chemical modification will block (inhibit) the amplification of these DNA molecules during PCR. At the same time crosslinking occurs, the remaining PMA in the solution reacts with water and becomes unreactive (Nocker et al., 2009).

Arcobacter is a new foodborne pathogen. It is related to *Campylobacter* that is one of the main causes of diarrhea in humans. *Arcobacter butzleri* has been the cause of enteritis outbreaks associated to the consumption of contaminated water and food in different countries (Collado and Figueras, 2011; Ferreira et al., 2016). Recently Ferreira et al. (2017) reported how *Arcobacter* is commonly isolated along the whole food production chain, including animals from farms, slaughterhouses and retail. Although *Arcobacter* spp. have been isolated from poultry, pork, dairy products, and vegetables (Collado et al., 2009; Wesley and Miller, 2010; Hsu and Lee, 2015; Ferreira et al., 2017), their prevalence rate in seafood products, some of which are consumed raw or undercooked, is relatively high compared to other foods, ranging from 14.6 to a 73.3% of positive samples (Collado et al., 2009; Nieva-Echevarria et al., 2013; Salas-Massó et al., 2016, 2018; Leoni et al., 2017). In these types of food samples *A. butzleri* has been shown to be the most prevalent species using conventional culture methods, however, other species may prevail using other approaches (Salas-Massó et al., 2016). So far, there are no official standard protocols for the isolation of these bacteria. Some of the developed methods are time consuming, as they require at least 48 h for growing cultures and a pre-enrichment step in a broth containing antibiotics (Levican et al., 2016; Salas-Massó et al., 2016). Therefore, advances in molecular tools for the study of these bacteria have been developed (Ferreira et al., 2017). So far, few publications report the use of DNA-intercalating dyes to study the viability of *Arcobacter* spp. cells. Hrušková et al. (2013) used a PMA methodology for detection of *A. butzleri* and *A. cryaerophilus* in biofilms and studied their composition in relation to the viability status of the cells. Recently, Webb et al. (2016) used ethidium monoazide (EMA) coupled with a qPCR to evaluate how wastewater treatments can affect the viability of *A. butzleri* cells. Despite the high prevalence of *Arcobacter* spp. in seafood, there are no studies that have used PMA to investigate these bacteria in shellfish. In fact, up to date only two PMA treatments have been developed for the study of other microbes in seafood samples. Zhu et al. (2012) assayed a PMA-qPCR in raw oysters to quantify viable cells of *Vibrio parahaemolyticus* positive for the thermostable direct hemolysin gene (*tdh*) which is associated to the pathogenicity of this organism. Quijada et al. (2016) developed a PMA-qPCR to detect and enumerate enteric RNA and DNA viruses in clams, both strategies showing promising results as alternatives to predicting the status of viability of these foodborne pathogens.

The aim of the study is to define the best conditions under which the PMA method can be used and to develop a PMA-qPCR protocol for the detection and enumeration of viable *Arcobacter* cells in seafood samples, comparing the results with those obtained with different culture approaches.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

A total of 38 bacterial species, 18 species comprising reference strains from eight different genera, like *Campylobacter* related to *Arcobacter* and others frequently recovered from shellfish (i.e., *Salmonella*, *Escherichia coli*, etc.), and 20 *Arcobacter* species. The bacterial strains were used to develop and examine the specificity of primers and probes (**Supplementary Table S1**). The different

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Nuria Salas Massó

strains were grown on tryptone soy agar at 37°C for 24 h (TSA, Difco, France), with the exception of *Arcobacter* species that were grown on Blood Agar (BA; TSA supplemented with 5% sheep blood BD Difco, Le Pont de Claix, France), and *A. marinus* and *A. halophilus*, that were grown on Marine Agar (MA; Scharlab, Barcelona, Spain) and were incubated under aerobic conditions at 30°C for 48 h. Species from the genus *Campylobacter* were inoculated on BA and incubated under microaerobic conditions (oxygen, 6 to 16%; carbon dioxide, 2 to 10%; and nitrogen, 80%; generated using the Gas Pak EZ Campy container sachets™ Becton Dickinson, Sparks, MD, United States) at 37°C for 48 h.

Sample Preparation and Analysis

Pure Culture Samples and Mixed Models

Twenty *Arcobacter* species (**Supplementary Table S1**) were used to determine the viability of cells, and evaluate the specificity of the developed assay. Initial bacterial suspensions were prepared in 0.9% (w/v) sterile saline solution (SS) up to an OD_{550} = 0.250. An aliquot of those live cell (LC) suspensions was used to obtain dead cells (DC) by thermal inactivation (100°C, 10 min). Four models, 100% LC; 50% LC + 50% DC; 10% LC + 90% DC and 100% DC in a final volume of 200 μl were tested for each species in duplicate. For quantification of CFU and confirm the efficacy of thermal inactivation, the LC and DC suspensions were plated on BA (with the exception of *A. marinus* and *A. halophilus* that were plated on MA) and incubated at 30°C for 48 h, (**Figure 1**). The procedure was continued as described in the sections "PMA treatment" and "DNA isolation." Additionally, LC treated with PMA were also plated on the media described above to check for any cytotoxic effects on the cells by the presence of this DNA-intercalating dye.

Artificially Contaminated Samples

Depurated oyster and mussel samples were collected from a depuration plant in Alfacs Bay (Ebro River Delta, Spain) and were scrubbed, shucked, and then homogenized with a stomacher (Lab · Blender 400). A mix of 270 mL of peptone water (PW) and 30 g of flesh and intervalval liquid from the seafood were homogenized (ISO/TS 16649-3:2005). Then, the homogenized mixtures (hereinafter referred to as direct samples) were aliquoted in 9 mL and were inoculated with 1 mL of a 10-fold dilution (from 10^0 to 10^{-6}) from an initial inoculum (1.26×10^8 CFU/ml) of *A. butzleri* (OD_{550} = 0.250). Additionally, a mix of living and dead *A. butzleri* cells (100% LC; 50% LC + 50% DC; 10% LC + 90% DC; 100% DC) were also inoculated in the equivalent homogenized mixtures of shellfish. The CFU number in each dilution was obtained by standard plate counting methods using BA plates, which were incubated for 48 h at 30°C. After seeding and agitating, bacterial cells were obtained following the washing and concentration protocol, previously described by Zhu et al. (2012). To test the presence of background *Arcobacter* spp. in the depurated samples, 3 mL aliquots of homogenized sample were transferred to 7 mL of Arcobacter-CAT broth and Arcobacter-CAT broth supplemented with 2.5% NaCl and incubated at 30°C for 48 h to collect cell pellets for qPCR testing (hereinafter referred to as blank samples). Additionally, 200 μL of the enrichment broth were plated on BA and MA for examining positive culture of *Arcobacter* spp. The final pellets were suspended in 1 mL of SS, for further analysis (see sections "PMA Optimization Protocol," "DNA Isolation," "Polymerase Chain Reaction (PCR)," "Quantitative PCR (qPCR)") A flow diagram is shown in **Figure 1**.

Natural Samples

To study the effectiveness of PMA-qPCR, 17 different raw seafood samples including oyster (*Crassostrea gigas*; n = 2), mussel (*Mytilus galloprovincialis*; n = 5), razor clam (*Ensis arcuatus*; n = 3) and wedge clam (*Donax trunculus*; n = 7) collected from Alfacs Bay were tested. Analysis was conducted within 24 h after the collection. The samples were scrubbed, shucked, and homogenized as mentioned above in Section "Artificially Contaminated Samples." After the washing treatment, the pellets were suspended in 1 mL of sterile salt water. The presence of background *Arcobacter* was performed for all the samples as mentioned in Section "Artificially Contaminated Samples."

PMA Treatment

PMA (Blu-V Viability PMA Kit, Qiagen, Germany) was suspended in 20% dimethyl sulfoxide (DMSO) according to the manufacturer's instructions to a final concentration of 2 mM, which was used as stock and was stored at −20°C in the dark. The PMA solution was added to 200 μL of sample in a 1.5 mL light-transparent micro-centrifuge tube to yield a final concentration of 20 μM. The tubes were incubated at room temperature in the dark for 5 min to allow PMA penetration into the damaged cells. Afterward, the samples were photoactivated using a PhAST-Blue lamp (450λ LED, GenIUL, Spain) for 15 min at room temperature. Duplicates of all these samples without the addition of PMA followed the same protocol.

PMA Optimization Protocol

For the optimization of the method, conventional PCR was used as a standard [see section "Polymerase Chain Reaction (PCR)"]. The PMA was added to a final volume of 50 μL of a 1 ng/μL solution of *A. butzleri* DNA, as previously described (Nocker et al., 2006) to obtain a series of final concentrations of the dye of: 0.02, 0.2, 2, and 20 μM. An incubation time of 5 min at room temperature and a photoactivation period of 15 min, were initially applied, following the manufacturer's instructions. Afterward, different DNA concentrations, ranging from: 1, 2, 10, and 20 ng/μL, were tested with PMA added to a final concentration of: 0.2, 2, and 20 μM, in a final volume of 50 μL.

To check whether the exposure time of the dye to light can influence the removal of the DNA signal at lower PMA concentrations, an experimental design was prepared. A series of tubes with a final volume of 50 μL containing 10 ng/μL of DNA and PMA to a final concentration of 0.2 μM were prepared. Photoactivation was performed during 7.5, 15, 30, and 60 min. Additionally, the reactivity of possible remaining excess PMA was assessed according to Nocker et al. (2006). Briefly, tubes containing 47.5 μL of PMA at 0.02, 0.2, 2, and 20 μM concentration were photoactivated

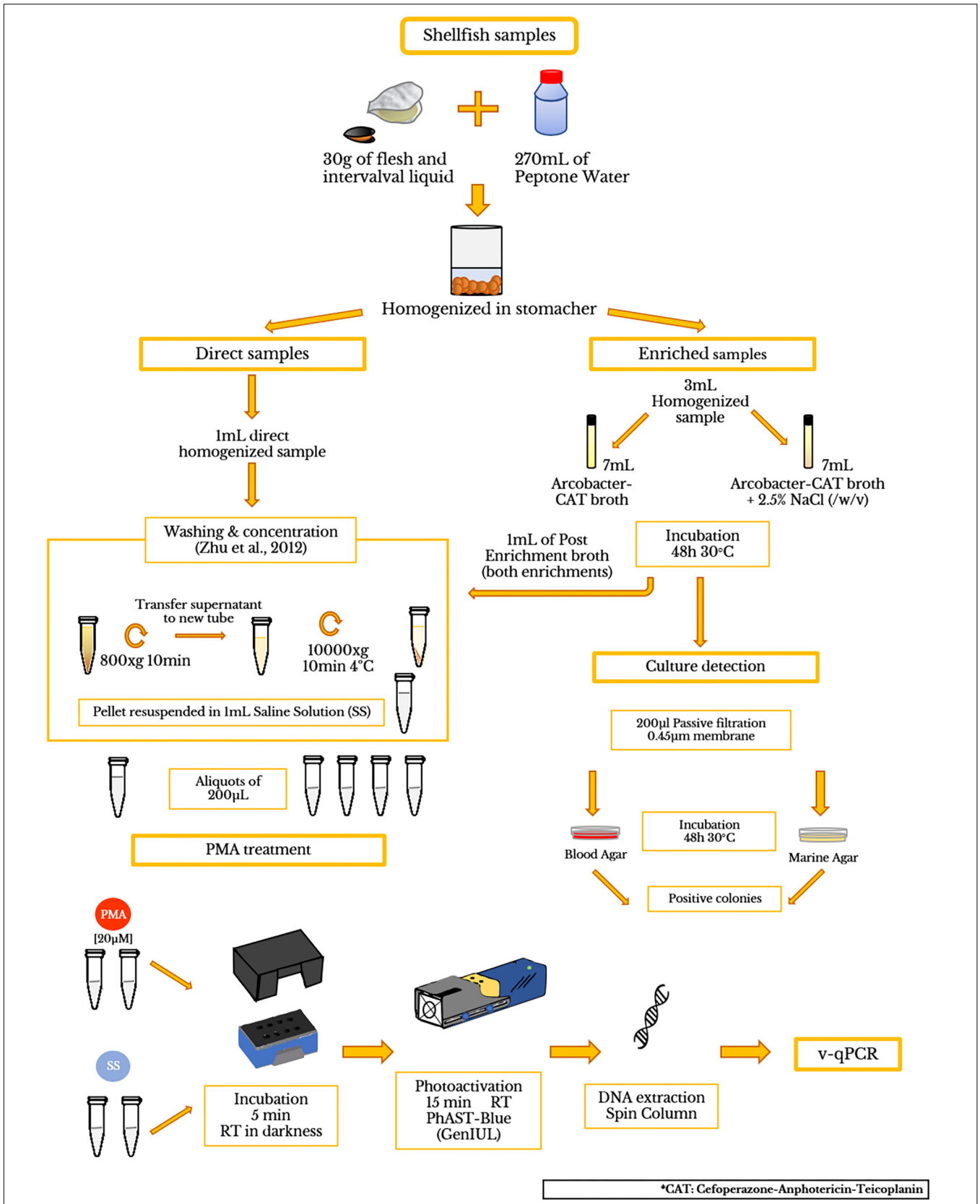

FIGURE 1 | Flow diagram showing the processing of shellfish samples and PMA treatment.

for 15 min. Afterward, 2.5 μL of 20 ng/μL of DNA was added and photoactivated again, to see if there was remaining cross-linking activity of PMA to DNA. All experiments described included a positive and negative control and were performed twice.

DNA Isolation

Total bacterial DNA was extracted from natural and artificially spiked samples (including direct samples and cell pellets from the enrichment in Arcobacter-CAT and Arcobacter-CAT supplemented with 2.5% NaCl; sections "Artificially Contaminated Samples" and "Natural Samples") with the isolation performed according to Zhu et al. (2012) using spin columns (QIAmp DNA MiniKit 250; Qiagen, Germany) and following the manufacture's instruction. DNA concentration was determined using a NanoDrop™ 2000 spectrophotometer (Thermo Scientific, Waltham, MA, United States).

Polymerase Chain Reaction (PCR)

Conventional PCR targeting 23S rRNA was performed to check specificity and to optimize the PMA protocol. The primers previously described by Hausdorf et al. (2013) with a modification (underlined) on the forward primer (23SF 5′-AACATATAAGCGCGATGTGGGGAC-3′; and the reverse primer: 23SR 5′-ACGGTACGGGCAACATATAATA-3′) were used. The PCR was performed in a T3 Thermocycle Biometra with PCR reaction mixtures containing 5 μL of 2X Phusion® Human Specimen PCR Buffer (Thermo Fisher Scientific) forward and reverse primers to a final concentration of 500 nM, 0.1 U of Phusion Hot Start II High Fidelity DNA polymerase (Thermo Fisher Scientific), 2 μL of the DNA or DNA-PMA mix, and water to a final volume of 10 μL. The PCR program of 24 cycles consisted of: (1) 5 min at 98°C, (2) 30 s at 98°C, (3) 20 s at 56°C, (4) 20 s at 72°C, without a final elongation step. The expected PCR amplicons of 233 bp were visualized in a 2.5% agarose gel stained with RedSafe™ (iNtRON Biotechnology, South Korea).

Quantitative PCR (qPCR)

Quantitative PCR amplifications were carried out in duplicate on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Alcobendas, Spain) in a final volume of 20 μL containing 2 μL of DNA or DNA-PMA mix, 900 nM of the modified 23S rRNA primer pair described above, 10 μL of SsoAdvanced™ Universal SYBR Green Supermix (Bio-Rad), and 1 U platinum Taq DNA polymerase (Invitrogen). All assays also systematically included a negative control. The PCR thermocycling was initiated at 98°C for 3 min, followed by 35 cycles of 15 s at 95°C, 30 s at 56°C, and 1 min at 60°C. Fluorescence data were collected at the end of each cycle. The limits of detection (LOD) and quantification (LOQ) were calculated as described before (Hausdorf et al., 2013). To evaluate the effect of PMA treatment on a sample, the ΔCt was calculated. The ΔCt of a sample is the difference between the Ct-value obtained with PMA treated sample and the Ct-value obtained with non-treated sample: ΔCt = (Ct sample w/PMA – Ct sample w/o PMA). An increase in Ct values is perceived as a reduction in PCR signal and will be described as such throughout the text.

Statistics Analysis

Ct values are presented as mean and standard deviations. Equalities of variance for the mean percentages for the model mixture experiments were assessed using the Levene's test. Statistically significant difference in group means was performed using a one-way ANOVA analysis. To evaluate where the differences occurred between species the *post hoc* test Games-Howell was run. All the analyses were performed using Software SPSS Statistical (IBM Analytics).

RESULTS

Development and Optimization of Conditions for Use of PMA to Study DNA of *Arcobacter* spp. in the Samples

It was necessary to develop and optimize a method to use the PMA for studying live and dead *Arcobacter* spp. cells. The developed method was first applied to pure DNA samples and afterward was used to discriminate live and dead cells. Initially, the minimum PMA concentration that can effectively remove the signals from 1 ng/μL of *A. butzleri* DNA was 0.2 μM (**Supplementary Figure S1A**). However, in the experiment testing different DNA concentrations, total removal of signal from the sample containing 20 ng/μL of *A. butzleri* DNA was achieved when using 20 μM PMA in a 50 μl volume reaction (**Supplementary Figures S1B,C**).

When the different photo-activation times were assessed, it was observed that periods longer than 15 min did not improve the removal of the DNA signal by PMA (**Supplementary Figure S1D**). As shown in **Supplementary Figure S1E**, after photoactivation any potential remaining excess PMA had reacted with water and was no longer effective.

Optimization of PMA Conditions for *Arcobacter* spp. Pure Cultures

The optimized conditions described for the DNA in Section "PMA Optimization Protocol" were applied to pure *A. butzleri* cultures. Initially, cells of *A. butzleri* were resuspended in SS solution (0.9%) to a final OD_{550} = 0.250 (McFarland 1). Two different samples consisting of live cells (LC) and dead cells (DC), the latter being obtained by heating at 100°C for 10 min as described previously (Hrušková et al., 2013), were treated with PMA in a final volume of 500 μL. The PMA prevented amplification of a signal from the DNA of the dead cells as shown in **Supplementary Figure S2A**. Afterward, the PMA protocol was also tested at higher concentrations of dead *A. butzleri* cells (OD_{550} = 0.8, 0.4, 0.250; **Supplementary Figure S2B**), and resulted in the inhibition of these higher concentrations. The same results were obtained for the other 19 *Arcobacter* spp. (data not shown). After plating both LC and LC + PMA, no cytotoxic effect of the 20 μM PMA was observed, as the CFU values were equal in both cases (data not shown).

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Nuria Salas Massó

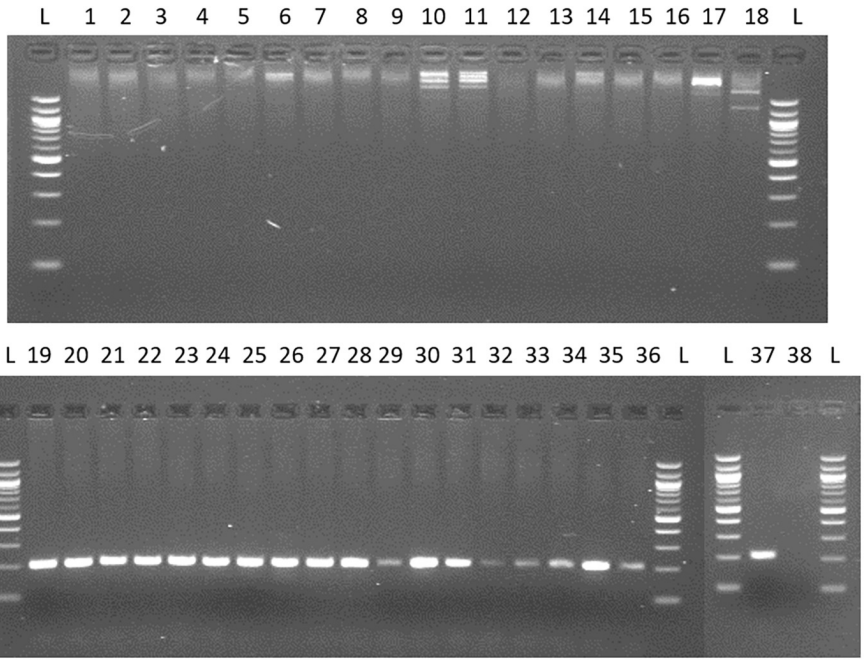

FIGURE 2 | Testing specificity of 23S primers with species from genus frequently recovered/related to *Arcobacter* and 20 species of the genus *Arcobacter*. Lane 1: *Campylobacter coli;* 2: *C. jejuni;* 3: *C. lari;* 4: *C. mucosalis;* 5: *C. upsaliensis*; 6: *C. sputorum ss.spo;* 7: *C. fetus subsq. Fetus*; 8: *C. concisus;* 9: *C. hyointestinalis;* 10: *Salmonella enteritidis;* 11: *S. typhimurium;* 12: *Enterococcus faecalis;* 13: *E. faecium;* 14: *Escherichia coli;* 15: *Streptococcus pneumoniae;* 16: *Proteus hauseri;* 17: *Citrobacter freundii;* 18: *Yersinia ruckeri*. 19. *A. butzleri*; 20. *A. skirrowii*; 21 *A. cryaerophilus;* 22: *A. nitrofigilis;* 23: *A. thereius*; 24: *A. cloacae*; 25: *A. trophiarum*; 26: *A. cibarius*; 27: *A. suis*; 28: *A. defluvii*; 29: *A. marinus*; 30: *A. aquamarinus*; 31: *A. halophilus*; 32: *A. ebronensis*; 33: *A. molluscorum*; 34: *A. venerupis*; 35: *A. ellisii*; 36: *A. mytili*; 37: *A. bivalviorum*; 38: negative control.

Specificity and Viability of *Arcobacter* Cells in Model Mixtures by v-qPCR

For the quantification of viable *Arcobacter* spp. cells in seafood, a modified 23S q-PCR coupled with PMA was used. As shown in **Figure 2**, other Gram-negative bacteria genera, different than *Arcobacter* gave no amplification, showing the specificity of the primers. The efficiency, LOD and LOQ of the v-qPCR are shown in **Table 1**.

Table 2 shows an average Ct value for *Arcobacter* species of 14.36, which ranged from 12.54 for *A. cryaerophilus*, to 17.99 for *A. nitrofigilis*. When the v-qPCR was used in model mixtures, it was observed that PMA was able to reduce the signal from dead cells, in all the different ratios that were tested (**Table 2**).

TABLE 1 | Specificity, efficiency and limit of detection of Arcobacter-23S v-qPCR assays developed in this study.

23S v-qPCR	
R^2	0.9764
Intercept	39.72
Slope	−3.5139
PCR efficiency (%)	100.03
Limit of detection (LOD; n° copies)	21
Limit of quantification (LOQ; n° copies)	458

Assays were tested on genomic DNA from the selected bacterial strains.

However, the model mixture 50% LC + 50% DC was the one which gave a higher standard deviation, wherein *A. suis* there was detected a percentage of copies that was statistically ($P < 0.05$) higher than the expected for the 50% LC (71.31%). On the other hand, *A. ellisii* and *A. molluscorum* showed a lower percentage of copies detected than the expected (30.02 and 24.99%, respectively; **Table 2**).

Quantification of *Arcobacter* spp. in Seafood

Mussel and oyster samples were artificially inoculated with *A. butzleri* live and dead cells, in order to assess the interference from the shellfish tissues to the reactivity of the PMA. Results showed that, in the first place, there was no background *Arcobacter* DNA in the blank mussel and oyster samples; in the second place, when the cells were dead, the resulting Ct values suggest that DNA may have been lost or degraded during the extraction process (**Supplementary Figure S3**). However, the use of PMA improved the reduction of the signal from DNA of dead cells achieving the corresponding percentages of copies.

To evaluate the usefulness of the v-qPCR method, a total of 17 different raw shellfish samples were tested for the presence of viable *Arcobacter* spp. cells and the results were compared with those obtained from two different enrichment broths in addition to direct culture isolation (**Figure 3**). Only 5.9% (1/17) of the samples were positive by direct plating on BA. Culture after pre-enrichment in blood agar and marine agar yielded a 29.4 and

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Nuria Salas Massó

TABLE 2 | Bacterial model mixtures for determining the efficiency of the Arcobacter-23S v-qPCR to detect DNA from live cells.

Species	Ct-value OD_{550} = 0.250	100% LC copy numbers	50% LC [a]	10% LC [a]	100% DC [a]
A. aquimarinus	14.23 ± 0.13	2.24×10^7	56.81 ± 22.45	15.43 ± 4.34	0.01 ± 0.01
A. bivalviorum	14.77 ± 0.18	1.41×10^7	66.04 ± 16.90	15.76 ± 5.26	0.01 ± 0.01
A. butzleri	13.33 ± 0.07	1.17×10^7	45.28 ± 5.54	11.85 ± 4.75	0.02 ± 0.01
A. cibarius	13.98 ± 0.32	3.11×10^7	59.11 ± 5.24	11.26 ± 1.56	0.04 ± 0.03
A. cloacae	13.46 ± 0.28	4.21×10^7	61.82 ± 11.73	14.58 ± 4.48	ND
A. cryaerophilus	12.54 ± 0.16	2.14×10^7	51.48 ± 4.54	8.39 ± 1.83	0.01 ± 0
A. defluvii	13.62 ± 0.21	3.96×10^7	40.08 ± 6.69	8.74 ± 1.01	0.24 ± 0.21
A. ebronensis	14.34 ± 0.21	1.88×10^7	38.56 ± 8.09	7.11 ± 1.06	ND
A. ellisii	12.83 ± 1.08	7.53×10^7	30.02* ± 4.53	8.16 ± 2.49	0.02 ± 0.01
A. halophilus	14.01 ± 0.14	2.10×10^7	67.42 ± 4.84	12.61 ± 1.17	ND
A. lanthieri	13.54 ± 0.12	4.97×10^7	47.85 ± 1.69	8.16 ± 0.62	0.01 ± 0
A. marinus	15.98 ± 0.13	5.62×10^6	71.43 ± 16.52	23.66 ± 1.96	0.5 ± 0.27
A. molluscorum	14.47 ± 0.46	1.80×10^7	24.99* ± 3.12	7.68 ± 6.09	0.12 ± 0.05
A. mytili	14.34 ± 0.03	2.57×10^7	56.98 ± 14.71	19.5 ± 15.6	0.63 ± 1.08
A. nitrofigilis	17.99 ± 0.21	3.52×10^6	58.06 ± 2.57	11.3 ± 0.96	0.03 ± 0.03
A. skirrowii	13.74 ± 0.21	1.12×10^7	50.32 ± 4.77	9.38 ± 1.85	ND
A. suis	14.78 ± 0.30	5.77×10^6	71.31* ± 4.2	19.09 ± 2.59	0.01 ± 0
A. thereius	15.54 ± 0.33	8.69×10^6	55.65 ± 13.2	9.04 ± 2.88	ND
A. trophiarum	14.94 ± 0.61	7.07×10^7	39.4 ± 23.57	6.96 ± 6.15	0.13 ± 0.21
A. venerupis	14.77 ± 0.22	2.06×10^7	30.28 ± 14.21	8.44 ± 4.88	ND
Average	14.36 ± 1.20	2.59×10^7	53.31 ± 21.97	12.32 ± 7.85	0.09 ± 0.27

[a]*Percentage of copy numbers detected in relation to the total copy number obtained from the bacterial model of 100% live cells. *Mean percentage was statistically different from the rest (P = 0.05). LC, live cells; DC, dead cells; ND, no detection.*

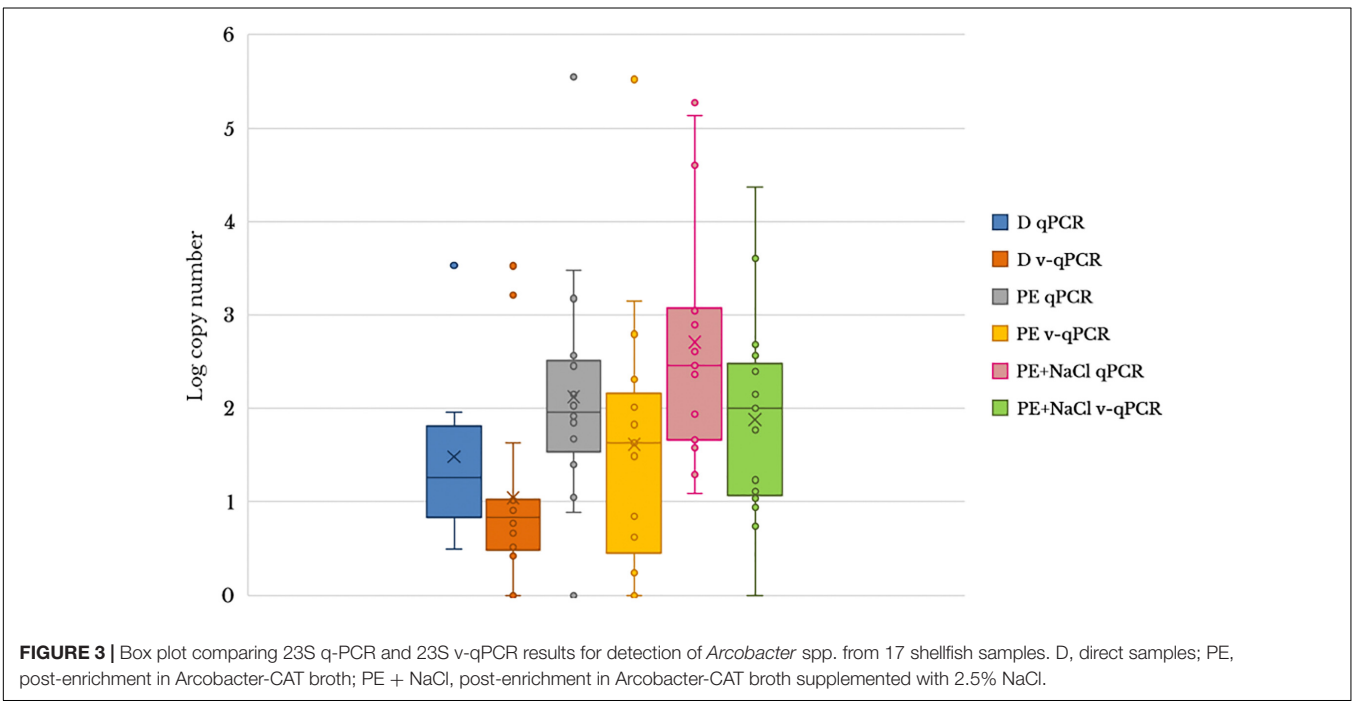

FIGURE 3 | Box plot comparing 23S q-PCR and 23S v-qPCR results for detection of *Arcobacter* spp. from 17 shellfish samples. D, direct samples; PE, post-enrichment in Arcobacter-CAT broth; PE + NaCl, post-enrichment in Arcobacter-CAT broth supplemented with 2.5% NaCl.

35.3% of positive samples, respectively. However, the number of positive samples increased when the v-qPCR was used. As expected, the number of copies/g of raw shellfish samples were lower than those from pre-enriched samples (PE). Differences in the number of copies were observed between samples treated or not treated with PMA, with a reduction in the copy number seen for those treated with PMA (**Figure 3**).

The effect of PMA on the reduction of qPCR signal from shellfish samples showed that the use of PMA reduced the signal and resulted in a lower copy number being detected (**Table 3**).

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Nuria Salas Massó

TABLE 3 | Quantitative detection of 23S copy numbers of *Arcobacter* spp. in 17 seafoods samples by qPCR and v-qPCR and comparison of results obtained by culture isolation.

	Log number of copies/g of flesh and intervalval liquid of shellfish						Culture			
Sample	D qPCR	D v-qPCR	PE qPCR	PE v-qPCR	PE + NaCl qPCR	PE + NaCl v-qPCR	D-BA[a]	D-MA[b]	PE-BA[c]	PE-MA[d]
Mussel 1	4.23	ND	5.20	5.19	5.63	5.36	–	–	–	–
Mussel 2	4.72	4.13	4.22	ND	8.45	6.78	–	–	–	–
Mussel 3	4.43	3.97	6.65	3.45	4.26	4.28	–	–	–	–
Mussel 4	4.61	3.84	4.06	3.41	8.31	7.55	–	–	–	–
Mussel 5	5.14	3.69	5.03	4.02	4.75	3.91	–	–	–	–
Oyster 1	3.67	3.60	5.34	5.01	5.55	5.18	–	–	–	–
Oyster 2	4.00	3.95	5.63	5.48	5.11	4.94	–	–	–	–
Razor clam 1	4.84	4.21	ND	ND	5.79	ND	–	–	–	–
Razor clam 2	4.32	3.63	4.58	4.81	4.84	4.41	–	–	–	+
Razor clam 3	3.70	ND	5.74	5.97	6.27	5.57	–	–	+	–
Wedge clam 1	6.71	6.70	6.35	6.32	5.54	5.32	+	–	–	+
Wedge clam 2	6.73	6.39	8.73	8.70	6.07	5.86	–	–	+	–
Wedge clam 3	4.91	4.81	5.09	3.80	7.78	5.74	–	–	+	+
Wedge clam 4	3.92	4.13	4.85	4.66	4.46	4.34	–	–	–	–
Wedge clam 5	5.06	4.01	5.13	5.00	4.84	4.21	–	–	+	+
Wedge clam 6	4.13	4.19	5.32	4.08	6.22	4.12	–	–	–	+
Wedge clam 7	4.01	4.09	5.02	5.19	6.13	5.23	–	–	+	+
Average	4.65	3.84	5.11	4.42	5.88	4.87				

D, direct samples; PE, post-enrichment in Arcobacter-CAT broth; PE + NaCl, post-enrichment in Arcobacter-CAT broth supplemented with 2.5% NaCl. [a]D-BA, homogenized samples directly cultured onto blood agar and incubated at 30°C for 48 h. [b]D-MA, homogenized samples directly cultured onto marine agar and incubated at 30°C for 48 h. [c]PE-BA, post-enrichment in Arcobacter-CAT broth cultured onto blood agar and incubated at 30°C for 48 h. [d]PE-MA, post-enrichment in Arcobacter-CAT broth supplemented with 2.5% NaCl cultured onto marine agar and incubated at 30°C for 48 h.

The reduction effect (ΔCt) from direct samples and those pre-enriched in Arcobacter-CAT broth were similar $\Delta Ct_{Dsamples} = 1.05$ and $\Delta Ct_{PEsamples} = 1.04$. However, a higher reduction is observed in the samples pre-enriched in Arcobacter-CAT broth supplemented with 2.5% NaCl $\Delta Ct_{PE+NaCl\ samples} = 3.27$.

DISCUSSION

The genus *Arcobacter* comprises species that have been considered zoonotic agents and emergent pathogens by the International Commission on Microbiological Specifications for Foods (ICMSF, 2002) i.e., *A. butzleri*. *Arcobacter* species have been recovered from a wide range of different food animals and food products worldwide (Collado and Figueras, 2011; Ferreira et al., 2016, 2017), among which seafood represents a reservoir of known and unknown *Arcobacter* species (Salas-Massó et al., 2016, 2018), posing a risk for the consumer. The detection of foodborne pathogens in shellfish is mainly based on culture techniques, which are time consuming and cannot detect VBNC cells; but also, in PCR, which cannot differentiate between DNA from live or dead cells, that could lead to unnecessary product recalls (Barbau-Piednoir et al., 2014; Gensberger et al., 2014; Zhang et al., 2016). Thus, for the first time in our study, the use of a viable-qPCR method based on specific primers for the detection of viable *Arcobacter* spp. cells in shellfish was developed.

Recently, Ferreira et al. (2017) reviewed the different molecular methods for the detection of *Arcobacter* spp.; most of them being multiplex PCR targeting only three species, *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii* [i.e., m-PCR by Harmon and Wesley (1997); m-PCR by Houf et al. (2000); m-PCR by Kabeya et al. (2003); PCR by González et al. (2014)]. So far, four quantitative PCR (qPCR) have already been developed for *Arcobacter*. De Boer et al. (2013) and Webb et al. (2016) designed a qPCR specific for *A. butzleri* targeting the genes *hsp60* (encoding for a heat shock protein) and the *qhnDH* (encoding for the gamma subunit of quinohemoprotein amine dehydrogenase). However, Hausdorf et al. (2013) developed two q-PCR assays targeting both, the 16S and the 23S rRNA genes. The 16S q-PCR showed a reduced efficiency detecting *A. halophilus* and *A. marinus*, both species being common in shellfish (Salas-Massó et al., 2016). Thus, the 23S q-PCR was chosen to be coupled with PMA because of its higher efficiency detecting *Arcobacter* spp., and because the amplification product (233 bp) had an appropriate length for PMA experiments as previously reviewed by Fittipaldi et al. (2012). Although the authors tested the specificity of their primers with other genera different from *Arcobacter*, they did not test other bacteria like *Campylobacter*, which is closely related to *Arcobacter*, nor *Salmonella,* which is also commonly found in different types of food, and only 15 *Arcobacter* species were tested. After a modification in the forward primer we observed that no amplification from other species different from *Arcobacter* were obtained and all the 20

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Nuria Salas Massó

Arcobacter species tested were detected with no signal from the other genera tested.

One of the main problems regarding v-qPCR is the comparison of results among the different studies available in the literature, mainly due to the different conditions used in the experiments. A clear example of this is the study of Josefsen et al. (2010), they developed a PMA-qPCR targeting *Campylobacter* spp. in broiler carcasses, obtaining good results in the discrimination of dead cells even at bacterial concentration of 10^6 CFU · mL^{-1}. However, Pacholewicz et al. (2013) using the same PMA-qPCR protocol of Josefsen's, did not obtain complete inhibition of the signal from dead *Campylobacter* cells at concentrations higher than 10^4 CFU · mL^{-1}. Similar results were obtained by Seinige et al. (2014) although they used a different qPCR protocol. Recently, Castro et al. (2018) evaluated the presence of *Campylobacter* cells in frozen and chilled broiler carcasses by means of a v-qPCR and they found a good discrimination between the different stages of the bacterial cells. Among other considerations, the differences between these studies could be attributed to the fact that none of the four studies mentioned above had the same PMA treatment. The light source varied in power and type (halogen lamps vs. LED devices); the PMA final concentration was different (i.e., 10, 20, 25.55, and 50 $\mu g \cdot mL^{-1}$); there were also variations in the incubation time and temperature, as well as the photoactivation time (1, 3, and 15 min). This is why the optimization and standardization of v-qPCR protocols are necessary. Additionally, the storage of a sample should also be taken into account, when comparing results, because as long as the storage step affects the viability of the cells, it would also influence the results obtained by the v-qPCR. In the study of Castro et al. (2018), they were led to the conclusion that *Campylobacter* spp. remain viable more frequently in chilled carcasses than in frozen ones. Further, Fernández-Piquer et al. (2012) showed that the number of *Arcobacter* cells are affected by the storage temperature of oysters. The number of *Arcobacter* cells increase after storage in comparison to the pre-storage oysters (Fernández-Piquer et al., 2012). Similar results were obtained for *Arcobacter buztleri* in broiler carcasses, indicating that this species can multiply during storage (Badilla-Ramírez et al., 2016).

Optimization of the use of DNA-intercalating dyes should be performed for each species due to the different sensitivity and integrity of the membrane and to diverse susceptibilities of different species to stress (Fittipaldi et al., 2012). Usually, the optimization of the protocol should include selection of dye (EMA or PMA), type of light used, time of photoactivation, concentration of the dye and of the bacteria, among other factors. In this work PMA was chosen as it has been demonstrated that PMA has a lower cytotoxicity than EMA (Fittipaldi et al., 2012 and references therein). Hrušková et al. (2013) performed a study where they evaluated the proportion of viable and dead cells of *A. butzleri* and *A. cryaerophilus* in biofilms. In that study they were the first researchers to test this genus using both EMA and PMA. Blockage of amplification of DNA from dead *A. butzleri* cells was achieved using EMA 25 $\mu g \cdot mL^{-1}$ or PMA 0.2 $\mu mol \cdot L^{-1}$. However, *A. cryaerophilus* was more sensitive to both dyes, i.e., EMA 1 $\mu g \cdot mL^{-1}$ or PMA 0.02 $\mu mol \cdot L^{-1}$. Recently, Webb et al. (2016) described a q-PCR for which they used an EMA final concentration of 100 $\mu g \cdot mL^{-1}$ as a pre-treatment for detecting *A. butzleri* cells in wastewater samples. Nevertheless, this protocol was originally designed for *Campylobacter jejuni*, and according to the results from Hrušková et al. (2013), this concentration would block amplification of DNA from viable cells leading to an underestimation of the number of live cells detected by their q-PCR. However, in our study we found that PMA at a concentration of 20 $\mu mol \cdot L^{-1}$ was optimal. According to Hrušková et al. (2013) this concentration would not block the signal from *A. cryaerophilus* cells, but it would from those of *A. butzleri*. On the contrary, we did not observe any reduction in the signal from live *A. butzleri* cells at 20 $\mu mol \cdot L^{-1}$. One of the possible reasons for this discrepancy could be the photoactivation source. In both works (Hrušková et al., 2013; Webb et al., 2016) they used a halogen lamp, which has shown to heat the samples leading to a possible higher susceptibility of the cells to the dyes. The use of devices like the one used in this study (Phast Blue which uses LED, thus generating only negligible heat) would make the use of viable dyes more standardized (Fittipaldi et al., 2012).

When the PMA-based v-qPCR was used for testing model mixtures of cells (live and/or dead), the percentages obtained for the different ratios of live and dead cells was in general terms good, which are in concordance with the observations reported by Hrušková et al. (2013) for *A. butzleri* and *A. cryaerophilus,* where the PMA method was good enough to discern viable cells from dead cells. These experiments with model mixtures have demonstrated the ability for differentiation of cell status by PMA in other bacterial species such as *Listeria innocua* (Løvdal et al., 2011; Soejima et al., 2011), and *Enterobacter sakazakii* (Cawthorn and Witthuhn, 2008). However, in the present work, when PMA was applied to the model 50% LC + 50% DC for *A. suis*, an overestimation of the proportion of live cells occurred, indicating that PMA at that ratio was not capable of binding to all the DNA present in dead or damaged cells. On the other hand, *A. ellisii* and *A. molluscorum* showed an underestimation in the percentage of live cells in the 50:50 model. Fittipaldi et al. (2012) in their review, showed that different proportions of dead cells in a sample can determine an increase/decrease in the Ct value of the samples treated with PMA. In this case for the species *A. suis*, *A. ellisii*, and *A. molluscorum* the ratio 50:50 altered the effectiveness of the method. Nevertheless, it should be taken into account that the v-qPCR has been designed for detecting the genus *Arcobacter* and not specific species. As a tool for food safety, on average the PMA method presented good results that should aid in preventing unnecessary, costly food recalls.

To date, only two studies have investigated the effectivity of a v-qPCR methodology in seafood samples. Quijada et al. (2016) showed that PMA activity was not affected by the processing of such a complex matrix as are clams. However, Zhu et al. (2012) when analyzing different seafood (including oysters, scallops and crabs) observed that samples with turbidities greater than 10 Nephelometric Turbidity Units (NTU) did not adequately inhibit the amplification of DNA from dead cells. Our results showed that, when processing *A. butzleri* spiked shellfish samples, the PMA method was effective for inhibiting the signal from the

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Nuria Salas Massó

dead cells, in concordance with the results obtained by Quijada et al. (2016). However, when PMA was not used, there was also a reduction in the number of copies detected from the model mixtures of live and dead cells, indicating that part of the dead cells and free DNA added to the spiked sample could have been lost during DNA extraction. It has been demonstrated that different DNA extraction protocols yield different quantity and/or quality of nucleic acid (Demeke et al., 2009 and references therein). Thus, DNA extraction may be an additional factor to include in future standardization of v-qPCR protocols. The v-qPCR method presented in this study, and tested in four different types of shellfish, could be used for studying *Arcobacter* in other different matrices, providing that the DNA extraction method is demonstrated to be sufficiently effective for the other matrix, as Quijada et al. (2016) have done for their enteric RNA and DNA viruses PMA protocol, that was applied in clams and "chorizo" sausages. Nevertheless, Fittipaldi et al. (2012) indicated in their review that EMA and PMA effectiveness is matrix dependent; this is why optimization of the protocols are highly recommended.

Some studies have used in parallel direct plating, post-enrichment culture and a direct multiplex-PCR for analyzing diverse types of samples (González et al., 2007; Collado and Figueras, 2011; Levican et al., 2016). In these studies, it was found that direct multiplex-PCR yielded the same or higher number of positive samples as culturing, with the exception of Levican et al. (2016) that reported just the opposite. In our study, we used specific qPCR and v-qPCR as tools for detection of *Arcobacter* in shellfish samples in parallel to four culturing approaches. As recommended by Salas-Massó et al. (2016), when analyzing seafood samples, in addition to the conventional approach as described in Levican et al. (2016), we included direct plating in marine agar and post-enrichment in Arcobacter-CAT broth + 2.5% NaCl and subculturing in marine agar. Salas-Massó et al. (2016) demonstrated that the use of this protocol in marine samples yielded c.a. 40% more positive culture samples for *Arcobacter* than when only the conventional approach was used for analyzing these samples. Through the use of culture-independent approaches (molecular biology), we obtained a higher number of positive samples than from the culture-based approaches. The presence of potential new unculturable *Arcobacter* species in marine samples have been demonstrated in several studies (Miller et al., 2007; Wesley and Miller, 2010; Collado and Figueras, 2011; Fernández-Piquer et al., 2012; King et al., 2012) that could favor the utilization of molecular tools over culture-based methodologies. While DNA-based approaches can detect cells in the VBNC state, a disadvantage is that free DNA is also detected from dead cells. Our results have shown that implementation of v-qPCR, using PMA, reduces the signal obtained from samples containing dead cells as compared to those analyzed by standard qPCR, indicating that amplification of free DNA, or that of dead cells, is being blocked as occurred for other bacterial species in many different samples (Kobayashi et al., 2009; Josefsen et al., 2010; Li and Chen, 2012; Zhu et al., 2012; Zhang et al., 2015). However, a greater reduction was observed in samples that corresponded to post-enrichment in Arcobacter-CAT broth + 2.5% NaCl. This phenomenon has been reported by Shi et al. (2011) where heat-killed cells were previously exposed to different concentrations of NaCl (0.125–10%), and they observed that the higher the osmotic shock, the greater is the signal reduction. This may be attributed to an osmotic destabilization of the cell membrane leading to more efficient dye uptake (Fittipaldi et al., 2012).

CONCLUSION

This is the first report on the development of a viable q-PCR for selectively amplifying DNA from viable *Arcobacter* spp. cells in shellfish samples. The PMA protocol was optimized for 20 species of the genus *Arcobacter* taking into account diverse factors like the concentration of PMA, incubation time and temperature, photo-activation time or cell concentration. The usefulness of PMA was then extrapolated to a v-qPCR where different mixed ratios of viable and dead cells were used, obtaining satisfactory inhibition of DNA amplification from the different proportions of dead cells in 85% of the *Arcobacter* species tested. The demonstrated efficiency of the PMA v-qPCR was applied to real seafood matrices such as raw oysters and mussels. A general decrease in the number of copies was detected in spiked samples treated with and without PMA, probably associated to DNA extraction procedures for shellfish samples. However, when PMA was applied, a significant reduction in the signal of *Arcobacter* DNA was observed and this reduction increased when the DNA was extracted from post-enrichment broth containing 2.5% NaCl, favoring the penetration of the PMA into damaged cells.

With this study, we encourage the use and standardization of viable qPCR for rapid, specific detection of viable microorganisms of public health concern in food products. Thus, this work, if applied to *Arcobacter* species along with other hazardous bacteria and viruses, could contribute to improve the database for food safety authorities, when regulating for food safety and risk analysis regarding shellfish consumption. Moreover, it opens a new way to better study the potential role of *Arcobacter*, not only in estuarine and marine environments, where its associations with shellfish could have other unexplored roles, but also in other food matrices or environments like sewage where *Arcobacter* spp. are frequently recovered, and even for studies focused in the clinical aspects of these microorganisms.

NOTE

A paper entitled "Revisiting the Taxonomy of the Genus *Arcobacter*: Getting Order From the Chaos" by Pérez-Cataluña et al. (2018) was published in Front. Microbiol. 2018 Sep 4; 9:2077. doi: 10.3389/fmicb.2018.02077, followed by an Erratum, while our manuscript was under review. Pérez-Cataluña et al. (2018) proposed the reassignment of several *Arcobacter* spp. to other genera including newly proposed taxa; however, we have retained presently valid nomenclature because the proposed names have not yet been validated.

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Nuria Salas Massó

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

DDB, AW, KA, MF, and MDF designed the work. NS-M, QL, and WC performed the optimization protocol for PMA. NS-M carried out the setup of the viable q-PCR and tested shellfish samples. NS-M, DDB, KA, MF, and MDF wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00368/full#supplementary-material

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UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Nuria Salas Massó

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

Figure S1A. Different concentration of PMA using 1ng/µl DNA. Lines 1 and 2: 20µM PMA; 3 and 4: 2µM PMA; 5 and 6: 0.2 µM PMA; 7 and 8: 0.02µM PMA; 9 and 10 no PMA; 11: negative control.

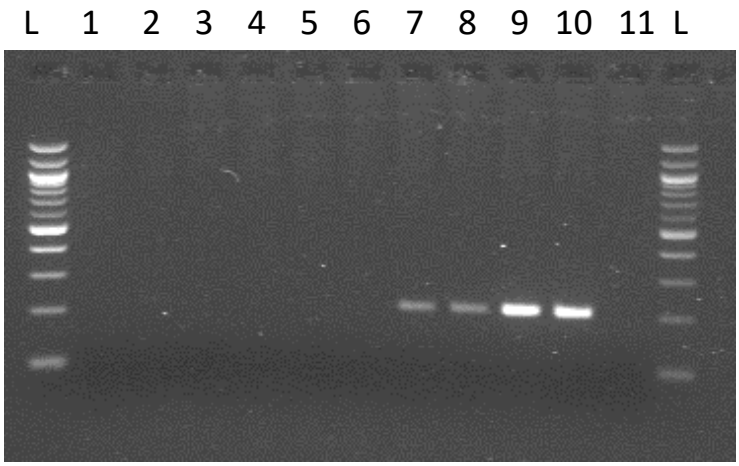

Figure S1B. Different concentration of *A. butzleri* DNA using 0.2 µM PMA. Lines1 and 2: 0 ng/µl DNA; 3 and 4: 1 ng/µl DNA; 5 and 6: 2 ng/µl DNA; 7 and 8: 10 ng/µl DNA; 9 and 10: 20 ng/µl; 11: 1 ng/µl of *A. butzleri* DNA but no PMA added; 12: negative control.

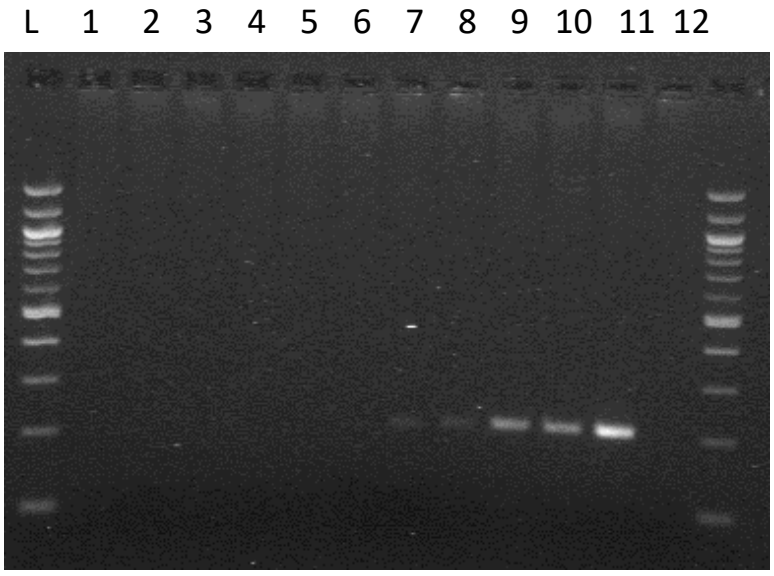

Figure S1C. Different concentration of PMA using different concentration of *A. butzleri* DNA. Lines 1 and 2: 10ng/µl DNA + 2µM PMA; 3 and 4: 20ng/µl DNA + 2µM PMA; 5 and 6: 10ng/µl DNA + 20µM PMA; 7 and 8: 20ng/µl DNA + 20µM PMA; 9: 1ng/µl DNA + no PMA; 11: negative control.

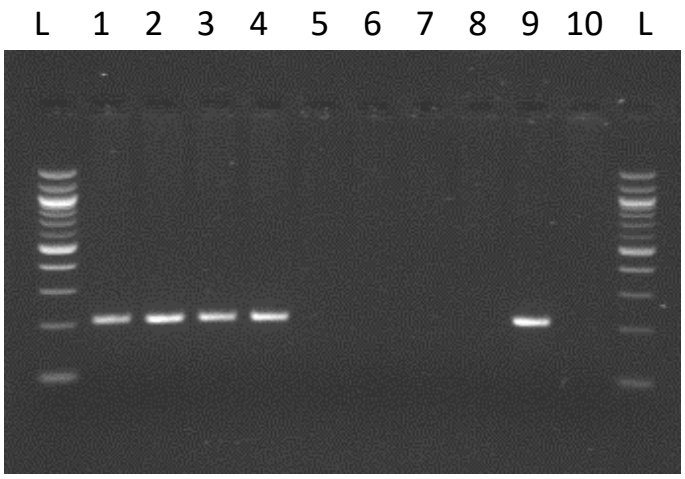

Figure S1D. Effect of time exposure to LED using 10ng/µl *A. butzleri* DNA and 0.2 µM PMA: 1 and 2: 7.5 min; 3 and 4: 15 min; 5 and 6: 30 min; 7 and 8: 60 min; 9 and 10: 60 min photoactivation without addition of PMA; 11: positive control 1 ng/µl *A. butzleri* DNA without PMA nor photoactivation; 12: negative control.

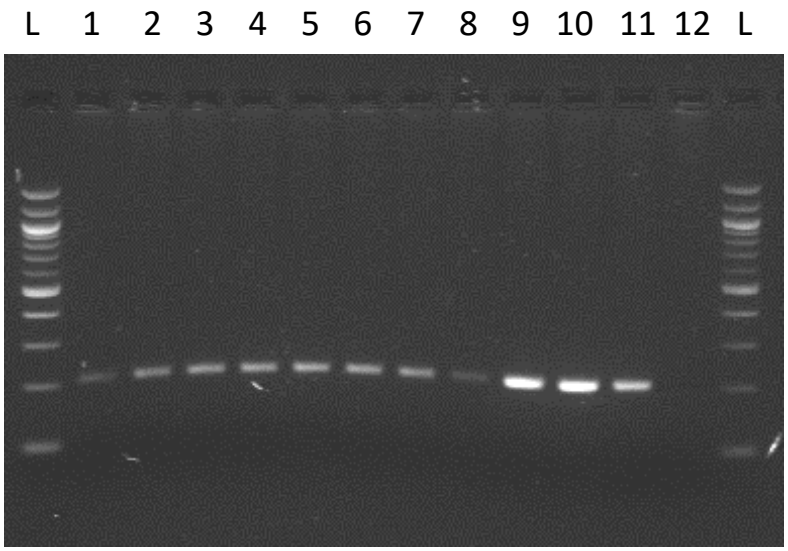

Figure S1E. Different concentration of PMA that were photoactivated with the following addition of 1ng/µl DNA. Lines 1: 20µM; 2: 2µM; 3: 0.2µM; 4: 0.02µM; 5: positive control 1ng/µl *A. butzleri* DNA no PMA; 6: negative control.

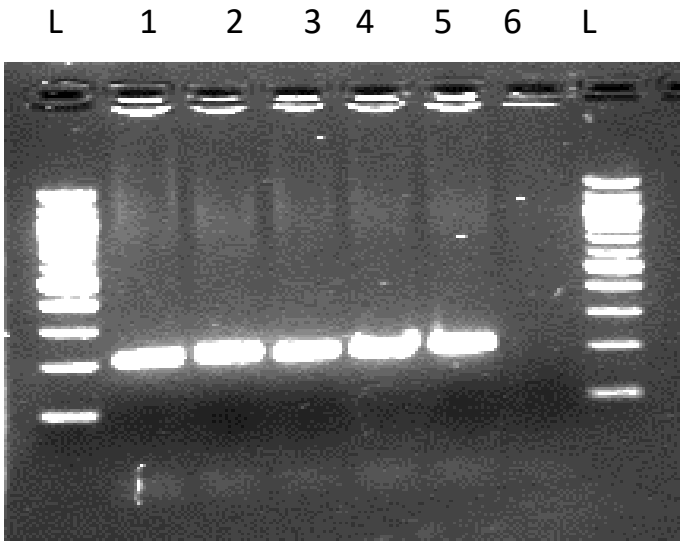

Figure S2A. Testing the efficiency of the PMA when working with DNA extracted from pure cultures of live (LC) or dead (DC) *A. butzleri* cells OD_{550}=0.250 in a final volume of 25µl and treated or not with PMA. Line 1: LC no PMA added; 2: LC + 50µM PMA; 3: LC + 20µM PMA; 4: LC + 2µM PMA; 5: LC + 0.2µM PMA; 6: DC no PMA added; 7: DC + 50µM PMA; 8: DC + 20µM PMA; 9: DC + 2µM PMA;10: DC + 0.2µM PMA; 11: positive control 10ng/µl DNA *A. butzleri*; 12: negative control.

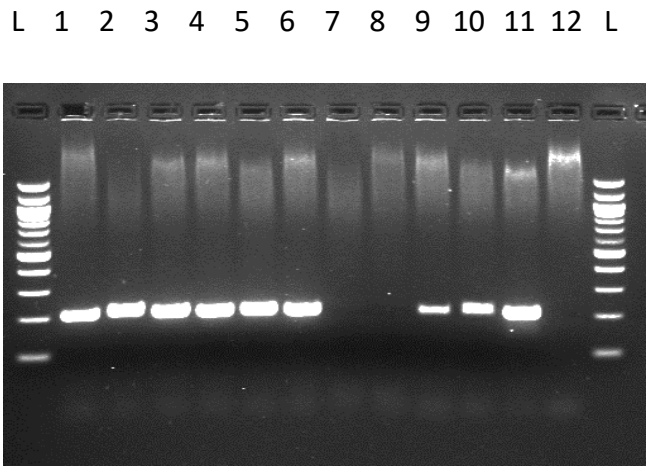

Figure S2B. Testing the efficiency of the PMA when working with DNA extracted from pure cultures of dead (DC) *A. butzleri* cells at different concentrations. Line 1: DC OD_{550}=0.8; 2: DC OD_{550}=0.4; 3: DC OD_{550}=0.250; 4: DC OD_{550}=0.8+ 20µM PMA; 5: DC OD_{550}=0.4+ 20µM PMA; 6: DC OD_{550}=0.250+ 20µM PMA; 7: positive control 10ng/µl DNA *A. butzleri*; 8: negative control.

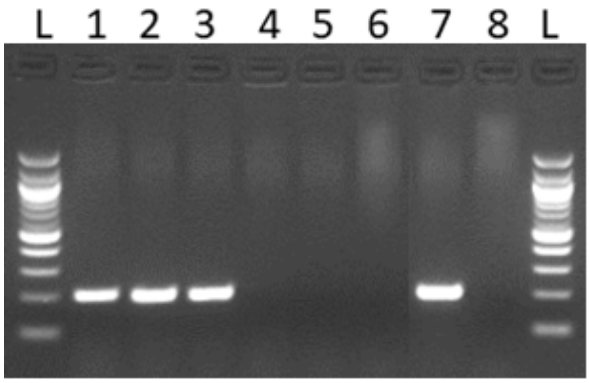

Figure S3. Percentage of detection of 23S *A. butzleri* copy numbers using qPCR and v-qPCR in mussels and oysters' tissues using model mixtures of an initial inoculum (OD_{550}=0.250) of live (LC) and dead (DC) cells, consisting in 100% LC; 50% LC + 50% DC and 100% DC.

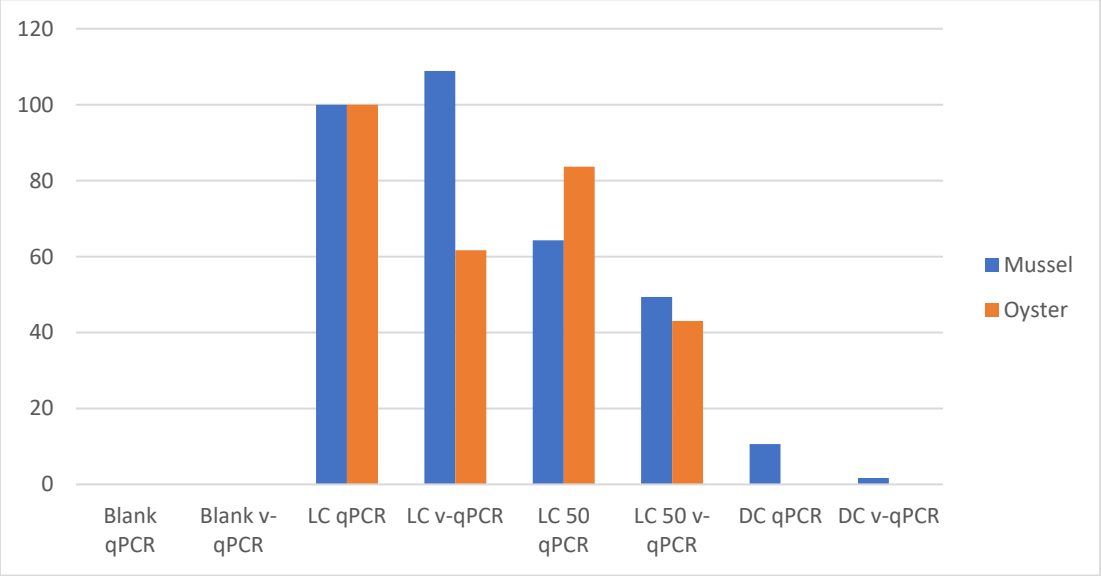

Table S1. Species used for testing the specificity of the primers.

Bacteria	Strain	Donor
Campylobacter coli	CCUG 11283	Provided by CCUG
Campylobacter jejuni	CCUG 11284	Provided by CCUG
Campylobacter lari	CCUG 18267	Provided by CCUG
Campylobacter mucosilis	CCUG 6822	Provided by CCUG
Campylobacter upsaliensis	CCUG 14913	Provided by CCUG
Campylobacter sputorum ss.spo	CCUG 9728	Provided by CCUG
Campylobacter fetus subsq. Fetus	CCUG 6823A	Provided by CCUG
Campylobacter concisus	CCUG 131444	Provided by CCUG
Campylobacter hyointestinalis	CCUG 19512	Provided by CCUG
Salmonella Enteritidis	92243/nybol 3L	Own strain
Salmonella Typhimurium	DVI-Jeo 3979 Jgt.110	Own strain
Enterococcus faecalis	ATCC 29212	Provided by ATCC
Enterococcus faecium	CCUG 47860	Provided by CCUG
Escherichia coli	CCUG 17620	Provided by CCUG
Streptococcus pneunomiae	ATCC 49619	Provided by ATCC
Proteus hauseri	CCUG 36761	Provided by CCUG
Citrobacter freundii	CCUG 418^{T}	Provided by CCUG
Yersinia ruckeri	ATCC 29473	Provided by ATCC
Arcobacter. aquimarinus	CECT 8442^{T}	Own strain
Arcobacter bivalviorum	CECT 7836^{T}	Own strain
Arcobacter butzleri	LMG 10828^{T}	Provided by LMG
Arcobacter cibarius	CECT 7203^{T}	Provided by CECT
Arcobacter cloacae	CECT 7834^{T}	Own strain

Arcobacter cryaerophilus	LMG 9904^T	Provided by LMG
Arcobacter defluvii	CECT 7697^T	Own strain
Arcobacter ebronensis	CECT 8441^T	Own strain
Arcobacter ellisii	CECT 7837^T	Own strain
Arcobacter halophilus	LA31B^T	Provided by Dr Maqsudul Alam (University of Hawaii)
Arcobacter lanthieri	LMG 28516^T	Provided by LMG
Arcobacter marinus	CECT 7727^T	Provided by CECT
Arcobacter molluscorum	CECT 7696^T	Own strain
Arcobacter mytili	CECT 7386^T	Own strain
Arcobacter nitrofigilis	CECT 7204^T	Provided by CECT
Arcobacter skirrowii	LMG 6621^T	Provided by LMG
Arcobacter suis	CECT 7833^T	Own strain
Arcobacter thereius	LMG 24486^T	Provided by LMG
Arcobacter trophiarum	LMG 25534^T	Provided by LMG
Arcobacter venerupis	CECT 7836^T	Own strain

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TAXONOMIC DESCRIPTION

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MICROBIOLOGY SOCIETY

Arcobacter canalis sp. nov., isolated from a water canal contaminated with urban sewage

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Abstract

Four bacterial strains recovered from shellfish (*n*=3) and from the water (*n*=1) of a canal contaminated with urban sewage were recognized as belonging to a novel species of the genus *Arcobacter* (represented by strain F138-33^T) by using a polyphasic characterization. All the new isolates required 2 % NaCl to grow. Phylogenetic analyses based on 16S rRNA gene sequences indicated that all strains clustered together, with the most closely related species being *Arcobacter marinus* and *Arcobacter molluscorum*. However, phylogenetic analyses using the concatenated sequences of housekeeping genes (*atpA*, *gyrB*, *hsp60*, *gyrA* and *rpoB*) showed that all the novel strains formed a distinct lineage within the genus *Arcobacter*. Results of *in silico* DNA–DNA hybridization and the average nucleotide identity between the genome of strain F138-33^T and those of the closely related species *A. marinus* and other relatively closely related species such as *A. molluscorum* and *Arcobacter halophilus* were all below 70 and 96 %, respectively. All the above results, together with the 15 physiological and biochemical tests that could distinguish the newly isolated strains from the closely related species, confirmed that these strains represent a novel species for which the name *Arcobacter canalis* sp. nov. is proposed, with the type strain F138-33^T (=CECT 8984^T=LMG 29148^T).

The taxonomy of the class *Epsilonproteobacteria* has recently been reviewed by Waite *et al.* [1] on the basis of 16S and 23S rRNA genes as well as 120 single-copy marker proteins. The authors proposed that the class *Epsilonproteobacteria* together with the order *Desulfurellales* should be considered a new phylum, *Epsilonbacteraeota*. In this new phylum, the genus *Arcobacter* within the order *Campylobacterales* has been proposed as the only known member of the new family *Arcobacteraceae*. This genus comprises Gram-stain-negative, campylobacter-like bacteria that are able to grow at low temperatures and under aerobic conditions, these being the features that differentiate the genus *Arcobacter* from the genus *Campylobacter* [2, 3]. Since the description of the genus *Arcobacter* in 1991 [2], 26 member species have been described [4–6].

In a recent survey on the prevalence of members of the genus *Arcobacter* in water and shellfish samples, 27 isolates were recovered (13 from mussels, 12 from oysters and 2 from water) with the typical colony morphology (small, translucent, beige to pale orange) of the genus *Arcobacter* on marine agar [7]. The samples came from a canal that receives untreated urban sewage from the village of Poble Nou (40° 38′ 30.8″ N 0° 41′ 37.2″ E), to which the shellfish were exposed for 72 h [7]. The isolation protocol involved an enrichment step in Arcobacter CAT (cefoperazone, amphotericin B and teicoplanin) broth supplemented with 2.5 % NaCl, followed by sub-culturing on marine agar at 30 °C under aerobic and microaerobic conditions [7]. All the 27 presumptive isolates of the genus *Arcobacter* were Gram-stain-negative, slightly curved rods with oxidase activity, as previously described for this genus [2, 3]. All isolates were genotyped with Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR) using previously described primers and conditions [8] to recognize potential clones [9]. Patterns that differed by one or more bands were considered different genotypes, as in other studies [3, 8]. Among the 13 isolates from mussels, 12 isolates from oysters and 2 from water, only four different ERIC-genotypes were recognized, represented by strains F190-2IL33 from mussels, F138-33^T and F181-1F33 from oysters and

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Keywords: *Arcobacter*; *A. canalis*; shellfish; MLPA; 16S rRNA; ANI; *is*DDH.
Abbreviations: ANI, average nucleotide identity; *is*DDH, in silico DNA-DNA hybridization; MLPA, multilocus phylogenetic analysis; m-PCR, multiplex PCR; TEM, transmission electron microscope.
†These authors contributed equally to this work.
The GenBank/EMTBL/DDBJ accession numbers for the 16S rRNA, *atpA*, *gyrB*, *hsp60*, *gyrA* and *rpoB* gene sequences of strain F138-33^T are MG015880, LT903675, LT903676, LT903678, LT903677 and LT903674, respectively. The accession number for the whole-genome sequence is NWVW00000000.
Nine supplementary figures are available with the online version of this article.

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W110-33 from water. The characterization of the four strains was initially attempted using identification methods specific for the genus *Arcobacter,* i.e. two multiplex-PCR (m-PCR) methods [10, 11] and one RFLP analysis of the 16S rRNA gene [12]. The four strains produced two amplicons of the expected size described for *Arcobacter cryaerophilus* and *Arcobacter skirrowii* with the m-PCR of Houf *et al.* [10]. However, with the m-PCR of Douidah *et al.* [11], the strains produced an amplicon corresponding to *Arcobacter butzleri*. By contrast, with the 16S rRNA gene RFLP identification method [12], the four strains produced the same pattern described for *Arcobacter marinus* using *Mse*I and *Mnl*I endonucleases. Considering the contradictory results, the *rpoB* (621 bp) genes of the four strains were sequenced using primers and conditions described previously [13, 14]. The phylogenetic tree reconstructed with the maximum-likelihood method [15] showed that the four strains clustered together forming a differential branch closely related to *A. marinus* (Fig. S1, available in the online version of this article). In order to further investigate these findings, the 16S rRNA gene and the housekeeping genes *atpA*, *gyrB, gyrA* and *hsp60* were also amplified and sequenced as described previously [16]. Alignments were performed using MEGA 6.0 [17] with the ClustalW algorithm [18]. The phylogenetic trees were reconstructed using the neighbour-joining [19, 20] (Figs 1 and 2) and/or the maximum-likelihood methods [15]. The trees reconstructed with the latter method with the five individual gene sequences and with the concatenated sequences are shown in Figs S1 to S7.

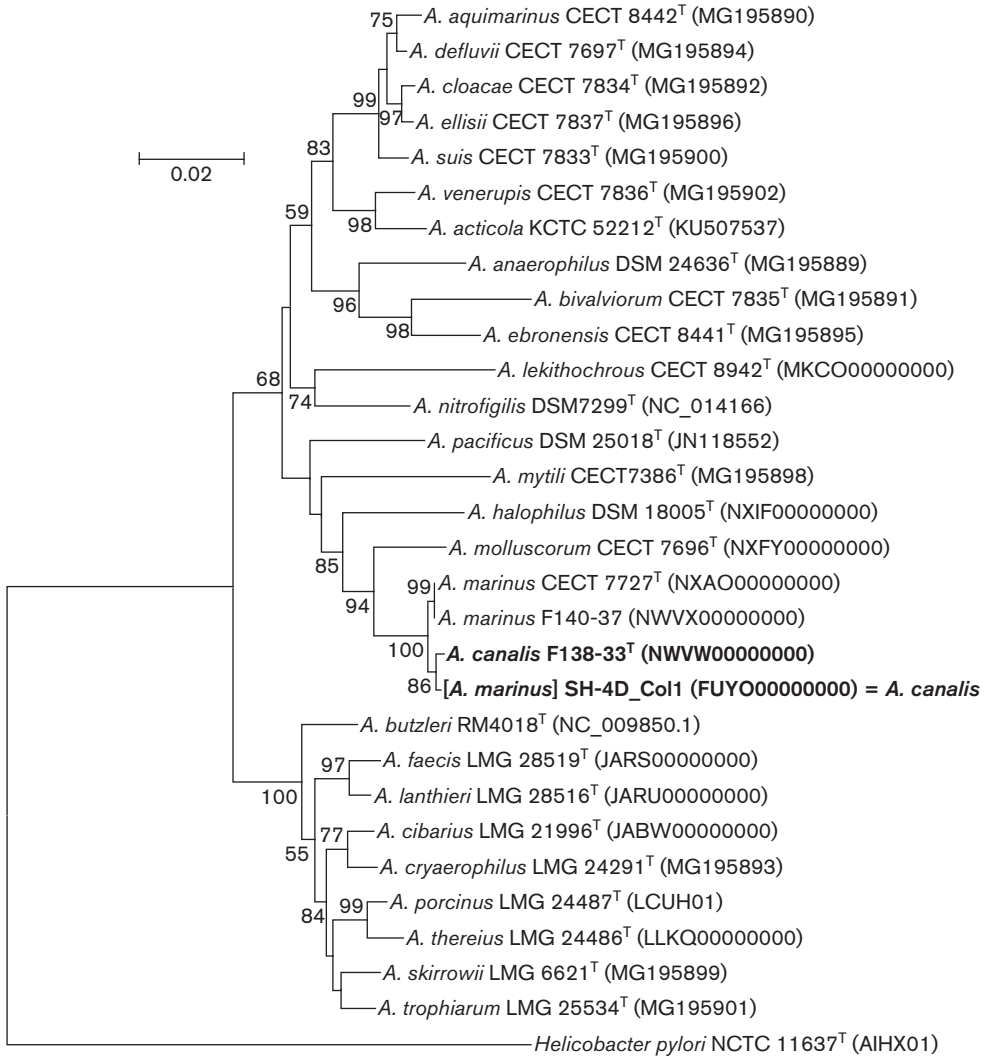

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences (1427 bp) showing the phylogenetic position of *A. canalis* sp. nov. within the genus *Arcobacter*. Notice that the genome deposited at the NCBI as [*A. marinus*] SH-4D_Col1 does not belong to the species *A. marinus* because it clusters with *A. canalis* sp. nov. Bootstrap values (>50 %) based on 1000 replications are shown at the nodes of the tree. Bar, 2 substitutions per 100 nt.

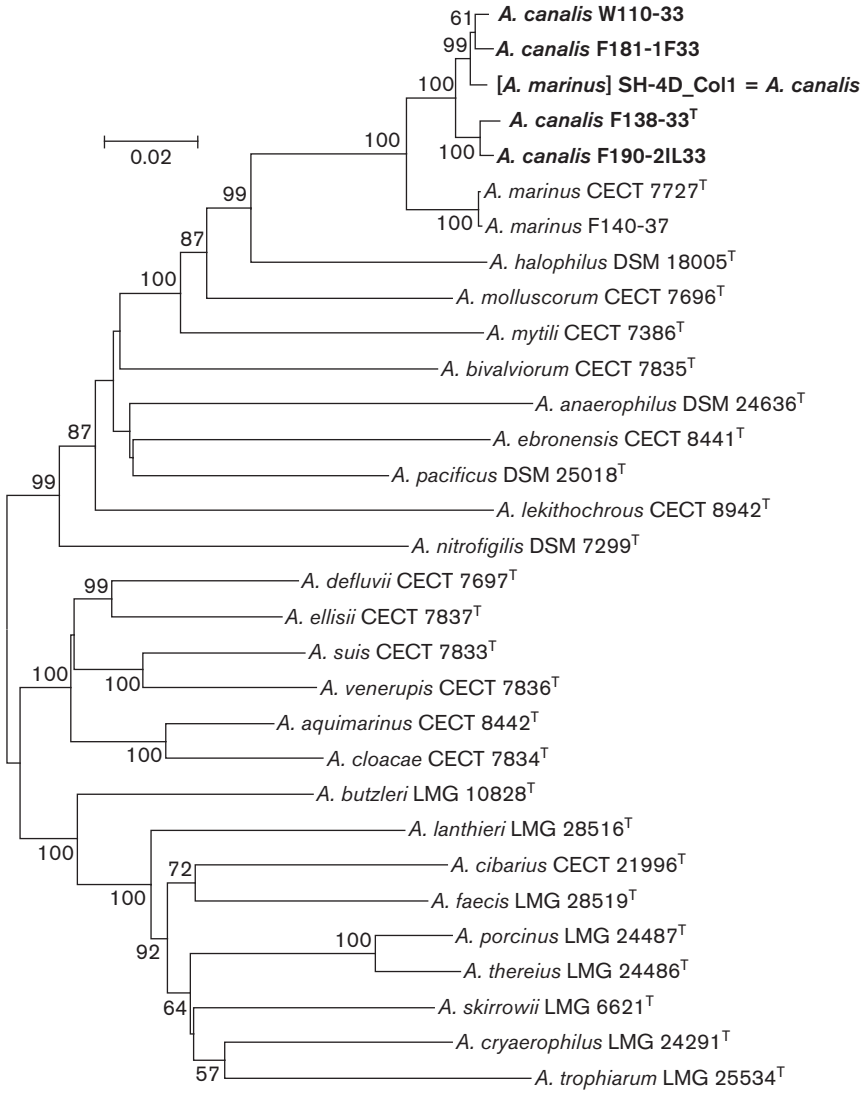

Fig. 2. Neighbour-joining tree based on the concatenated sequences of *atpA*, *gyrB*, *hsp60*, *rpoB* and *gyrA* (3039 bp) genes showing the phylogenetic position of *A. canalis* sp. nov. strains within the genus *Arcobacter*. Notice that the genome deposited at the NCBI as [*A. marinus*] SH-4D_Col1, from where the gene sequences were extracted, does not belong to the species *A. marinus* because it clusters with *A. canalis* sp. nov. Bootstrap values (>50 %) based on 1000 replications are shown at the nodes of the tree. Bar indicates 2 substitutions per 100 nt.

The 16S rRNA gene of strain F138-33^T (chosen as a representative of the four strains) clustered in the phylogenetic tree with the type strain of the species *A. marinus*, CECT 7727^T, and with a sequence that came from a genome deposited at the NCBI as *A. marinus* SH-4D_Col1 (FUYO00000000) (Fig. 1). In addition, the strain *A. marinus* F140-37, isolated in a previous study [7], also clustered in the group. The similarity of the 16S rRNA genes between the potential novel species represented by strain F138-33^T (1503 bp) and the *A. marinus* strains CECT 7727^T and F140-37 was 99.7 %, while with *Arcobacter molluscorum* CECT 7696^T and *Arcobacter halophilus* DSM 18005^T it was much lower, i.e. 97.6 and 96.8 %, respectively. Interestingly, a 16S rRNA gene sequence similarity of 99.8 % was obtained between strain F138-33^T and the gene extracted from the genome of *A. marinus* SH-4D_Col1. The multilocus phylogenetic analysis (MLPA) performed with the concatenated sequences of the five housekeeping genes (*atpA*, *gyrB*, *hsp60*, *gyrA* and *rpoB*, 3039 bp) confirmed that the new cluster formed by the four newly isolated strains (F138-33^T, W110-33, F181-1F33 and F190-2IL33) represented a well separated linage from the species *A. marinus* (Fig. 2). Notably, this new cluster also included the concatenated sequences of the five genes mentioned that were obtained from the genome labelled *A. marinus* SH-4D_Col1 (FUYO00000000). This, together with the 99.8 % sequence similarity observed with

strain F138-33^T, indicated that the genome SH-4D_Col1 belongs to the novel species.

In order to further confirm the latter finding and that we were dealing with a novel species, the genomes of five strains i.e. F138-33^T (NWVW00000000); *A. marinus* strains F140-37 (NWVX00000000) and CECT 7727^T (NXAO00000000); *A. halophilus* DSM 18005^T (NXIF00000000) and *A. molluscorum* CECT 7696^T (NXFY00000000) were obtained using the MiSeq Illumina platform and assembled with SPAdes software [21]. The average nucleotide identity (ANI) and the *in silico* DNA–DNA hybridization (*is*DDH) values were used to compare the genomes of strain F138-33^T and the species *A. marinus*, *A. molluscorum* and *A. halophilus* using OrthoANI [22] and GGDC [23] software, respectively. Table 1 shows that the ANI and *is*DDH values obtained between the genome of the newly proposed species (represented by F138-33^T) and those of *A. marinus* (strains CECT 7727^T, F140-37 and SH-4D_Col1), *A. molluscorum* CECT 7696^T and *A. halophilus* DSM 18005^T were all lower than 96 and 70 %, respectively, with the only exception being the values obtained with the genome of *A. marinus* SH-4D_Col1, which were 97.7 and 79.3 %, respectively (Table 1). In general, for separation of species, an ANI below 95–96 % [22] and an *is*DDH below 70 % [23] have been recommended. However, for the genus *Arcobacter*, ANI values above 96 % were shown to be the ones that correlated better with *is*DDH results above 70 % [5]. The ANI and *is*DDH results confirmed that the genome of *A. marinus* SH-4D_Col1 belongs to the novel species as shown by the phylogeny of the 16S rRNA gene and MLPA (Figs 1, 2). Therefore, the name of the genome sequence *A. marinus* SH-4D_Col1 should be changed in the NCBI database to *Arcobacter canalis* SH-4D_Col1 because this genome corresponds to the proposed novel species. It is clear that before the description of our novel species, the most closely related species to SH-4D_Col1 in the databases was *A. marinus* CECT 7727^T, with a 16S rRNA gene sequence similarity of 99.51 % (1417 bp). However, if identification had been done with the *rpoB* phylogeny, a branch separated from *A. marinus* (Fig. S1) could have been detected indicating that genome SH-4D_Col1 represented a distinct taxon. The genomic information derived from ANI and the *is*DDH was shown to have a higher resolution than that from the 16S rRNA gene for differentiating new species of the genus *Arcobacter*.

The DNA G+C content of strain F138-33^T was 27.5 %, in agreement with the values for *A. marinus* (27 %) and other species of the genus *Arcobacter*, which range between 26.6 and 31.9 % [24, 25].

The colony morphology, temperature and atmospheric conditions for growth as well as the biochemical properties and resistance to antimicrobial agents were evaluated following the recommendations in the minimal standards for describing new taxa of the family *Campylobacteraceae* [26], which were recently updated [27]. All tests were carried out at least twice for the four newly isolated strains and for the type strains of *A. marinus* CECT 7727^T and *A. molluscorum* CECT 7696^T and the strains of the species used as positive and negative controls for each test. Phenotypical characteristics for *A. halophilus* DSM 18005^T were derived from the description of the species [28] and from the recent re-evaluation of this species done in our laboratory [7]. Colony morphology was analysed from the growth obtained on marine agar at 30 °C under aerobic conditions for 48 h. A total of 39 tests were carried out, 12 testing growth conditions and 27 testing biochemical properties. Growth conditions were evaluated on marine agar at 22–25 °C, 30 °C, 37 and 42 °C in three different atmospheres: aerobiosis, microaerobiosis and anaerobiosis. The biochemical properties were evaluated at 30 °C on blood agar supplemented with 2 % NaCl for each condition. The biochemical properties tested included oxidase, catalase and urease activity, nitrate reduction, glucose fermentation in triple-sugar iron agar, hydrolysis of indoxyl acetate, casein, lecithin and starch, growth in media supplemented with 0.5 and 4 % NaCl, 1 % oxgall, 0.1 % sodium deoxycholate, 1 % glycine, 0.05 % safranin, 0.005 fucsine, crystal violet, brilliant green, 0.1, 0.01 and 0.04 % triphenyl tetrazolium chloride, and growth in charcoal cefoperazone deoxycholate agar (CCDA), minimal media and MacConkey agar. Resistance to nalidixic acid (30 μg l^{-1}), cefalotin (30 μg l^{-1}) and cefoperazone (64 mg l^{-1}) was also tested using TSA supplemented with 2 % NaCl and with each antimicrobial. The bacterial morphology, cell size and the presence of flagella were determined using a transmission

Table 1. Results (percentages) of ANI and *is*DDH between the genome of *A. canalis* sp. nov. F138-33^T and those of the most closely related species

The ANI values are displayed in bold type (down-left) and the *is*DDH values are in italics (up-right); values below 96 and 70 % indicate that the genomes belong to different species. Notice that ANI and *is*DDH values show that the genome [*A. marinus*] SH-4D_Col1 does not belong to this species* but to *A. canalis* sp. nov.,† in agreement with the phylogenetic results of Fig. 2.

		DNA G+C content (mol%)	1	2	3	4	5	6
1	*A. canalis* sp. nov. F138-33^T	27.3	***	*79.3†*	*63.6*	*63.5*	*22.8*	*30.4*
2	[*A. marinus*] SH-4D_Col1	27.1	**97.7†**	***	*63.3**	*63.8**	*23.0*	*30.5*
3	*A. marinus* CECT 7727^T	27.0	**95.4**	**95.3***	***	*93.9*	*22.9*	*31.3*
4	*A. marinus* F140-37	27.0	**95.4**	**95.4***	**99.2**	***	*22.8*	*30.7*
5	*A. molluscorum* CECT 7696^T	26.1	**80.3**	**80.5**	**80.0**	**80.2**	***	22.8
6	*A. halophillus* DMS 18005^T	27.4	**86.2**	**86.2**	**86.6**	**86.3**	**80.2**	***

electron microscope (TEM), JEOL 1011. Cells were grown for 24 h in marine agar and suspended for fixation in 2 % glutaraldehyde in 0.1 % phosphate buffer for 30 min. The suspension was mounted in a copper grid and negatively stained with 2 % phosphotungstic acid (pH 7.5) for 1 min. Strain F138-33^T showed a single polar flagellum under the TEM (Fig. S8), and all the newly isolated strains showed motility under the phase contrast microscope. Fifteen tests could be considered diagnostic because they enabled differentiation of the four novel strains, F138-33^T, W110-33, F181-1F33 and F190-2IL33, from the most closely related species, *A. marinus*, and also from *A. molluscorum* and *A. halophilus* (Table 2). Differentiation from *A. marinus* was based on the capacity of the novel species to grow in marine agar at 42 °C under microaerobiosis, with 2 % NaCl and in media with 0.05 % safranin or crystal violet supplemented with NaCl and an inability to produce H_2S in triple-sugar iron media supplemented with NaCl, to reduce nitrate or to grow with 0.1 % oxgall (Table 2). Phenotypical differentiation of the novel species from the species *A. molluscorum* was based on the inability of the novel species to grow with 0.5 % NaCl, 0.1 % sodium deoxycholate, 1 % oxgall or 0.01 % triphenyl tetrazolium chloride, to resist cefoperazone (64 mg l^{-1}) or to reduce nitrate. Another differential characteristic between *A. molluscorum* and *A. canalis* sp. nov. was the capacity of the novel species to grow at 22–25 °C, 30 and 37 °C under anaerobiosis, to grow in minimal medium and to produce H_2S in triple-sugar iron media supplemented with NaCl. Differentiation between the novel species and *A. halophilus* could be demonstrated because the latter does not grow in marine agar at 42 °C under microaerobiosis, or in media supplemented with 0.05 % safranin or crystal violet, or minimal media or MacConkey agar, among several other differential characteristics, which are listed in Table 2.

The six genomes studied (F138-33^T, F140-37, SH-4D-Col1, CECT 7727^T, CECT 7696^T and DSM 18005^T) were annotated using RAST [29], and genes encoding for polar lipid syntheses were searched for. All the genomes possessed the genes that encode phosphatidylglycerolphosphatase A (*pspA*, EC3.1.3.27) and phosphatidase cytidylyltransferase (*cdsA*, EC 2.7.7.41), related with the synthesis of phosphatidylglycerol. Additionally, the genomes also contained the gene phosphatidylserine descarboxilase (*psd*, EC4.1.1.65) involved in the synthesis of phosphatidylethanolamine. These results agree with the polar lipids found experimentally in other species of the genus *Arcobacter*, such as *Arcobacter pacificus*, *Arcobacter acticola* and *Arcobacter haliotis* [4, 30, 31].

Considering that during the preparation of this paper an additional m-PCR method for the characterization of six species of the genus *Arcobacter* was described by Khan *et al.* [32], the method was tested on the type strain F138-33^T of the novel species and in parallel on *A. marinus* CECT 7727^T, *A. molluscorum* CECT 7696^T and *A. halophilus* DSM 18005^T. Strain F138-33^T and the type strains of the other three species showed an amplicon of 654 bp, which was similar to the one expected for *A. skirrowii* according to Khan *et al.* [32]. Results obtained with this method and with the m-PCRs of Houf *et al.* [10] and Douidah *et al.* [11] and the 16S rRNA gene RFLP [12] are shown in Fig. S9.

This study has demonstrated the existence of a novel *Arcobacter* species, for which the name *A. canalis* sp. nov. is proposed.

Table 2. Differential characteristics of *Arcobacter canalis* sp. nov. and type strains of the most closely related species of the genus *Arcobacter*

Taxa: 1, *Arcobacter canalis* sp. nov. (*n*=4); 2, *A. marinus* CECT 7727^T; 3, *A. molluscorum* CECT 7696^T; 4, *A. halophilus* DSM 18005^T. Unless otherwise indicated: +,≥95 % strains positive; −,≤11 % strains positive; V, 12–94 % strains positive; ND, not determined. All strains show catalase activity and to grow they require the media to be supplemented with 2 % NaCl. In addition, all strains grow in TSA with 4 % NaCl and in marine agar under aerobiosis and microaerobiosis at 22–25 °C, 30, 37 and 42 °C. None of the strains grow under anaerobiosis at 42 °C, in TSA with 1 % glycine, 0.01–0.1 % triphenyl tetrazolium chloride (TTC), nor in CCDA. None of the strains hydrolyse starch, casein or lecithin nor show urease activity or resistance to nalidixic acid.

Characteristic	1	2	3	4*
Growth at/with/on:				
42 °C (microaerobic)†	+	−	+	−
22–25 °C (anaerobiosis)†	+	+	−	+
30 °C (anaerobiosis)†	+	+	−	+
37 °C (anaerobiosis)†	+	+	−	+
0.5 % NaCl (w/v)	−	−	+	−
2 % NaCl (w/v)	+	−	+	+
0.05 % Safranin‡	+	−	+	−
0.005 % Basic fuchsine‡	V	−	+	−
Crystal violet‡	+	−	+	−
Brilliant green‡	V	−	−	ND
0.1 % Sodium deoxycholate‡	−	−	+	−
1 % (w/v) Oxgall‡	−	+	+	ND
0.01 % TTC‡	−	−	+	ND
Minimal medium‡	+	+	−	−
MacConkey‡	V	+	+	−
Triple-sugar iron+NaCl	+	−	−	−
Resistance to:				
Cefoperazone (64 mg l^{-1})‡	−	−	+	−
Cefalotin (30 mg l^{-1})‡	V	−	+	−
Enzyme activity				
Nitrate reduction‡	−	+	+	+
Indoxyl acetate hydrolysis§	−	+	−	+
Catalase	+	−	+	−

*Information extracted from Donachie *et al.* [28] and Salas-Massó *et al.* [7].

†These tests were carried out on marine agar.

‡These tests were carried out on TSA supplemented with 2 % NaCl.

§This test was performed under aerobic and microaerophilic conditions and produced the same results, except for *A. marinus* CECT 7727^T, which was only positive under microaerophilic conditions.

DESCRIPTION OF *ARCOBACTER CANALIS* SP. NOV.

Arcobacter canalis (ca.na′lis. L. gen. n. *canalis* of a canal).

Cells are Gram-stain-negative, slightly curved rods, non-encapsulated, non-spore forming, 0.2–0.4 µm wide and 1.2–2.8 µm long. They are motile by a single polar flagellum. Colonies on marine agar incubated under aerobic conditions at 30 °C for 48 h are 2–4 mm in diameter, pale yellow to pale orange, circular with entire margins, convex and non-swarming. Pigments are not produced in marine agar. Strains grow on marine agar at 22–25, 30 and 37 °C under aerobic, microaerobic and anaerobic conditions, and at 42 °C under aerobic and microaerobic conditions. After 48 h, colonies are smaller at 37 and 42 °C than at room temperature or 30 °C. No growth is observed in blood agar at the different temperatures tested nor under the different atmospheric conditions. Produces oxidase and catalase activity. Does not hydrolyse indoxyl acetate, urea, casein, lecithin or starch. Not able to produce acid from glucose by oxidization or fermentation, but produces hydrogen sulphide in triple-sugar iron agar medium, and is not able to reduce nitrate. Under aerobic conditions at 30 °C, grows on minimal medium with 2 % NaCl and on nutrient medium supplemented with 5 % sheep blood and 2 % NaCl containing 0.05 % safranin or 0.005 % crystal violet. Additionally, strains W110-33 and F181-1F33 grow on 0.005 % basic fuchsine, 0.001 % brilliant green and MacConkey agar supplemented with 2 % NaCl, while the other strains (F138-33^T and F190-2IL33) are unable to grow in these media. No growth occurs on campylobacter charcoal deoxycholate agar (CCDA), on nutrient medium supplemented with 5 % sheep blood containing 0.5 % NaCl, 1 % oxgall, 0.1 % sodium deoxycholate, 1 % glycine or 0.01–0.1 % 2,3,5-triphenyltetrazolium chloride. Strains W110-33 and F181-1F33 are resistant to 30 µg cefalotin l^{-1} while strains F138-33^T and F190-2IL33 are susceptible. All strains are susceptible to 64 mg cefoperazone l^{-1} and 30 µg nalidixic acid l^{-1}.

The type strain is F138-33^T (=CECT 8984^T=LMG 29148^T), isolated from oysters exposed for 72 h to untreated urban sewage in Poble Nou canal, Catalonia, Spain.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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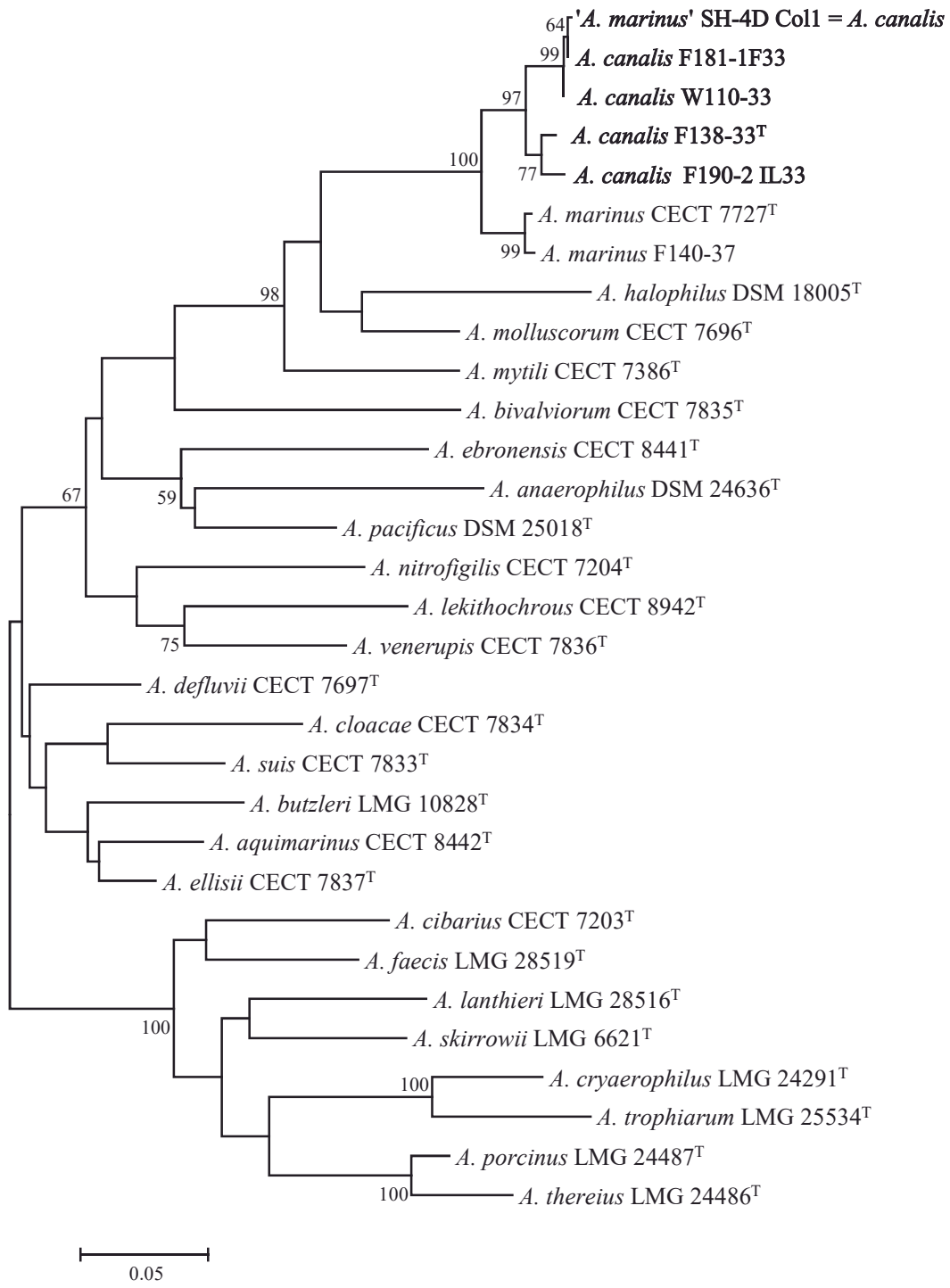
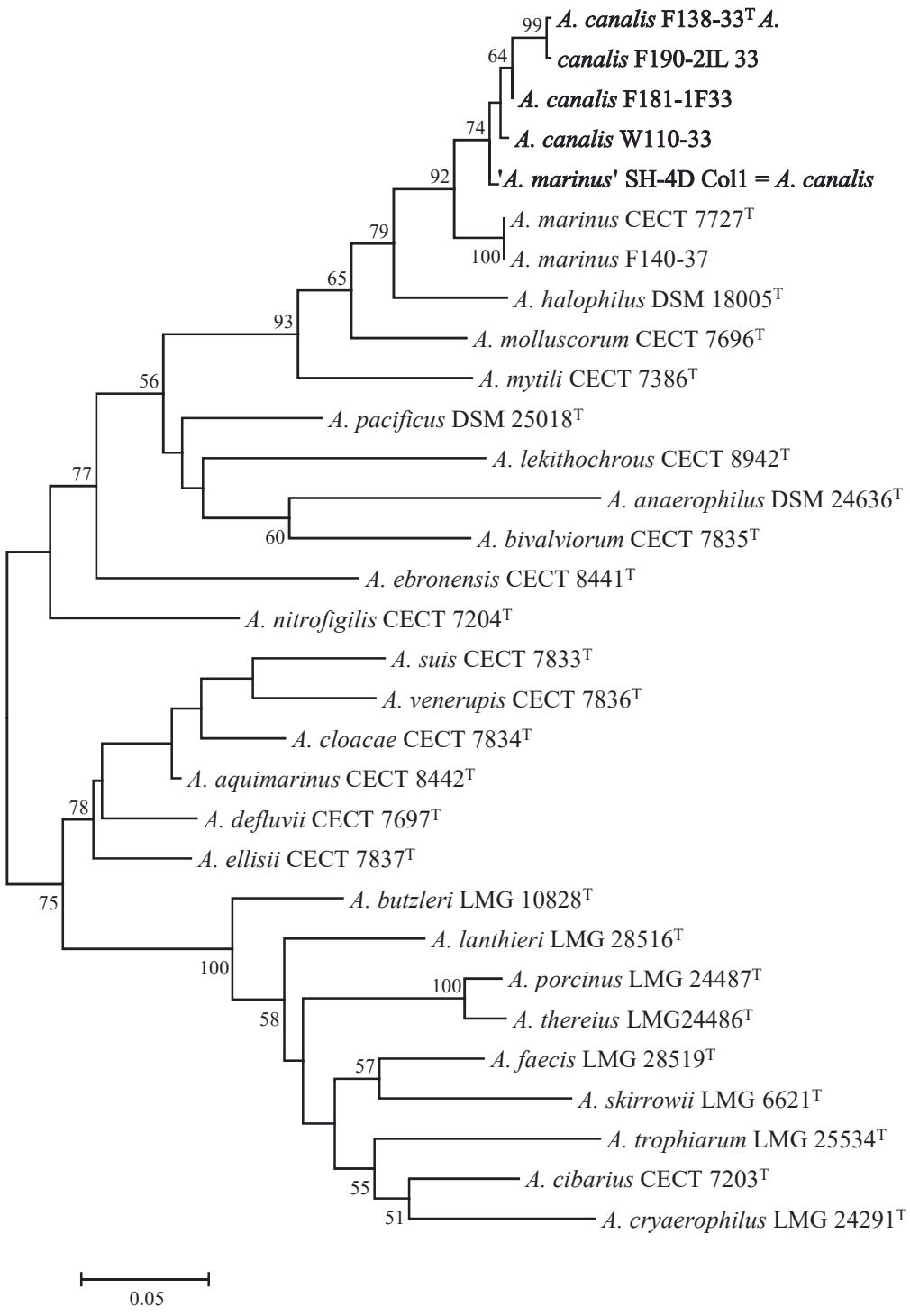
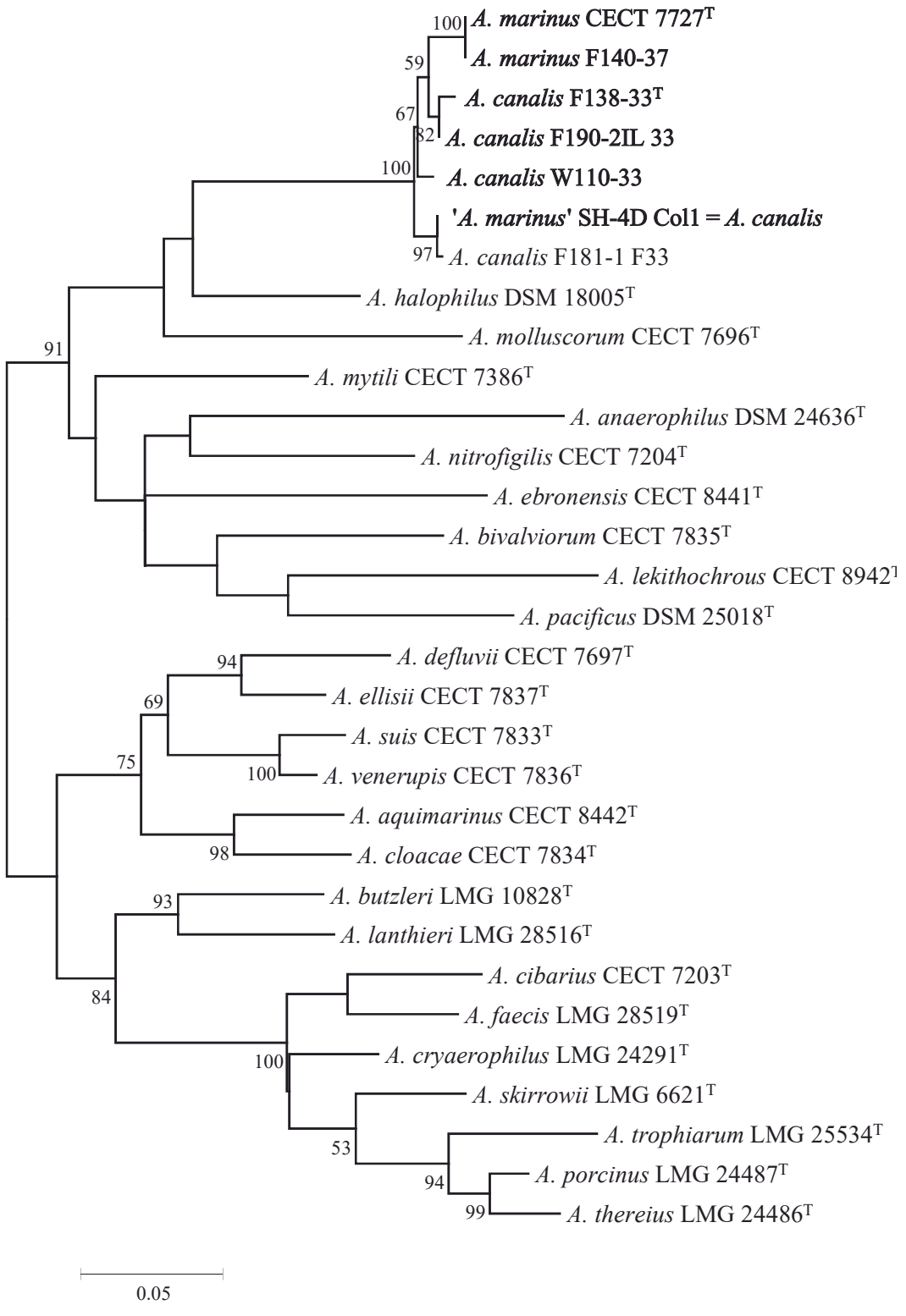
Supplementary Material



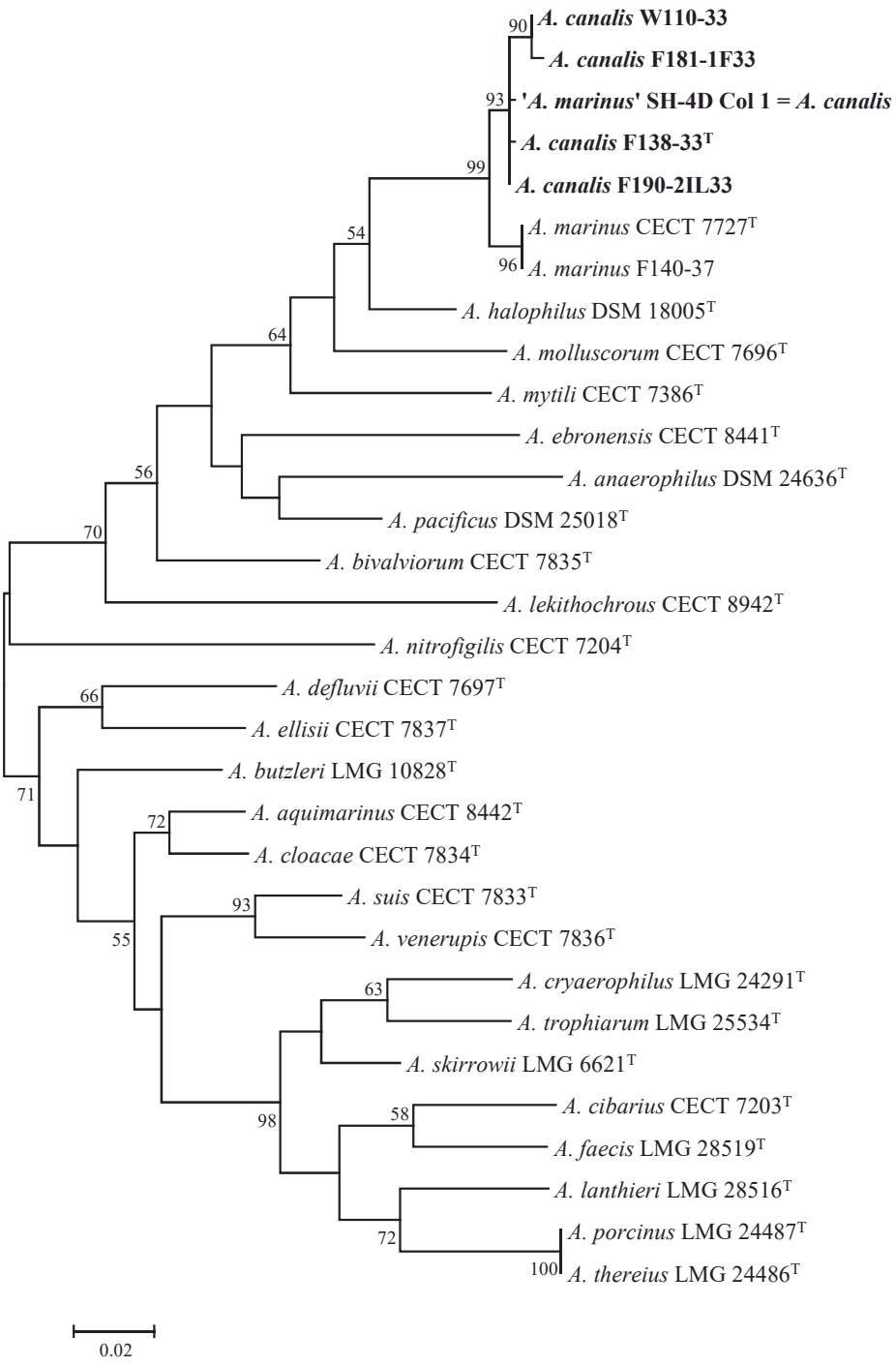
Supplementary figure S1. Maximum Likelihood tree (model GTR+G+I) based on the *rpoB* gene sequence (619 bp) showing the phylogenetic position of *A. canalis* sp. nov. strains within the genus *Arcobacter*. Bootstrap values (>50%) based on 1000 replications are shown at the nodes of the tree. Bar indicates 5 substitutions per 100 nt.



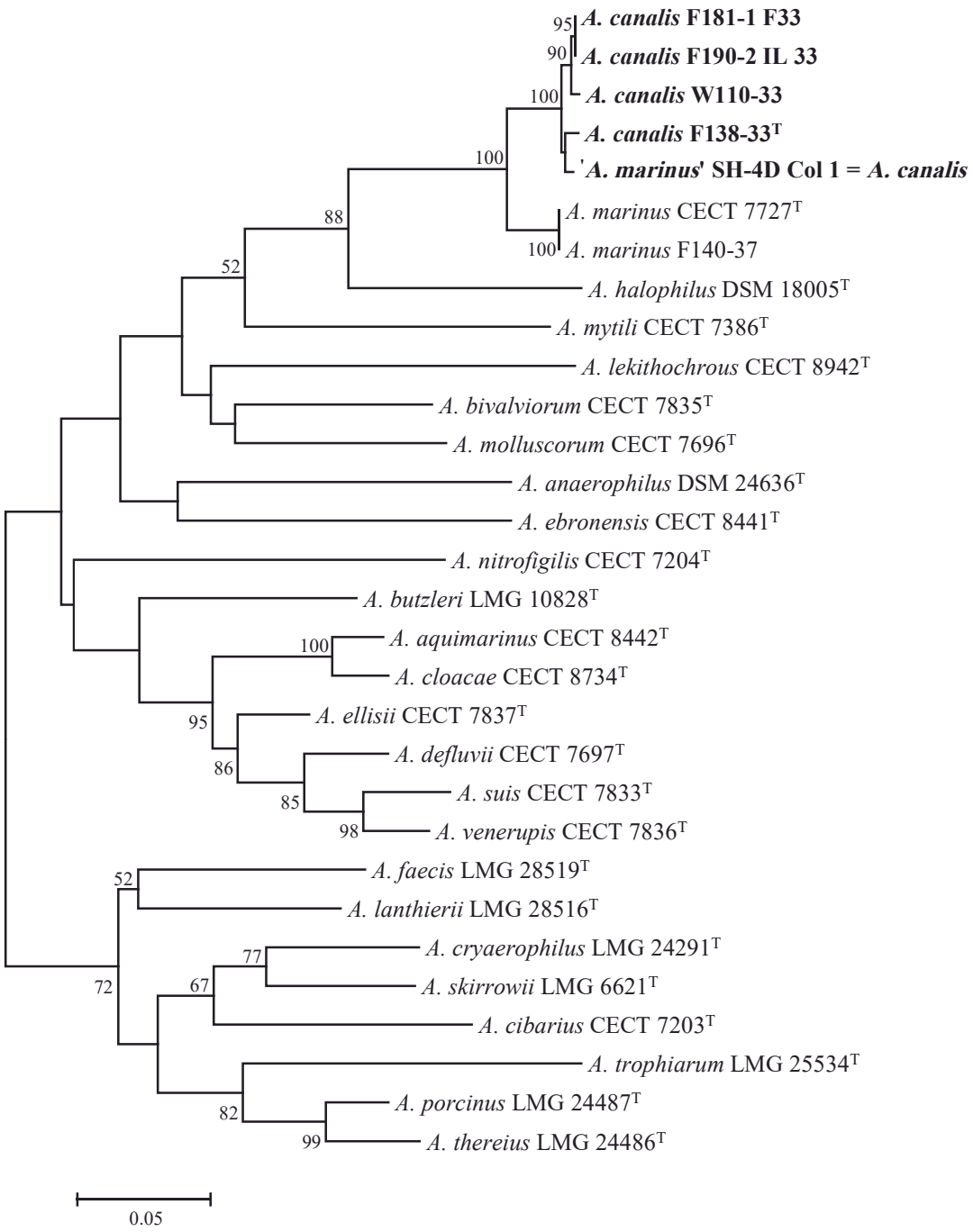
Supplementary figure S2. Maximum Likelihood tree (model GTR+G) based on the *gyrB* gene sequence (617 bp) showing the phylogenetic position of *A. canalis* sp. nov. strains within the genus *Arcobacter*. Bootstrap values (>50%) based on 1000 replications are shown at the nodes of the tree. Bar indicates 5 substitutions per 100 nt.



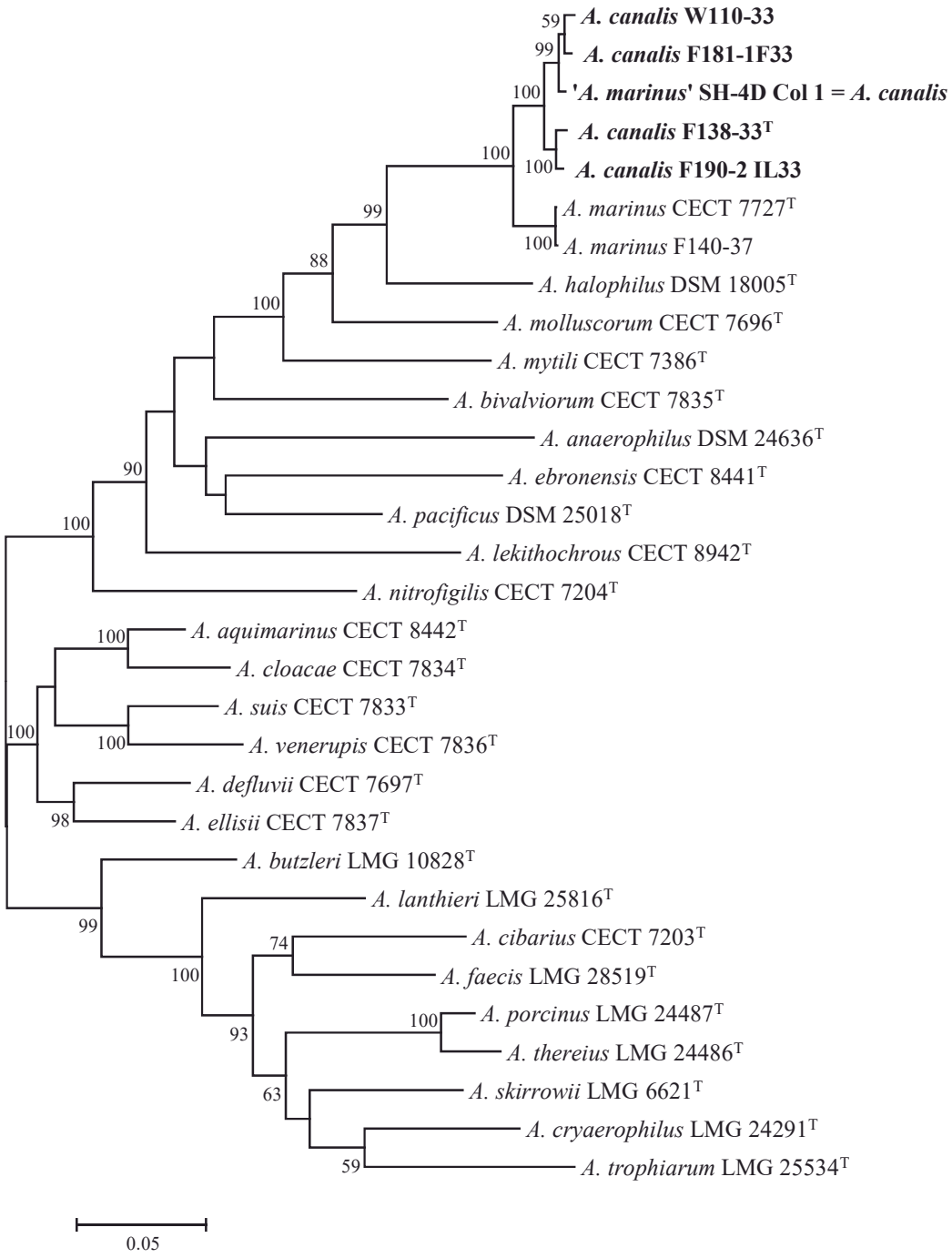
Supplementary figure S3. Maximum Likelihood tree (model GTR+G+I) based on the *hsp60* gene sequence (545 bp) showing the phylogenetic position of *A. canalis* sp. nov. strains within the genus *Arcobacter*. Bootstrap values (>50%) based on 1000 replications are shown at the nodes of the tree. Bar indicates 5 substitutions per 100 nt.



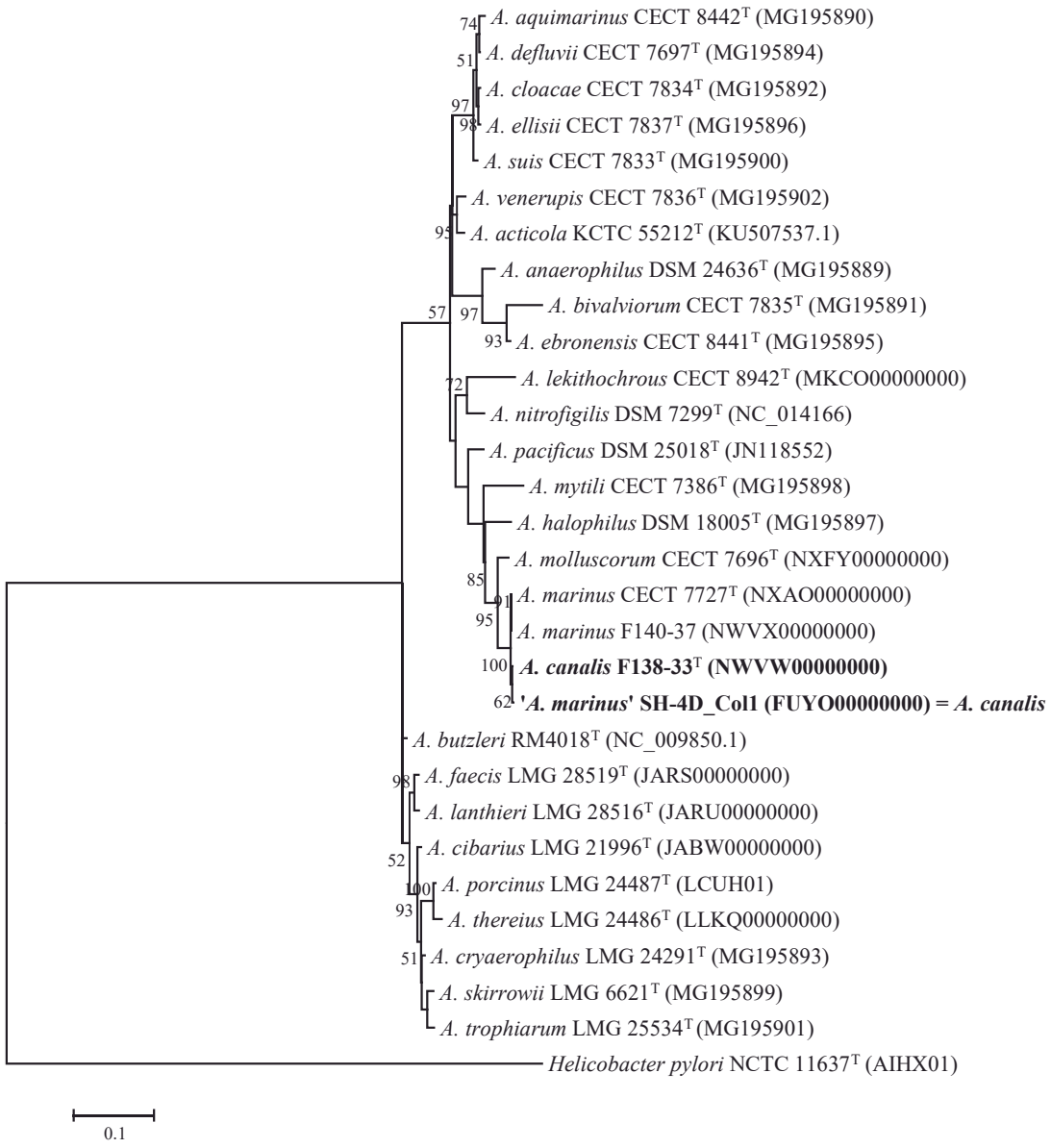
Supplementary figure S4. Maximum Likelihood tree (model GTR+G) based on the *atpA* gene sequence (613 bp) showing the phylogenetic position of *A. canalis* sp. nov. strains within the genus *Arcobacter*. Bootstrap values (>50%) based on 1000 replications are shown at the nodes of the tree. Bar indicates 2 substitutions per 100 nt



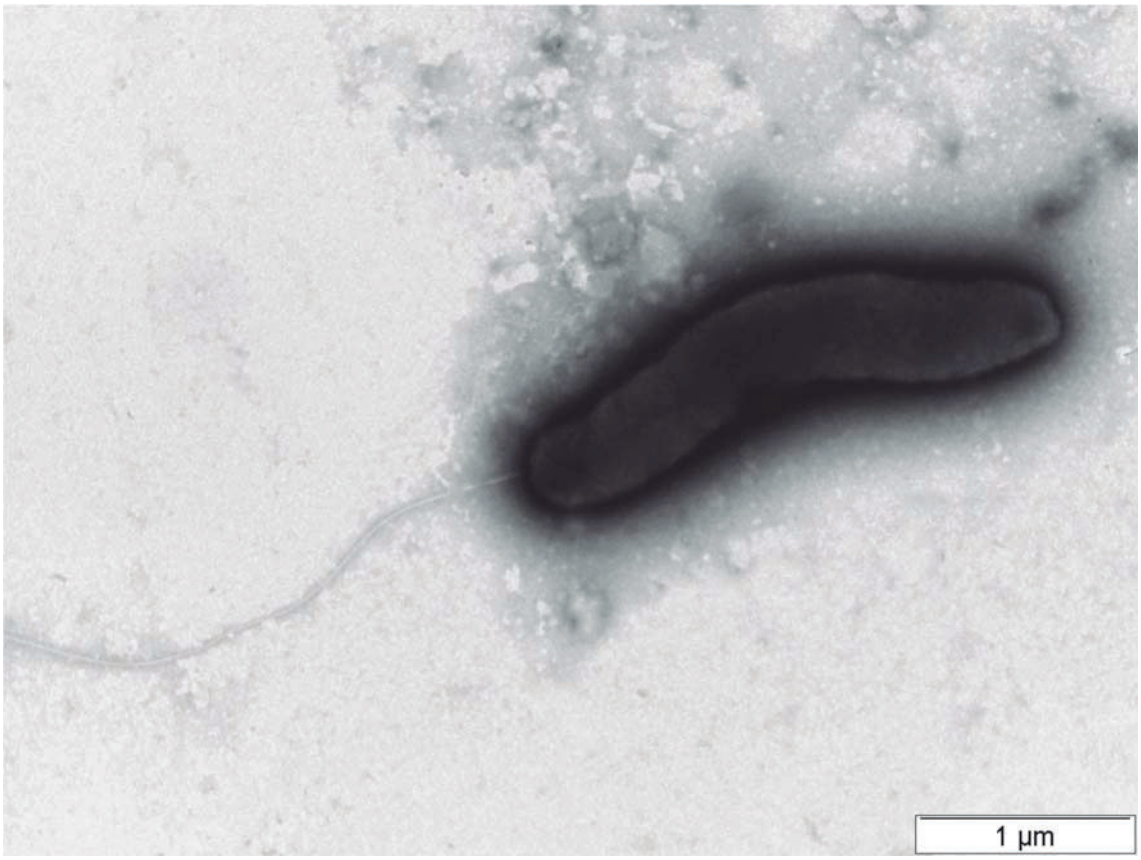
Supplementary figure S5. Maximum Likelihood tree (model GTR+G+I) based on the *gyrA* gene sequence (647 bp) showing the phylogenetic position of *A. canalis* sp. nov. strains within the genus *Arcobacter*. *Arcobacter pacificus* has not been added to the phylogeny because the gene is not available. Bootstrap values (>50%) based on 1000 replications are shown at the nodes of the tree. Bar indicates 5 substitutions per 100 nt



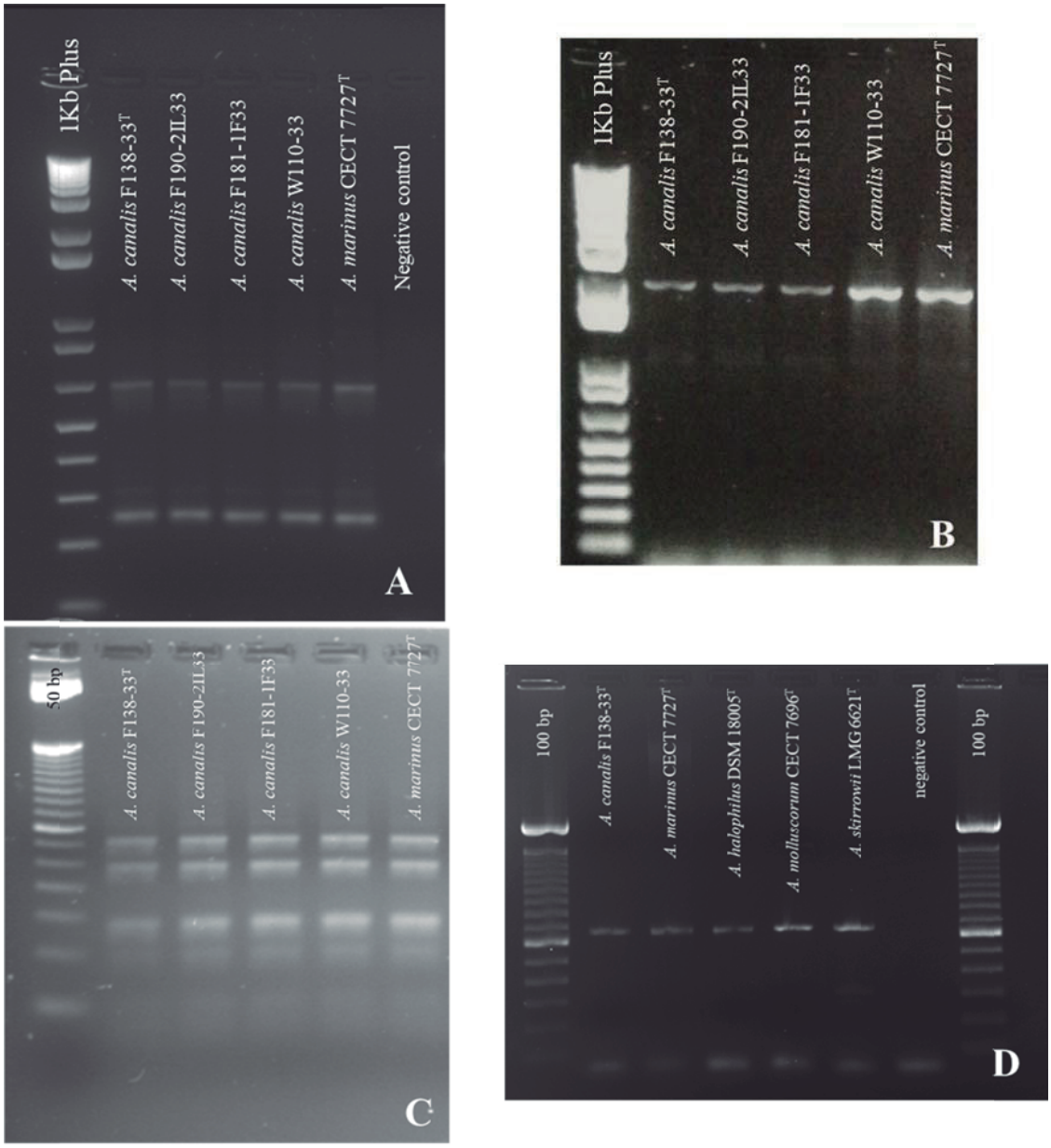
Supplementary figure S6. Maximum Likelihood tree based on the concatenated sequences of *atpA*, *gyrB*, *hsp60*, *rpoB* and *gyrA* (3041 bp) genes showing the phylogenetic position of *A. canalis* sp. nov. strains within the genus *Arcobacter*. The region of the *gyrA* gene of *A. pacificus* available at the NCBI database is not the same of the other *Arcobacter* species and was not added to the phylogenetic analysis. Bootstrap values (>50%) based on 1000 replications are shown at the nodes of the tree. Bar indicates 2 substitutions per 100 nt.



Supplementary figure S7. Maximum Likelihood tree (model GTR+G+I) based on the 16S rRNA gene sequence (1427 bp) showing the phylogenetic position of *A. canalis* sp. nov. strains within the genus *Arcobacter*. Bootstrap values (>50%) based on 1000 replications are shown at the nodes of the tree. Bar indicates 10 substitutions per 100 nt



Supplementary figure S8. Transmission electron microscopy image of a cell of the strain F138-33^{T} negatively stained. Bar, 1 µm.



Supplementary figure S9. Results obtained for the new species *A. canalis* with four molecular methods (A-D) used for the identification of *Arcobacter* spp. [10-12, 32]: A, The amplicons obtained for the four strains of *A. canalis* with the mPCR of Houf *et al*. [10] were of the expected sizes described for *A. cryaerophilus* (257 bp) and *A. skirrowii* (641bp); B, The amplicon of the four strains was of the size described for *A. butzleri* (1590 bp) with m-PCR of Douidah *et al*. [11]; C, The 16S rDNA-RFLP patter obtained for the four strains was identical to the one described for *A. marinus* [12]; Notice that the latter species produced identical amplicons than *A. canalis* with the A, B mPCR methods [10, 11]. D. With the mPCR of Khan *et al*. [32] the type strain of *A. canalis* showed, as occurred with the nearest species *A. marinus*, *A. molluscorum* and *A. halophilus*, an amplicon of the size (654bp) described for *A. skirrowii* [32]. Ladders from Invitrogen A and B 1Kb plus, C 50 bp and D 100 bp

3.7 Description of six new species within the family *Campylobacteraceae* from the Ebro River Delta and proposal of *Aliarcobacter lacus* comb. nov. and *Pseudoarcobacter caeni* comb. nov. (Basonyms, *Arcobacter lacus* and *Arcobacter caen*i, Pérez-Cataluña et al., 2018). Salas-Massó N, Pérez-Cataluña A, Andree KB, Furones MD; Figueras MJ.(In preparation)

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

Description of six new species within the family *Campylobacteraceae* from the Ebro River Delta and proposal of *Aliarcobacter lacus* comb. nov. and *Pseudoarcobacter caeni* comb. nov. (Basonyms, *Arcobacter lacus* and *Arcobacter caeni*, Pérez-Cataluña et al., 2018).

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene of strains W112-28^T, F154-34^T, F161-33^T, F155-33^T, F146-38^T and F142-34^T are LT629995, LT904749, LT629993, LT629992, LT629989 and LT708302, respectively. The accession numbers for the genomes of strains W112-28^T, F154-34^T, F161-33^T, F155-33^T, F146-38^T and F142-34^T are PDKN00000000, NXIE00000000, PDKF00000000, PDKE00000000, PDKG00000000 and PDKC00000000, respectively.

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Abstract

A recent re-evaluation of members of the genus *Arcobacter* revealed that the genus embraced at least seven genera. The present study aims to characterize with a polyphasic taxonomic approach six strains recovered from a study that investigated the diversity of *Arcobacter* spp. in shellfish (mussels and oysters) and water from the Ebro River Delta (Spain). The low similarity of the16S rRNA gene of strain (W112-28^T) with known *Arcobacter* species (95.8%), suggested that this strain belonged to a novel genus. However, the other five strains represented new species belonging to genus *Halarcobacter* and *Malaciobacter* based on the 16S rRNA similarity and other phylogenetic studies. The phylogenetic analysis of the core genome corroborated that the new genus is well separated from all genera related to *Arcobacter*. Three strains (F161-33^T, F155-33^T and F156-34^T) formed separated branches that cluster with species of the genus *Halarcobacter* and the other two (F146-38^T and F146-34^T) cluster with the species of the genus *Malaciobacter*. Values of Average Nucleotide Identity and in silico DNA-DNA hybridization between these strains and their nearest species were below 96% and 70%, respectively confirming that they corresponded to new species. Based on distinct phenotypic and genotypic properties strain W112-28^T (=CECT 8987^T=LMG 29147^T) was classified as the type strain of the novel genus, *Arcomarinus aquaticus* gen. nov., sp. nov. and the other five strains as the new species *Halarcobacter mediterraneus* (F156-34^T=CECT 9197^T= LMG 29913^T), *Halarcobacter ponticus* (F161-33^T=CECT 8983^T=LMG 29149^T), *Halarcobacter salis* (F155-33^T=CECT 8989^T=LMG 29056^T), *Malaciobacter neptunis* (F146-38^T=CECT 8986^T=LMG 29054^T), and *Malaciobacter viscosus* (F142-34^T=CECT 8985^T=LMG 29053^T). Additionally, according to the new taxonomy of the genus *Arcobacter*, the recently described species *Arcobacter lacus* and *Arcobacter caeni* were renamed as *Aliarcobacter lacus* and *Pseudarcobacter caeni*, respectively.

Keywords: *Arcomarinus*, *Halarcobacter*, *Malaciobacter*, shellfish, seawater, core genome, 16S rRNA, ANI, *is*DDH

Abbreviations: ANI, Average Nucleotide Identity; isDDH, in silico DNA-DNA hybridization; TEM, Transmission Electron Microscope.

The genus *Arcobacter* was created by Vandamme et al. in 1991[1] and embraces Gram negative, motile and oxidase positive, bacteria. In 2017, Waite et al. [2] reviewed the taxonomy of the ε-proteobacteria using the 16S and 23S rRNA genes and more than 4,000 genomes, along with 120 single-copy marker proteins and proposed a reclassification of *Epsilonproteobacteria* and *Desulfurellales* to the phylum Epsilonbacteraeota. These authors also proposed the creation of the new family *Arcobacteraceae* only composed by the genus *Arcobacter.* On the other hand, Dieguez et al. [3] realized, while describing the new species *Arcobacter lekithochrous*, that the 16S rRNA gene similarity with the other species was far below the 95% similarity established to delimit different genera [4]. In this sense, it was suggested that such species could represent new genera and that the taxonomy of *Arcobacter* should be clarified. Recently, with the objective to throw light on the taxonomy of *Arcobacter*, Pérez-Cataluña et al. [5] have performed an extensive work analyzing 55 *Arcobacter* genomes, including those of the type strains of the 27 known *Arcobacter* species and of strains considered potential new species. The study included analysis of ribosomal genes (16S and 23S rDNA), 13 housekeeping genes (*atp*A, *atp*D, *dna*A, *dna*J, *dna*K, *fts*Z, *gyr*A, *hsp*60, *rad*A, *rec*A, *rpo*B, *rpo*D, and *tsf*) and core genomes. Additionally, several indices were calculated and used to compare genomes: Average Nucleotide Identity (ANI), *in silico* DNA–DNA hybridization (*is*DDH), Average Amino-acid Identity (AAI), Percentage of Conserved Proteins (POCPs), and Relative Synonymous Codon Usage (RSCU). All these data enabled the division of the genus *Arcobacter* into at least seven different genera for which the names *Arcobacter*, *Aliarcobacter* gen. nov., *Pseudarcobacter* gen. nov., *Halarcobacter* gen. nov., *Malaciobacter* gen. nov., *Poseidonibacter* gen. nov., and Candidate '*Arcomarinus'* gen. nov. were proposed. The purpose of the present study is to formally describe 6 new species that were potentially recognized as such in the study of Perez-Cataluña et al. [5]. One of these species corresponds to the type and single strain of the candidate genus '*Arcomarinus'* gen. nov., 3 species correspond to the recently proposed genus *Halarcobacter* and the remaining 2 species to the genus *Malaciobacter*. When the re-evaluation of the genus *Arcobacter* was published [5], the species descriptions for *Arcobacter lacus* and *Arcobacter caeni* had not been published, although their genomes were used in the study of Pérez-Cataluña et al. [6]. The description of these two species was made before the names of the genera were officially validated, therefore, they were published as members of the genus *Arcobacter*. Because of this, another aim of this paper is to propose the new names for this species according to their respective genera, *Aliarcobacter* and *Pseudarcobacter*, based on the taxonomical position and genomic indices described by Pérez-Cataluña et al. [5].

Source and isolation conditions

The 6 strains investigated in this study belonged to a group of 43 isolates (2 from sewage, 20 from mussels, 8 from oysters, 6 from clams and 7 from cockles) recovered between March and December 2013 in the Alfacs Bay in the Ebro River Delta (40.572897N, 0.653600E; Spain) in which a new methodology for the isolation of *Arcobacter* from seawater, sewage and shellfish, was evaluated [7]. The isolation protocol involved two methodologies: i) the conventional one consisting in an enrichment step in Arcobacter CAT (Cefoperazone, Amphotericin B, and Teicoplanin) broth, followed by sub-culturing on blood agar at 30°C under aerobic and microaerobic conditions, (strain W112-28^T was recovered using this approach); and a new approach ii) involving an enrichment step in Arcobacter CAT broth supplemented with 2.5% NaCl, followed by sub-culturing on marine agar and incubated at the same conditions mentioned before, the remaining 5 type strains were recovered using this methodology. The colonies considered as presumptive *Arcobacter* by their morphology (small, translucent, beige to off-white on blood agar and beige to pale orange on marine agar) were Gram-stained and tested for oxidase activity. The 43 isolates were Gram-stain-negative curved rods under the microscope and presented oxidase activity, in agreement with the described characteristics of the genus [1,8]. These isolates were genotyped with the Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR) using primers and conditions previously described [9] to recognize potential clones. Patterns that differed by one or more bands were considered different genotypes, as in other studies [9, 10]. Among the 43 isolates 20 different ERIC-genotypes were recognized and represented by the strains shown in Table S1. The strains W112-28^T, F156-34^T, F146-38^T, F161-33^T, F155-33^T and F142-34^T were chosen as type strains.

Phylogeny

In order to infer the species mostly closely related to the 6 strains, alignments of the 16S rRNA gene was performed with ClustalW [11] and phylogenetic trees were constructed with MEGA 6.0 [12] for genome and phenotypic comparison purposes. (Fig. 1). The resulting trees with the Neighbour- Joining (NJ; Fig. 2) [13,14] and the Maximum Likehood (ML; Fig. S1) [15] methods corroborated the data obtained by Pérez-Cataluña et al. [5], because one strain (W112-28^T) formed an independent branch from the known genera and the remaining five strains clustered with the known species of the genera *Malaciobacter* and *Halarcobacter.*

Table 1 shows the 16S rRNA gene similarities between the six potential new species and the closest related species that were calculated using MegAlign version 7.0.0 (DNASTAR®,

Madison, WI) [5]. The strain W112-28^T showed the highest similarity of 95.8% with four species, 2 already described species *Pseudarcobacter venerupis* CECT 7836^T and *Malaciobacter molluscorum* CECT 7696^T and the two new proposed species, *Malaciobacter neptunis* sp. nov. and *Malaciobacter viscosus* sp. nov. However, the similarity with the other species of the family ranged from 92.2% with *Aliarcobacter faecis* LMG 28519^T to 95.4% with *Malaciobacter marinus* CECT 7727^T and *Malaciobacter canalis* CECT 8984^T (data obtained from Table S1 of Pérez-Cataluña et al. [5]).

Strain F156-34^T showed a 16S gene similarity that ranged from 91.8% with *Aliarcobacter skirrowii* LMG 6621^T to 96.9% with the new strain F161-33^T. The latter strain showed the highest 16S rRNA similarity (99.5%) with the new strain F155-33^T. According to the results obtained by Pérez-Cataluña [5], similarity of strain F161-33^T would range between 91.4% with 3 species of the genus *Aliarcobacter: A. skirrowii* LMG6621^T*, A. cryaerophilus* ATCC 43158^T and *A. faecis* LMG 28519^T and 98.6% with the species *Halarcobacter bivalviorum* CECT 7835^T. This range of similarity for strain F155-33T was between 91.1% with *A. faecis* LMG 28519^T to 98.9% with *Halarcobacter bivalviorum* CECT 7835^T [5].

The highest 16S rRNA gene similarity for the strains F142-34^T and F146-38^T was between each other with a value of 98.6%, followed by a 98.5% and 98.4%, respectively, with *Malaciobacter molluscorum* CECT 7696^T.

Genomic characterization

The genomes of the new strains W112-28^T (PDKN00000000); F156-34^T (NXIE00000000); F161-33^T (PDKF00000000); F155-33^T (PDKE00000000); F146-38^T (PDKG00000000) and F142-34^T (PDKC00000000), along with the genomes of the closest related species were sequenced, assembled and annotated as described by Pérez-Cataluña et al. [5]. The genomes of the new strains were compared with their nearest species using the ANI and *is*DDH calculated with JSpeciesWS [5, 16] and GGDC [5, 17], respectively. The values of ANI and *is*DDH between each strain and their nearest species were below 96% and 70%, respectively (Table 1). The phylogenetic analysis with the sequences of the core genomes (87681bp), that was extracted with Roary software [18] using a cut-off value for BLASTp search of 70% is shown in Fig. 2. The observed clustering demonstrated that the 6 strains represent new species of 3 different genera within the family *Campylobacteraceae: Arcomarinus aquaticus* gen. nov., sp. nov. (strain W112-28^T), *Halarcobacter mediterraneus* sp. nov., *H. ponticus* sp. nov. and *H. salis* sp. nov. (F156-34^T, F161-33^T and F155-33^T, respectively) and *Malaciobacter neptunis* sp. nov. and *M. viscosus* sp. nov. (F146-38^T and F142-34^T). Additionally, the analysis shows that *Arcobacter lacus* strain RW43-9^T clusters within the genus *Aliarcobacter* and *Arcobacter caeni* strain RW17-10^T within the genus *Pseudarcobacter*, therefore these represent the new combined Linnaean nomenclature for *Aliarcobacter lacus* comb. nov. and *Pseudoarcobacter caeni* comb. nov., respectively.

The G+C values of the genome of the strain W112-28^T was 34.9 mol% being the highest among the genera forming the proposed family *Arcobacteraceae* [5]. However, the G+C values of strains F156-34^T, F161-33^T and F155-33^T were 27.3. 28.1, and 29.0 mol%, respectively, and within the range of the genus *Halarcobacter* (27.3-29.9 mol%). Strains F142-34^T and F146-38^T (26.6 and 27.1 mol%, respectively) showed values of G+C similar to those described for other species of the genus *Malacobacter* (26.1 - 27.3 mol%) [5].

Phenotypic characterization

Phenotypic characterization of the 20 strains comprised in the 6 new species was performed according to the recommended minimal standards for the description of new *Campylobacteraceae* species by Ursing et al. [19] and updated by On et al. [20]. The results were compared in parallel with those of the type strains of the nearest species *P. venerupis* CECT 7836^T, *H. bivalviorum* CECT 7835^T and *M. molluscorum* CECT 7696^T. The following

characters were investigated: morphology of the colonies, growth at different temperatures and atmospheric conditions, biochemical properties and resistance to antimicrobials. Each phenotypic characteristic was tested at least twice. A total of 39 tests were performed, including 27 tests for biochemical properties (i.e. oxidase, catalase, and urease activity, nitrate reduction and glucose fermentation in triple-sugar iron agar (TSI), indoxyl acetate hydrolysis, casein, lecithin and starch, growth in media supplemented with 2% and 4% NaCl, 1% oxgall, 0.1% sodium deoxycholate, 1% glycine, 0.05% safranin, 0.005% basic fuchsine, 0.0005% crystal violet, 0.001% brilliant green, 0.1, 0.01 and 0.04% 2,3,5-triphenyltetrazolium chloride (TTC), and growth in charcoal cefoperazone deoxycholate agar (CCDA), minimal media and MacConkey agar) and 12 tests of growth conditions (i.e. 22-25ºC, 30ºC, 37ºC and 42ºC in aerobic, microaerobic and anaerobic conditions). For the assessment of the colony and bacteria morphology the strains were incubated on marine agar at 30ºC in aerobiosis, with the exception of strain W112-28^T, that did not grow on marine agar and was grown on blood agar. The morphology of the bacteria, the presence of flagella and cell size were evaluated with the transmission electron microscope (TEM) JEOL 1011. For this purpose, bacteria were fixed in a solution of 2% glutaraldehyde-0.1% phosphate buffer. The suspended cells were mounted on a copper grid and negative stained with 2% phosphotungstic acid (pH 7.5). All the strains showed the presence of a single polar flagellum (Fig. S2-S7). Additionally, the strains showed motility using the phase contrast microscope.

A total of 12 tests enabled differentiation of strain W112-28^T from the species *P. venerupis* CECT 7836^T and 15 tests to differentiate it from *M. molluscorum* CECT 7696^T (Table 2). Some of the tests are shared in the discrimination of strain W112-28^T from the two mentioned species and are the following: the ability of strain W112-28^T to grow in the presence of 0.005% basic fuchsine and on blood agar under anaerobic conditions at 30ºC; and the inability of this strain to reduce nitrate, to grow on MacConkey agar and to produce catalase or oxidase (Table 2).

The number of tests that differentiated the three strains F156-34^T, F161-33^T and F155-33^T from *H. bivalviorum* CECT 7835^T were their incapacity to grow on media containing 0.5 and 2% of NaCl. Additionally, strain F161-33^T did not grow under anaerobic conditions at room temperature and 30ºC and did not produce oxidase and strain F155-33^T did not grow under microaerobic conditions at 18-22ºC (Table 2). Strain F156-34^T grew under aerobic and microaerobic conditions at 42ºC and under anaerobic conditions at 37 and 42ºC; strain F161-

33^T grew at 42ºC under aerobic and microaerobic conditions; and strain F155-33^T showed the ability to grow under aerobic conditions at 42ºC, but growth was weak under microaerobic conditions at 42ºC and under anaerobic conditions at 37ºC (Table 2).

Strains F146-38^T and F142-34^T shared 13 phenotypical characteristics out of the 14 total tests that enabled differentiation of them from their nearest species, *M. molluscorum* CECT 7696^T. These shared tests are shown in Table 2. However, strain F146-38^T differed from *M. molluscorum* CECT 7696^T and at the same time from strain F142-34^T in the ability to grow under anaerobic conditions at 37ºC. On the other hand, the test that differentiate strain F142-34^T from *M. molluscorum* CECT 7696^T, but also from strain F146-38^T was the ability to grow under anaerobic conditions at room temperature (Table 2).

The genomes of the six strains selected as type strains were annotated with RAST [21]. All the genomes showed the following polar lipids: phosphatidylglycerolphosphatase A (*psp*A, EC3.1.3.27) and phosphatidase cytidylyltransferase (*cds*A, EC 2.7.7.41) genes, with the exception of strain W112-28^T, involved in the synthesis of phosphatidilglycerol; and the gene phosphatidylserine descarboxilase (*psd*, EC4.1.1.65) involved in the synthesis of phosphatidylethanolamine. These polar lipids have been found using experimental chromatopraphic detection [22-24], as well as with genomic tools, in other *Arcobacter* species [6, 25, 26].

Description of *Arcomarinus* gen. nov.

Arcomarinus (Ar'co. ma.ri′nus L. n. arcus, bow; L. masc. adj. marinus referring to the sea, bow shaped rod from the sea).

Cells are Gram-negative, curved rods and motile. Does not swarm. Oxidase and catalase negative. No growth occurs at 4% NaCl. Growth occurs at 25-42ºC in microaerobic and anaerobic conditions in marine agar. Carbohydrates are not fermented. Nitrate is not reduced to nitrite. Negative for the hydrolysis of indoxyl acetate and urease. Growth does not occur in the presence 2,3,5-triphenyltetrazolium chloride (0.04%, wt/vol) or glycine (1% wt/vol). Growth occurs in the presence of safranin (0,05% wt/vol) or fuchsine (0.005% wt/vol). Resistant to cefoperazone (60 μg) and cefalotin (30 μg). DNA G+C content is 34.9 mol%.

The type species is *Arcomarinus aquaticus*.

Description of *Arcomarinus aquaticus* sp. nov.

Arcomarinus aquaticus (a.qua'ti.cus. L. masc. adj. *aquaticus* living, growing or found in or by water, aquatic)

Cells are Gram-stain-negative, slightly curved rods, non-encapsulated, non-spore forming, 0.6-1.0 µm wide and 3.2-4.6 µm long. Motile by a single polar flagellum. Colonies grown on blood agar at 30ºC in aerobiosis for 48 hours are 2-4 mm in diameter, beige to off-white, convex, circular with entire margins and non-swarming. Pigments or hemolysis are not produced on blood agar. The strain grows on blood agar at 25-37ºC in aerobiosis, anaerobiosis and microaerobiosis, but not at 42ºC. Colonies grow in marine agar when incubated at 25-42ºC under microaerobiosis and anaerobiosis. No growth occurs in marine agar under aerobic conditions for any of the temperatures tested. The strain does not produce oxidase or catalase activities and does not reduce nitrates. Indoxyl acetate, urea, casein, lecithin or starch are not hydrolyzed. The strain cannot produce hydrogen sulphide nor acid from glucose in TSI. Growth occurs in media with 2% NaCl and on nutrient medium supplemented with 5% sheep blood and 2% NaCl containing 0.05% safranin, 0.0005% crystal violet, 0.005% basic fuchsine, in CCDA, and in minimal medium. No growth occurs in media with 4% NaCl, 1% Oxgall, 0.1% sodium deoxycholate, 1% glycine, 0.001% brilliant green, 0.1-0.04% TTC, in MacConkey agar. Resistant to cephalothin (30 mg l^{-1}) and cephoperazone (64 mg l^{-1}).

The type strain is W112-28^T (=CECT 8987^T =LMG 29147^T) isolated from domestic sewage from Poble Nou, Catalonia, Spain.

Description of *Halarcobacter mediterraneus* sp. nov.

Halarcobacter mediterraneus (med.i. ter.ra.ne'us. N. L. adj. *mediterraneus* of the Mediterranean Sea)

Cells are Gram-stain-negative, slightly curved rods, non-encapsulated, non-spore forming, 0.4-0.7 µm wide and 1.6-2.2 µm long. They are motile by a single polar flagellum. Colonies on marine agar incubated under aerobic conditions at 30ºC for 48 hours are 1-3 mm in diameter, pale yellow to pale orange, circular with entire margins, convex, and non-swarming. Pigments are not produced. All the strains grow on marine agar at 25-42ºC in aerobiosis, anaerobiosis and microaerobiosis. No growth occurs in blood agar at the temperatures and atmospheres mentioned above. Produce oxidase but no catalase activities and

nitrate is not reduced. Hydrolyze indoxyl acetate but not urea, casein, lecithin or starch. The strain cannot produce hydrogen sulphide nor acid from glucose in TSI. Growth occurs only in media with 4% NaCl. No growth occurs in media with 0.5 and 2% NaCl, 1% Oxgall, 0.1% sodium deoxycholate,1% glycine, and in 0.1%, 0.01 or 0.04% TTC, 0.05% safranin, 0.005% basic fuchsine, 0.0005% crystal violet, 0.001% brilliant green, in CCDA and MacConkey, and in minimal medium. Sensitive to nalidixic acid (30 mg l^{-1}), cefalotin (30 mg l^{-1}) and cefoperazone (64 mg l^{-1}).

The type strain is F156-34^T (=CECT 9197^T = LMG 29913^T) isolated from mussels from Alfacs Bay, Ebro River Delta, Spain.

Description of *Halarcobacter ponticus* sp. nov.

Halarcobacter ponticus (pon'ti.cus. L. masc. adj. *ponticus*, pertaining to the sea)

Cells of *H. ponticus* are Gram-stain-negative, slightly curved rods, non-spore forming, non-encapsulated, 0.3-0.6 µm wide and 2.1-3.3 µm long. Motile by a single polar flagellum. Colonies grown on marine agar at 30ºC in aerobiosis for 48 hours are 2.5-4 mm in diameter, pale yellow to pale orange, circular with entire margins, convex, and non-swarming. Pigments are not produced. The strains grow on marine agar at 25-42ºC in aerobiosis, and microaerobiosis; no growth was observed under anaerobic conditions. Does not reduce nitrate and there is no production of catalase activity, but oxidase is produced. Can hydrolyze indoxyl acetate, but not urea, casein, lecithin or starch. Hydrogen sulphide is not produced in TSI agar medium. Growth occurs in media with 4% NaCl with the exception of strain F161-42. No growth occurs in media with 0.5 and 2% NaCl, 1% Oxgall, 0.1% sodium deoxycholate,1% glycine, and in 0.1%, 0.01 or 0.04% TTC, 0.05% safranin, 0.005% basic fuchsine, 0.0005% crystal violet, 0.001% brilliant green, in CCDA and MacConkey, and in minimal medium. The strain F153-42 grows on nutrient medium supplemented with 5% sheep blood and 2%NaCl containing 1% glycine, 0.005% basic fuchsine and 0.01% TTC. Sensitive to nalidixic acid (30 mg l^{-1}), cefalotin (30 mg l^{-1}) and cefoperazone (64 mg l^{-1}). Strain F153-42 is resistant to nalidixic acid (30 mg l^{-1}).

The type strain is F161-33^T (=CECT 8983^T =LMG 29149^T) isolated from cockles from Alfacs Bay, Ebro River Delta, Spain.

Description of *Halarcobacter salis* sp. nov.

Halarcobacter salis (sal'is. L. gen. n. *salis* of salt)

Cells are Gram-stain-negative, slightly curved rods, non-encapsulated, non-spore forming, 0.4-0.8 µm wide and 1.7-3.8 µm long. Motile by a single polar flagellum. Colonies grown on marine agar at 30ºC in aerobiosis for 48 hours are 2-3.5 mm in diameter, pale yellow to pale orange, circular with entire margins, convex, and non-swarming. No production of pigments in marine agar. The strains grow on marine agar at 25-42ºC in aerobiosis (strain F157-43 does not grow at 42ºC). Under microaerobiosis no growth was observed at 25ºC, but the strains grow at 30 and 37ºC and weak growth was observed at 42ºC. The strains grow at 25 and 30ºC under anaerobic conditions, weak growth was observed at 37ºC and no growth occurred at 42ºC. Does not reduce nitrate and no production of catalase activity but does produce oxidase. Can hydrolyze indoxyl acetate, but not urea, casein, lecithin or starch. Hydrogen sulphide is not produced on TSI agar medium. Growth occurs in media with 4% NaCl. No growth occurs in media with 0.5 and 2% NaCl, 1% Oxgall, 0.1% sodium deoxycholate,1% glycine, and in 0.1%, 0.01 or 0.04% TTC, 0.05% safranin, 0.005% basic fuchsine, 0.0005% crystal violet, 0.001% brilliant green, in CCDA and MacConkey, and in minimal medium. Sensitive to nalidixic acid (30 mg l^{-1}), cefalotin (30 mg l^{-1}) and cefoperazone (64 mg l^{-1}).

The type strain is F155-33^T (=CECT 8989^T =LMG 29056^T) isolated from oysters exposed for 72h to untreated urban sewage in Poble Nou canal, Catalonia, Spain.

Description of *Malaciobacter neptunis* sp. nov.

Malaciobacter neptunis (nep.tu′ni.us. L. masc. adj. *neptunius* of Neptune, the Roman god of the sea)

Cells are Gram-stain-negative, slightly curved rods, non-encapsulated, non-spore forming, 0.2-0.4 µm wide and 0.9-1.9 µm long. Motile by a single polar flagellum. Colonies on marine agar incubated under aerobic conditions at 30ºC for 48 hours are 1-3 mm in diameter, pale yellow to pale orange, circular with entire margins, convex, and non-swarming. Pigments are not produced. The strain grows on marine agar at 25-42ºC in aerobiosis, and microaerobiosis and at 37ºC in anaerobiosis. No growth occurs in blood agar at the temperatures and atmospheres mentioned above. Produces oxidase, but no catalase activities and nitrate is not reduced. Can hydrolyze indoxyl acetate but not urea, casein, lecithin or starch. The strain cannot

produce hydrogen sulphide nor acid from glucose in TSI. Growth occurs only in media with 4% NaCl. No growth occurs in media with 0.5 and 2% NaCl, 1% Oxgall, 0.1% sodium deoxycholate,1% glycine, and in 0.1%, 0.01 or 0.04% TTC, 0.05% safranin, 0.005% basic fuchsine, 0.0005% crystal violet, 0.001% brilliant green, in CCDA and MacConkey, and in minimal medium. Sensitive to nalidixic acid (30 mg l^{-1}), cefalotin (30 mg l^{-1}) and cefoperazone (64 mg l^{-1}).

The type strain is F146-38^T (=CECT 8986^T =LMG 29054^T) isolated from mussels from Alfacs Bay, Ebro River Delta, Spain.

Description of *Malaciobacter viscosus* sp. nov.

Malaciobacter viscosus (vis.co' sus. L. masc. adj. *viscosus* viscous, because of its thread-forming, adherent colonies)

Cells are Gram-stain-negative, slightly curved rods, non-encapsulated, non-spore forming, 0.3-0.4 µm wide and 1.2-2.0 µm long. Motile by a single polar flagellum. Colonies on marine agar incubated under aerobic conditions at 30ºC for 48 hours are 1-3 mm in diameter, pale yellow to pale orange, circular with entire margins, convex, and non-swarming. No pigments are produced. All the strains grow on marine agar at 25-42ºC in aerobiosis and microaerobiosis and at 25ºC in aerobiosis. No growth occurs in blood agar at the temperatures and atmospheres mentioned above. Produces oxidase, but no catalase activities and nitrate is not reduced. Can hydrolyze indoxyl acetate but not urea, casein, lecithin or starch. The strain cannot produce hydrogen sulphide nor acid from glucose in TSI. Growth occurs only in media with 4% NaCl. No growth occurs in media with 0.5 and 2% NaCl, 1% Oxgall, 0.1% sodium deoxycholate,1% glycine, and in 0.1%, 0.01 or 0.04% TTC, 0.05% safranin, 0.005% basic fuchsine, 0.0005% crystal violet, 0.001% brilliant green (with the exception of strain F136-41), in CCDA and MacConkey, and in minimal medium. Sensitive to nalidixic acid (30 mg l^{-1}), cefalotin (30 mg l^{-1}) and cefoperazone (64 mg l^{-1}).

The type strain is F142-34^T (=CECT 8985^T =LMG 29053^T) isolated from mussels exposed for 72h to untreated urban sewage in Poble Nou canal, Catalonia, Spain.

Description of *Aliarcobacter lacus* comb. nov.

Basonym: *Arcobacter lacus* Pérez-Cataluña et al., 2018.

The description is the same given by Pérez-Cataluña et al. [5]. The type strain is RW43-9^T (=CECT 8994^T=LMG 29062^T)

Description of *Pseudarcobacter caeni* comb. nov.

Basonym: *Arcobacter caeni* Pérez-Cataluña et al., 2018.

The description is the same given by Pérez-Cataluña et al. [5]. The type strain is RW17-10^T (=CECT 9140^T=LMG 29151^T).

Conflict of Interest

The authors have no conflict of interest to declare.

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Table 1. Closest species based on the 16S rRNA similarity and results (in percentage) of ANI and *is*DDH between the genomes of the 6 new *Arcobacteraceae* species and the genomes of the most closely related species. Values of ANI and *is*DDH below 96% and 70%, respectively, indicate that the genomes correspond to different species. Data extracted from Pérez-Cataluña et al. [4].

Species	**Closest 16S rRNA species**	**% 16S rRNA similiraity**	**% ANI**	***is*DDH**
***Arcomarinus aquaticus* gen. nov., sp. nov. W112-28^T**	*Pseudarcobacter venerupis* CECT 7836^T	95.8	71.1	18.8
	Malaciobacter molluscorum CECT 7696^T	95.8	71.5	18.5
	Malaciobacter neptunis sp. nov. F146-38^T	95.8	71.3	18.5
	Malaciobacter viscosus sp. nov. F142-34^T	95.8	71.4	18.7
***Halarcobacter mediterraneus* sp. nov. F156-34^T**	*Halarcobacter ponticus* sp. nov. F161-33^T	96.9	82.2	25.6
	Halarcobacter bivalviorum CECT 7835^T	96.6	81.8	24.8
***Halarcobacter ponticus* sp. nov. F161-33^T**	*Halarcobacter salis* sp. nov. F155-33^T	99.5	89.0	37.4
	Halarcobacter bivalviorum CECT 7835^T	98.6	84.2	28.2
***Halarcobacter salis* sp. nov. F155-33^T**	*Halarcobacter bivalviorum* CECT 7835^T	98.9	84.9	29.4
***Malaciobacter neptunis* sp. nov. F146-38^T**	*Malaciobacter viscosus* sp. nov. F142-34^T	98.6	85.3	29.1
	Malaciobacter molluscorum CECT 7696^T	98.4	90.3	40.4
***Malaciobacter viscosus* sp. nov. F142-34^T**	*Malaciobacter molluscorum* CECT 7696^T	98.5	85.6	29.4

Table 2. Differential characteristics of the six new species of the family *Campylobacteraceae* and type strain of the closest species of the genus *Arcobacter*. Taxa: 1, *Arcomarinus aquaticus* gen. nov., sp. nov. (n=1); 2, *Halarcobacer mediterraneus* sp. nov. (n=2); 3, *Halarcobacer ponticus* sp. nov. (n=4); 4, *Halarcobacer salis* sp. nov. (n=9); 5, *Malaciobacer neptunis* sp. nov. (n=1); 6, *Malacobacer viscosus* sp. nov. (n=3); 7, *Pseudarcobacter venerupis* CECT 7836^T; 8, *Malaciobacter molluscorum* CECT 7696^T; 9, *Halarcobacter bivalviorum* CECT 7835^T. Unless otherwise indicated: +, ≥95% strains positive; −, ≤11% strains positive; V, 12–94% strains positive. All strains were positive to growth in marine agar in aerobiosis at room temperature (18-22ºC) and at 30ºC, and in microaerobiosis at 37ºC. All strains were negative to growth in 0.1% and 0.04% TTC, to hydrolyze starch, casein and lecithin and to resist Nalidixic acid. RT= room temperature (18-22ºC). w= weak growth.

Characteristics	**1**	**2***	**3***	**4***	**5***	**6***	**7**	**8**	**9**
Growth in/on									
Aerobiosis at:									
37°C	+	+	+	+	+	+	-	+	+
42°C	-	+	+	V (+)	+	+	-	w	-
Microaerobiosis at:									
RT	+	+	+	-	+	+	+	+	+
30°C	+	+	+	+	+	+	+	+	+
42°C	+†	+	+	w	+	+	-	+	-
Anaerobiosis at:									
RT	+	+	-	+	-	+	+	-	+
30°C	+	+	-	+	-	-	-	w	+
37°C	+	+	-	w	+	-	+	-	-
42°C	+†	+	-	-	-	-	+	-	-
0.5% NaCl (w/v)	+	-	V (-)	-	-	-	+	+	+
2% NaCl (w/v)	+	-	V (-)	-	-	-	+	+	+
4% NaCl (w/v)	-	+	V (+)	+	+	+	-	+	+
1% (w/v) Oxgall	-	-	-	-	-	-	-	+	-
0.1% sodium deoxycholate	-	-	-	-	-	-	-	+	-
1% glycine	-	-	V (-)	-	-	-	-	-	-
0.05% safranin	+	-	-	-	-	-	-	+	-
0.005% basic fuchsine	+	-	V (-)	-	-	-	-	-	-
0.0005% crystal violet	+	-	-	-	-	-	-	+	-
0.001% brilliant green	-	-	V (-)	-	-	V (-)	-	-	-
0.01% TTC	-	-	V (-)	-	-	-	-	+	-
Minimal medium	+	-	-	-	-	-	+	-	-
MacConkey	-	-	-	-	-	-	+	+	-
CCDA	+†	-	-	-	-	-	+	-	-

Table 2. Continuation

Characteristics	1	2*	3*	4*	5*	6*	7	8	9
Resistance to:									
Cefoperazone 64mg/l	+†	-	V (-)	-	-	-	-	+	-
Enzyme activity									
Nitrate reduction	-	-	-	-	-	-	+	+	-
Catalase	-	-	-	-	-	-	+	+	-
Oxidase	-	+	-	+	+	+	+	+	+
Urease	-	-	-	-	-	-	+	-	-
Indoxyl acetate hydrolysis	-	+	+	+	+	+	+	-	+

* For these strains, the tests were carried out on media supplemented with 2% NaCl, with the exception of 0.5 and 4% (w/v) NaCl, catalase and indoxyl acetate hydrolysis

† These tests were carried out on marine agar.

Figure 1. Neighbour-joining tree based on 16S rRNA gene sequences (1409 bp) showing the phylogenetic position of *Arcomarinus aquaticus* gen. nov., sp. nov; *Halarcobacter mediterraneus* sp. nov., *Halarcobacter ponticus* sp. nov., *Halarcobacter salis* sp. nov., *Malaciobacter neptunis* sp. nov. and *Malaciobacter viscosus* sp. nov. and the proposal of *Aliarcobacter lacus* comb. nov. and *Pseudoarcobacter caeni* comb. nov. within the family *Arcobacteraceae.* Bootstrap values (>50 %) based on 1000 replications are shown at the nodes of the tree. Bar, 5 substitutions per 1000 nt.

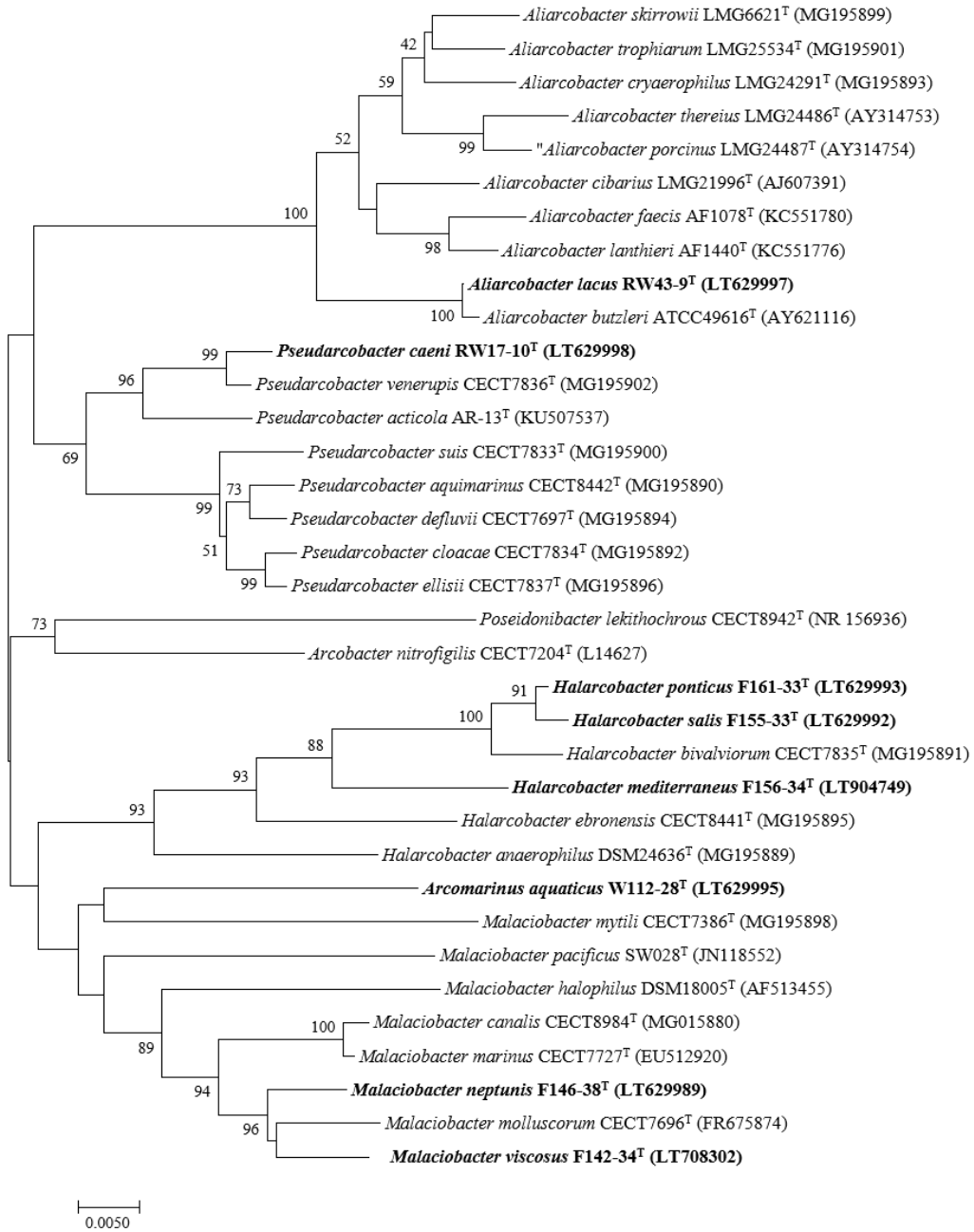
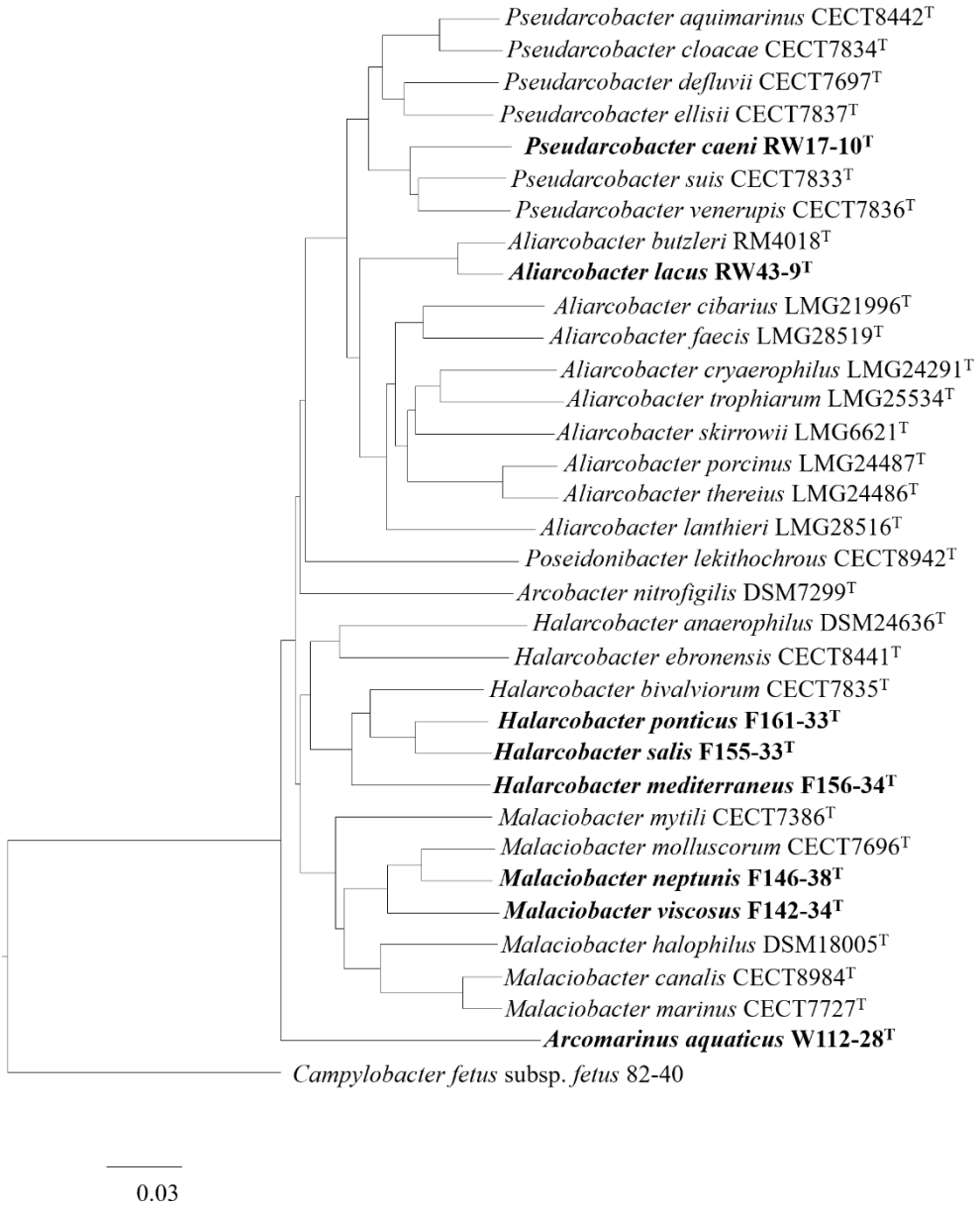


Figure 2. Neighbour-joining tree based on the core genome (87681bp) of 33 species previously grouped in the genus *Arcobacter*, and now corresponding to 7 different genera. It shows the phylogenetic position of *Arcomarinus aquaticus* gen. nov., sp. nov; *Halarcobacter mediterraneus* sp. nov., *Halarcobacter ponticus* sp. nov., *Halarcobacter salis* sp. nov., *Malaciobacter neptunis* sp. nov, *Malaciobacter viscosus* sp. nov, *Aliarcobacter lacus* comb. nov. and *Pseudoarcobacter caeni* comb. nov. *Campylobacter fetus* subsp *fetus* 82-40 (NC_008599.1) was used as outgroup. Bar indicates 3 substitutions per 100 nt.

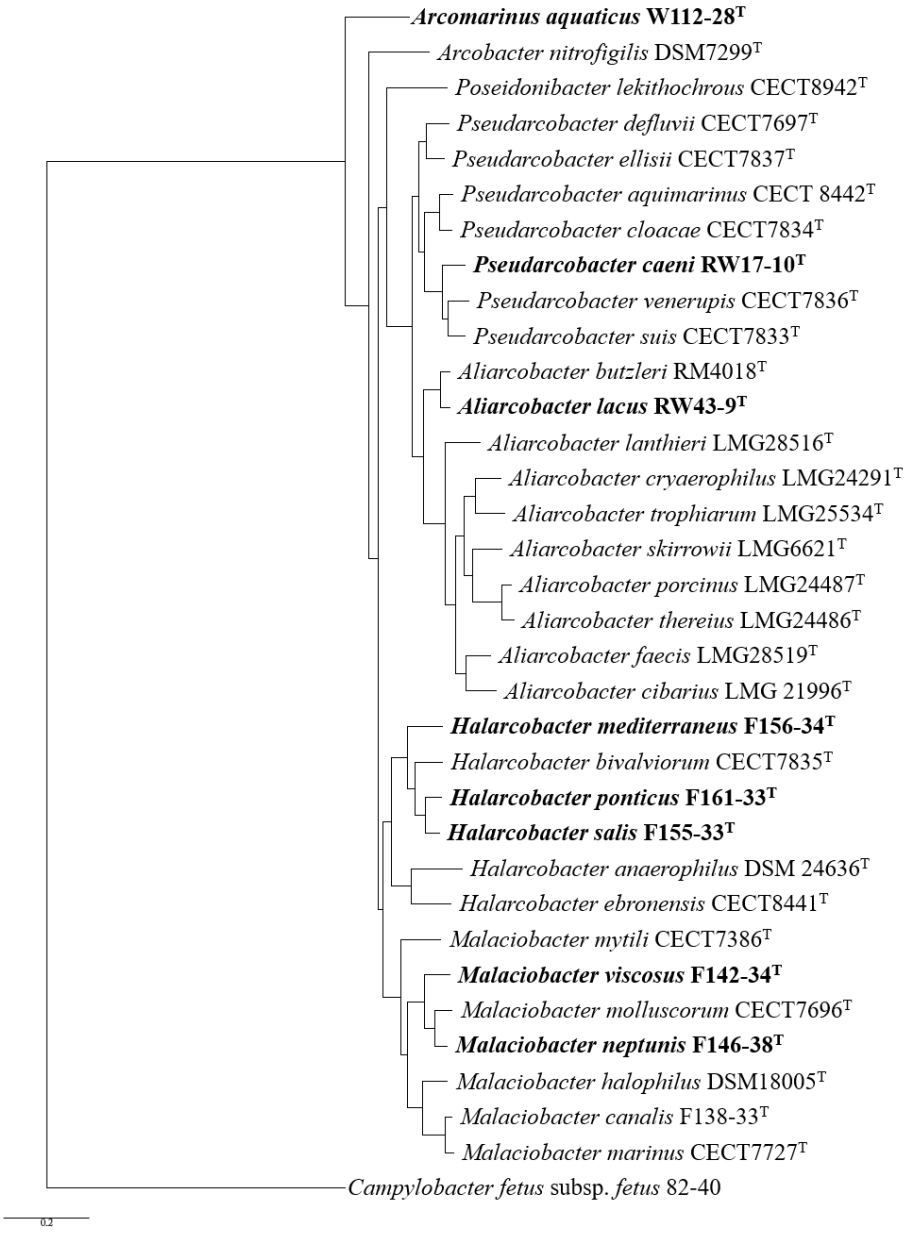


Supplementary data

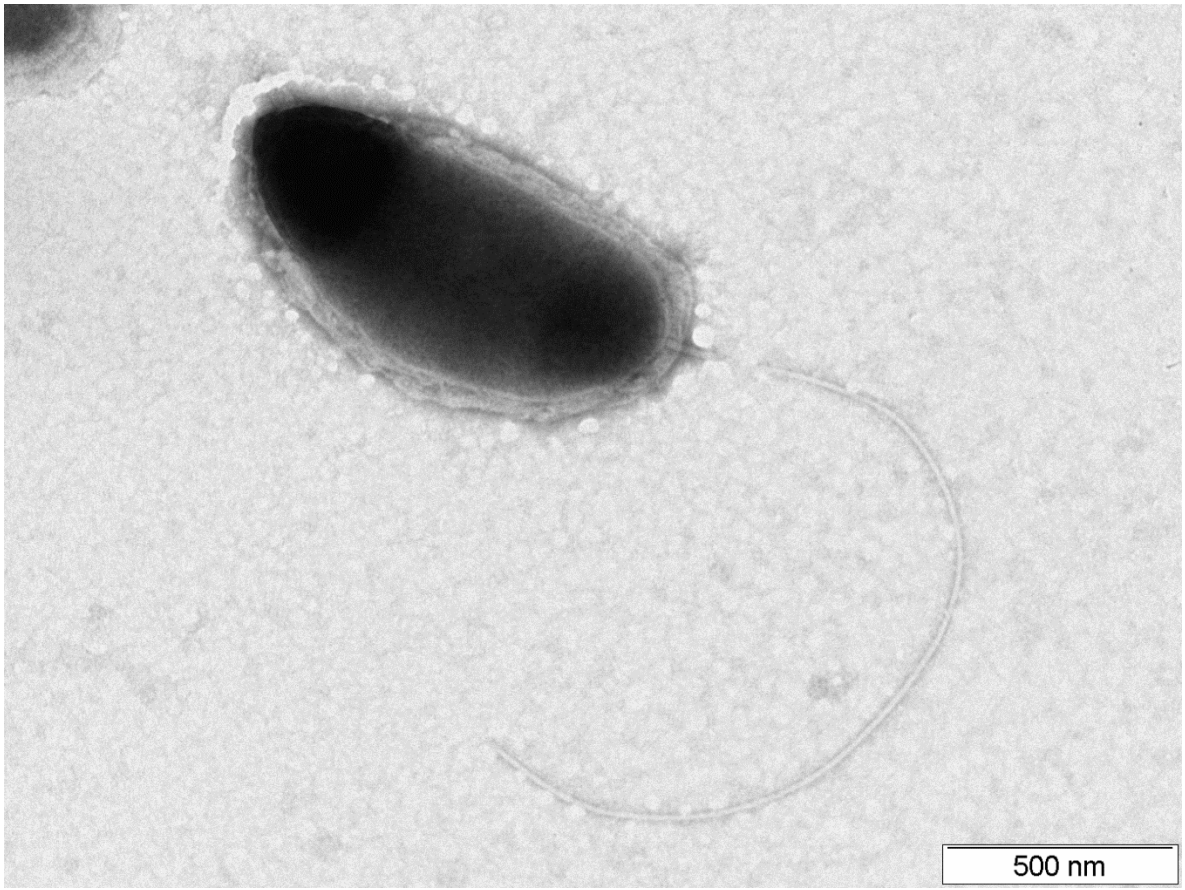
Supplementary table S1. Number of isolates, genotypes and origin of the *Arcomarinus aquaticus. Haloarcobacer. mediterraneus, H. ponticus, H. salis, Malacobacter neptunis* and *M. visocus isolates*.

Species	Number of isolates	Strain	Sample	Month
Arcomarinus aquaticus	n= 3			
	1	W112-28^T	Sewage	April
Haloarcobacer mediterraneus	n= 2			
	1	F156-34^T	Mussel	August
	1	W143-33	Sewage	April
Haloarcobacer ponticus	n= 9			
	2	F153-42	Oyster	August
	1	F161-33^T	Cockle	October
	5	F161-35	Cockle	October
	1	F161-42	Cockle	October
Haloarcobacer salis	n= 18			
	5	F155-33^T	Oyster	August
	1	F157-33	Clam	August
	1	F157-42	Clam	August
	1	F157-43	Clam	August
	1	F157-44	Clam	August
	5	F158-33	Mussel	August
	2	F158-36	Mussel	August
	1	F160-43	Clam	August
	1	F160-46	Clam	August
Malacobacer neptunis	n= 10			
	1	F145-41T	Oyster	June
	1	F146-33	Mussel	July
	8	F146-38	Mussel	July
Malacobacer viscosus	n= 5			
	2	F136-41	Mussel	March
	1	F139-33	Oyster	March
	2	F142-34^T	Mussel	June

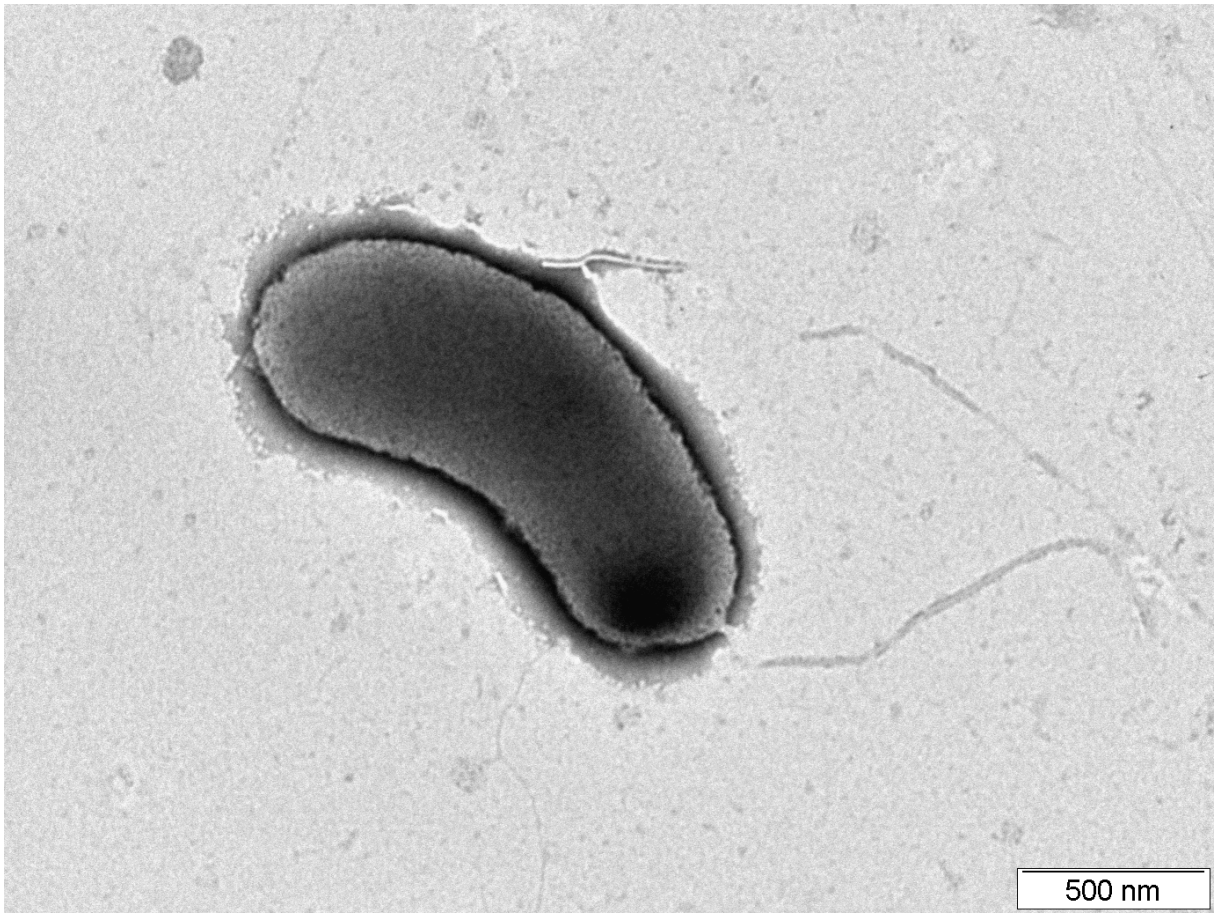
Supplementary figure S1. Maximum Likelihood tree (model GTR+G+I) based on the core genome (87681bp) of 33 species previously grouped in the genus *Arcobacter*, and now corresponding to 7 different genera. It shows the phylogenetic position of *Arcomarinus aquaticus* gen. nov., sp. nov; *Halarcobacter mediterraneus* sp. nov., *Halarcobacter ponticus* sp. nov., *Halarcobacter salis* sp. nov., *Malaciobacter neptunis* sp. nov, *Malaciobacter viscosus* sp. nov, *Aliarcobacter lacus* comb. nov. and *Pseudoarcobacter caeni* comb. nov. *Campylobacter fetus* subsp *fetus* 82-40 (NC_008599.1) was used as outgroup. Bar indicates 2 substitutions per 10 nt.



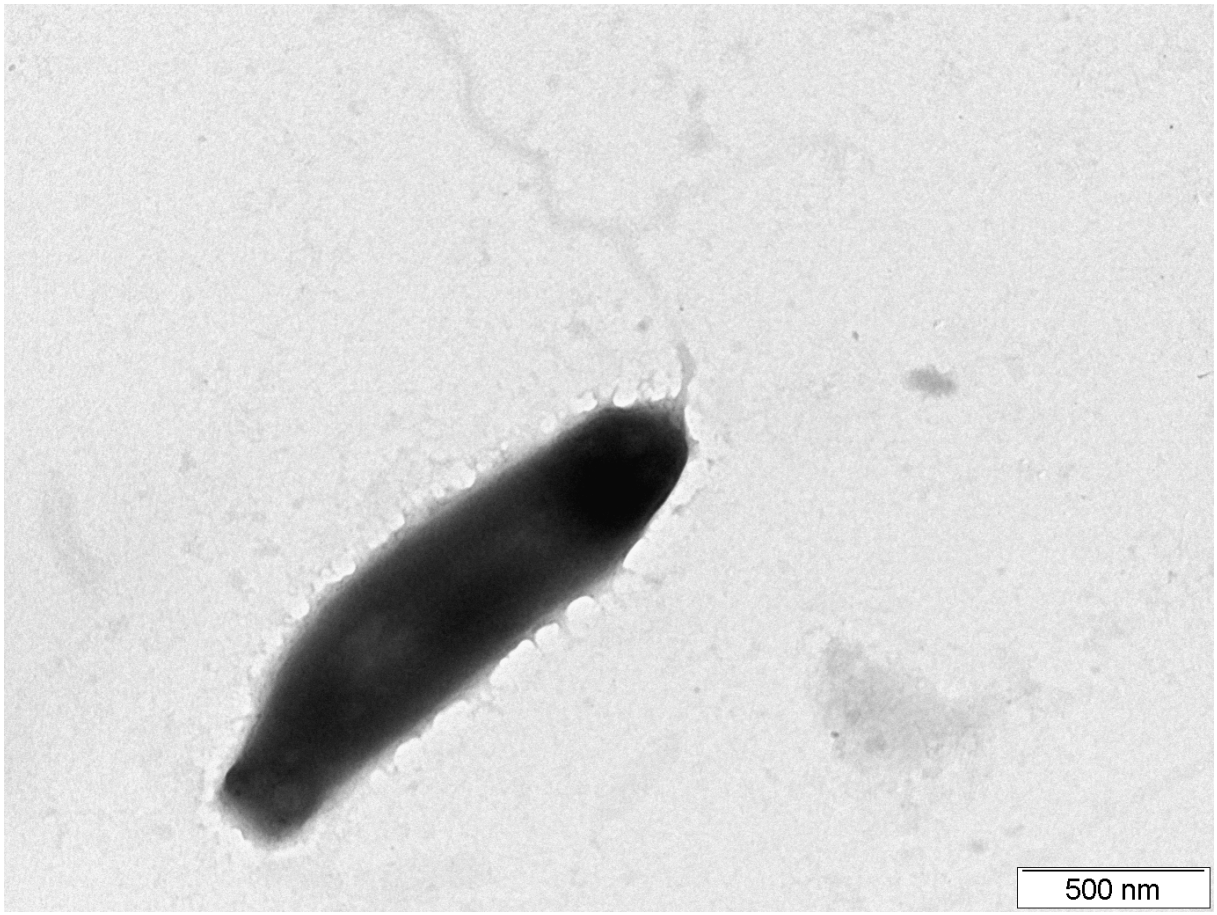
Supplementary figure S2. Transmission electron microscopy image of a cell of the strain W112-28^{T} negatively stained. Bar, 0.5 µm.



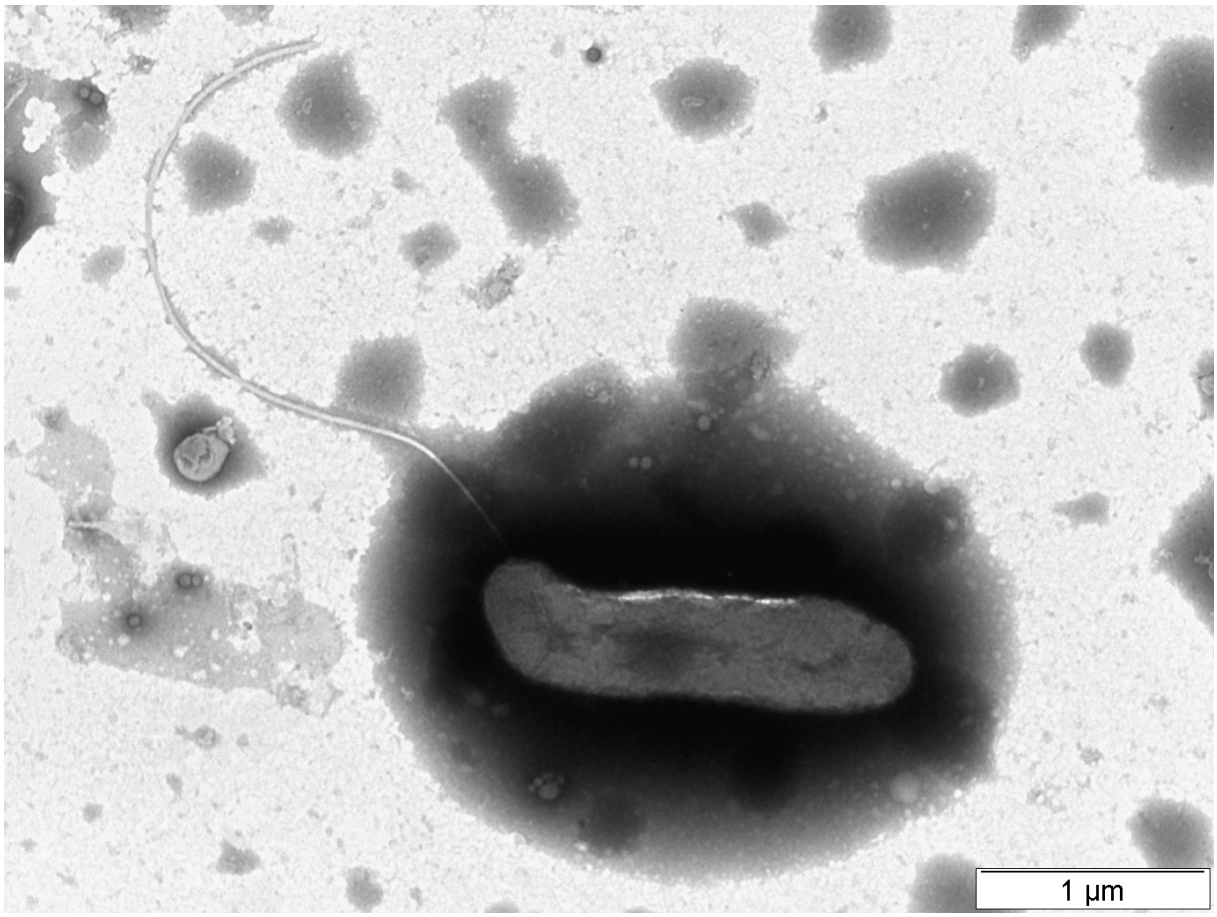
Supplementary figure S3. Transmission electron microscopy image of a cell of the strain F156-34^{T} negatively stained. Bar, 0.5 µm.



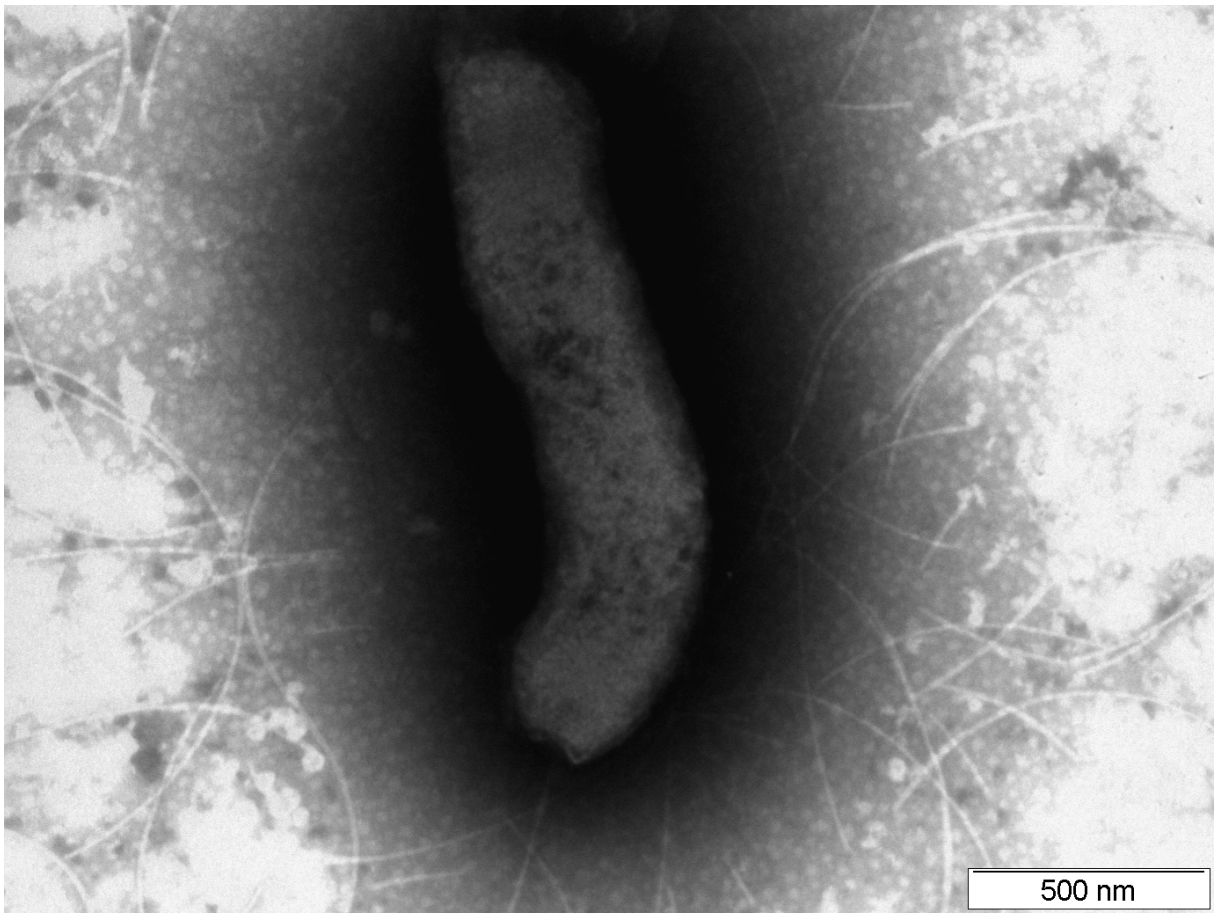
Supplementary figure S4. Transmission electron microscopy image of a cell of the strain F161-33^{T} negatively stained. Bar, 0.5 µm.



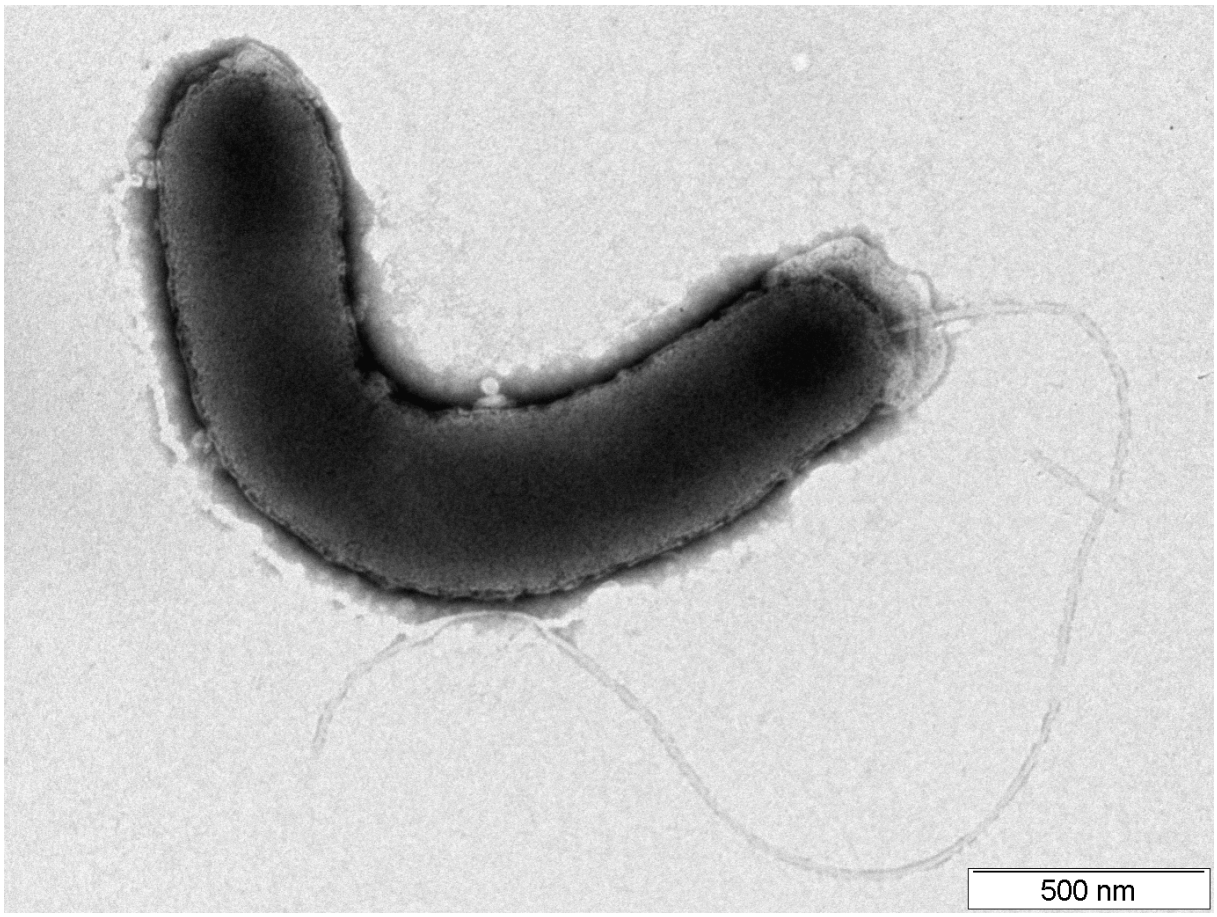
Supplementary figure S5. Transmission electron microscopy image of a cell of the strain F155-33^{T} negatively stained. Bar, 1 µm.



Supplementary figure S6. Transmission electron microscopy image of a cell of the strain F146- 38^{T} negatively stained. Bar, 0.5 µm.



Supplementary figure S7. Transmission electron microscopy image of a cell of the strain F142-34^{T} negatively stained. Bar, 0.5 µm.



UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

The development of this thesis has led to the development of the works presented above and the publication of other three scientific publications, which are the result of the collaboration with different institutions and researchers.

Giacometti F, Salas-Massó N, Serraino A, Figueras MJ. (2015) Characterization of Arcobacter suis isolated from water buffalo (Bubalus bubalis) milk. *Food Microbiology*, 51: 186-191.

Figueras MJ, Pérez-Cataluña A, Salas-Massó N, Levican A. and Collado L. (2017) '*Arcobacter porcinus*' sp. nov., a novel Arcobacter species uncovered by *Arcobacter thereius*. *New Microbes New Infections*, 15: 104–106.

Pérez-Cataluña A, Salas-Massó N, Figueras MJ. (2018) *Arcobacter lacus* sp. nov. and *Arcobacter caeni* sp. nov., two novel species isolated from reclaimed water. *International Journal of Systematic and Evolutionary Microbiology*,. doi: 10.1099/ijsem.0.003101.

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GENERAL DISCUSSION

UNIVERSITAT ROVIRA I VIRGILI
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GENERAL DISCUSSION

The genus *Arcobacter*, as it was described by Vandamme et al. (1991), comprises species that have been considered zoonotic agents and emergent pathogens (*Aliarcobacter butzleri*) by the International Commission on Microbiological Specifications for Foods (ICMSF, 2002). *Arcobacter* species have been recovered from a wide range of animals and food products worldwide, among which seafood presents a high positivity for the presence of this bacteria, posing a risk for the consumer as some seafood products are consumed raw or slightly cooked (Collado and Figueras, 2011; Levican et al., 2014; Ferreira et al., 2016, 2017). In addition to this latter fact, the presence of *Arcobacter*-related species in seawater and shellfish samples have been demonstrated in several studies, which would make this group, including known and potential new unculturable species, to be considered as indigenous from these environments (Miller et al., 2007; Wesley and Miller, 2010; Collado and Figueras, 2011; Fernández-Piquer et al., 2012; King et al., 2012). For these reasons, our first objective in the present thesis was to develop an improved culture protocol for recovering *Arcobacter* species from these types of marine samples (**Study 3.1**). Our new approach, which consisted of a simple modification of the conventional method used for the isolation of *Arcobacter* (Collado et al., 2008), led to very interesting results. First, the percentage of positive *Arcobacter* related spp. detected in shellfish and water samples was statistically higher in comparison with the use of the conventional broth and culturing on blood agar (69.7% vs 30.3%), when 2.5% NaCl was added to the enrichment medium followed by culturing on marine agar. Therefore, taking in consideration this promising result, this methodology was used in parallel with the conventional one in the rest of the studies performed in the present Ph.D. thesis, and the results observed in the following studies were similar. In **Study 3.2**, in which we evaluated the relationship of *Arcobacter*-related spp. with *E. coli*, the percentages of positive samples in Alfacs Bay samples were 81% vs. 19% for water and, 69.6% vs. 26.1% for shellfish. In **Study 3.3**, where we analyzed the compartmentalization of *Arcobacter*-related spp. in shellfish tissues, the samples from Alfacs Bay presented a higher number of positive samples obtained by the new approach than with the traditional one, resulting in 65.4% versus 49%, respectively. In the **Study 3.5** the development of a viable-qPCR is presented, and we observed that when we analyzed the Alfacs Bay shellfish samples, the difference between the methods was not as high as in the other studies, and yet culturing on marine agar after the pre-enrichment in Arcobacter-CAT broth supplemented with NaCl yielded 35.3% of positive samples vs. 29.4% obtained by enriching in Arcobacter-CAT broth and posterior culture in blood agar. The exception in this study was

that, in addition to mussels and oysters, other shellfish were studied, such as razor clams and bean clams.

The second effect of the use of this new culturing approach with NaCl supplement was that the diversity of species recovered from the samples increased and species like *Halarcobacter bivalviorum*, *Malaciobacter molluscorum*, and *Malaciobacter mytili* were recovered more often, representing 53% of the strains recovered in **Study 3.1** giving further evidence of their common natural association with bivalves (Levican et al., 2014). Additionally, the species *Malaciobacter halophilus* (Donachie et al., 2005), and *Malaciobacter marinus* (Kim et al., 2010) were isolated for the first time, since their description exclusively using the new method, indicated that the recovery of these species is favored by adding extra salt in the media. Moreover, the use of this new methodology improved the time of isolation of these species, from 2 weeks for *M. halophilus* and 12 days for *M. marinus*, to only 4 days with our method. Additionally, using the culture media with NaCl resulted in the isolation of 7 potentially new *Arcobacter* related species (Table 1, **Study 3.1**). The conventional approach presented an established view of the diversity of *Arcobacter* spp., with *A. butzleri* being the most prevalent species in water and shellfish samples from both origins studied in the present thesis (**Study 3.1**, **Study 3.2**, **Study 3.3**), followed usually by *Aliarcobacter cryaerophilus* (Maugeri et al., 2000; Fernández et al., 2001; Collado et al., 2009b, 2014; Levican et al., 2014; Mottola et al., 2016), but this species was not isolated by using the media with NaCl. This was associated with the fact that *A. cryaerophilus* does not appear in association with marine water or in the presence of salt. Several authors have demonstrated that the enrichment step could cause a bias, and *A. cryaerophilus* may be the predominant species when analyzing samples without enrichment, but after the enrichment step, *A. butzleri* would become the most prevalent one (Ho et al., 2008; Levican et al., 2016). In fact, *A. cryaerophilus* was found to be the dominant species recovered from wastewater by using a metagenomic approach (Fisher et al., 2015)

Once preliminary results on the diversity of *Arcobacter* species and their high prevalence in the marine environment and bivalves were obtained by developing improved culture tools, (**Study 3.1)**, the next step of the thesis aimed to analyze the relationship between *Arcobacter*-related spp. and *E. coli*, which is the reference fecal indicator bacteria in the EU regulation (EU 854/2004) used to assess if bivalve mollusks are safe for human consumption. In order to increase knowledge about the ecology of *Arcobacter*-related spp., the relationship between *Arcobacter* and *E. coli* was studied in two completely different scenarios: a shellfish

production area, officially classified as a B harvesting area (Alfacs Bay), and heavily fecally polluted brackish water from a drainage channel (Poble Nou Channel; PNC). These two environments with differing levels of pollution were used as study sites for three studies **(Studies 3.2, 3.3 and 3.4).** performed in this thesis, to achieve a consistent background of the bacterial ecology.

The correlation between the presence of *E. coli* and *Arcobacter*-related spp. (with special attention to pathogenic species like *A. butzleri* and *A. cryaerophilus*) was evaluated in **Study 3.2** where, for the first time, the presence of both, *Arcobacter*-related spp. and *E. coli,* was simultaneously investigated in shellfish and in their surrounding waters. The shellfish accumulation capacity was corroborated by the higher prevalence of positive samples for both bacteria in shellfish (78.3%) when compared with water (52.4%). The results showed that *E. coli* was not detected in the water and shellfish summer samples, but *Arcobacter* was. Previous reports have also shown, that fecal indicator bacteria decrease when the temperature of the water increases (Chigbu et al., 2005; Leight et al., 2016). These results suggest that, in shellfish harvested from category B production areas, when the temperature of the water was above 26.2 °C, the MPN of *E. coli* would fail to predict the presence of *A. butzleri* and *Aliarcobacter skirrowii*, among other species (Table 1; **Study 3.2**). In contraposition to *E. coli*, certain species of *Arcobacter* seem to prevail at colder temperatures, i.e. *A. cryaerophilus* and *A. skirrowii* are more prevalent at temperatures between 9.8 and 19.8 °C, than at warmer ones (20–29.5 °C), where *A. butzleri* prevails (Fisher et al., 2014; Levican et al., 2014). This tendency was also observed in **Study 3.3**, in which *A. cryaerophilus* was favored by the lower salinity and temperatures of the PNC during the winter-spring periods.

A lack of significant correlation was observed between the MPN of *E. coli* and *Arcobacter,* in any type of sample from Alfacs Bay, however, significant correlation was found in the PNC samples. On average, in this scenario of the PNC, the concentration of *Arcobacter*-related spp. (4.5×10^5 MPN/100 mL) was one log higher than that of *E. coli* (4.1×10^4 MPN/100 mL; P=0.05), and the concentration of both bacteria correlated with the results described by Collado et al. (2008) for contaminated freshwater. Later studies have hypothesized that the high prevalence of *Arcobacter* spp. found in sewage, is due to the capacity of *Arcobacter* to multiply in such environments (McLellan et al., 2010; Vandewalle et al., 2012; Fisher et al., 2014; McLellan and Roguet, 2019), rather than associating their presence to contamination with

human feces, as it was originally thought in other studies (Moreno et al., 2003; Collado et al., 2008; Merga et al., 2014).

A deeper analysis, considering the different *Arcobacter* species recovered from all the water and shellfish samples, showed a correlation between *E. coli* and specific species of *Arcobacter*, and it was dependent upon the load of *E. coli* in the samples (Tables S2 and S3; **Study 3.2**). For instance, the presence of *E. coli* in shellfish was associated with the presence of the dominant species *A. butzleri,* and *A. cryaerophilus.* These findings, in agreement with results of Leoni et al. (2017) (Tables S2 and S3; **Study 3.2**), suggest these species are introduced in the seawater with fecal pollution (Maugeri et al., 2000; Wirsen et al., 2002; Fera et al., 2004; Collado et al., 2009; McLellan and Roguet, 2019). A similar distribution pattern of species was also observed in **Study 3.3**, in which class C shellfish, were dominated by the species *A. butzleri*, and in class D species like *A. cryaerophilus*, *Pseudarcobacter cloacae* and *Pseudarcobacter defluvii* prevailed.

However, species recovered from shellfish and seawater like *M. molluscorum* and *M. mytili* showed a higher prevalence when the levels of *E. coli* in water and shellfish were low, suggesting these species could be of marine indigenous origin (Tables S2 and S3; **Study 3.2**).

Since water and shellfish samples were studied concurrently, it allowed us to determine that the detection of *Arcobacter* in water may predict its presence in shellfish, independently of the concentration of the bacteria in the water, as the correlation coefficients obtained in both AB (ρ=0.527) and PNC (ρ=0.472) were statistically significant. Thus, whenever *Arcobacter*-related spp. were present in the water, it will be very likely that these species will be also found in shellfish.

One of the objectives of the study was to evaluate the potential correlation between *E. coli* and the pathogenic species of *Arcobacter*, the methodology of the MPN used was based on the conventional approach for the isolation of these species, even though this combination of media can lead to bias in the detection of environmental species (Table 1, **Study 3.2**). For instance, species like *H. bivalviorum*, *Halarcobacter ebronensis*, *M. marinus,* and *M. mytili*, would not be so easily detected with the MPN method used in **Study 3.2**, as they were previously more frequently recovered with the method supplemented with NaCl (Salas-Massó et al., 2016; **Study 3.1**). However, for the purpose of generating the relevant data in the context

of the EU system for shellfish safety, maintaining the standard protocol to detect *Arcobacter* was considered a priority.

Once the relationship of *Arcobacter*-related spp. with *E. coli* was determined, and it was observed that it was dependent on the levels of fecal pollution, water temperature, and salinity, together with the previous results that demonstrated some species recovered were autochthonous marine species, we developed the scheme for **Study 3.3**. This study was undertaken to investigate the distribution of *Arcobacter*-related spp. within the compartments and tissues of mussels (*Mytilus galloprovincialis*) and oysters (*Crassostrea gigas*), and to examine their relationship with their presence in water with different levels of fecal pollution (AB and PNC). To achieve this result and to evaluate the abundance of samples positive for *Arcobacter*-related spp., five different approaches were assessed: i) multiplex PCR (m-PCR) from DNA obtained directly from the raw homogenized tissues (m-PCR-D), ii) m-PCR from DNA obtained from the pre-enrichment Arcobacter-CAT broth (m-PCR-PE1), iii) m-PCR from DNA obtained from the pre-enrichment Arcobacter-CAT broth supplemented with 2.5% NaCl (m-PCR-PE2), iv) positive isolation on blood agar (BA-C), and v) positive isolation on Marine agar (MA-C). The two m-PCR performed with the DNA obtained from the two enrichment broths were the methodologies that resulted in higher number of positive tissue samples. These results could be explained by two main factors: i) the DNA was obtained from a pre-enrichment step, where more bacteria could be present as a result of the enrichment than in a direct non-enriched sample; and ii) the positives could come from either dead or viable-but-not-culturable bacteria (VBNC), that would not have been detected when culture-dependent approaches were used. A better performance was also reported by González et al. (2007) and Collado et al. (2008), when comparing the m-PCR after pre-enrichment, to culturing from wastewater samples. Additionally, Levican et al. (2016) also observed that m-PCR-D performed worse than m-PCR from enriched samples and culture, which agrees with this work. Therefore, pre-enrichment is an important step in the evaluation of *Arcobacter* in shellfish samples.

The distribution of *Arcobacter*-related genera among shellfish tissues was evaluated from different perspectives. The first one, was the influence of the origin of the samples. In samples from AB (category B production area), the intervalval liquid (IL), was the compartment which presented the highest percentage of positivity i.e. 65.4% in mussels and 84.6% in oysters, and therefore, this compartment was potentially the main source of *Arcobacter*-related species in shellfish. The digestive gland (DG) and the remaining tissues (RT) of both shellfish were

also positive for the presence of *Arcobacter*-related species, meaning that these species were internalized by the bivalves. In the PNC samples, however, it was observed that the RT samples were slightly more positive than the IL, although not significant differences were observed. In this case, the high levels of fecal pollution recorded in the PNC (**Study 3.2**) may have saturated the bivalves, leading to a plateau for the maximum microbiota accumulated within the shellfish. This would not allow discriminating any potential differences among tissues to host *Arcobacter*-related spp.

The distribution of *Arcobacter* species within the tissues of bivalves, could indicate their ecological relationship. Thus, if *Arcobacter* would pass through with the water, in the process of filter feeding, they are considered allochthonous bacteria (Romero et al., 2002); but if they would become part of the shellfish microbiota, supplying nutrient factors to promote metabolism, they are considered autochthonous bacteria (Pujalte et al.,1999; Zurel et al., 2011; Wang et al., 2014). In fact, Wang et al. (2014), suggested that all the allochthonous species would be on the gills, but not inside other tissues. Interestingly, none of the AB gill (G) samples were positive for *Arcobacter* by any of the methodologies tested in our study, therefore, the *Arcobacter* species recovered in AB samples could be considered autochthonous species of mussels and oysters, i.e. *A. butzleri*, *Malaciobacter canalis, M. marinus*, and *M. mytili*. Moreover, Romero et al. (2002) showed that *Arcobacter* species were an abundant and common component of the oyster microbiota. Additionally, more recent studies reinforced the idea of *Arcobacter* species being autochthonous bacteria in shellfish and having a major role, as opportunistic pathogens, when the health of the animals is being compromised. Moribund oysters, which status can be caused by either infections or by increased water temperature, are characterized by a low microbial diversity, but a high abundance of *Arcobacter* species, suggesting that these species could act as an indicator of impaired animal health (Lokmer and Wegner, 2015; Li et al., 2018). Recently, Lasa et al. (2019) reported the concomitant presence of *Arcobacter* and *Vibrio aestuarianus* during mortality episodes of *C. gigas* in European shellfish productions areas. Therefore, further studies are needed to study the potential pathogenic role of *Arcobacter* related spp. in shellfish and other marine fauna.

The second factor that would affect the distribution of *Arcobacter* spp within the shellfish tissues is the species of bivalves studied. In the AB samples, the distribution of the four *Arcobacter*-related species recovered (i.e. *A. butzleri*, *M. canalis, M. marinus,* and *M. mytili*) during sampling in **Study 3.3** was different depending on the type of bivalve under

study. These results might indicate a different retention of these species by mussels and oysters as it has been previously pointed out (**Study 3.2**), and to the different capability of these bacteria to survive the immune system defenses of the shellfish (Pruzzo et al., 2005; Canesi et al., 2016). However, the relationship between the immune system of bivalves and *Arcobacter*-related spp. has not yet been studied, and this could be a new research line that could provide complementary information.

In **Study 3.3**, we also evaluated how the presence of one *Arcobacter*-related species in shellfish, would predict the presence of other species from the same or different genera in the same type of shellfish. The results showed that species belonging to the genera *Halarcobacter* and *Malaciobacter* would favor the presence of other species of these same genera in the same tissues. Additionally, when *H. bivalviorum* was isolated, it was very likely that *A. butzleri* was also recovered, and vice versa. However, an opposite relationship between *A. butzleri* and *M. marinus* was observed, as far as we are concerned, this is the first study that has addressed this issue. It is likely that when the nature and the relationship of these species become better known, such knowledge could provide, among other insights, improved evaluation of the health status of shellfish, leading to the design of better strategies for avoiding shellfish mortalities.

The isolates of *Arcobacter*-related spp recovered in **Study 3.3**, showed a genetic diversity based on the different patterns of ERIC-PCR of 45.8%, which agreed with previous results of Collado et al. (2014) and Levican et al. (2014). The strains most represented in the samples differed depending on the season, with few exceptions, i.e. the same genotype of *M. canalis* was recovered in February and March. The fact that the same genotypes of *A. butzleri* and *M. marinus*, were found both in the water and within the shellfish confirms that these species are taken from the water by the bivalves, and then they are incorporated into their tissues by the filtration feeding process.

The results obtained from **Study 3.3,** in shellfish from Alfacs Bay, showed that the compartment with higher prevalence of *Arcobacter* was the intervalval liquid, which meant that they were not internalized in the shellfish tissues. This result suggests that the compulsory 24-42 hours depuration process for the category B shellfish, prior to going to the market for consumption (Order APA/3228/2005), may be enough for removing *Arcobacter* from the animals. Depuration is the most common practice used in several countries for elimination of bacterial loads from bivalves prior to human consumption (Lopez-Joven et al., 2011; Wang et al., 2010; Shen et al., 2019). Therefore, to validate our hypothesis we developed the **Study 3.4**

in which we investigated the depuration of *E. coli, A. butzleri,* and *M. molluscorum* in mussels and oysters during two seasons i.e. in summer (June) and in winter (December). The main objective was to assess the efficacy of the depuration process to eliminate *Arcobacter* from shellfish, under different scenarios of bacterial load and composition, as well as under different key environmental parameters, such as water temperature.

Our first finding showed that the commercial shellfish that came from a harvesting area of category B, which should have been depurated for 24 hours to assure *E. coli* levels are below the limit established for category A (≤230 MPN/100g), had *E. coli* levels above that limit in mussels at both sampling periods. However, the levels of *E. coli* in oysters in both seasons, were under the legal limits to be sold. Additionally, the levels of *Arcobacter*-related species in these samples were two logs higher than those of *E. coli* (**Study 3.2**). Depuration has been reported to being less than 100% efficient in the elimination of pathogenic microorganisms, i.e. *Vibrio* spp. (FAO, 2008; Martínez et al., 2009; Rong et al., 2014; Pereira et al., 2017). For this reason, and considering the fact that in summer shellfish samples as described in **Study 3.2** showed no detection of *E. coli,* but included presence of *Arcobacter,* we believed that *Arcobacter*-related spp. could be persistent in bivalve samples. Therefore, considering that some *Arcobacter*-related species can act as pathogens for humans and that *E. coli,* the official fecal indicator bacteria, cannot predict their presence, the consumption of these bivalves could represent a risk for public health.

The oysters and mussels that were contaminated by 24 hours immersion in the PNC water achieved an *E. coli* concentration of category C and after 120 hours in clear water, they reached the level of category A, for both types of shellfish and in the two seasons tested. Moreover, there was noted a tendency for depuration of mussels to be more effective than for oysters, for removal of both bacteria. Interestingly, the log reductions achieved in the PNC depuration tanks were higher than in the experimentally contaminated shellfish with pure culture mixes of *E. coli* and *A. butzleri,* or *M. molluscorum*. These differences could be due to the physiological or genetic nature of the strains that accumulated within the shellfish. In the case of the PNC tanks, the predominant species are mainly related to fecal pollution, like *A. butzleri*, *A. cryaerophilus* or *P. cloacae* (as seen in **Studies 3.2 and 3.3**), and the salinity levels of the PNC (10.7‰, in summer and 16.8‰ in winter) were lower than those of the depuration system (34.5‰. for both seasons) and, therefore, their physiological status might have been compromised, thereby improving depuration, because their survival could have been affected

by an osmotic shock (D'Sa and Harrsion, 2005). On the contrary, in the controlled experimental conditions, although we were using a strain of *A. butzleri*, together with a strain of *M. molluscorum*, both of these strains were originally isolated from mussels harvested in Alfacs Bay, which is suggestive that these strains could be better adapted to the marine environment of the depuration tanks than those bacteria from the PNC. Something similar occurred with *E. coli;* despite its initial load in the artificially contaminated tanks being lower than those of the PNC, its concentration barely changed over time. This difference could be explained by the fact that culture collection strains may accumulate mutations which make them behave in a totally different way from their environmental equivalents (Palková, 2004).

The temperature seemed to have a greater effect on depuration efficiency than the type of shellfish from which they originated. Thus, at 20ºC, the log reductions were higher (between 4 and 4.99 logs) than at 14ºC (between 2.61 and 3.68 logs). This relationship between depuration rates and temperature has been reported by some authors (Shen et al., 2019 and references therein). However, the possible effect of the temperature in our depuration trials was not supported by the statistical tests performed, probably due to the low number of experiments. Therefore, an important recommendation, linked also with animal welfare issues, is to avoid stress to shellfish by setting the temperature and salinity close to those of their harvesting areas (McMenemy et al., 2018). We are aware of the limited scope of **Study 3.4**, due to the restricted number of trials performed, and the difficulties to achieve the desired inoculum load for the experiments using artificially contaminated shellfish. However, we value the pioneering aspect of the work, since we have thrown some needed light into this neglected topic, such as the longer depuration times required for *Arcobacter*-related spp. and the possible temperature dependence for its depuration from bivalves. Further studies will be required to assess the optimum temperature conditions for depuration of *Arcobacter* for every type of shellfish, and co-infection with other pathogens together with other species of *Arcobacter*-related spp. would be of interest.

After performing the studies on the relationship between *Arcobacter* and shellfish, we conclude that i) *E. coli* has limitations as an indicator for the detection of *Arcobacter* in shellfish, specifically for mussels and oysters (**Study 3.2**), and that ii) conventional depuration may not fully eliminate *Arcobacter* from shellfish, which may take between 24 and 48 hours longer than for *E. coli*. Additionally, it is quite likely that it would be a temperature-dependent process (**Study 3.4**). All this work was based on labor-intensive and time-consuming

methodologies, therefore, the objective for the next step was to develop a molecular procedure in which the detection of *Arcobacter*-related spp. could be done in a faster way. Usually the detection of foodborne pathogens in shellfish is mainly based on culture techniques, and specifically in the case of *Arcobacter*-related spp. it requires at least 4 days. Although, several molecular methodologies are commonly used for the detection of pathogens in food, (typically PCR) frequently, these techniques cannot differentiate whether the pathogen is alive or not. Therefore, in **Study 3.5** we developed a viable qPCR (v-qPCR) method based on specific primers for the detection of viable cells of *Arcobacter* spp. in different shellfish matrixes that would allow us to differentiate between DNA from live or dead cells.

The work for developing a viable qPCR begun by deciding the different genes to be targeted. We chose the 23S rRNA, because a q-PCR protocol with promising specificity had already been developed by Hausdorf et al. (2013), and the length of the amplicon (233 bp) was appropriate for PMA experiments (Fittipaldi et al., 2012). However, these primers had not been tested against related species like *Campylobacter*. For that reason, we made a modification in the forward primer, and no amplification from other species different from the 20 *Arcobacter*-related species tested was obtained. Following the designing of the primers, we optimized the PMA protocol, which is specific for the species tested. In this case, we had to optimize the protocol for a whole genus. The lack of optimization of PMA protocols in the literature and many different conditions used in published experiments (Fittipaldi et al., 2012), has made the comparison of results among the different studies difficult. Finally, our protocol consisted of a PMA concentration of 20 $mmol \cdot L^{-1}$, incubation of the dye for 5 minutes in the dark and photoactivation for 15 min by using a LED device.

Once the protocol for the PMA-based v-qPCR was established, models of mixtures of live and/or dead cells were tested. The percentages obtained for the different ratios of live and dead cells was in general terms good, but for some species like *P. suis, P. ellisii*, and *M. molluscorum* the ratio 50:50 altered the effectiveness of the method. As commented above, the PMA protocols are species-specific due to the differences in their cellular membranes (Fittipaldi et al., 2012). As our v-qPCR was designed to detect several *Arcobacter*-relates spp. at the same time, but not for a single specific species, the final efficiency of the v-qPCR was 85%, and for that reason focus was placed on achieving the highest possible specificity rather than sensitivity.

When shellfish samples were analyzed, wild-caught shellfish were spiked with *A. butzleri* mixtures of live and dead bacteria, and later the different species of shellfish were

studied directly. Our results showed that the developed v-qPCR was effective at inhibiting the signal from dead cells as compared to those analyzed by standard qPCR, in concordance with previous results (Quijada et al., 2016; Zhu et al., 2012; Zhang et al., 2015). However, when PMA was not used, a reduction in the number of copies detected from the model mixtures of live and dead cells was observed. Thus, part of the dead cells and free DNA added to the spiked sample could have been lost during DNA extraction, this phenomenon has been reported (Demeke et al., 2009 and references therein), and DNA extraction may be an additional factor to include in future standardization of v-qPCR protocols. Overall, as a tool for food safety, the PMA method presented good results, which should help in preventing unnecessary illness and costly food recalls.

As presented above, following the sampling experiments that took place during 2 years in the Ebro Delta environment (**Studies 3.1, 3.2, and 3.3**), the isolation procedures of *Arcobacter*-related species that were performed in parallel by using the conventional culture method and the new NaCl enriched approach presented in **Study 3.1**, that led to the recovery of several isolates (see **Annex II**) from our shellfish and water samples that could represent known or potentially new unknown species. The use of the new approach with NaCl allowed us to recover species of *Arcobacter* that had been described on the basis of only one strain, and had never been isolated again since their description, such as *M. halophilus* and *M. marinus* (**Study 3.1**); for these re-isolated species we provided an update on their taxonomical description. Moreover, new species of *Arcobacter* were isolated (**Studies 3.6, 3.7, Annex IV**). The establishment of the taxonomic position of all these strains has led to a major contribution to the taxonomy of the genus *Arcobacter*, because the genomic study of all these strains, together with the new species that were described since this Ph.D. thesis started, led to the division of the genus *Arcobacter* into 7 new genera (**Annex IV**; Pérez-Cataluña et al., 2018). It is interesting to notice how species from the same environment clustered together in these new proposed genera. For instance, we found genera related to marine environments such as *Halarcobacter*, *Malaciobacter* or *Poseidonibacter,* whose members are more frequently recovered when the media is supplemented with NaCl. Although this approach was very simple, and consisted simply in the addition of 2.5% NaCl to the enrichment broth and later culture on marine agar (**Study 3.1**), it gave us very significant results that have improved the resolution of the reconstruction of the evolutionary history for these species to better understand the phylogeny of these new genera and it gave us the opportunity to get closer to the reality of what is happening in the marine environments.

Additionally, during the development of this thesis, we had a collaboration between our laboratory and the Department of Veterinary Medical Sciences of the University of Bologna (Italy), in order to identify a new isolate of the species *Arcobacter suis* from water buffalo milk (Giacommeti et al., 2013) by means of the Multilocus Phylogenetic analysis (MLPA). This species was described in our laboratory based on a single strain recovered from pig meat (Levican et al., 2013), and therefore the new isolate was recognized for its relevance of this new species. At the beginning of this thesis one of the best tools for the identification of the species of *Arcobacter* was the 16S rRNA-RFLP and was able to discriminate between the 17 species described up to that moment (year 2013). However, the rapid increase in the number of new species of the genus, as seen in the introduction, has shown that some RFLP patterns were shared between some species like *Aliarcobacter lanthieri* and *A. butzleri,* or *M. marinus* and *M. canalis*, and sequencing the *rpoB* gene was necessary to verify the identity and to establish the phylogenetic position of all these new strains. Several studies have confirmed that the use of this gene is an excellent tool for species level identification (Collado et al., 2010; Figueras et al., 2014; Pérez-Cataluña, 2018). Furthermore, all the new species and genera proposed relied on the application of new genetic tools derived from the analysis of the genomes obtained from each of the new species (Diéguez et al., 2017; Waite et al., 2017; Chun et al., 2018; Pérez-Cataluña et al, 2018). Collectively, the results herein have expanded our knowledge of the ecology and systematics of these genera, and provided solid foundations to improve the surveillance of seafoods that could potentially ameliorate food intoxications and expensive product recalls.

CONCLUSIONS

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

CONCLUSIONS

1. The supplementation of the Arcobacter-CAT broth enrichment medium with 2.5% NaCl and posterior culture on marine agar enhanced significantly the number of positive samples for *Arcobacter* related species found in shellfish and in the surrounding marine and brackish water.

2. This approach enabled the recovery, for the first time since their description, of different strains of the species *Malaciobacter marinus* and *Malaciobacter halophilus* that confirmed their phenotypic characteristics. In addition, 7 potentially new *Arcobacter* related species were recognized for the first time from water or shellfish.

3. The levels of the fecal indicator *Escherichia coli* used in the European Union regulation to classify the shellfish production areas as category A (not requiring depuration) and B to D (requiring different depuration times) correlated with the presence of two potentially pathogenic *Arcobacter* related species, i.e. *Aliarcobacter butzleri* and *Aliarcobacter cryaerophilus*. The only exception was the lack of prediction for *A. butzleri*, and *Aliarcobacter skirrowii* among other species, that occurred in 26.1% of the shellfish samples harvested from Alfacs Bay during the warmer months, when the temperature of the water was above 26.2ºC.

4. In Alfacs Bay, the marine shellfish production areas of category A or B, showed a lower diversity of *Arcobacter*-related species than the heavily fecally polluted brackish water classified as category C or D.

5. The study of the distribution of *Arcobacter*-related species in the different shellfish compartments and tissues showed that the intervalval liquid presented a significantly higher percentage of positive samples than the investigated tissues.

6. In the study comparing the *Arcobacter*-related species found in water and in shellfish it was demonstrated that some species (*Aliarcobacter butzleri*, *Malaciobacter marinus*, and *Malaciobacter canalis*) were commonly found in the shellfish compartments and tissues in the marine water, whereas others (*Haliarcobacter ebronensis*) were found

only in the contaminated brackish water, or in the marine water (*Arcobacter nitrofigilis* and *Malaciobacter molluscorum*).

7. The EU standards for depuration of shellfish from category B harvesting areas, is based on the reduction of the *Escherichia coli* after 24-42 h to levels of category A. However, this decrease does not correlate with what was observed for the *Arcobacter*- related species, which is slower, requiring at least 24 to 48 extra hours.

8. The use of Propidium Monoazide coupled with a viable qPCR protocol was optimized for the detection of living cells of *Arcobacter*-related species achieving a satisfactory inhibition of the DNA amplification from the different proportions of dead cells in 85% of the *Arcobacter*-related species tested.

9. The comparison of the results from the q-PCR and the v-qPCR in the studied shellfish samples showed that, on average, 1 log of the copy number detected corresponded to dead cells.

10. A new genus and seven new *Arcobacter*-related species, belonging to three recently described genera, have been described in this thesis by using a polyphasic approach, for which the names *Arcobacter canalis*, *Arcomarinus aquaticus*, *Halarcobacter mediterraneus*, *Halarcobacter ponticus*, *Halarcobacter salis*, *Malaciobacter neptunis*, and *Malaciobacter viscosus* have been proposed.

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ANNEX

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

ANNEX I: Type strains used in this work

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Table 1. Type strains used in the present thesis.

Taxa	**Strain**	**Origen**	**Reference**
Arcobacter nitrofigilis	DSM 7299^T	Roots of Spartina alterniflora	McLung et al. (1983)
Aliarcobacter cryaerophilus	LMG 24291^T	Bovine abortion foetuses	Neill et al. (1985)
Aliarcobacter butzleri	LMG 10828^T	Human faeces	Vandamme et al. (1992)
Aliarcobacter skirrowii	LMG 6621^T	Sheep faeces	Vandamme et al. (1992)
Aliarcobacter cibarius	LMG 21996^T	Chiken meat	Houf et al. (2005)
Malaciobacter halophilus	DSM 18005^T	Hypersaline lagoon	Donachie et al. (2005)
Malaciobacter mytili	CECT 7386^T	Mussels	Collado et al. (2009)
Aliarcobacter thereius	LMG 25534^T	Porcine abortions	Houf et al. (2009)
Malaciobacter marinus	CECT 7727^T	Mix of seaweed and a starfish	Kim et al. (2010)
Aliarcobacter trophiarum	LMG 25534^T	Faeces of fattening pigs	De Smet et al. (2010)
Pseudarcobacter defluvii	CECT 7697^T	Sewage	Collado et al. (2010)
Malaciobacter molluscorum	CECT 7696^T	Mussels	Figueras et al. (2011)
Pseudarcobacter ellisii	CECT 7837^T	Mussels	Figueras et al. (2011)
Halarcobacter bivalviorum	CECT 7835^T	Mussels	Levican et al. (2012)
Pseudarcobacter venerupis	CECT 7836^T	Clams	Levican et al. (2012)
Pseudarcobacter cloacae	CECT 7834^T	Mussels and sewage	Levican et al. (2013)
Pseudarcobacter suis	CECT 7833^T	Pork meat	Levican et al. (2013)
Halarcobacter anaerophilus	DSM 24636^T	Estuarine sediment	Jyothsna et al. (2015)
Pseudarcobacter aquimarinus	CECT 8442^T	Sea water	Levican et al. (2015)
Halarcobacter ebronensis	CECT 8441^T	Mussels	Levican et al. (2015)
Aliarcobacter lanthieri	LMG 28516^T	Pig and dairy cattle manure	Whiteduck-Léveillée et al. (2015)
Aliarcobacter faecis	LMG 28519^T	Human waste septic tank	Whiteduck-Léveillée et al. (2016)

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

ANNEX II: Strains of environmental origin isolated and identified in this study.

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
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Table 1. Strains recovered in the present thesis under two different atmospheres (aerobiosis and microaerophilia and using two culturing approaches pre-enrichement in Arcobacter-CAT broth followed by culturing on Blood Agar (CAT-broth + BA) and Arcobacter-CAT broth supplemented with 2.5 %NaCl followed by culturing on Marine Agar (CAT-broth+NaCl + MA). Bay: Alfacs Bay; PNC: Poble Nou Channel

Genus	Reference strain	Nº isolates	Atmosphere	Culture media	Origen	Sample	Month	Final identification
Aliarcobacter	F136-19	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth + BA	PNC	Mussel	March	*A. butzleri*
	F137-18	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth + BA	PNC	Mussel	March	*A. butzleri*
	F137-21	1	Aerophilia	CAT-broth + BA	PNC	Mussel	March	*A. butzleri*
	F137-25	1	Microaerophilia	CAT-broth + BA	PNC	Mussel	March	*A. butzleri*
	F137-26	1	Microaerophilia	CAT-broth + BA	PNC	Mussel	March	*A. butzleri*
	F137-29	1	Microaerophilia	CAT-broth + BA	PNC	Mussel	March	*A. butzleri*
	F138-17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
	F138-25	1	Microaerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
	F138-26	1	Microaerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
	F138-27	1	Microaerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
	F139-17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
	F139-18	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
	F139-27	1	Microaerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
	F139-28	1	Microaerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
	F139-29	1	Microaerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
	F142-17	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth + BA	PNC	Mussel	June	*A. butzleri*
	F142-18	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth + BA	PNC	Mussel	June	*A. butzleri*
	F142-20	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth + BA	PNC	Mussel n=1 Oyster n=1	June	*A. butzleri*
	F142-25	1	Microaerophilia	CAT-broth + BA	PNC	Mussel	June	*A. butzleri*
	F142-29	1	Microaerophilia	CAT-broth + BA	PNC	Mussel	June	*A. butzleri*
	F142-30	1	Microaerophilia	CAT-broth + BA	PNC	Mussel	June	*A. butzleri*
	F143-17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
	F143-18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
	F143-20	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
	F143-22	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
	F143-25	1	Microaerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
	F143-27	1	Microaerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
	F143-29	1	Microaerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
	F146-25	1	Microaerophilia	CAT-broth + BA	Bay	Mussel	July	*A. butzleri*
	F146-28	1	Microaerophilia	CAT-broth + BA	Bay	Mussel	July	*A. butzleri*
	F153-43	3	Aerophilia n=2 Microaerophilia n=1	CAT-broth + BA n=1 CAT-broth+NaCl + MA n=2	Bay n=1 PNC n=2	Oyster n=1 Mussel n=1 Water n=1	August/ July	*A. butzleri*
	F154-17	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth + BA	PNC	Mussel	August	*A. butzleri*
	F154-18	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth + BA	PNC	Mussel	August	*A. butzleri*
	F154-27	1	Microaerophilia	CAT-broth + BA	PNC	Mussel	August	*A. butzleri*
	F154-28	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth + BA	PNC	Mussel n=1 Water n=1	August/July	*A. butzleri*
	F158-17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	August	*A. butzleri*
	F159-17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	August	*A. butzleri*
	F159-21	1	Aerophilia	CAT-broth + BA	PNC	Oyster	August	*A. butzleri*
	F161-25	1	Microaerophilia	CAT-broth + BA	Bay	Cockle	October	*A. butzleri*
	F161-27	1	Microaerophilia	CAT-broth + BA	Bay	Cockle	October	*A. butzleri*
	F164-17	1	Aerophilia	CAT-broth + BA	Bay	Oyster	November	*A. butzleri*
	F164-19	1	Aerophilia	CAT-broth + BA	Bay	Oyster	November	*A. butzleri*
	F164-20	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth + BA	Bay n=1 PNC n=1	Oyster n=1 Water n=1	November	*A. butzleri*
	F164-21	1	Aerophilia	CAT-broth + BA	Bay	Oyster	November	*A. butzleri*
	F164-22	1	Aerophilia	CAT-broth + BA	Bay	Oyster	November	*A. butzleri*
	F164-25	1	Microaerophilia	CAT-broth + BA	Bay	Oyster	November	*A. butzleri*
	F164-26	1	Microaerophilia	CAT-broth + BA	Bay	Oyster	November	*A. butzleri*
	F164-27	1	Microaerophilia	CAT-broth + BA	Bay	Oyster	November	*A. butzleri*
	F164-28	1	Microaerophilia	CAT-broth + BA	Bay	Oyster	November	*A. butzleri*
	F164-29	1	Microaerophilia	CAT-broth + BA	Bay	Oyster	November	*A. butzleri*
	F164-30	1	Microaerophilia	CAT-broth + BA	Bay	Oyster	November	*A. butzleri*
	F166-17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	December	*A. butzleri*
	F166-18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	December	*A. butzleri*
	F166-20	1	Aerophilia	CAT-broth + BA	PNC	Oyster	December	*A. butzleri*
	F166-21	1	Aerophilia	CAT-broth + BA	PNC	Oyster	December	*A. butzleri*
	F166-25	1	Microaerophilia	CAT-broth + BA	PNC	Oyster	December	*A. butzleri*
	F166-26	1	Microaerophilia	CAT-broth + BA	PNC	Oyster	December	*A. butzleri*
	F166-27	1	Microaerophilia	CAT-broth + BA	PNC	Oyster	December	*A. butzleri*
	F167 DG 17	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth + BA	Bay	Mussel	January	*A. butzleri*
	F167 DG 22	3	Aerophilia n=2 Microaerophilia n=1	CAT-broth + BA n=2 CAT-broth+NaCl + MA n=1	Bay n=2 PNC n=1	Mussel n=1 Water n=2	January	*A. butzleri*
	F169 DG 19	2	Aerophilia	CAT-broth + BA n=1 CAT-broth+NaCl + MA n=1	PNC	Mussel	January	*A. butzleri*
	F169 IL 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	January	*A. butzleri*
	F169 RW 19	1	Aerophilia	CAT-broth + BA	PNC	Mussel	January	*A. butzleri*
	F169-1 F 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	January	*A. butzleri*
	F169-1 F 18	1	Aerophilia	CAT-broth + BA	PNC	Mussel	January	*A. butzleri*
	F169-1 F 19	1	Aerophilia	CAT-broth + BA	PNC	Mussel	January	*A. butzleri*

F169-1 F 20	1	Aerophilia	CAT-broth + BA	PNC	Mussel	January	*A. butzleri*
F169-1 IL 18	1	Aerophilia	CAT-broth + BA	PNC	Mussel	January	*A. butzleri*
F169-1 IL 19	1	Aerophilia	CAT-broth + BA	PNC	Mussel	January	*A. butzleri*
F169-1 IL 20	1	Aerophilia	CAT-broth + BA	PNC	Mussel	January	*A. butzleri*
F169-1 RW 18	1	Aerophilia	CAT-broth + BA	PNC	Mussel	January	*A. butzleri*
F169-1 RW 19	1	Aerophilia	CAT-broth + BA	PNC	Mussel	January	*A. butzleri*
F169-2 DG 17	2	Aerophilia	CAT-broth + BA n=1 CAT-broth+NaCl + MA n=1	PNC	Mussel	January	*A. butzleri*
F169-2 DG 18	1	Aerophilia	CAT-broth + BA	PNC	Mussel	January	*A. butzleri*
F169-2 G 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	January	*A. butzleri*
F169-2 G 33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	January	*A. butzleri*
F169-2 IL 18	1	Aerophilia	CAT-broth + BA	PNC	Mussel	January	*A. butzleri*
F169-2 IL 19	1	Aerophilia	CAT-broth + BA	PNC	Mussel	January	*A. butzleri*
F169-3 DG 17	2	Aerophilia	CAT-broth + BA n=1 CAT-broth+NaCl + MA n=1	PNC	Mussel	January	*A. butzleri*
F169-3 IL 19	2	Aerophilia	CAT-broth + BA n=1 CAT-broth+NaCl + MA n=1	PNC	Mussel	January	*A. butzleri*
F169-4 DG 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	January	*A. butzleri*
F169-4 DG 18	1	Aerophilia	CAT-broth + BA	PNC	Mussel	January	*A. butzleri*
F169-4 DG 19	1	Aerophilia	CAT-broth + BA	PNC	Mussel	January	*A. butzleri*
F169-4 F 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	January	*A. butzleri*
F169-4 F 18	1	Aerophilia	CAT-broth + BA	PNC	Mussel	January	*A. butzleri*
F169-4 F 19	1	Aerophilia	CAT-broth + BA	PNC	Mussel	January	*A. butzleri*
F169-4 F 20	1	Aerophilia	CAT-broth + BA	PNC	Mussel	January	*A. butzleri*
F169-4 G 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	January	*A. butzleri*
F170 DG 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	January	*A. butzleri*
F170 F 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	January	*A. butzleri*
F170 F 18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	January	*A. butzleri*
F170 G 18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	January	*A. butzleri*
F170-1 DG 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	January	*A. butzleri*
F170-1 G18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	January	*A. butzleri*
F170-3 DG 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	January	*A. butzleri*
F170-3 G 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	January	*A. butzleri*
F170-3 G 18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	January	*A. butzleri*
F170-3 IL 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	January	*A. butzleri*
F170-3 IL 18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	January	*A. butzleri*
F170-4 DG 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	January	*A. butzleri*
F170-4 DG 18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	January	*A. butzleri*
F170-4 F 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	January	*A. butzleri*
F170-4 F 18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	January	*A. butzleri*
F170-4 IL 17	2	Aerophilia	CAT-broth + BA n=1 CAT-broth+NaCl + MA n=1	PNC	Oyster	January	*A. butzleri*
F170-4 RW 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	January	*A. butzleri*
F170-4 RW 20	1	Aerophilia	CAT-broth + BA	PNC	Oyster	January	*A. butzleri*
F173 DG 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173 DG 21	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173 F 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173 F 20	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173 G 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173 G 18	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173 IL 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173 IL 18	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173 IL 19	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173 IL 20	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173 IL 22	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173-1 F 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173-1 F 19	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173-1 F 22	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173-1 G 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173-1 G 18	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173-1 G 19	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173-1 G 21	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173-1 G 22	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173-1 IL 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173-2 DG 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173-2 DG 18	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173-2 F 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173-2 F 18	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173-2 F 19	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173-2 F 21	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173-2 G 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173-2 G 19	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173-2 G 21	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173-2 IL 19	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173-2 IL 20	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173-3 DG 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173-3 DG 21	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173-3 DG 22	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173-3 F 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173-3 F 18	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173-3 G 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173-3 G 19	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173-3 G 20	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173-3 G 21	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*

F173-3 G 22	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173-3 IL 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173-3 IL 18	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173-4 IL 20	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173-4 DG 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173-4 DG 18	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173-4 DG 20	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F173-4 G 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. butzleri*
F174 F 19	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174 F 22	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174 F 36	2	Aerophilia	CAT-broth + BA n=1 CAT-broth+NaCl + MA n=1	PNC	Oyster	February	*A. butzleri*
F174 G 18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174 G 22	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174 IL 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174 IL 18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174 IL 19	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174 IL 21	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174 IL 22	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174-1 DG 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174-1 DG 19	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174-1 DG 21	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174-1 DG 22	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174-1 G 18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174-1 G 20	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174-1 IL 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174-1 IL 21	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174-1 IL 34	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	February	*A. butzleri*
F174-2 DG 22	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174-2 DG 34	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	February	*A. butzleri*
F174-2 F 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174-2 F 21	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174-2 G 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174-2 G 19	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174-2 IL 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174-2 IL 18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174-2 IL 20	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174-211	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174-3 F 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174-3 F 18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174-3 F 19	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174-3 F 21	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174-3 G 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174-3 G 21	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174-3 IL 18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174-3 IL 20	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174-3 IL 21	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174-3 IL 22	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174-4 DG 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174-4 G 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174-4 G 19	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174-4 G 21	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174-4 G 22	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174-4 G 33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	February	*A. butzleri*
F174-4 G 35	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	February	*A. butzleri*
F174-4 IL 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F174-4 IL 18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. butzleri*
F177 DG 18	1	Aerophilia	CAT-broth + BA	PNC	Mussel	March	*A. butzleri*
F177 DG 21	1	Aerophilia	CAT-broth + BA	PNC	Mussel	March	*A. butzleri*
F177 F 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	March	*A. butzleri*
F177 F 20	1	Aerophilia	CAT-broth + BA	PNC	Mussel	March	*A. butzleri*
F177 F 22	1	Aerophilia	CAT-broth + BA	PNC	Mussel	March	*A. butzleri*
F177 G 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	March	*A. butzleri*
F177 IL 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	March	*A. butzleri*
F177 IL 18	1	Aerophilia	CAT-broth + BA	PNC	Mussel	March	*A. butzleri*
F177-1 IL 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	March	*A. butzleri*
F177-1 IL 18	1	Aerophilia	CAT-broth + BA	PNC	Mussel	March	*A. butzleri*
F177-1 IL 19	1	Aerophilia	CAT-broth + BA	PNC	Mussel	March	*A. butzleri*
F177-171	1	Aerophilia	CAT-broth + BA	PNC	Mussel	March	*A. butzleri*
F177-181	1	Aerophilia	CAT-broth + BA	PNC	Mussel	March	*A. butzleri*
F177-2 DG 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	March	*A. butzleri*
F177-2 DG 18	1	Aerophilia	CAT-broth + BA	PNC	Mussel	March	*A. butzleri*
F177-2 DG 21	1	Aerophilia	CAT-broth + BA	PNC	Mussel	March	*A. butzleri*
F177-2 F 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	March	*A. butzleri*
F177-2 F 18	1	Aerophilia	CAT-broth + BA	PNC	Mussel	March	*A. butzleri*
F177-2 G 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	March	*A. butzleri*
F177-2 G 19	1	Aerophilia	CAT-broth + BA	PNC	Mussel	March	*A. butzleri*
F177-201	1	Aerophilia	CAT-broth + BA	PNC	Mussel	March	*A. butzleri*
F177-3 DG 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	March	*A. butzleri*
F177-3 DG 19	1	Aerophilia	CAT-broth + BA	PNC	Mussel	March	*A. butzleri*
F177-3 DG 22	1	Aerophilia	CAT-broth + BA	PNC	Mussel	March	*A. butzleri*
F177-3 F 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	March	*A. butzleri*
F177-3 IL 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	March	*A. butzleri*
F177-4 DG 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	March	*A. butzleri*

F177-4 IL 21	1	Aerophilia	CAT-broth + BA	PNC	Mussel	March	*A. butzleri*
F178 DG 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
F178 DG 20	1	Aerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
F178 F 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
F178 G 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
F178 G 19	1	Aerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
F178-1 DG 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
F178-1 DG 18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
F178-1 IL 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
F178-1 IL 18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
F178-1 IL 19	1	Aerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
F178-1 IL 21	1	Aerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
F178-181	1	Aerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
F178-191	1	Aerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
F178-2 DG 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
F178-2 G 18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
F178-2 IL 18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
F178-2 IL 21	1	Aerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
F178-3 DG 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
F178-3 DG 18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
F178-3 F 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
F178-3 F 18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
F178-3 G 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
F178-4 DG 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
F178-4 DG 19	1	Aerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
F178-4 F 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
F178-4 F 19	1	Aerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
F178-4 G 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
F178-4 IL 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
F178-4 IL 19	1	Aerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
F178-4 IL 20	1	Aerophilia	CAT-broth + BA	PNC	Oyster	March	*A. butzleri*
F180-2 F 17	2	Aerophilia	CAT-broth + BA n=1 CAT-broth+NaCl + MA n=1	Bay	Oyster	April	*A. butzleri*
F181 DG 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	April	*A. butzleri*
F181 F 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	April	*A. butzleri*
F181 F 20	1	Aerophilia	CAT-broth + BA	PNC	Mussel	April	*A. butzleri*
F181 G 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	April	*A. butzleri*
F181 G 20	1	Aerophilia	CAT-broth + BA	PNC	Mussel	April	*A. butzleri*
F181 G 22	1	Aerophilia	CAT-broth + BA	PNC	Mussel	April	*A. butzleri*
F181 IL 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	April	*A. butzleri*
F181 IL 18	1	Aerophilia	CAT-broth + BA	PNC	Mussel	April	*A. butzleri*
F181 IL 19	1	Aerophilia	CAT-broth + BA	PNC	Mussel	April	*A. butzleri*
F181 IL 20	1	Aerophilia	CAT-broth + BA	PNC	Mussel	April	*A. butzleri*
F181 IL 22	1	Aerophilia	CAT-broth + BA	PNC	Mussel	April	*A. butzleri*
F181-1 F 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	April	*A. butzleri*
F181-1 F 20	1	Aerophilia	CAT-broth + BA	PNC	Mussel	April	*A. butzleri*
F181-1 G 19	1	Aerophilia	CAT-broth + BA	PNC	Mussel	April	*A. butzleri*
F181-1 G 20	1	Aerophilia	CAT-broth + BA	PNC	Mussel	April	*A. butzleri*
F181-1 G 21	1	Aerophilia	CAT-broth + BA	PNC	Mussel	April	*A. butzleri*
F181-171	1	Aerophilia	CAT-broth + BA	PNC	Mussel	April	*A. butzleri*
F181-191	1	Aerophilia	CAT-broth + BA	PNC	Mussel	April	*A. butzleri*
F181-2 F 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	April	*A. butzleri*
F181-2 F 19	1	Aerophilia	CAT-broth + BA	PNC	Mussel	April	*A. butzleri*
F181-2 F 20	1	Aerophilia	CAT-broth + BA	PNC	Mussel	April	*A. butzleri*
F181-3 DG 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	April	*A. butzleri*
F181-3 DG 19	1	Aerophilia	CAT-broth + BA	PNC	Mussel	April	*A. butzleri*
F181-3 DG 20	1	Aerophilia	CAT-broth + BA	PNC	Mussel	April	*A. butzleri*
F181-3 F 18	1	Aerophilia	CAT-broth + BA	PNC	Mussel	April	*A. butzleri*
F181-3 IL 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	April	*A. butzleri*
F181-4 DG 19	1	Aerophilia	CAT-broth + BA	PNC	Mussel	April	*A. butzleri*
F185-201	1	Aerophilia	CAT-broth + BA	PNC	Mussel	May	*A. butzleri*
F185-211	1	Aerophilia	CAT-broth + BA	PNC	Mussel	May	*A. butzleri*
F186-171	1	Aerophilia	CAT-broth + BA	PNC	Oyster	May	*A. butzleri*
F189 DG 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	June	*A. butzleri*
F189 DG 18	1	Aerophilia	CAT-broth + BA	PNC	Mussel	June	*A. butzleri*
F189 F 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	June	*A. butzleri*
F189 F 18	1	Aerophilia	CAT-broth + BA	PNC	Mussel	June	*A. butzleri*
F189 F 19	1	Aerophilia	CAT-broth + BA	PNC	Mussel	June	*A. butzleri*
F189 IL 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	June	*A. butzleri*
F189 IL 18	1	Aerophilia	CAT-broth + BA	PNC	Mussel	June	*A. butzleri*
F189 IL 19	1	Aerophilia	CAT-broth + BA	PNC	Mussel	June	*A. butzleri*
F189 IL 20	1	Aerophilia	CAT-broth + BA	PNC	Mussel	June	*A. butzleri*
F189 IL 21	1	Aerophilia	CAT-broth + BA	PNC	Mussel	June	*A. butzleri*
F189 IL 22	1	Aerophilia	CAT-broth + BA	PNC	Mussel	June	*A. butzleri*
F189-1 F 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	June	*A. butzleri*
F189-1 F 19	1	Aerophilia	CAT-broth + BA	PNC	Mussel	June	*A. butzleri*
F189-1 IL 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	June	*A. butzleri*
F189-1 IL 18	1	Aerophilia	CAT-broth + BA	PNC	Mussel	June	*A. butzleri*
F189-1 IL 20	1	Aerophilia	CAT-broth + BA	PNC	Mussel	June	*A. butzleri*
F189-2 DG 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	June	*A. butzleri*
F189-2 DG 18	1	Aerophilia	CAT-broth + BA	PNC	Mussel	June	*A. butzleri*
F189-2 IL 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	June	*A. butzleri*
F189-3 F 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	June	*A. butzleri*
F189-3 F 18	1	Aerophilia	CAT-broth + BA	PNC	Mussel	June	*A. butzleri*

F189-3 IL 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	June	*A. butzleri*
F189-3 IL 18	1	Aerophilia	CAT-broth + BA	PNC	Mussel	June	*A. butzleri*
F189-3 IL 21	1	Aerophilia	CAT-broth + BA	PNC	Mussel	June	*A. butzleri*
F189-4 DG 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	June	*A. butzleri*
F189-4 F17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	June	*A. butzleri*
F189-4 F22	1	Aerophilia	CAT-broth + BA	PNC	Mussel	June	*A. butzleri*
F189-4 IL 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	June	*A. butzleri*
F190 DG 20	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190 F 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190 F 18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190 IL 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-1 DG 18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-1 DG 19	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-1 G 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-1 G 18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-1 G 19	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-1 G 21	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-1 IL 19	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-1 IL 20	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-1 IL 22	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-2 DG 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-2 DG 19	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-2 F 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-2 F 18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-2 F 21	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-2 G 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-2 IL 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-3 DG 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-3 DG 18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-3 F 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-3 F 18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-3 F 20	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-3 G 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-3 G 18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-3 G 21	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-3 G 22	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-3 IL 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-3 IL 19	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-3 IL 20	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-4 DG 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-4 F 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-4 F 18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-4 F 19	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-4 F 21	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-4 F 22	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-4 G 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-4 G 18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-4 G 19	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
F190-4 G 20	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. butzleri*
W108-25	1	Microaerophilia	CAT-broth + BA	PNC	Water	March	*A. butzleri*
W108-26	1	Microaerophilia	CAT-broth + BA	PNC	Water	March	*A. butzleri*
W108-28	1	Microaerophilia	CAT-broth + BA	PNC	Water	March	*A. butzleri*
W108-29	1	Microaerophilia	CAT-broth + BA	PNC	Water	March	*A. butzleri*
W110-17	1	Aerophilia	CAT-broth + BA	PNC	Water	March	*A. butzleri*
W110-18	1	Aerophilia	CAT-broth + BA	PNC	Water	March	*A. butzleri*
W110-19	1	Aerophilia	CAT-broth + BA	PNC	Water	March	*A. butzleri*
W110-20	1	Aerophilia	CAT-broth + BA	PNC	Water	March	*A. butzleri*
W110-21	1	Aerophilia	CAT-broth + BA	PNC	Water	March	*A. butzleri*
W110-23	1	Aerophilia	CAT-broth + BA	PNC	Water	March	*A. butzleri*
W110-24	1	Aerophilia	CAT-broth + BA	PNC	Water	March	*A. butzleri*
W112-17	1	Aerophilia	CAT-broth + BA	PNC	Water	April	*A. butzleri*
W112-25	1	Microaerophilia	CAT-broth + BA	PNC	Water	April	*A. butzleri*
W112-27	1	Microaerophilia	CAT-broth + BA	PNC	Water	April	*A. butzleri*
W119-17	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth + BA	Bay n=1 PNC n=1	Water	July	*A. butzleri*
W120-18	1	Aerophilia	CAT-broth + BA	PNC	Water	July	*A. butzleri*
W120-20	1	Aerophilia	CAT-broth + BA	PNC	Water	July	*A. butzleri*
W120-21	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth + BA	PNC	Water	July	*A. butzleri*
W120-22	1	Aerophilia	CAT-broth + BA	PNC	Water	July	*A. butzleri*
W120-25	1	Microaerophilia	CAT-broth + BA	PNC	Water	July	*A. butzleri*
W121-17	1	Aerophilia	CAT-broth + BA	PNC	Water	July	*A. butzleri*
W125-22	1	Microaerophilia	CAT-broth + BA	PNC	Water	August	*A. butzleri*
W129-17	1	Aerophilia	CAT-broth + BA	PNC	Water	November	*A. butzleri*
W129-18	1	Aerophilia	CAT-broth + BA	PNC	Water	November	*A. butzleri*
W129-19	1	Aerophilia	CAT-broth + BA	PNC	Water	November	*A. butzleri*
W129-20	1	Aerophilia	CAT-broth + BA	PNC	Water	November	*A. butzleri*
W129-26	1	Microaerophilia	CAT-broth + BA	PNC	Water	November	*A. butzleri*
W129-27	1	Microaerophilia	CAT-broth + BA	PNC	Water	November	*A. butzleri*
W129-29	1	Microaerophilia	CAT-broth + BA	PNC	Water	November	*A. butzleri*
W129-30	1	Microaerophilia	CAT-broth + BA	PNC	Water	November	*A. butzleri*
W129-31	1	Microaerophilia	CAT-broth + BA	PNC	Water	November	*A. butzleri*
W129-33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Water	November	*A. butzleri*
W129-36	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Water	November	*A. butzleri*

W129-38	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Water	November	*A. butzleri*
W129-42	1	Microaerophilia	CAT-broth+NaCl + MA	PNC	Water	November	*A. butzleri*
W129-46	1	Microaerophilia	CAT-broth+NaCl + MA	PNC	Water	November	*A. butzleri*
W131-17	1	Aerophilia	CAT-broth + BA	PNC	Water	December	*A. butzleri*
W131-25	1	Microaerophilia	CAT-broth + BA	PNC	Water	December	*A. butzleri*
W131-27	1	Microaerophilia	CAT-broth + BA	PNC	Water	December	*A. butzleri*
W134-17	1	Aerophilia	CAT-broth + BA	PNC	Water	January	*A. butzleri*
W134-20	1	Aerophilia	CAT-broth + BA	PNC	Water	January	*A. butzleri*
W135-35	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Water	February	*A. butzleri*
W137-17	1	Aerophilia	CAT-broth + BA	PNC	Water	February	*A. butzleri*
W137-19	1	Aerophilia	CAT-broth + BA	PNC	Water	February	*A. butzleri*
W137-20	1	Aerophilia	CAT-broth + BA	PNC	Water	February	*A. butzleri*
W140-171	1	Aerophilia	CAT-broth + BA	PNC	Water	March	*A. butzleri*
W140-181	1	Aerophilia	CAT-broth + BA	PNC	Water	March	*A. butzleri*
W143-181	1	Aerophilia	CAT-broth + BA	PNC	Water	April	*A. butzleri*
W143-201	1	Aerophilia	CAT-broth + BA	PNC	Water	April	*A. butzleri*
W145-33	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Water	May	*A. butzleri*
F169-1 G 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	January	*A. cryaerophilus*
F169-1 G 18	1	Aerophilia	CAT-broth + BA	PNC	Mussel	January	*A. cryaerophilus*
F169-2 IL 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	January	*A. cryaerophilus*
F169-2 IL 20	1	Aerophilia	CAT-broth + BA	PNC	Mussel	January	*A. cryaerophilus*
F170-1 F 18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	January	*A. cryaerophilus*
F170-2 RW 18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	January	*A. cryaerophilus*
F170-3 F 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	January	*A. cryaerophilus*
F170-3 F 19	1	Aerophilia	CAT-broth + BA	PNC	Oyster	January	*A. cryaerophilus*
F173-171	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. cryaerophilus*
F173-181	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. cryaerophilus*
F173-191	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. cryaerophilus*
F173-2 G 18	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. cryaerophilus*
F173-2 IL 21	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. cryaerophilus*
F173-211	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. cryaerophilus*
F173-221	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*A. cryaerophilus*
F174 DG 36	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	February	*A. cryaerophilus*
F174-171	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. cryaerophilus*
F174-181	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. cryaerophilus*
F174-2 F 18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. cryaerophilus*
F174-2 F 20	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. cryaerophilus*
F174-2 F 22	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. cryaerophilus*
F174-2 G 18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. cryaerophilus*
F174-2 G 22	2	Aerophilia	CAT-broth + BA n=1 CAT-broth+NaCl + MA n=1	PNC	Oyster	February	*A. cryaerophilus*
F174-2 IL 19	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. cryaerophilus*
F174-201	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. cryaerophilus*
F174-3 G 18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. cryaerophilus*
F174-3 G 19	1	Aerophilia	CAT-broth + BA	PNC	Oyster	February	*A. cryaerophilus*
F177 DG 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	March	*A. cryaerophilus*
F177-3 F 22	1	Aerophilia	CAT-broth + BA	PNC	Mussel	March	*A. cryaerophilus*
F178-171	1	Aerophilia	CAT-broth + BA	PNC	Oyster	March	*A. cryaerophilus*
F181 F 18	1	Aerophilia	CAT-broth + BA	PNC	Mussel	April	*A. cryaerophilus*
F181-3 DG 21	1	Aerophilia	CAT-broth + BA	PNC	Mussel	April	*A. cryaerophilus*
F181-4 G 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	April	*A. cryaerophilus*
F181-4 G 18	1	Aerophilia	CAT-broth + BA	PNC	Mussel	April	*A. cryaerophilus*
F182-171	1	Aerophilia	CAT-broth + BA	PNC	Oyster	April	*A. cryaerophilus*
F182-211	1	Aerophilia	CAT-broth + BA	PNC	Oyster	April	*A. cryaerophilus*
F185-191	1	Aerophilia	CAT-broth + BA	PNC	Mussel	May	*A. cryaerophilus*
F189 G 17	1	Aerophilia	CAT-broth + BA	PNC	Mussel	June	*A. cryaerophilus*
F189 G 20	1	Aerophilia	CAT-broth + BA	PNC	Mussel	June	*A. cryaerophilus*
F190 G 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. cryaerophilus*
F190-1 IL 17	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. cryaerophilus*
F190-1 IL 18	1	Aerophilia	CAT-broth + BA	PNC	Oyster	June	*A. cryaerophilus*
W108-1	1	Aerophilia	CAT-broth + BA	PNC	water	March	*A. cryaerophilus*
W108-2	1	Aerophilia	CAT-broth + BA	PNC	Water	March	*A. cryaerophilus*
W108-4	1	Aerophilia	CAT-broth + BA	PNC	Water	March	*A. cryaerophilus*
W109-1	1	Aerophilia	CAT-broth + BA	PNC	Water	March	*A. cryaerophilus*
W109-10	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth + BA	PNC	Water	March	*A. cryaerophilus*
W109-11	1	Microaerophilia	CAT-broth + BA	PNC	Water	March	*A. cryaerophilus*
W109-13	1	Microaerophilia	CAT-broth + BA	PNC	Water	March	*A. cryaerophilus*
W109-14	1	Microaerophilia	CAT-broth + BA	PNC	Water	March	*A. cryaerophilus*
W109-2	1	Aerophilia	CAT-broth + BA	PNC	Water	March	*A. cryaerophilus*
W109-3	1	Aerophilia	CAT-broth + BA	PNC	Water	March	*A. cryaerophilus*
W109-4	1	Aerophilia	CAT-broth + BA	PNC	Water	March	*A. cryaerophilus*
W109-6	1	Aerophilia	CAT-broth + BA	PNC	Water	March	*A. cryaerophilus*
W109-7	1	Aerophilia	CAT-broth + BA	PNC	Water	March	*A. cryaerophilus*
W109-8	1	Aerophilia	CAT-broth + BA	PNC	Water	March	*A. cryaerophilus*
W110-13	1	Microaerophilia	CAT-broth + BA	PNC	Water	March	*A. cryaerophilus*
W113-1	1	Aerophilia	CAT-broth + BA	PNC	Water	June	*A. cryaerophilus*
W113-10	1	Microaerophilia	CAT-broth + BA	PNC	Water	June	*A. cryaerophilus*
W113-2	1	Aerophilia	CAT-broth + BA	PNC	Water	June	*A. cryaerophilus*
W113-4	1	Aerophilia	CAT-broth + BA	PNC	Water	June	*A. cryaerophilus*
W113-5	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth + BA	PNC	Water	June	*A. cryaerophilus*
W113-6	1	Aerophilia	CAT-broth + BA	PNC	Water	June	*A. cryaerophilus*

	W122-25	1	Microaerophilia	CAT-broth + BA	PNC	Water	August	*A. cryaerophilus*
	W122-26	1	Microaerophilia	CAT-broth + BA	PNC	Water	August	*A. cryaerophilus*
	W122-28	1	Microaerophilia	CAT-broth + BA	PNC	Water	August	*A. cryaerophilus*
	W122-29	1	Microaerophilia	CAT-broth + BA	PNC	Water	August	*A. cryaerophilus*
	W129-25	1	Microaerophilia	CAT-broth + BA	PNC	Water	November	*A. cryaerophilus*
	W129-28	1	Microaerophilia	CAT-broth + BA	PNC	Water	November	*A. cryaerophilus*
	W137-171	1	Aerophilia	CAT-broth + BA	PNC	Water	February	*A. cryaerophilus*
	W137-181	1	Aerophilia	CAT-broth + BA	PNC	Water	February	*A. cryaerophilus*
	W137-191	1	Aerophilia	CAT-broth + BA	PNC	Water	February	*A. cryaerophilus*
	W137-201	1	Aerophilia	CAT-broth + BA	PNC	Water	February	*A. cryaerophilus*
	W137-211	1	Aerophilia	CAT-broth + BA	PNC	Water	February	*A. cryaerophilus*
	W137-22	1	Aerophilia	CAT-broth + BA	PNC	Water	February	*A. cryaerophilus*
	W137-221	1	Aerophilia	CAT-broth + BA	PNC	Water	February	*A. cryaerophilus*
	W140-17	1	Aerophilia	CAT-broth + BA	PNC	Water	March	*A. cryaerophilus*
	W140-191	1	Aerophilia	CAT-broth + BA	PNC	Water	March	*A. cryaerophilus*
	W143-171	1	Aerophilia	CAT-broth + BA	PNC	Water	April	*A. cryaerophilus*
	W143-221	1	Aerophilia	CAT-broth + BA	PNC	Water	April	*A. cryaerophilus*
	F164-18	1	Aerophilia	CAT-broth + BA	Bay	Oyster	November	*A. hispanicus*
	W125-41	2	Microaerophilia	CAT-broth+NaCl + MA	PNC	Water	August	*A. skirrowii*
	F178-4 DG 21	1	Aerophilia	CAT-broth + BA	PNC	Oyster	March	*A. lacus*
Arcobacter	F139-46	1	Microaerophilia	CAT-broth+NaCl + MA	PNC	Oyster	March	*A. nitrofigilis*
	W111-35	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Water	March	*A. nitrofigilis*
	W111-43	1	Microaerophilia	CAT-broth+NaCl + MA	PNC	Water	March	*A. nitrofigilis*
	W138-33	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Water	March	*A. nitrofigilis*
Arcomarinus	W112-28	3	Microaerophilia	CAT-broth + BA	PNC	Water	April	*A. aquaticus*
Halarcobacter	F141-41	2	Microaerophilia	CAT-broth+NaCl + MA	Bay	Oyster	June	*H. bivalviorum*
	F142-48	1	Microaerophilia	CAT-broth+NaCl + MA	PNC	Mussel	June	*H. bivalviorum*
	F148-33	4	Aerophilia n=2 Microaerophilia n=2	CAT-broth+NaCl + MA	PNC	Oyster	July	*H. bivalviorum*
	F148-35	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	July	*H. bivalviorum*
	F148-43	1	Microaerophilia	CAT-broth+NaCl + MA	PNC	Oyster	July	*H. bivalviorum*
	F148-44	1	Microaerophilia	CAT-broth+NaCl + MA	PNC	Oyster	July	*H. bivalviorum*
	F149-33	3	Aerophilia n=2 Microaerophilia n=1	CAT-broth+NaCl + MA	Bay	Mussel	July	*H. bivalviorum*
	F151-36	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	July	*H. bivalviorum*
	F157-34	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Clam	August	*H. bivalviorum*
	F158-34	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	August	*H. bivalviorum*
	F158-41	1	Microaerophilia	CAT-broth+NaCl + MA	PNC	Mussel	August	*H. bivalviorum*
	F158-44	3	Aerophilia n=1 Microaerophilia n=2	CAT-broth+NaCl + MA	PNC	Clam n=1 Mussel n=1 Oyster n=1	August	*H. bivalviorum*
	F159-36	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	August	*H. bivalviorum*
	F160-35	4	Aerophilia n=3 Microaerophilia n=1	CAT-broth+NaCl + MA	PNC	Clam	August	*H. bivalviorum*
	F160-44	2	Microaerophilia	CAT-broth+NaCl + MA	PNC	Clam	August	*H. bivalviorum*
	F161-17	2	Aerophilia	CAT-broth + BA	Bay	Cockle	October	*H. bivalviorum*
	F161-26	3	Microaerophilia	CAT-broth + BA	Bay	Cockle	October	*H. bivalviorum*
	F164-36	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Oyster	November	*H. bivalviorum*
	F164-37	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Oyster	November	*H. bivalviorum*
	F164-45	1	Microaerophilia	CAT-broth+NaCl + MA	Bay	Oyster	November	*H. bivalviorum*
	F166-47	1	Microaerophilia	CAT-broth+NaCl + MA	PNC	Oyster	December	*H. bivalviorum*
	F173 DG 33	3	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	February	*H. bivalviorum*
	F173 DG 34	9	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	February	*H. bivalviorum*
	F173 G 34	4	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	February	*H. bivalviorum*
	F173-3 DG 33	27	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	February	*H. bivalviorum*
	F173-3 F 38	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	February	*H. bivalviorum*
	F173-331	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	February	*H. bivalviorum*
	F174 G 17	3	Aerophilia	CAT-broth + BA n=2 CAT-broth+NaCl + Ma n=1	PNC	Oyster	February	*H. bivalviorum*
	F174-3 DG 33	2	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	February	*H. bivalviorum*
	F189-1 IL 33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	June	*H. bivalviorum*
	F189-1 IL 34	5	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	June	*H. bivalviorum*
	F189-2 F 34	2	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	June	*H. bivalviorum*
	F189-3 DG 33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	June	*H. bivalviorum*
	F189-3 DG 35	2	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	June	*H. bivalviorum*
	F189-3 F 34	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	June	*H. bivalviorum*
	F189-3 F 36	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	June	*H. bivalviorum*
	F190-1 IL 35	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	June	*H. bivalviorum*
	F190-2 IL 36	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	June	*H. bivalviorum*
	F190-2 IL 38	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	June	*H. bivalviorum*
	W114-33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Water	June	*H. bivalviorum*
	W114-34	7	Aerophilia n=4 Microaerophilia n=3	CAT-broth+NaCl + MA	PNC	Water	June	*H. bivalviorum*
	W114-42	4	Microaerophilia	CAT-broth+NaCl + MA	PNC	Water	June	*H. bivalviorum*
	W119-33	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Water	July	*H. bivalviorum*
	W120-43	1	Microaerophilia	CAT-broth+NaCl + MA	PNC	Water	July	*H. bivalviorum*
	W129-35	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Water	November	*H. bivalviorum*

	W129-37	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	PNC	Water	November	*H. bivalviorum*
	W131-33	11	Aerophilia n=5 Microaerophilia n=6	CAT-broth+NaCl + MA	PNC	Water	December	*H. bivalviorum*
	W128-33	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	Bay	Water	November	*H. ebronensis*
	W129-34	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Water	November	*H. ebronensis*
	W135-38	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Water	February	*H. ebronensis*
	W136-34	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Water	February	*H. ebronensis*
	W136-36	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Water	February	*H. ebronensis*
	W140-331	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Water	March	*H. ebronensis*
	W145-331	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Water	May	*H. ebronensis*
	W145-34	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Water	May	*H. ebronensis*
	W145-351	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Water	May	*H. ebronensis*
	W145-361	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Water	May	*H. ebronensis*
	F156-34	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Mussel	August	*H. mediterraneus*
	W143-33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Water	April	*H. mediterraneus*
	F153-42	1	Microaerophilia	CAT-broth+NaCl + MA	Bay	Oyster	August	*H. ponticus*
	F161-33	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	Bay	Cockle	October	*H. ponticus*
	F161-35	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Cockle	October	*H. ponticus*
	F161-42	1	Microaerophilia	CAT-broth+NaCl + MA	Bay	Cockle	October	*H. ponticus*
	F155-33	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	PNC	Oyster	August	*H. salis*
	F157-33	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Clam	August	*H. salis*
	F157-42	1	Microaerophilia	CAT-broth+NaCl + MA	Bay	Clam	August	*H. salis*
	F157-43	1	Microaerophilia	CAT-broth+NaCl + MA	Bay	Clam	August	*H. salis*
	F157-44	1	Microaerophilia	CAT-broth+NaCl + MA	Bay	Clam	August	*H. salis*
	F158-33	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	PNC	Mussel	August	*H. salis*
	F158-36	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	PNC	Mussel	August	*H. salis*
	F158-48	1	Microaerophilia	CAT-broth+NaCl + MA	PNC	Mussel	August	*H. salis*
	F160-34	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Clam	August	*H. salis*
	F160-42	1	Microaerophilia	CAT-broth+NaCl + MA	PNC	Clam	August	*H. salis*
	F160-43	1	Microaerophilia	CAT-broth+NaCl + MA	PNC	Clam	August	*H. salis*
	F160-46	1	Microaerophilia	CAT-broth+NaCl + MA	PNC	Clam	August	*H. salis*
Malaciobacter	F138-33	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	PNC	Oyster	March	*M. canalis*
	F159-35	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	August	*M. canalis*
	F164-33	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Oyster	November	*M. canalis*
	F167 F 33	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Mussel	January	*M. canalis*
	F169 DG 35	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	January	*M. canalis*
	F173 F 33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	February	*M. canalis*
	F173-3 F 33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	February	*M. canalis*
	F173-3 F 36	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	February	*M. canalis*
	F174-351	2	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	February	*M. canalis*
	F174-381	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	February	*M. canalis*
	F178-1 IL 33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	March	*M. canalis*
	F181-1 F 33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	April	*M. canalis*
	F181-3 IL 34	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	April	*M. canalis*
	F181-331	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	April	*M. canalis*
	F182-331	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	April	*M. canalis*
	F190-1 IL 33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	June	*M. canalis*
	F190-1 IL 36	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	June	*M. canalis*
	F190-2 IL 33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	June	*M. canalis*
	W110-33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Water	March	*M. canalis*
	W110-37	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Water	March	*M. canalis*
	W110-38	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Water	March	*M. canalis*
	W110-42	1	Microaerophilia	CAT-broth+NaCl + MA	PNC	Water	March	*M. canalis*
	W111-33	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	PNC	Water	March	*M. canalis*
	W111-44	1	Microaerophilia	CAT-broth+NaCl + MA	PNC	Water	March	*M. canalis*
	W112-37	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Water	April	*M. canalis*
	W112-45	1	Microaerophilia	CAT-broth+NaCl + MA	PNC	Water	April	*M. canalis*
	W113-33	1	Microaerophilia	CAT-broth+NaCl + MA	PNC	Water	June	*M. canalis*
	W113-34	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	PNC	Water	June	*M. canalis*
	W117-34	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Water	June	*M. canalis*
	W117-37	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Water	June	*M. canalis*
	W131-34	3	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	PNC	Water n=2 Oyster n=1	December	*M. canalis*
	W137-33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Water	February	*M. canalis*
	W137-38	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Water	February	*M. canalis*
	W140-33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Water	March	*M. canalis*
	W140-34	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Water	March	*M. canalis*
	W140-381	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Water	March	*M. canalis*
	W142-341	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Water	April	*M. canalis*
	W143-331	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Water	April	*M. canalis*

W143-34	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Water	April	*M. canalis*
W143-341	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Water	April	*M. canalis*
W143-36	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Water	April	*M. canalis*
F166-37	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	PNC	Oyster	December	*M. halophilus*
W119-41	1	Microaerophilia	CAT-broth+NaCl + MA	Bay	Water	July	*M. halophilus*
F137-42	1	Microaerophilia	CAT-broth+NaCl + MA	PNC	Mussel	March	*M. marinus*
F139-34	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	PNC	Oyster	March	*M. marinus*
F139-44	1	Microaerophilia	CAT-broth+NaCl + MA	PNC	Oyster	March	*M. marinus*
F140-37	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Clam	April	*M. marinus*
F140-38	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	Bay	Clam	April	*M. marinus*
F140-46	1	Microaerophilia	CAT-broth+NaCl + MA	Bay	Clam	April	*M. marinus*
F140-48	1	Microaerophilia	CAT-broth+NaCl + MA	Bay	Clam	April	*M. marinus*
F162-33	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	Bay	Oyster	November	*M. marinus*
F165-33	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	Bay	Oyster	December	*M. marinus*
F165-36	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	Bay	Oyster	December	*M. marinus*
F165-45	1	Microaerophilia	CAT-broth+NaCl + MA	Bay	Oyster	December	*M. marinus*
F167 DG 33	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	Bay	Mussel	January	*M. marinus*
F167 F 34	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	Bay	Mussel	January	*M. marinus*
F167 F 37	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Mussel	January	*M. marinus*
F167 F 43	1	Microaerophilia	CAT-broth+NaCl + MA	Bay	Mussel	January	*M. marinus*
F167 F 44	1	Microaerophilia	CAT-broth+NaCl + MA	Bay	Mussel	January	*M. marinus*
F172 IL 33	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Oyster	February	*M. marinus*
F172-3 IL 33	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Oyster	February	*M. marinus*
F172-3 IL 34	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Oyster	February	*M. marinus*
F172-331	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Oyster	February	*M. marinus*
F173-1 F 33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	February	*M. marinus*
F173-2 F 33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	February	*M. marinus*
F173-2 F 38	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	February	*M. marinus*
F173-3 G 33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	February	*M. marinus*
F173-3 G 34	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	February	*M. marinus*
F173-3 G 37	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	February	*M. marinus*
F173-3 IL 33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	February	*M. marinus*
F174-331	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	February	*M. marinus*
F174-4 F 33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	February	*M. marinus*
F174-4 F 34	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	February	*M. marinus*
F177 IL 33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	March	*M. marinus*
F177-1 DG 33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	March	*M. marinus*
F177-1 IL 38	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	March	*M. marinus*
F177-2 IL 33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	March	*M. marinus*
F177-2 IL 36	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	March	*M. marinus*
F177-3 IL 33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	March	*M. marinus*
F177-3 IL 34	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	March	*M. marinus*
F177-331	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	March	*M. marinus*
F178 IL 33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	March	*M. marinus*
F178 IL 34	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	March	*M. marinus*
F178-1 G 33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	March	*M. marinus*
F178-1 IL 35	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	March	*M. marinus*
F178-1 IL 36	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	March	*M. marinus*
F178-2 G 33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	March	*M. marinus*
F178-2 IL 34	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	March	*M. marinus*
F178-3 G 33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	March	*M. marinus*
F178-3 IL 33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	March	*M. marinus*
F178-331	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	March	*M. marinus*
F178-341	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	March	*M. marinus*
F178-4 G 33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	March	*M. marinus*
F178-4 IL 33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	March	*M. marinus*
F178-4 IL 34	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	March	*M. marinus*
F178-4 IL 36	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	March	*M. marinus*
F180-2 IL 33	2	Aerophilia	CAT-broth+NaCl + MA	Bay	Oyster	April	*M. marinus*
F181-1 IL 33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	April	*M. marinus*
F181-1 IL 36	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	April	*M. marinus*
F181-1 IL 37	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	April	*M. marinus*
F181-2 IL 33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	April	*M. marinus*
F184-4 DG 33	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Oyster	May	*M. marinus*
F184-4 DG 34	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Oyster	May	*M. marinus*
F188 DG 33	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Oyster	June	*M. marinus*
F188 DG 34	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Oyster	June	*M. marinus*
F188 DG 35	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Oyster	June	*M. marinus*
F188 DG 37	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Oyster	June	*M. marinus*
F188 DG 38	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Oyster	June	*M. marinus*
F188 F 33	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Oyster	June	*M. marinus*
F188 IL 33	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Oyster	June	*M. marinus*
F188 IL 38	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Oyster	June	*M. marinus*
F188-1 IL 33	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Oyster	June	*M. marinus*

F188-1 IL 34	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Oyster	June	*M. marinus*
F188-4 DG 33	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Oyster	June	*M. marinus*
F190-1 IL 34	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	June	*M. marinus*
F190-1 IL 37	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	June	*M. marinus*
W110-35	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Water	March	*M. marinus*
W110-41	1	Microaerophilia	CAT-broth+NaCl + MA	PNC	Water	March	*M. marinus*
W110-43	1	Microaerophilia	CAT-broth+NaCl + MA	PNC	Water	March	*M. marinus*
W112-47	1	Microaerophilia	CAT-broth+NaCl + MA	PNC	Water	April	*M. marinus*
W130-33	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	Bay	Water	December	*M. marinus*
W132-33	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	Bay	Water	January	*M. marinus*
W132-34	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	Bay	Water	January	*M. marinus*
W137-331	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Water	February	*M. marinus*
W137-34	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Water	February	*M. marinus*
W137-37	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Water	February	*M. marinus*
W139-33	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Water	March	*M. marinus*
W140-341	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Water	March	*M. marinus*
W140-351	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Water	March	*M. marinus*
W141-33	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Water	April	*M. marinus*
W141-34	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Water	April	*M. marinus*
W142-33	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Water	April	*M. marinus*
W142-331	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Water	April	*M. marinus*
W148-331	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Water	June	*M. marinus*
W148-341	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Water	June	*M. marinus*
W148-381	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Water	June	*M. marinus*
F142-35	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	PNC	Mussel	June	*M. molluscorum*
F142-36	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	PNC	Mussel	June	*M. molluscorum*
F142-45	1	Microaerophilia	CAT-broth+NaCl + MA	PNC	Mussel	June	*M. molluscorum*
F144-33	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	Bay	Mussel n=1 Oyster n=1	June	*M. molluscorum*
F145-33	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	Bay	Oyster	June	*M. molluscorum*
F145-35	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Oyster	June	*M. molluscorum*
F146-34	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Mussel	July	*M. molluscorum*
F146-41	1	Microaerophilia	CAT-broth+NaCl + MA	Bay	Mussel	July	*M. molluscorum*
F147-36	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Oyster	July	*M. molluscorum*
F147-37	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	Bay n=1 PNC n=1	Oyster n=1 Water n=1	July	*M. molluscorum*
F147-38	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Oyster	July	*M. molluscorum*
F147-39	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Oyster	July	*M. molluscorum*
F147-41	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	Bay	Oyster n=1 Water n=1	July	*M. molluscorum*
F149-46	1	Microaerophilia	CAT-broth+NaCl + MA	Bay	Mussel	July	*M. molluscorum*
F150-33	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	Bay	Oyster	July	*M. molluscorum*
F150-35	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	Bay	Oyster	July	*M. molluscorum*
F153-41	1	Microaerophilia	CAT-broth+NaCl + MA	Bay	Oyster	August	*M. molluscorum*
F156-41	1	Microaerophilia	CAT-broth+NaCl + MA	Bay	Mussel	August	*M. molluscorum*
F158-46	1	Microaerophilia	CAT-broth+NaCl + MA	PNC	Mussel	August	*M. molluscorum*
F159-33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	August	*M. molluscorum*
F159-34	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	August	*M. molluscorum*
F159-41	1	Microaerophilia	CAT-broth+NaCl + MA	PNC	Oyster	August	*M. molluscorum*
F160-45	1	Microaerophilia	CAT-broth+NaCl + MA	PNC	Clam	August	*M. molluscorum*
F161-45	1	Microaerophilia	CAT-broth+NaCl + MA	Bay	Cockle	October	*M. molluscorum*
F164-34	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	Bay	Oyster	November	*M. molluscorum*
F164-43	1	Microaerophilia	CAT-broth+NaCl + MA	Bay	Oyster	November	*M. molluscorum*
F181-3 IL 35	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	April	*M. molluscorum*
F189 DG 33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	June	*M. molluscorum*
F189-2 IL 34	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	June	*M. molluscorum*
W115-45	1	Microaerophilia	CAT-broth+NaCl + MA	Bay	Water	June	*M. molluscorum*
W116-33	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Water	June	*M. molluscorum*
W116-34	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	Bay	Water	June	*M. molluscorum*
W116-35	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	Bay	Water	June	*M. molluscorum*
W116-41	1	Microaerophilia	CAT-broth+NaCl + MA	Bay	Water	June	*M. molluscorum*
W116-42	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	Bay n=1 PNC n=1	Water	June	*M. molluscorum*
W118-41	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	Bay n=1 PNC n=1	Water	July	*M. molluscorum*
W119-34	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Water	July	*M. molluscorum*
W119-44	1	Microaerophilia	CAT-broth+NaCl + MA	Bay	Water	July	*M. molluscorum*
W128-17	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth + BA	Bay	Water	November	*M. molluscorum*
W129-43	1	Microaerophilia	CAT-broth+NaCl + MA	PNC	Water	November	*M. molluscorum*
W147-171	1	Aerophilia	CAT-broth + BA	Bay	Water	June	*M. molluscorum*

	F167 IL 34	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Mussel	January	*M. mytili*
	F167 IL 41	1	Microaerophilia	CAT-broth+NaCl + MA	Bay	Mussel	January	*M. mytili*
	F142-44	1	Microaerophilia	CAT-broth+NaCl + MA	PNC	Mussel	June	*M. mytili*
	F143-33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	June	*M. mytili*
	F143-34	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	PNC	Oyster	June	*M. mytili*
	F146-30	1	Microaerophilia	CAT-broth + BA	Bay	Mussel	July	*M. mytili*
	F147-30	1	Microaerophilia	CAT-broth + BA	Bay	Oyster	July	*M. mytili*
	F149-34	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	Bay	Mussel	July	*M. mytili*
	F149-43	1	Microaerophilia	CAT-broth+NaCl + MA	Bay	Mussel	July	*M. mytili*
	F151-34	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	July	*M. mytili*
	F151-38	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	PNC	Oyster	July	*M. mytili*
	F151-43	1	Microaerophilia	CAT-broth+NaCl + MA	PNC	Oyster	July	*M. mytili*
	F151-44	1	Microaerophilia	CAT-broth+NaCl + MA	PNC	Oyster	July	*M. mytili*
	F152-35	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Mussel	August	*M. mytili*
	F153-17	1	Aerophilia	CAT-broth + BA	Bay	Oyster	August	*M. mytili*
	F155-42	1	Microaerophilia	CAT-broth+NaCl + MA	PNC	Oyster	August	*M. mytili*
	F156-25	1	Microaerophilia	CAT-broth + BA	Bay	Mussel	August	*M. mytili*
	F156-26	1	Microaerophilia	CAT-broth + BA	Bay	Mussel	August	*M. mytili*
	F167 IL 43	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth + BA n=1 CAT-broth+NaCl + MA n=1	Bay n=1 PNC n=1	Mussel	January	*M. mytili*
	F172-1 IL 34	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Oyster	February	*M. mytili*
	F190 F 34	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	June	*M. mytili*
	F190-2 DG 33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	June	*M. mytili*
	W118-25	1	Microaerophilia	CAT-broth + BA	Bay	Water	July	*M. mytili*
	W123-33	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	Bay	Water	August	*M. mytili*
	W123-34	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Water	August	*M. mytili*
	W124-33	1	Microaerophilia	CAT-broth+NaCl + MA	Bay	Water	August	*M. mytili*
	F145-41	1	Microaerophilia	CAT-broth+NaCl + MA	Bay	Oyster	June	*M. neptunis*
	F142-33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	June	*M. neptunis*
	F146-33	2	Aerophilia n=1 Microaerophilia n=1	CAT-broth+NaCl + MA	Bay	Mussel	July	*M. neptunis*
	F146-38	1	Aerophilia	CAT-broth+NaCl + MA	Bay	Mussel	July	*M. neptunis*
	F136-44	1	Microaerophilia	CAT-broth+NaCl + MA	PNC	Mussel	March	*M. viscosus*
	F136-41	1	Microaerophilia	CAT-broth+NaCl + MA	PNC	Mussel	March	*M. viscosus*
	F139-33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	March	*M. viscosus*
	F139-36	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Oyster	March	*M. viscosus*
	F142-34	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	June	*M. viscosus*
	F142-43	1	Microaerophilia	CAT-broth+NaCl + MA	PNC	Mussel	June	*M. viscosus*
	F165-18	1	Aerophilia	CAT-broth + BA	Bay	Oyster	December	*M. viscosus*
Pseudarcobacter	F185-171	2	Aerophilia	CAT-broth + BA	PNC	Mussel	May	*P. aquimarinus*
	F173-2 IL 33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Mussel	February	*P. cloacae*
	F186-201	1	Aerophilia	CAT-broth + BA	PNC	Oyster	Ago	*P. cloacae*
	F157-41	1	Microaerophilia	CAT-broth+NaCl + MA	Bay	Clam	August	*P. cloacae*
	W124-41	1	Microaerophilia	CAT-broth+NaCl + MA	Bay	Water	August	*P. cloacae*
	F178-1 IL 20	1	Aerophilia	CAT-broth + BA	PNC	Oyster	March	*P. defluvii*
	W112-19	1	Aerophilia	CAT-broth + BA	PNC	Water	April	*P. defluvii*
	W112-20	2	Aerophilia	CAT-broth + BA n=1 CAT-broth+NaCl + MA n=1	PNC	Water	April	*P. defluvii*
	W112-33	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Water	April	*P. defluvii*
	W112-36	1	Aerophilia	CAT-broth+NaCl + MA	PNC	Water	April	*P. defluvii*
	W112-41	1	Microaerophilia	CAT-broth+NaCl + MA	PNC	Water	April	*P. defluvii*
	W131-18	1	Aerophilia	CAT-broth + BA	PNC	Water	December	*P. defluvii*
	W131-28	1	Microaerophilia	CAT-broth + BA	PNC	Water	December	*P. defluvii*
	F140-17	3	Aerophilia n=2 Microaerophilia n=1	CAT-broth + BA n=2 CAT-broth+NaCl + MA n=1	Bay	Clam	April	*P. ellisii*
	F173-2 IL 18	1	Aerophilia	CAT-broth + BA	PNC	Mussel	February	*P. ellisii*

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EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

ANNEX III: Primers used in this work

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Table 1 Primers used for the genotyping and identification of *Arcobacter*-related spp. isolates and detection of viable *Arcobacter*-related spp. cells.

Objective	Method	Forward primer	Reverse primer	Target	Size (bp)	Reference
Genotyping	**ERIC-PCR**	ERIC 1R	ERIC 2	Genome	NA	Houf et al. (2002)
Identification	**m-PCR**	BUTZ	ARCO	16S rRNA	401	Houf et al. (2000)
		SKIRR	ARCO	16S rRNA	641	Houf et al. (2000)
		CRY1	CRY2	23S rRNA	257	Houf et al. (2000)
	l6S rDNA-RFLP	CAH16S1a	CAH16S1b	16S rRNA	1026	Figueras et al. (2008)
	l6S rRNA gene	Anti 1	S	16S rRNA	1500	Martínez-Murcia et al. (1992)
Housekeeping genes	***rpoB***	rpoB-Arc15F	rpoB-Arc24R	*rpoB*	900	Levican (2013)
	atpA	atpA-Arc5F	atpA-Arc12R	*atpA*	751	Levican (2013)
	gyrA	gyrA-Arc4F	gyrA-Arc13R	*gyrA*	1014	Levican (2013)
	gyrB	gyrB-Arc-7F	gyrB-Arc-14R	*gyrB*	722	Levican (2013)
	hsp60	cpn60-Arc2F	cpn60-Arc8R	*hsp60*	570	Levican (2013)
Detection of viable cells	**v-qPCR**	23SF	23SR	23S rRNA	233	**(Study 3.5)**

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

ANNEX IV: Revisiting the taxonomy of the genus *Arcobacter*: Getting order from the chaos Pérez-Cataluña A, Salas-Massó N, Diéguez AL, Balboa S, Lema A, Romalde JL, Figueras MJ. *Frontiers in Microbiology*. 2018 Sep 4;9:2077. Erratum in: *Frontiers in Microbiology*. 2018 Dec 21;9:3123.

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
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Revisiting the Taxonomy of the Genus *Arcobacter*: Getting Order From the Chaos

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Since the description of the genus *Arcobacter* in 1991, a total of 27 species have been described, although some species have shown 16S rRNA similarities below 95%, which is the cut-off that usually separates species that belong to different genera. The objective of the present study was to reassess the taxonomy of the genus *Arcobacter* using information derived from the core genome (286 genes), a Multilocus Sequence Analysis (MLSA) with 13 housekeeping genes, as well as different genomic indexes like Average Nucleotide Identity (ANI), *in silico* DNA–DNA hybridization (*is*DDH), Average Amino-acid Identity (AAI), Percentage of Conserved Proteins (POCPs), and Relative Synonymous Codon Usage (RSCU). The study included a total of 39 strains that represent all the 27 species included in the genus *Arcobacter* together with 13 strains that are potentially new species, and the analysis of 57 genomes. The different phylogenetic analyses showed that the *Arcobacter* species grouped into four clusters. In addition, *A. lekithochrous* and the candidatus species '*A. aquaticus*' appeared, as did *A. nitrofigilis,* the type species of the genus, in separate branches. Furthermore, the genomic indices ANI and *is*DDH not only confirmed that all the species were well-defined, but also the coherence of the clusters. The AAI and POCP values showed intra-cluster ranges above the respective cut-off values of 60% and 50% described for species belonging to the same genus. Phenotypic analysis showed that certain test combinations could allow the differentiation of the four clusters and the three orphan species established by the phylogenetic and genomic analyses. The origin of the strains showed that each of the clusters embraced species recovered from a common or related environment. The results obtained enable the division of the current genus *Arcobacter* in at least seven different genera, for which the names *Arcobacter*, *Aliiarcobacter* gen. nov., *Pseudoarcobacter* gen. nov., *Haloarcobacter* gen. nov., *Malacobacter* gen. nov., *Poseidonibacter* gen. nov., and Candidate '*Arcomarinus*' gen. nov. are proposed.

Keywords: *Arcobacter*, *Aliiarcobacter* gen. nov., *Pseudoarcobacter* gen. nov., *Haloarcobacter* gen. nov., *Malacobacter* gen. nov., *Poseidonibacter* gen. nov., taxonomic criteria

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

INTRODUCTION

The genus *Arcobacter* was created by Vandamme et al. (1991) to accommodate Gram-negative, curved-shaped bacteria belonging to two species *Campylobacter cryaerophila* (now *Arcobacter cryaerophilus*) and *Campylobacter nitrofigilis* (now *A. nitrofigilis*), considered atypical campylobacters due to their ability to grow at lower temperatures (15°C–30°C) and without microaerophilic conditions (Vandamme et al., 1991). The latter species was selected as the type species for the new genus (Vandamme et al., 1991). One year later the genus was enlarged with the addition of two new species, *A. skirrowii* with an animal origin being isolated from aborted ovine, porcine and bovine fetuses, and from lambs with diarrhea, and *A. butzleri,* which was recovered from cases of human and animal diarrhea (Vandamme et al., 1992). Another two new species were incorporated into the genus in 2005. *A. halophilus* was isolated from water from a hypersaline lagoon in Hawaii (Donachie et al., 2005), and *A. cibarius* was isolated from broiled carcasses in Belgium (Houf et al., 2005). These species were assigned to the genus *Arcobacter* on the basis of the 16S rRNA gene similarity (94% and 95% for *A. nitrofigilis* with *A. halophilus* and *A. cibarius,* respectively). However, these values are equal, or even below, the cut-off of 95% for genus definition (Rosselló-Mora and Amann, 2001; Yarza et al., 2008, 2014; Tindall et al., 2010).

From 2009 onward, new species were being described year-by-year, reaching a total number of 27 in 2017. In some of these descriptions, the similarity of the 16S rRNA gene was the decisive character for taxonomic assignation at genus level, although phylogeny based on housekeeping genes (*rpo*B first and then *gyr*B and *hsp*60) was also included as additional, more discriminatory tools for the species (Collado et al., 2009a, 2011; De Smet et al., 2011). Using this approach, *A. molluscorum*, *A. ellisii*, *A. defluvii*, or *A. bivalviorum* were defined, among others (Collado et al., 2009a, 2011; Figueras et al., 2011a,b; Levican et al., 2012), which showed 16S rRNA similarities ranging from 91.1 to 94.7%, not supporting their common affiliation. On the other hand, the most closely related species, which showed a similarity of 99.1% were *A. ellisii* and *A. defluvii* (Collado et al., 2011), giving evidence for the first time of the poor resolution of the 16S rRNA gene for separating closely related species in the genus *Arcobacter*. However, the phylogenetic analysis based on the concatenated sequences of *gyr*B, *rpo*B, and *cpn*60 genes, together with the DNA–DNA hybridization results, clearly supported the existence of these two differentiated taxa (Figueras et al., 2011a). Also in 2011, *A. trophiarum* was discovered from the intestinal tract of healthy fattening pigs, which interestingly showed the closest similarities (≥97.4%) with the other species also recovered from humans or animals, i.e., *A. cryaerophilus*, *A. thereius*, *A. cibarius,* or *A. skirrowii* (De Smet et al., 2011; Figueras et al., 2014; Van den Abeele et al., 2014).

In 2013, the species *A. cloacae* and *A. suis* were described, using a Multilocus Sequence Analysis (MLSA) approach including five housekeeping genes (Levican et al., 2013) for the first time. Simultaneously, and due to the highest 16S rRNA gene similarity with *A. marinus* (95.5%), the species *A. anaerophilus* was incorporated to the genus (Sasi-Jyothsna et al., 2013). However, this species showed atypical characteristics, including lack of motility and obligate anaerobic metabolism, which led to the original description of the genus *Arcobacter* being emended (Sasi-Jyothsna et al., 2013). The most recently described species from shellfish are *A. lekithochrous*, *A. haliotis*, and *A. canalis* (Diéguez et al., 2017; Tanaka et al., 2017; Pérez-Cataluña et al., 2018a). The first one included several isolates recovered from scallop larvae and from tank seawater of a Norwegian hatchery (Diéguez et al., 2017), the second species came from an abalone of Japan (Tanaka et al., 2017) and the third from oysters submerged in a water channel contaminated with wastewater (Pérez-Cataluña et al., 2018a). However, Diéguez et al. (2018) evidenced that the species *A. haliotis* is a later heterotypic synonym of *A. lekithochrous*. Additionally, the low 16S rRNA gene similarity of *A. lekithochrous* with the known *Arcobacter* species (91.0–94.8%) found in the *A. lekithochrous* description made Diéguez et al. (2017) suggest that certain species might belong to other genera and recommend that a profound revision of the genus might clarify the taxonomy.

On the other hand, adding 2.5% NaCl to the enrichment medium and subculturing on marine agar, Salas-Massó et al. (2016) recognized seven potential new species from water and shellfish (mussels and/or oysters), and recovered new isolates of *A. halophilus* and *A. marinus* of which only the type strains had been known. In addition, during the characterization of the most recently described species *A. canalis* (Pérez-Cataluña et al., 2018a) and when trying to define the seven mentioned new species, we observed that the *Arcobacter* species formed several different clusters distant enough to suspect they might correspond to different genera, in agreement with Diéguez et al. (2017).

There are clear criteria for describing new bacterial species (Tindall et al., 2010; Figueras et al., 2011a,b). However, the description of a genus is usually based on a cut-off of <95% similarity in the 16S rRNA gene sequence, and a G+C (% mol) content differing by more than 10% (Rosselló-Mora and Amann, 2001; Yarza et al., 2008; Tindall et al., 2010; Yarza et al., 2014). Nowadays, genomic data like the Average Nucleotide Identity (ANI) and the *in silico* DNA–DNA hybridization (*is*DDH) are used to define bacterial species, although have not yet been fully explored for delineating genera (Konstantinidis and Tiedje, 2005; Goris et al., 2007; Richter and Rosselló-Móra, 2009; Qin et al., 2014; Chun et al., 2018).

A percentage of Average Amino-acid Identity (AAI) ranging from 60 to 80% between the compared genomes of species or strains and a Percentage of Conserved Proteins (POCPs) above 50% has been proposed if they are to belong to the same genus (Konstantinidis and Tiedje, 2005; Qin et al., 2014). Finally, the Relative Synonymous Codon Usage (RSCU) has also been used by some authors to infer evolutionary and ecological links among bacterial species (Ma et al., 2015; Farooqi et al., 2016).

Very recently, Waite et al. (2017) carried out a comparative genomic analysis of the class *Epsilonproteobacteria*. Using 16S and 23S rRNA, 120 single-copy marker proteins and AAI analysis they proposed its reclassification as the new phylum Epsilonbacteraeota. In that study, Waite et al. (2017) also proposed a reclassification of the genus *Arcobacter* as a new

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Nuria Salas Massó

Family *Arcobacteraceae*, within the class *Campylobacteria*, order *Campylobacterales*. One weakness of this study, specifically regarding the genus *Arcobacter*, is that only seven validated species were included in the analysis. The new family therefore comprised only the genus *Arcobacter*. However, these findings also support the need for a clarification of the taxonomy of the current genus *Arcobacter*.

The rise of genome sequencing has dramatically changed the landscape of systematics of prokaryotes, improving different aspects such as the identification of species, the functional characterization for resolving taxonomic groups, and the resolution of the phylogeny of higher taxa (Whitman, 2015). It seems clear that the incorporation of genomics into the taxonomy will boost its credibility providing reproducible, reliable, highly informative means to infer phylogenetic relationships among prokaryotes, and avoiding unreliable methods and subjective difficult-to-replicate data (Chun and Rainey, 2014; Chun et al., 2018).

Within this modern taxonomy context, the objective of the present study was to reassess the taxonomy of the known and newly recognized *Arcobacter* species by using a MLSA of 13 housekeeping genes, the whole genome sequences and the derived genomic analysis. The latter analysis included ANI, *is*DDH, AAI, POCP, and RSCU of all *Arcobacter* type strains. In addition, phylogenies based on 16S and 23S rRNA gene sequences were also performed with comparative purposes. The new taxonomic criteria were stable when including whole genome sequences of a second strain of each species or of unassigned sequences obtained from the public databases.

MATERIALS AND METHODS

Bacterial Strains

All 27 valid species included in the genus *Arcobacter* have been studied. They are represented by 39 strains, and 13 strains that are potentially new species (**Table 1**). Furthermore, 50 genomes of *Arcobacter* strains identified at species level were investigated, 39 of which were obtained in our laboratory (27 from known species and 13 from potentially new species) and the others from the public databases[1,2]. Five genomes that had been deposited as *Arcobacter* sp. in the databases were also included in the study. If there was more than one strain of a known *Arcobacter* species, two representative genomes for each species were included in the analysis. The only exceptions were: *A. acticola* (Park et al., 2016) and *A. pacificus* (Zhang et al., 2015), whose taxonomic positions were only inferred by the phylogenetic analysis of the 16S rRNA gene sequences published in their species descriptions, together with a MLSA of three housekeeping genes (*atp*A, *gyr*B, and *rpo*B) for *A. pacificus* (Zhang et al., 2015; Park et al., 2016). The strains considered potentially new species, and named hereafter as 'candidate species,' had been recognized with an MLSA analysis of five housekeeping genes (*atp*A, *gyr*A, *gyr*B, *hsp*60, and *rpo*B) (data not shown).

Culturing for genome sequencing was carried out either on blood agar (DIFCO, Madrid, Spain) or marine agar (Scharlau, Sentmenat, Spain) at 30°C in aerobiosis for 24–72 h, depending on the requirements. DNA was extracted using Easy-DNA™ gDNA Purification kit (Invitrogen, Madrid, Spain) following the manufacturer's instructions. The integrity of the DNA was evaluated by electrophoresis of 10 μl of the sample in a 1.5% agarose gel. The total amount of DNA was quantified using Qubit™ with the dsDNA Broad Range Assay kit (Invitrogen). Paired-end libraries were constructed with 50 ng of DNA using Nextera DNA Library Preparation Kit (Illumina, Lisbon, Portugal) and sequenced with MiSeq platform (Illumina). Sequencing generated 2 × 300 bp paired-end reads. Clean reads were assembled with SPAdes (Nurk et al., 2013) and the CGE assembler (Larsen et al., 2012) in order to select the better assembly. Before depositing the genomes in the NCBI database, FASTA files were screened for eukaryotic and prokaryotic sequences using BLASTn, and for adaptors with VecScreen standalone software[3]. The five housekeeping genes used in the first MLSA analysis (*atp*A, *gyr*A, *gyr*B, *hsp*60, and *rpo*B) were extracted from each genome and compared with the Sanger sequences of these genes obtained originally for the identification of the strain. The existence of a single and identical copy of these genes confirmed that the genomes were not contaminated and belonged to the correct strain. Finally, contigs were deleted if they had less than 200 bp. The genomes were deposited in the GenBank database and **Table 1** lists the accession numbers.

The 55 genomes were annotated with a local installation of Prokka v1.2 (Seemann, 2014) using an e-value of 1e-06. The annotation was performed with Prokka, with the prediction tools Prodigal v2.6 (Hyatt et al., 2010) and ARAGORN v1.2 (Laslett and Canback, 2004). The prediction tool Barrnap v0.6[4] included in Prokka v1.2 was used for the annotation of rRNA genes. Coding sequences (CDS) were annotated, combining the Rapid Annotation Subsystems Technology (RAST) (Overbeek et al., 2014) using the *classic RAST* scheme and the Annotation Tools of PATRIC server (Wattam et al., 2017). The characteristics of each genome (i.e., N50, number of contigs, number of CDS, G+C content) were obtained from NCBI annotations.

Analysis of Housekeeping Genes, Ribosomal Genes, and Core Genome

Thirteen housekeeping genes (*atp*A, *atp*D, *dna*A, *dna*J, *dna*K, *fts*Z, *gyr*A, *hsp*60, *rad*A, *rec*A, *rpo*B, *rpo*D, and *tsf*) were obtained from the genomes using BLASTn search. Sequence similarities of housekeeping genes were determined using the MegAlign program (DNASTAR®, Madison, WI, United States). Genes were aligned using ClustalW (Larkin et al., 2007) and phylogenies based on individual genes and on the concatenated sequences was constructed with MEGA version 6.0 (Tamura et al., 2013) using the Neighbor-Joining (NJ) and Maximum-Likelihood (ML) algorithms.

[1] https://www.ncbi.nlm.nih.gov/genome/
[2] https://gold.jgi.doe.gov/
[3] ftp://ftp.ncbi.nlm.nih.gov/blast/demo/
[4] http://www.vicbioinformatics.com/software.barrnap.shtml

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Núria Salas Massó

The phylogenetic analysis of the core genome was assessed with the Roary software (Page et al., 2015) using 80% as cut-off for the BLASTp search. The core genome alignment was extracted with the latter software and the phylogeny was inferred using SplitsTree version 4.14.2 as described in Sawabe et al. (2007) using SplitsTree version 4.14.2, with a neighbor net drawing and Jukes-Cantor correction (Bandelt and Dress, 1992; Huson and Bryant, 2005).

Furthermore, the 16S and 23S rRNA genes of each genome were obtained using RNammer (Lagesen et al., 2007). In some cases, 16S rRNA gene sequences were obtained in our laboratories by Sanger sequencing or from the GenBank. The similarity of the 16S rRNA genes was calculated using MegAlign version 7.0.0 (DNASTAR®, Madison, WI, United States). Phylogenetic trees were reconstructed with MEGA version 6.0 (Tamura et al., 2013) also using the NJ and ML algorithms. Alignments obtained for both genes were visually analyzed in order to localize signature sequences for strains or groups of strains.

Genomic Indices

In order to ensure the correct assignation at species level of each analyzed genome, the ANI and the *is*DDH were calculated between all the genomes (Konstantinidis and Tiedje, 2005; Richter and Rosselló-Móra, 2009; Qin et al., 2014). The ANIb was calculated using JSpeciesWS (Richter et al., 2016), the resulting matrix was clustered and visualized using ggplot2 2.2.1 package (Wickham, 2009) and the *is*DDH was calculated with the GGDC software using results obtained with the formula 2 (Meier-Kolthoff et al., 2013). Two other indices (AAI and POCP) described for genus classification (Konstantinidis and Tiedje, 2005; Luo et al., 2014; Qin et al., 2014) were calculated among the genomes that corresponded to the type strains of the accepted species and the reference strains of the candidate species. The AAI was calculated with the Lycoming College Newman Lab AAIr Calculator[5] using the Sequence-Based Comparison Tools output file from RAST (Overbeek et al., 2014). The POCP was determined as described by Qin et al. (2014) using the following parameters to consider a peptide as a conserved protein: an e-value lower than 1e-5 and an identity percentage higher than 40% from an aligned region higher than 50%.

Finally, the RSCU was computed using the Codon Adaptation Index (CAI) developed by Sharp and Li (1987) through the CAIcal web-server (Puigbò et al., 2008). Statistical differences in the RSCU were assessed by a multinomial regression approach using the R software environment (R Core Team, 2015). The principal component analysis (PCA) was performed by the R software environment (R Core Team, 2015, and visualized using ggplot2 2.2.1 and ggfortify 0.4.4 (Wickham, 2009; Horikoshi and Tang, 2015; Tang et al., 2016) or pca3d 0.10 (Weiner, 2017) packages.

Phenotypic Analysis and Metabolic Inference

Phenotypic characterization of each described species was obtained from this study, from the original descriptions or from the summary published by On et al. (2017). For the potentially new *Arcobacter* species, the phenotype was characterized following the recommended minimal standards described for new taxa of the family *Campylobacteraceae* (Ursing et al., 1994; On et al., 2017) and with complementary tests used in the description of other *Arcobacter* species (Levican et al., 2013).

Inference of the metabolic routes from the genome sequences was performed with the software package Traitar (Microbial Trait Analyzer) (Weimann et al., 2016), using the protein coding genes files obtained with Prokka v1.2 (Seemann, 2014). Traitar software is based on phenotypic data extracted from the Global Infectious Disease and Epidemiology Online Network (GIDEON) and Bergey's Systematic Bacteriology. The software uses two prediction models: the phypat classifier, which predicts the presence/absence of proteins found in the phenotype of 234 bacterial species; and the phypat+PGL classifier, which uses the same information as the phypat combined with the information of the acquisition and loss of protein families and phenotypes during evolutive events. A total of 67 traits available within the software, related to oxygen requirement, enzymatic activities, proteolysis, antibiotic resistance, morphology and motility and the use of different carbon sources, were tested and the combined results of the two predictors were analyzed using a heat map.

RESULTS AND DISCUSSION

Strains and Genomes

All the 27 species currently included in the genus *Arcobacter* and 13 candidate species have been investigated in the present study, which has analyzed 55 genomes, 16 of them from the public databases and 39 sequenced in this study (**Tables 1**, **2**). It was not possible to analyze the genomes from *A. acticola* and *A. pacificus* because we were unable to get the type strains of the species. The contigs obtained and the N50 values complied with the recently proposed minimal standards for the use of genomes in taxonomic studies (Chun et al., 2018). The genome size ranged from 1.81 Mb for *A. skirrowii* F28 to 3.60 Mb for *A. lekithochrous* CECT 8942^T (**Table 2**). The G+C content ranged from 26.1% in *A. molluscorum* CECT 7696^T to 34.9% in '*A. aquaticus*' W112-28. The G+C values agree with the range from 24.6% (which corresponded to the type strain of *A. anaerophilus*) to 31% indicated for the genus *Arcobacter* in the recent emended description by Sasi-Jyothsna et al. (2013). Interestingly, 26 genomes (47.3%) showed the presence of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) and CRISPR-associated genes, related with the immune response of the bacteria.

Taxonomic and Phylogenetic Analysis

Similarities in the 16S rRNA gene sequences among type and representative strains of the different *Arcobacter* species (all the 27 species currently included in the genus and the 13 new candidate species) showed a wide range of values (**Supplementary Tables S1, S4**). They ranged from 90.8% (observed between *A. anaerophilus* and *A. faecis*) to 99.9% (between *A. butzleri and* '*A. lacus*'). The lower range of

[5]http://lycofs01.lycoming.edu/~newman/AAI/

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Nuria Salas Massó

TABLE 1 | Strains used in this study, source of isolation and accession numbers of the available genomes.

Species	Strain	Source	Acc. No. Genome	Species	Strain	Source	Acc. No. Genome
A. acticola	KCTC 52212^T	Seawater	NA[a]	*A. mytili*	T234	Seawater	PDJW00[b]
A. anaerophilus	DSM 24636^T	Estuarine sediment	PDKO00[b]	*A. nitrofigilis*	DSM7299^T	Marshland plant	NC014166[c]
	IR-1	Utsira aquifer	NZ_JXXG00[c]	*A. pacificus*	DSM 25018^T	Seawater	NA[a]
A. aquimarinus	CECT 8442^T	Mediterranean Sea	NXIJ00[b]	*A. skirrowii*	LMG 6621^T	Diarrheic lamb	NXIC00[b]
A. bivalviorum	CECT 7835^T	Mussels	PDKM00[b]		F28	Wild pig	PDJT00[b]
	F118-4	Mussels	PDKL00[b]	*A. suis*	CECT 7833^T	Pork meat	NREO00[b]
A. butzleri	RM4018^T	Human (Clinical)	NC_009850[c]	*A. thereius*	LMG 24486^T	Aborted pig foetus	LLKQ01[c]
	ED1	Microbial fuel cell	NC_017187[c]		DU22	Duck cloaca	LCUJ01[c]
A. canalis	F138-33	Oyster PNC[e]	NWVW01[b]	*A. trophiarum*	LMG 25534^T	Piglet feces	PDKD00[b]
	SH-4D_Col1	Unknown	FUYO00[c]		CECT 7650	Chicken cloacal swab	PDJS00[b]
A. cibarius	LMG 21996^T	Broiler, skin	NZ_JABW00[c]	*A. venerupis*	CECT 7836^T	Clams	NREP00[b]
A. cloacae	CECT 7834^T	Sewage	NXII00[b]	*Arcobacter* sp.	L	Microbial fuel cell	NC_017192[c]
	F26	Mussels	PDJZ00[b]		AF1028	Human feces	JART01[c]
A. cryaerophilus	LMG 24291^T	Aborted bovine foetus	NXGK00[b]		CAB	Marine	Go0012496[d]
A. defluvii	CECT 7697^T	Sewage	NXIH00[b]		LA11	Marine	BDIR01[c]
A. ebronensis	CECT 8441^T	Mussels	PDKK00[b]		LPB0137	Environmental	CP019070[c]
	CECT 8993	Seawater	PDKJ00[b]				
A. ellisii	CECT 7837^T	Mussels	NXIG00[b]	'*A. aquaticus*'	W112-28	Freshwater PNC[e]	PDKN00[b]
A. faecis	LMG 28519^T	Human septic tank	NZ_JARS00[c]	'*A. caeni*'	RW17-10	Recycled wastewater	MUXE00[b]
A. halophillus	DSM 18005^T	Hypersaline lagoon	PDJY00[b]	'*A. hispanicus*'	FW-54	Wastewater	PDKI00[b]
	F166-45	Oyster PNC[e]	PDJY00[b]	'*A. lacus*'	RW43-9	Recycled wastewater	MUXF00[b]
A. lanthieri	LMG 28516^T	Pig manure	JARU01[c]	'*A. mediterraneus*'	F156-34	Mussels Alfacs Bay	NXIE00[b]
	LMG 28517	Dairy cattle manure	JARV01[c]	'*A. miroungae*'	9Ant[f]	Cloaca elephant seal	PDKH00[b]
A. lekithochrous	CECT 8942^T	Great scallop larvae	NZ_MKCO00[b]	'*A. neptunis*'	F146-38	Mussels Alfacs Bay	PDKG00[b]
	LMG 28652	Abalon	PZYW00[c]	'*A. porcinus*'	LMG 24487^T	Aborted pig foetus	LCUH01[c]
A. marinus	CECT 7727^T	Seawater	NXAO01[b]	'*A. ponticus*'	F161-33	Cockle Alfacs Bay	PDKF00[b]
	F140-37	Clams Alfacs Bay	NWVX01[b]	'*A. salis*'	F155-33	Oyster PNC[e]	PDKE00[b]
A. molluscorum	CECT 7696^T	Mussels	NZ_NXFY00[b]	'*A. viscosus*'	F142-34[g]	Mussels PNC[e]	PDKC00[b]
	F91	Mussels	PDJX00[b]	'*A. vitoriensis*'	FW59[g]	Wastewater	PDKB00[b]
A. mytili	CECT 7386^T	Mussels	NXID00[b]	*Arcobacter* sp.	F2176	Mussels	PDJV00[b]

[a]*Genome not available;* [b]*Genome sequenced in this study;* [c]*Genome obtained from NCBI database;* [d]*Genome obtained from JGI Gold atabase;* [e]*PNC means PobleNou Channel, which is a freshwater channel heavily (geometric mean of E. coli counts 4.1 × 10^4 c.f.u./100ml) contaminated with wastewater where shellfish were exposed for 72h (Salas-Massó et al., 2016, 2018).* [f]*This strain was obtained from F.J. García from the Laboratorio Central de Veterinaria de Algete, MAGRAMA, Madrid, Spain;* [g]*These strains were recovered at the Faculty of Pharmacy, University of the Basque Country (UPV-EHU), Vitoria-Gasteiz, Spain, by R. Alonso, I. Martinez-Malaxetxebarria and A. Fernández-Astorga.*

similarity (90.8%) is due to the fact that those species, as occurred with others, were assigned within the genus based on the premise that 16S rRNA gene similarity was higher with any type strain of *Arcobacter* than with other taxa. However, in some cases being below the 95% cut-off value for genus delimitation (Rosselló-Mora and Amann, 2001; Yarza et al., 2008; Tindall et al., 2010; Figueras et al., 2011a,b). It is interesting to point out that 16S rRNA gene sequence similarities among *A. nitrofigilis*, the type species of the genus, and the other described species ranged from 93.2% (with *A. thereius*) to 95.9% (with *A. venerupis*). Furthermore, *A. nitrofigilis* showed higher similarities than the threshold value of 95% with only seven species (*A. acticola*, '*A. caeni*,' *A. cloacae*, *A. defluvii, A. ellisii*, *A. suis*, and *A. venerupis*) out of the 27 accepted species. In any case, from the analysis of the similarities in the 16S rRNA gene sequences among the *Arcobacter* species it is clear that this gene has limited value and that other approaches available in the genomic era of taxonomy are needed for their study.

Phylogenetic analysis based on the core genome made up of 286 genes (**Figure 1** and **Supplementary Table S5**) and also on the concatenated sequences of 13 housekeeping genes of the representative *Arcobacter* strains (**Figure 2**) revealed that the *Arcobacter* species could be grouped into 4 major monophyletic clusters. Cluster 1, comprised seven validated species: *A. butzleri*, *A. cibarius*, *A. cryaerophilus*, *A. lanthieri*, *A. skirrowii*, *A. thereius*, and *A. trophiarum*, together with *A. faecis* (species described but not validated yet) and five candidate taxa '*A. hispanicus,*' '*A. lacus,*' '*A. miroungae,*' '*A. porcinus,*' and '*A. vitoriensis*' (**Figure 1**). Cluster 2 embraced the species *A. aquimarinus*, *A. cloacae*, *A. defluvii*, *A. ellisii*, *A. suis*, and *A. venerupis*, as well as the non-validated *A. acticola* and the candidatus '*A. caeni*.' Cluster 3 included five species, *A. canalis*, *A. halophilus*, *A. marinus*, *A. molluscorum*,

TABLE 2 | Genome characteristics and annotation results. Source of whole genome sequences as indicated in **Table 1**.

Species	No. Contigs	N50 (Kb)	CDS (Total)	CDS (Coding)	RNA Genes	tRNAs	ncRNAs	CRISPR Arrays	G+C (%)	Size (Mb)
A. anaerophilus DSM 24636^T	40	186	2,938	2,922	45	40	2	1	29.9	2.98
A. anaerophilus IR1	7	1,179	3,360	3,024	61	47	2	3	30.2	3.25
'A. aquaticus' W112-28^T	20	370	2,500	2,487	55	45	3	0	34.9	2.53
A. aquimarinus CECT 8442^T	68	75	2,473	2,463	46	42	2	0	26.6	2.46
A. bivalviorum CECT 7835^T	179	461	2,786	2,728	50	41	3	0	28.2	2.75
A. bivalviorum F118-4	26	209	2,652	2,652	47	38	3	0	28.1	2.71
A. butzleri RM4018^T	1	–	2,261	2,256	71	54	2	0	27.0	2.34
A. butzleri ED1	1	–	2,151	2,145	71	54	2	0	27.1	2.26
'A. caeni RW17-10^T	59	123	2,357	2,337	58	51	3	0	27.1	2.42
A. canalis CECT8984^T	50	166	2,733	2,720	53	48	2	1	27.3	2.78
A. canalis SH-4D_Col1	69	72	2,716	2,663	63	52	2	1	27.1	2.82
A. cibarius LMG 21996^T	44	119	2,156	2,110	68	46	2	0	27.1	2.20
A. cloacae CECT 7834^T	135	135	2,826	2,795	58	51	2	3	26.8	2.78
A. cloacae F26	40	218	2,470	2,459	53	44	2	1	26.9	2.51
A. cryaerophilus LMG 24291^T	91	54	2,092	2,081	49	40	3	0	27.2	2.06
A. defluvii CECT 7697^T	80	166	2,921	2,894	57	49	2	2	26.3	2.94
A. ebronensis CECT 8441^T	103	188	3,089	3,072	47	39	3	1	29.2	3.15
A. ebronensis W129-34	126	217	3,206	3,171	46	40	3	2	29.2	3.23
A. ellisii CECT 7837^T	135	177	2,875	2,840	64	52	2	1	26.9	2.80
A. faecis LMG 28519^T	55	127	2,429	2,376	76	53	2	1	27.2	2.50
A. halophilus DSM 18005^T	111	56	2,677	2,660	54	46	3	2	27.4	2.75
A. halophilus F166-45	90	56	2,879	2,864	59	51	2	2	27.0	2.96
'A. hispanicus' FW54^T	76	148	2,228	2,207	46	40	3	1	26.4	2.21
'A. lacus' RW43-9^T	24	295	2,194	2,182	47	40	2	0	26.8	2.22
A. lanthieri LMG 28516^T	29	466	2,223	2,190	73	52	3	1	26.7	2.29
A. lanthieri AF1581	24	353	2,199	2,186	88	57	3	0	26.8	2.26
A. lekithochrous CECT 8942^T	436	343	3,628	3,316	88	75	3	0	28.6	3.61
A. lekithochrous LMG 28652	82	343	3,499	3,330	61	55	3	0	28.2	3.50
A. marinus CECT 7727^T	162	54	2,809	2,781	55	50	2	0	27.0	2.87
A. marinus F140-37	76	67	2,725	2,652	59	48	2	0	27.0	2.78
'A. mediterraneus' F156-34^T	29	689	2,769	2,750	47	41	3	1	27.3	2.83
'A. miroungae' 9Ant^T	35	363	1,868	1,847	46	41	2	1	28.1	1.84
A. molluscorum CECT 7696^T	117	121	2,746	2,736	58	49	3	6	26.1	2.76

(Continued)

TABLE 2 | Continued

Species	No. Contigs	N50 (Kb)	CDS (Total)	CDS (Coding)	RNA Genes	tRNAs	ncRNAs	CRISPR Arrays	G+C (%)	Size (Mb)
A. molluscorum F91	240	150	2,951	2,889	71	58	3	2	26.3	2.89
A. mytili CECT 7386^T	126	70	2,950	2,934	58	48	3	1	26.3	2.97
A. mytili T234	145	37	2,735	2,723	54	48	3	0	26.4	2.77
'A. neptunis' F146-38^T	36	267	2,627	2,614	57	45	3	0	27.1	2.65
A. nitrofigilis DSM 7299^T	1	–	3,101	3,086	69	55	2	1	28.4	3.19
'A. ponticus' F161-33	24	597	2,632	2,621	46	36	3	0	28.1	2.74
'A. porcinus' LMG 24487^T	70	123	2,186	2,112	47	41	2	0	27.0	2.14
'A. salis' F155-33^T	153	169	2,932	2,904	50	43	3	0	29.0	2.93
A. skirrowii LMG 6621^T	62	306	2,029	2,006	48	42	2	2	27.7	1.97
A. skirrowii F28	110	40	1,911	1,897	46	41	2	0	27.8	1.81
A. suis CECT 7833^T	122	142	2,646	2,613	57	52	2	0	27.3	2.62
A. thereius LMG 24486^T	2	1,039	1,896	1,883	57	46	2	3	27.0	1.91
A. thereius DU22	19	252	2,006	1,983	47	42	2	1	26.8	2.01
A. trophiarum CECT 7650	37	152	1,911	1,894	48	37	3	0	28.0	1.90
A. trophiarum LMG 25534^T	266	86	2,167	2,071	49	41	3	0	29.4	2.00
A. venerupis CECT 7836^T	234	182	3,319	3,267	64	52	2	0	28.0	3.28
'A. viscosus' F142-34^T	82	65	2,772	2,756	55	48	3	1	26.6	2.79
'A. vitoriensis' FW59^T	144	179	2,617	2,570	53	46	2	0	27.4	2.58
Arcobacter sp. CAB	367	20	3,596	3,392	NA	31	NA	NA	28.2	3.48
Arcobacter sp. F2176	99	178	3,212	3,186	67	57	2	0	28.1	3.27
Arcobacter sp. LA11	53	229	3,006	2,961	49	43	3	0	27.9	3.10
Arcobacter sp. LPB0137	1	–	2,731	2,698	85	64	2	0	27.7	2.87
Arcobacter sp. L[a]	1	–	2,847	2,834	73	56	2	1	26.6	2.95
Arcobacter sp. AF1028[b]	46	148	2,336	2,285	71	51	2	1	27.2	2.41

[a]*Genome sequenced in this study;* [b]*Genome obtained from NCBI database;* [c]*Genome obtained from JGI Gold database. Our results show that these strains belong to the species.* [d]*A. defluvii and* [e]*A. faecis.*

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Nuria Salas Massó

and *A. mytili*, together with two candidates, '*A. neptunis*' and '*A. viscosus.*' Finally, Cluster 4 included the species *A. anaerophilus*, *A. bivalviorum*, and *A. ebronensis*, as well as the candidates '*A. mediterraneus,*' '*A. ponticus,*' and '*A. salis.*' The split decomposition network analysis of the core genome showed that the species *A. lekithochrous* CECT 8942^T and *A. nitrofigilis* DSM 7299^T appeared as orphan species. Furthermore, with this analysis the candidatus '*A. aquaticus*' W112-28 also appeared in a separate branch near to *A. nitrofigilis* DSM 7299^T. On the other hand, both analyses, MLSA and core genome, confirmed the existence of two sub-clusters in Cluster 1 (again *A. butzleri* and '*A. lacus*' were located in the most distant branch within the cluster), and also two subgroups could be observed in Cluster 4, one comprising the species *A. anaerophilus* and *A. ebronensis*, and the other including the rest of species within this cluster (**Figures 1**, **2**). All the clusters and sub-clusters showed a similarity in the concatenated sequences of the 13 housekeeping genes higher than 85% (**Figure 2**).

Phylogenies based on the 16S and 23S rRNA gene sequences, undertaken with the NJ and ML approacheserealso constructed with comparative purposes. 16S rRNA based tree showed also the four major clusters although less defined (**Supplementary Figure S1A**). Species within Cluster 1, showed 16S rRNA gene sequence similarities ranging from 96.1 to 99.9%. Cluster 2 yielded similarities among species for the 16S rRNA gene between 96.7 and 99.6%, whereas within Cluster 3 ranged between 93.0 and 99.1%. Finally, Cluster 4 included species with a range of 16S rRNA sequence similarity from 94.0 to 99.5%. With the exception of Cluster 3, similarity values within the clusters (>94–95%) were within the classical boundaries for genus assignation in bacterial taxonomy (Rosselló-Mora and Amann, 2001; Yarza et al., 2008, 2014; Tindall et al., 2010; Figueras et al., 2011a,b). Our results agree with those from a recent study by Yarza et al. (2014), who investigated 568 taxa and described a threshold in 16S rRNA sequence identity of 94.5% for genus delineation.

Similar groups and topology, with only minor differences, were obtained when the 23S rRNA gene sequences were used to analyze the phylogeny of the genus (**Supplementary Figure S2**). In this analysis, the recently described species *A. acticola*, and *A. pacificus* could not be included because of the unavailability of the type strains and/or whole genome sequences. The same four major clusters formed in the 23S rRNA gene phylogenetic tree, and the species *A. lekithochrous* and *A. nitrofigilis* appeared also as orphan species (**Supplementary Figure S2**). Within Cluster 1 two subgroups could also be obtained, differentiating the species *A. butzleri* and '*A. lacus*' from the rest of the species. Similarly, the species *A. anaerophilus* and *A. ebronensis* formed a differentiated subgroup in Cluster 4.

The visual analysis of the alignments obtained with the sequences of the 16S and 23S rRNA genes allowed the localization of signature motifs, especially in the 16S rRNA gene, for the different clusters established in the phylogenetic analysis. In these sequences, a total of 16 locations were found, presenting nucleotide combinations characteristic for the clusters (**Supplementary Figure S3**). Some of these motifs were located in helix regions as interactions with proteins of the ribosomal 30S subunit, such as helix 21 (region V4) or helix 28/44 (region V9), and therefore had a considerable level of protection against mutations (Adilakshmi et al., 2008; Kitahara et al., 2012). There are some studies on the presence of signature regions with taxonomic/phylogenetic implications in the ribosomal genes (Martínez-Murcia et al., 1992, 2007; Ue et al., 2011;Řeháková et al., 2014; Martínez-Murcia and Lamy, 2015). Some regions with signature motifs detected in the present study have also shown implications for phylogenetic analysis in cyanobacteria, including regions H15, H17, H21, H22-H23, H41, and H44 (Řeháková et al., 2014). A tree was also constructed weighting such positions (**Supplementary Figure S1B**), which allowed a better definition of the main clusters observed with the whole 16S rRNA sequences although, as expected, differentiation among species within each cluster was lower. Two sub-clusters were observed in Cluster 1, where the species *A. butzleri* and '*A. lacus*' grouped into a well-differentiated branch with respect to the other species in the cluster (**Supplementary Figure S1B**). In this analysis, *A. pacificus* was clearly located in the Cluster 3, whereas in Cluster 4, *A anaerophilus* was the borderline species, while *A. ebronensis* and '*A. mediterraneus*' were located in an independent branch (**Supplementary Figure S1B**). Therefore, the signature motifs described here might be a new tool for identification of the different clusters and/or genus.

Genomic Indices

The results of the calculations of the ANI and the *is*DDH among the 36 studied genomes are given in the **Supplementary Table S2** and **Supplementary Figure S4**. The results of the ANI and *is*DDH calculations showed that the genomes grouped into the same clusters observed by the analyses of the MLSA of the 13 housekeeping and core genes (**Figures 1**, **2**). Ranges of ANI within each cluster were from 75.2 to 95.4%, whereas *is*DDH values were between 19.5 and 65.4% (**Figure 2** and **Table 3**). These results confirm the phylogenetic analysis for the 13 new candidate species because all of them showed ANI and *is*DDH values of <96% and <70%, respectively, which are the cut-off values proposed for the delineation of new species (Konstantinidis and Tiedje, 2005; Goris et al., 2007; Richter and Rosselló-Móra, 2009; Figueras et al., 2017). As discussed in other studies, the ANI and *is*DDH indices provided reliable information for the delineation of *Arcobacter* species and are also included in the minimal guidelines to define species using genomes (Whiteduck-Léveillée et al., 2015, 2016; Figueras et al., 2017; Chun et al., 2018). Although those indices are not considered useful for delimiting genera, each of the four clusters showed values that ranged between 75.2 and 81.8% as their lowest ANI, which might be the suitable range for separating different, closely related genera. These values are relatively similar to those reported by Qin et al. (2014) that found 68–82% interspecies ANI values among the genera that they studied. Values of ANI obtained for the candidate species '*A. aquaticus*' were lower than the other results, from 70.0% with *A. cryaerophilus* LMG 24291^T to 71.9% with *A. bivalviorum* CECT 7835^T and more in line with the Qin et al. (2014) results of 68% (**Supplementary Table S2**). In the case of the *is*DDH the lower values among species in the same cluster ranged between 19.5 and 24.8%, and again these might be the levels associated to different genera.

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Nuria Salas Massó

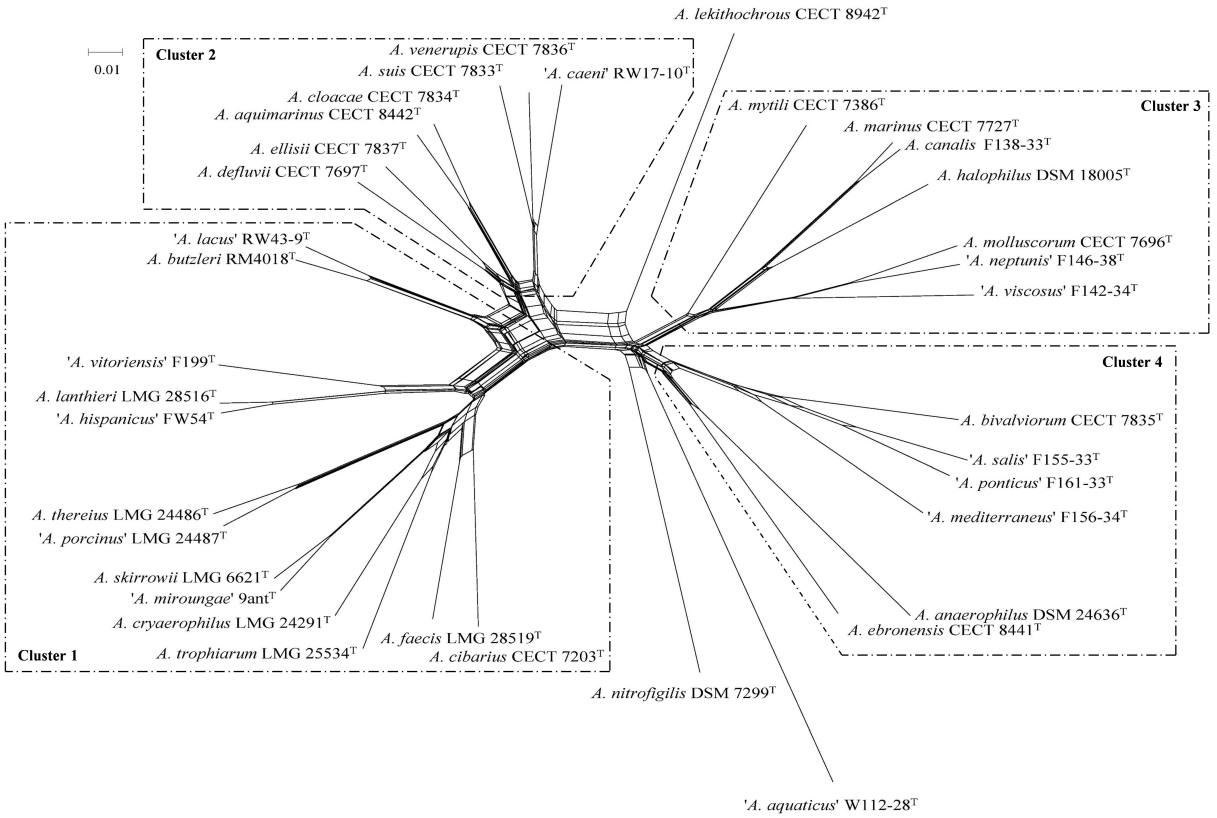

FIGURE 1 | Split decomposition network constructed with the concatenated sequences of 284 core genes from the genomes of 36 type and representative strains of *Arcobacter*. Scale bar, base substitutions per site.

With the aim of confirming if the clusters observed might represent different genera, as suggested by the phylogenetic analyses, the similarity indices AAI and POCP were also calculated (**Supplementary Table S3**). In agreement with the 60–80% AAI that have been described for species belonging to the same genus (Konstantinidis and Tiedje, 2005) all our clusters showed lower ranges of between 67.6 to 80.3% (**Table 3**). All the clusters also complied with the POCP proposed for genus separation above 50% (Luo et al., 2014; Qin et al., 2014) because as shown in **Table 3** all clusters showed the lowest values from 67.0 to 75.4%.

It is widely known that synonymous codon usage varies among organisms and that it is related to differences in G+C content, replication strand skew, or gene expression (Suzuki et al., 2008; Farooqi et al., 2016). The interaction of these factors may vary among species depending on their evolutionary process (Ma et al., 2015). It has also been suggested that the extent of codon usage bias plays a role in the adaptation of prokaryotic organisms to their environments and lifestyles (Botzman and Margalit, 2011). To analyze the overall codon usage trends of the *Arcobacter* species, the frequencies of the different codons were obtained from the whole genomes and the RSCU was computed using the CAI, which is a useful tool for estimating codon usage bias (Ma et al., 2015; Farooqi et al., 2016). A first finding was that all the *Arcobacter* species presented a preferential use of the codons finishing in A or T (**Supplementary Figure S5**), which might be expected due to their low G+C% content. The characteristic pattern showed by *A. aquaticus* is noteworthy (**Supplementary Figure S5**), which supports its differentiation from the other species in Cluster 3 as well as its unique taxonomy. Such difference was the only statistically significant ($p < 0.05$) in the multinomial regression analysis carried out.

Next, the codon usage trends were analyzed by PCA to reveal possible evolutionary relationships. Interestingly, different groups of strains could be observed in the three-dimensional graphic (**Figure 3**), which correlated with those clusters established in the different phylogenetic analyses, as shown above. As reported previously for different species of *Mycoplasma* (Marenda et al., 2005; Ma et al., 2015), PCA provides an additional pathway to investigate the evolutionary direction of the *Arcobacter* species. In addition, similarities in the synonymous codon usage patterns might reflect similar lifestyles (pathogenic vs. non-pathogenic) and adaptation to certain environments (marine water, shellfish, etc.).

Metabolic Inference and Phenotypic Analysis

Phylogenetic and genomic analysis confirmed the existence of four clusters among the validated and candidate *Arcobacter*

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Nuria Salas Massó

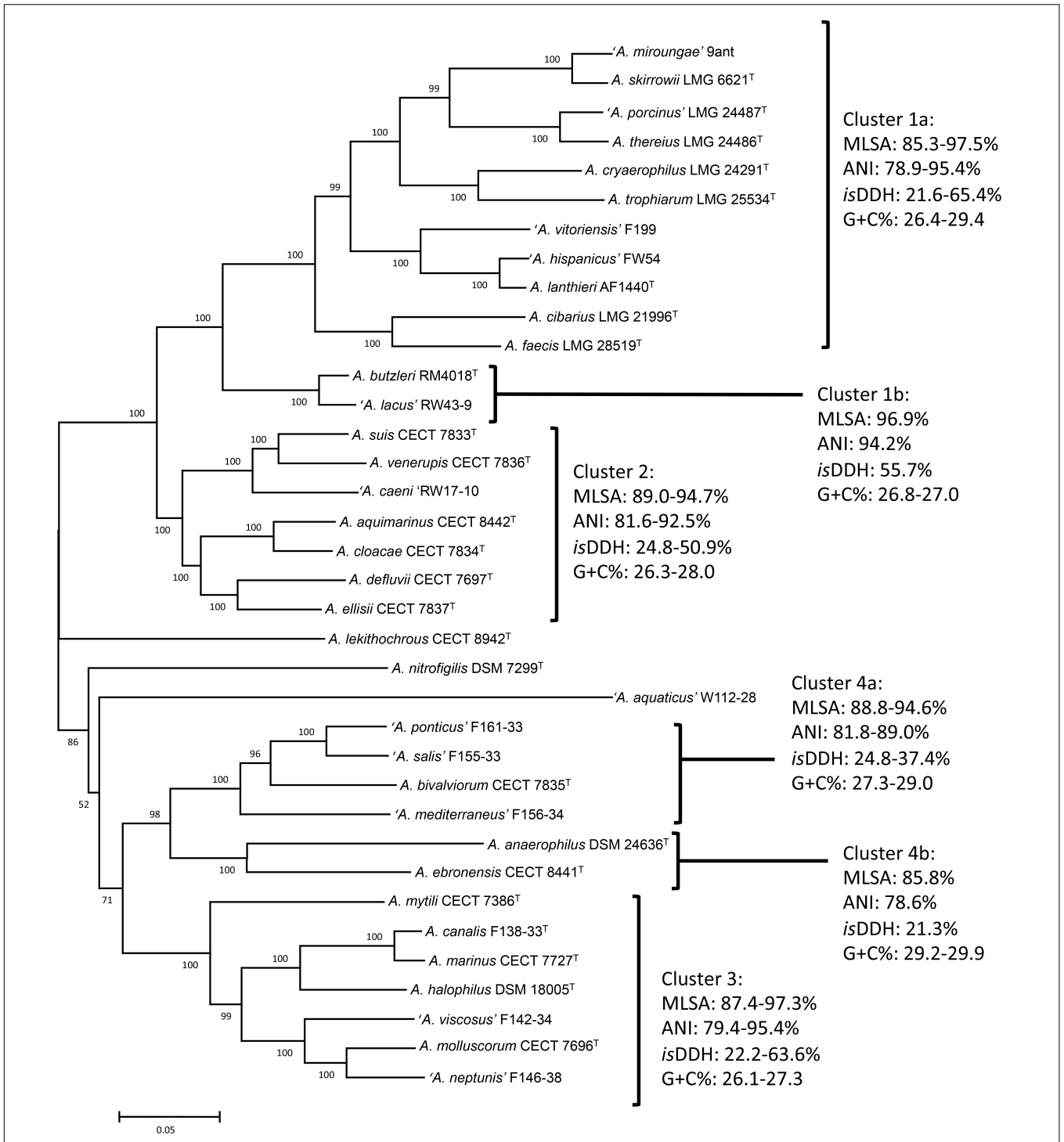

FIGURE 2 | Phylogenetic tree constructed with 36 type and representative strains of *Arcobacter* species based on concatenated sequences of 13 housekeeping genes by the Maximum-Likelihood algorithm (model GTR+G+I). Numbers at nodes denote the level of bootstrap based on 1,000 replicates; only values greater than 50% are shown. Scale bar, base substitutions per site.

species, which comply with the cut-off values established for the differentiation of independent genera. A thorough phenotypic analysis was therefore carried out to determine if the description of new taxa at genus level was possible or if such clusters were only clades or genomovars within the genus *Arcobacter*. In fact, this is what has occurred in a recent polyphasic study of 52 *A. cryaerophilus* strains (including genome information) in which, despite four different genomospecies

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Nuria Salas Massó

TABLE 3 | Intra-cluster similarities (%) obtained for the 16S rRNA gene and for the different genomic indexes analyzed.

	16S RNA gene		MLSA		ANI		*is*DDH		AAI		POCP		G+C% (mol)	
Cluster 1a	96.8–99.8	96.1–99.9	85.3–97.5	85.3–97.5	78.9–95.4	77.7–95.4	21.6–65.4	20.5–65.4	72.5–95.0	71.5–95.0	68.2–95.6	67.0–95.6	26.4–29.4	26.4–29.4
Cluster 1b	99.9		96.9		94.2		55.7		93.7		84.3		26.8–27.0	
Cluster 2	96.7–99.6		89.0–94.7		81.6–92.5		24.8–50.9		73.1–93.5		71.7–87.5		26.3–28.0	
Cluster 3	94.2–99.1		87.4–97.3		79.4–95.4		22.2–63.6		67.6–95.7		75.4–91.4		26.1–27.3	
Cluster 4a	96.6–99.5	94.0–99.5	88.8–94.6	85.8–94.6	81.8–89.0	75.2–89.0	24.8–37.4	19.5–37.4	80.3–83.4	68.7–83.4	74.4–90.7	71.6–90.7	27.3–29.0	27.3–29.9
Cluster 4b	96.9		85.8		78.6		21.3		78.4		77.9		29.2–29.9	

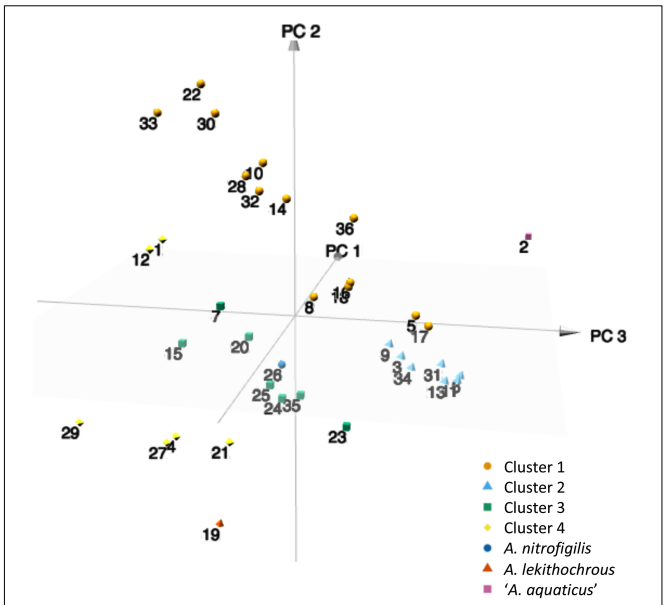

FIGURE 3 | 3D plot of the three major axes generated by principal component analysis (PCA) of the RSCU values computed for the 36 type and representative strains of *Arcobacter* species. 1, *A. anaerophilus* DSM 24636^T; 2, '*A. aquaticus*' W112-28; 3, *A. aquimarinus* CECT 8442^T; 4, *A. bivalviorum*; 5, *A. butzleri* RM4018^T; 6, '*A. caeni*' RW17-10; 7, *A. canalis* CECT 8984^T; 8 *A. cibarius* LMG 21996^T; 9, *A. cloacae* CECT 7834^T; 10, *A. cryaerophilus* LMG 24291^T; 11, *A. defluvii* CECT 7697^T; 12, *A. ebronensis* F128-2^T; 13, *A. ellisii* CECT 7837^T; 14, *A. faecis* AF1078^T; 15, *A. halophilus* DSM 18005^T; 16, '*A. hispanicus*' FW54; 17, '*A. lacus*' RW43-9; 18, *A. lanthieri* AF1440^T; 19, *A. lekithochrous* LFT1.7^T; 20, *A. marinus* CECT 7727^T; 21, '*A. mediterraneus*' F156-34; 22, '*A. miroungae*' 9Ant; 23, *A. molluscorum* CECT 7696^T; 24, *A. mytili* W112-28; 25, '*A. neptunis*' F146-38; 26, *A. nitrofrigilis* DSM 7299^T; 27, '*A. ponticus*' F161-33; 28, '*A. porcinus*' LMG 24487; 29, '*A. salis*' F155-33; 30, *A. skirrowii* LMG 6621^T; 31, *A. suis* CECT 7833^T; 32, *A. thereius* LMG 24486^T; 33, *A. trophiarum* LMG 25534^T; 34, *A. venerupis* CECT 7836^T; 35, '*A. viscosus*' F142-34; 36, '*A. vitoriensis*' F199.

being recognized, the phenotypic characterization did not allow their differentiation into separate species and were therefore considered genomovars (Pérez-Cataluña et al., 2018a).

Phenotypic inference using Traitar confirmed the lack of reaction of *Arcobacter* species to most of the tests commonly used for bacterial identification (**Supplementary Figure S6**). Thus, all the type and representative strains rendered negative results, regardless of the predictor employed, for use as the sole carbon source of sugars (D-Mannitol, D-Mannose, Salicin, or Trehalose, among others) and carboxylic acids (Citrate or Malonate). Such results have been previously reported in the original descriptions of the species (see review of On et al., 2017). On the other hand, there was some incongruence between results from Traitar and those obtained by classical characterization for some tests, including growth on MacConkey agar or urea hydrolysis (data not shown). A possible explanation is related with the macro-accuracy of the predictors employed in the Traitar analysis (82.6–85.5%), as reported in the original description of the microbial trait analyzer (Weimann et al., 2016). The fact that

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Nuria Salas Massó

some of the *Arcobacter* species studied are halophilic cannot be ignored, since some of the media usually employed in the wet-lab characterization are developed for non-halophilic microorganisms.

The heat maps built from the combined results of both predictors in the Traitar analysis revealed the existence of similarity groups regarding the metabolic characteristics of the *Arcobacter* type strains (**Supplementary Figure S6**). In most case, clustering of strains supported the groups obtained with genomic tools, although some incongruence was also observed, such as for *A. butzleri* (better related here to *A. defluvii, A. ellisii* or *A. cloacae*), *A. mytili* (closest Traitar species '*A. caeni*') or *A. venerupis* (forming a branch with *A. ebronensis* and '*A. ponticus*'). In any case, Traitar might be helpful as a first-step method for phenotypic inference, although further verification should be made, especially in environmental bacterial species with special growth requirements (i.e., halophilic conditions).

A deep review of the characteristics reported in the original descriptions of the *Arcobacter* species, together with results obtained in our respective laboratories, allowing phenotypic traits to differentiate the clusters established by the phylogenetic and genomic analyses (**Table 4**). Growth at 37°C in microaerophilic condition, the halophilic character, the ability to grow in presence of glycine, safranin, oxgall, or triphenyltetrazolium chloride (TTC), the presence of some enzymatic activities, such as catalase, urease or indoxyl acetate hydrolysis, and resistance to cefoperazone among others, were the main differentiating traits. Most of these characters are included in the minimal standards for describing new species in the families *Campylobacteraceae* and *Helicobacteraceae* (On et al., 2017), and they should, therefore, also be maintained for the new family *Arcobacteraceae* proposed by Waite et al. (2017), once this taxonomical change is validated. The phenotypic differentiation proposed in **Table 4** enabled to further describe the new genera that corresponded to the different clusters of *Arcobacter* species determined in the present study.

Stability of the Genomic-Based Clustering

In order to test the stability of the new taxonomical scheme proposed, we analyzed the whole genome sequences using second strains from each species or from unassigned sequences obtained from the public databases. That analysis is shown in **Supplementary Figure S7** and included 55 genomes. These new phylogenetic analyses of the core genome also using a Split network showed that the four clusters were maintained, but the two clusters (Clusters 3 and 4) that include species able to grow in media containing 2.5% NaCl appeared in the right place (**Supplementary Figure S7**). The genome of *Arcobacter* sp. LPB0137 obtained from the NCBI database grouped with the species *A. lekithochrous* CECT 8942^T, while the genomes *Arcobacter* sp. LA11 and CAB grouped together in a separate branch near to Cluster 4. Interestingly, the ANI and *is*DDH values of 91.4% and 45.8% between strain F2176, previously identified as *A. nitrofigilis* (Figueras et al., 2008), and the type strains of this species along with the phylogenetic position (**Supplementary Figure S7**), revealed that this strain belonged to another potentially new species. Furthermore, strains L and AF1028, deposited at the NCBI database as *Arcobacter* sp. were identified as *A. defluvii* and *A. faecis*, respectively, because they clustered with the type strains of those species (**Supplementary Figure S7**). This was also confirmed by the ANI and *is*DDH results being above 96% and 70%, respectively.

Collado and Figueras (2011), in their review about the epidemiology and clinical significance of the genus *Arcobacter*, reported that these bacteria should be considered quite atypical within the class *Epsilonproteobacteria* because of the great diversity of hosts and habitats from which they have been isolated. In order to show if the clusters obtained have a relationship with their ecological habitat, the origin of each strain is also given in **Supplementary Figure S7**. Despite the fact that only two strains from each species were included in the analysis, each of the clusters embraced species that had been recovered from common or related origins. Cluster 1 included by strains isolated from humans and animals, from wastewater and from broiler skin (*A. cibarius* CECT 7203^T). The fact that some strains isolated from wastewater that was contaminated by humans or animal excreta, gives evidence of the relationship of these sources. This finding agrees with the high abundance of *Arcobacter* in wastewater and in water contaminated with fecal pollution (Collado et al., 2008, 2010). Among the species of Cluster 1, both by metagenomics analysis or direct plating without enrichment (Fisher et al., 2014; Levican et al., 2016), the species *A. cryaerophilus* was the prevalent species in wastewater, while the species *A. butzleri* is normally predominant in studies that investigate water and food samples of animal origin, such as different types of meats using an enrichment step (Collado et al., 2009b; Collado and Figueras, 2011; Hsu and Lee, 2015; and references therein). So far, only the species *A. cryaerophilus*, *A. thereius*, *A. trophiarum, A. cibarius* or *A. skirrowii* have been recovered from humans or animals (De Smet et al., 2011; Figueras et al., 2014; Van den Abeele et al., 2014) and all these species are as commented in the same cluster.

Cluster 2 included strains from different origins but was dominated by species that came from wastewater, shellfish or food products. In this sense, *A. defluvii* CECT 7697^T and '*A. caeni*' RW17-10 were isolated from wastewater, while the strain *A. defluvii* L was recovered from a microbial fuel cell. Strains of *A. defluvii* have also been recovered from shellfish in other studies (Levican et al., 2014; Salas-Massó et al., 2016). The strain *A. suis* CECT 7833^T was isolated from pork meat, but other isolates have also been obtained from buffalo milk in Italy (Levican et al., 2013; Giacometti et al., 2015). The other five strains in the cluster were isolated from shellfish, wastewater and seawater (**Table 1** and **Supplementary Figure S7**). The other two clusters (Clusters 3 and 4) included strains isolated from seawater shellfish giving evidence of the marine origin of these clusters. The orphan species (*A. nitrofigilis* DSM7299^T, *A. lekithochrous* CECT 8942^T, and '*A. aquaticus*' W112-28) also corresponded

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Nuria Salas Massó

TABLE 4 | Differential phenotypic traits among the different clusters of *Arcobacter* species obtained on the basis of the characteristics of the type and representative strains of the species included in each group.

Test	*A. nitrofigilis*	Cluster 1	Cluster 2	Cluster 3	Cluster 4	*A. lekithochrous*	*A. aquaticus*
Growth at/on							
CO_2 37°C	−	V	V	+	V	−	+
0.5% NaCl	−	+[a]	+	V	−[b]	−[c]	+
4% NaCl	+	−	−	+	+	−	−
1% Glycine	−	V	−	V	V	−	−
0.05% Safranin	−	+	V	V	V	+	+
0.04 TTC	−	V	−	−	−	+	−
1% Oxgall	−	V	V	−	−[d]	−	−
CCDA	−	V	V	−[e]	−	+	+
Enzymatic activities							
Catalase	−	+[f]	+	V	V	+	−
Urease	+	−	V	−	−[d]	−	−
Indoxyl acetate hydrolysis	+	+[f]	+	V	V	−	−
Nitrate reduction	+	V	+	−[g]	V	−	−
Resistance to cefoperazone (64 mg/l)	ND	V	−	V	−	−	+

+, positive result; −, negative result; V, variable result in all the species of the cluster; [a]With the exception of A. skirrowii; [b]With the exception of A. pacificus; [c]A. lekithochrous needs sea salts to grow; [d]With the exception of A. ebronensis; [e]With the exception of A. molluscorum; [f]With the exception of A. cibarius; [g]With the exception of A. anaerophilus. ND, not determined.

to strains isolated from marine environments and their phylogenetic position was close to the two marine clusters (3 and 4).

As indicated in the review by Collado and Figueras (2011), there are many uncultured or not-yet-described species of *Arcobacter*, which have been recognized on the basis of nearly full-length 16S rRNA gene sequences, and which probably outnumber those species that were already known at that time. Their hosts and/or habitats are very diverse and include cod larvae, cyanobacterial mats, activated sludge, tidal and marine sediments, estuarine and river water, plankton, coral, tubeworms, snails, etc. (Collado et al., 2011; and references therein). In the near future new species can be expected to emerge that will reinforce the value of the different genera proposed in this study.

CONCLUSION

Genomic information obtained through next-generation sequencing leads to great advances in the systematics of prokaryotes (Whitman, 2015), not only to the general understanding of prokaryotic biology but also for the resolution of the phylogeny of taxa higher than species. Single gene phylogeny, including 16S rRNA gene, has often limitations that analysis of complete genome sequences can overcome. The study aims to use this modern taxonomy approach to clarify the relationships of the diverse *Arcobacter* species.

The results obtained in the present study confirmed the opinion of some authors on the need for a clarification of the taxonomy of the genus *Arcobacter*. The phylogenetic analyses derived from the MLSA of 13 genes and of the core genome as well as the existence of signature regions in the 16S rRNA gene have shown, together with the genomic indexes ANI (75.2–81.8%), *is*DDH (19.5–24.8%), AAI (67.6–80.3%), and POCP (67.0–75.4%), to be useful tools for delimiting several genomic and phylogenetic groups within this genus. The intra-genus ranges and cut-off values established here might also be helpful for future taxonomic studies in other bacterial groups.

Such genomic variability, together with the determination of combinations of differentiating phenotypic traits allowed the division of the current genus *Arcobacter* in at least six different genera for which the names *Aliiarcobacter* gen. nov., *Pseudoarcobacter* gen. nov., *Haloarcobacter* gen. nov., *Malacobacter* gen. nov., and *Poseidonibacter* gen. nov. are proposed. In addition, the candidate species '*A. aquaticus*' also constitutes a new genus for which the name Candidate '*Arcomarinus*' gen. nov. is proposed, although such proposal should be formulated in parallel to the formal description of the species.

According to Tindall et al. (2010) "*the type strain of a genus is the most important reference organism to which a novel species has to be compared.*" In the case of the genus *Arcobacter*, the type species has rarely been isolated (Collado et al., 2009b; Toh et al., 2011; Levican et al., 2016; Salas-Massó et al., 2016) and in fact, all the analyses show that *A. nitrofigilis* is an orphan species and the only representative of the genus *Arcobacter*, for which an emended description is provided.

The other genera are described here while taking into account the species validated at the time of writing but with the confidence that the formal description of the candidate species would fit in such descriptions. Thus, the genus *Aliiarcobacter* gen. nov. is described comprising seven species *Aliiarcobacter cryaerophilus* comb. nov., *A. butzleri* comb. nov., *A. skirrowii* comb. nov., *A. cibarius* comb. nov., *A. thereius* comb. nov., *A. trophiarum* comb. nov., *A. lanthieri* comb. nov., and *A. faecis* comb. nov. On the other hand, the genus *Pseudoarcobacter* gen. nov. includes

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Nuria Salas Massó

the species *Pseudoarcobacter defluvii* comb. nov., *P. ellisii* comb. nov., *P. venerupis* comb. nov., *P. cloacae* comb. nov., *P. suis* comb. nov., *P. aquimarinus* comb. nov., and *P. acticola* comb. nov. Four species, *Malacobacter halophilus* comb. nov., *M. mytili* comb. nov., *M. marinus* comb. nov., *M. molluscorum* comb. nov., and *M. pacificus* comb. nov. are compiled in the new genus *Malacobacter* gen. nov., whereas the genus *Haloarcobacter* gen. nov. comprises three species *Haloarcobacter bivalviorum* comb. nov., *H. anaerophilus* comb. nov., and *H. ebronensis* comb. nov. Finally, the genus *Poseidonibacter* gen. nov. has a unique species *Poseidonibacter lekithochrous* comb. nov.

Emended Description of the Genus *Arcobacter* Vandamme et al., 1991 emend. Vandamme et al., 1992 and Sasi-Jyothsna et al., 2013

Arcobacter (Ar'co.bac.ter. L. n. *arcus*, bow; Gr. n. *bacter*, rod; M. L. masc. n. *Arcobacter*, bow-shaped rod).

Cells are Gram-negative, curved rods 0.2–0.9 μm in diameter and 1–3 μm long. Coccoid bodies are found in old cultures but are not rapidly produced under aerobic conditions. Motile with a rapid corkscrew motion. Each cell possesses a single polar flagellum. Does not swarm. Chemoorganotrophic. Utilizes organic and amino acids as carbon sources, but not carbohydrates. Respiratory metabolism with oxygen as the terminal electron acceptor; anaerobic growth with aspartate and fumarate, but not with nitrate. Nitrate usually reduced to nitrite. Requires NaCl for growth. Grows at temperatures of 10°C–35°C but not at 42°C. Catalase, oxidase, urease, and nitrogenase positive. Phosphatase, sulfatase and indole negative. Does not hydrolyze esculin, casein, DNA, gelatine, hippurate or starch. Fluorescent pigments are not produced. Unable to grow with glycine (1% wt/vol), safranin (0.05% wt/vol), oxgall (1% wt/vol), or 2,3,5-triphenyltetrazolium chloride (0.04%, wt/vol). Positive for the hydrolysis of indoxyl acetate. Poly-β-hydroxybutyrate not produced.

The base composition of the DNA is 28.1–28.4% G+C as determined from the genomes.

The type species is *Arcobacter nitrofigilis*.

Description of *Aliiarcobacter* gen. nov.

Aliiarcobacter (A.li.i.ar.co.bac'ter, L. pronoun *alius* other, another; N.L. masc. n. *Arcobacter* a bacterial generic name; N.L. masc. n. *Aliiarcobacter* the other *Arcobacter*).

Cells are Gram-negative, curved rods 0.2–0.5 μm in diameter and 1–3 μm long. Motile by single polar flagellum. Does not swarm. Chemoorganotrophic. Oxidase and catalase positive. No growth occur at 4% NaCl. Growth occurs at 15°C–42°C. Carbohydrates are not fermented. Nitrate usually reduced to nitrite. Positive for the hydrolysis of indoxyl acetate and negative for urease. Growth does not occur in the presence 2,3,5-triphenyltetrazolium chloride (0.04%, wt/vol) or glycine (1% wt/vol). Some species may grow in the presence of safranin (0.05% wt/vol) or oxgall (1% wt/vol). Fluorescent pigments are not produced. Some species are sensitive to cefoperazone (64 mg/l). Range of DNA G+C content is 26.4–29.4 mol%.

The type species is *Aliiarcobacter cryaerophilus*.

Description of *Aliiarcobacter cryaerophilus* comb. nov.

Basonym: *Campylobacter cryaerophila* Neill et al., 1985.

Other synonym: *Arcobacter cryaerophilus* Vandamme et al., 1991.

The description is the same given by Neill et al. (1985). The type strain is A169/B^T (= NCTC 1185^T = ATCC 43158^T).

Description of *Aliiarcobacter butzleri* comb. nov.

Basonym: *Campylobacter butzleri* Kiehlbauch et al., 1991.

Other synonym: *Arcobacter butzleri* Vandamme et al., 1992.

The description is the same given by Vandamme et al. (1992). The type strain is LMG 10828^T (= CDC D2686^T = ATCC 49616^T).

Description of *Aliiarcobacter skirrowii* comb. nov.

Basonym: *Arcobacter skirrowii* Vandamme et al., 1992.

The description is the same given by Vandamme et al. (1992). The type strain is Skirrow 449/80^T (= LMG 6621^T = CCUG 10374^T).

Description of *Aliiarcobacter cibarius* comb. nov.

Basonym: *Arcobacter cibarius* Houf et al., 2005.

The description is the same given by Houf et al. (2005). The type strain is LMG 21996^T (= CCUG 48482^T).

Description of *Aliiarcobacter thereius* comb. nov.

Basonym: *Arcobacter thereius* Houf et al., 2009.

The description is the same given by Houf et al. (2009). The type strain is LMG 24486^T (= CCUG 56902^T).

Description of *Aliiarcobacter trophiarum* comb. nov.

Basonym: *Arcobacter trophiarum* De Smet et al., 2011.

The description is the same given by De Smet et al. (2011). The type strain is 64^T (= LMG 25534^T = CCUG 59229^T).

Description of *Aliiarcobacter lanthieri* comb. nov.

Basonym: *Arcobacter lanthieri* Whiteduck-Léveillée et al., 2015.

The description is the same given by Whiteduck-Léveillée et al. (2015). The type strain is AF1440^T (= LMG 28516^T = CCUG 66485^T).

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Nuria Salas Massó

Description of *Aliiarcobacter faecis* comb. nov.

Basonym: *Arcobacter faecis* Whiteduck-Léveillée et al., 2016.

The description is the same given by Whiteduck-Léveillée et al. (2016). The type strain is AF1078^T (= LMG 28519^T = CCUG 66484^T).

Description of *Pseudoarcobacter* gen. nov.

Pseudoarcobacter (Pseu.do.ar.co.bac'ter, Gr. adj. *pseudes*, false; N.L. masc. n. *Arcobacter* a bacterial generic name; N.L. masc. n. *Pseudoarcobacter*, false *Arcobacter*).

Gram-negative, cells are rod shaped and motile. Cell size 0.2–0.9 μm in diameter and 0.4–2.2 μm long. Some species may present cells up to 10 μm in length. Oxidase and catalase positive. No growth occurs at 4% NaCl. Growth occurs at 15–37°C, but not at 42°C. Carbohydrates are not fermented. Reduce nitrate to nitrite. Positive for the hydrolysis of indoxyl acetate. Some species may hydrolyze urea. Growth does not occur in the presence 2,3,5-triphenyltetrazolium chloride (0.04%, wt/vol) or glycine (1% wt/vol). Some species may grow in the presence of safranin (0.05% wt/vol) or oxgall (1% wt/vol). Sensitive to cefoperazone (64 mg/l). Range of DNA G+C content is 26.3–28.0 mol%.

The type species is *Pseudoarcobacter defluvii.*

Description of *Pseudoarcobacter defluvii* comb. nov.

Basonym: *Arcobacter defluvii* Collado et al., 2011.

The description is the same given by Collado et al. (2011). The type strain is SW28-11^T (= CECT 7697^T = LMG 25694^T).

Description of *Pseudoarcobacter ellisii* comb. nov.

Basonym: *Arcobacter ellisii* Figueras et al., 2011b.

The description is the same given by Figueras et al. (2011b). The type strain is F79-6^T (= CECT 7837^T = LMG 26155^T).

Description of *Pseudoarcobacter venerupis* comb. nov.

Basonym: *Arcobacter venerupis* Levican et al., 2012.

The description is the same given by Levican et al. (2012). The type strain is F67-11^T (= CECT 7836^T = LMG 26156^T).

Description of *Pseudoarcobacter cloacae* comb. nov.

Basonym: *Arcobacter cloacae* Levican et al., 2013.

The description is the same given by Levican et al. (2013). The type strain is SW28-13^T (= CECT 7834^T = LMG 26153^T)

Description of *Pseudoarcobacter suis* comb. nov.

Basonym: *Arcobacter suis* Levican et al., 2013.

The description is the same given by Levican et al. (2013). The type strain is F41^T (= CECT 7833^T = LMG 26152^T).

Description of *Pseudoarcobacter aquimarinus* comb. nov.

Basonym: *Arcobacter aquimarinus* Levican et al., 2015.

The description is the same given by Levican et al. (2015). The type strain is W63^T (= CECT 8442^T = LMG 27923^T).

Description of *Pseudoarcobacter acticola* comb. nov.

Basonym: *Arcobacter acticola* Park et al., 2016.

The description is the same given by Park et al. (2016). The type strain is AR-13^T (= KCTC 52212^T = NBRC 112272^T).

Description of *Malacobacter* gen. nov.

Malacobacter (Ma.la.co.bac'ter; Gr. n. *malaco*, soft, with soft boy, mollusc; Gr. n. *bacter*, rod; N.L. masc. n. *Malacobacter*, bacteria isolated from molluscs).

Gram-negative, cells are rod shaped and motile. Cell size 0.1–0.6 μm wide and 0.5–3.6 μm long. Oxidase positive and catalase variable among species. Halophilic, no growth can be obtained without NaCl and capable to grow up to 4% NaCl. Growth occurs at 15°C–37°C. Does not grow at 37°C in microaerophilic conditions nor at 42°C in anaerobiosis. Carbohydrates are not fermented. Does not reduce nitrate to nitrite. Negative for the hydrolysis of urea. Some species may hydrolyze indoxyl acetate. Growth does not occur in the presence of oxgall (1% wt/vol) or 2,3,5-triphenyltetrazolium chloride (0.04%, wt/vol). Some species may grow in the presence of glycine (1% wt/vol) or safranin (0.05% wt/vol). Sensitive to cefoperazone (64 mg/l) variable among species. Range of DNA G+C content is 26.1–27.3 mol%.

The type species is *Malacobacter halophilus.*

Description of *Malacobacter halophilus* comb. nov.

Basonym: *Arcobacter halophilus* Donachie et al., 2005.

The description is the same given by Donachie et al. (2005). The type strain is LA31B^T (= ATCC BAA-1022^T = CIP 108450^T).

Description of *Malacobacter mytili* comb. nov.

Basonym: *Arcobacter mytili* Collado et al., 2009a.

The description is the same given by Collado et al. (2009a). The type strain is F2075^T (= CECT 7386^T = LMG 24559^T).

Description of *Malacobacter marinus* comb. nov.

Basonym: *Arcobacter marinus* Kim et al., 2010.

The description is the same given by Kim et al. (2010), with the exception of variable result among strains for the hydrolysis of the indoxyl-acetate under microaerobic conditions (Salas-Massó et al., 2016). The type strain is CL-S1^T (= KCCM 90072^T = JCM 15502^T).

UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Nuria Salas Massó

Description of *Malacobacter canalis* comb. nov.

Basonym: *Arcobacter canalis* Pérez-Cataluña et al., 2018b.

The description is the same given by Pérez-Cataluña et al. (2018b). The type strain is F138-33^T (= CECT 8984^T = LMG 29148^T).

Description of *Malacobacter molluscorum* comb. nov.

Basonym: *Arcobacter molluscorum* Figueras et al., 2011a.

The description is the same given by Figueras et al. (2011a). The type strain is F98-3^T (= CECT 7696^T = LMG 25693^T).

Description of *Malacobacter pacificus* comb. nov.

Basonym: *Arcobacter pacificus* Zhang et al., 2015.

The description is the same given by Zhang et al. (2015). The type strain is SW028^T (= DSM 25018T = JCM 17857^T = LMG 26638^T).

Description of *Haloarcobacter* gen. nov.

Haloarcobacter (Ha.lo.ar.co.bac'ter, Gr. n. *halo*, salt; N.L. masc. n. *Arcobacter*, a bacterial generic name; N.L. masc. n. *Haloarcobacter, Arcobacter* salt loving).

Gram-negative, cells are rod shaped and motile. Cell size 0.1–0.5 μm in diameter and 0.9–2.5 μm in length. Oxidase positive and catalase variable among species. Halophilic, growth can be obtained within the range of 0.5% (variable among species) and up to 4% NaCl. Growth occurs at 15–42°C. Growth at 37°C in microaerophilic conditions or at 42°C in anaerobiosis variable among species. Carbohydrates are not fermented. Some species may reduce nitrate to nitrite. Negative for the hydrolysis of urea (with the exception of *H. ebronensis*). Some species may hydrolyze indoxyl acetate. Growth does not occur in the presence of oxgall (1% wt/vol) (with the exception of *H. molluscorum*) or 2,3,5-triphenyltetrazolium chloride (0.04%, wt/vol). No growth on CCDA. Some species may grow in the presence of glycine (1% wt/vol) or safranin (0.05% wt/vol). Sensitive to cefoperazone (64 mg/l). Range of DNA G+C content is 27.3–29.9 mol%.

The type species is *Haloarcobacter bivalviorum*.

Description of *Haloarcobacter bivalviorum* comb. nov.

Basonym: *Arcobacter bivalviorum* Levican et al., 2012.

The description is the same given by Levican et al. (2012). The type strain is F4^T (= CECT 7835^T = LMG 26154^T).

Description of *Haloarcobacter anaerophilus* comb. nov.

Basonym: *Arcobacter anaerophilus* Sasi-Jyothsna et al., 2013.

The description is the same given by Sasi-Jyothsna et al. (2013). The type strain is JC84^T (= KCTC 15071^T = MTCC 10956^T = DSM 24636^T).

Description of *Haloarcobacter ebronensis* comb. nov.

Basonym: *Arcobacter ebronensis* Levican et al., 2015.

The description is the same given by Levican et al. (2015). The type strain is F128-2^T (= CECT 8441^T = LMG 27922^T).

Description of *Poseidonibacter* gen. nov.

Poseidonibacter (Po.se.i.do.ni.bac'ter, Gr. n. *Poseidon*, God of the sea; Gr. n. *bacter*, rod; N.L. masc. n. *Poseidonibacter* referring to the marine habitat of this bacteria).

Gram-negative, cells are rod shaped and motile. Oxidase and catalase positive. Halophilic, no growth can be obtained without seawater or the addition of combined marine salts to the medium. Growth occurs at 15°C–25°C, but not at 37°C or 42°C. Range of pH for growth is 6–8. Carbohydrates are not fermented. Reduce nitrate to nitrite. Negative for the hydrolysis of indoxyl acetate and urea. Growth occurs in the presence of safranin (0.05% wt/vol), and 2,3,5-triphenyltetrazolium chloride (0.04%, wt/vol), but not in the presence of glycine (1% wt/vol) sensitive to cefoperazone (30 μg). Possess ubiquinone MK-6 as a respiratory quinone. DNA G+C content is 28.7 mol%.

The type species is *Poseidonibacter lekithochrous*.

Description of *Poseidonibacter lekithochrous* comb. nov.

Basonym: *Arcobacter lekithochrous* Diéguez et al., 2017.

The description is the same given by Diéguez et al. (2017). The type strain is LFT1.7^T (= CECT 8942^T = DSM 100870^T).

AUTHOR CONTRIBUTIONS

MF and JR designed the work. AP-C, NS-M, and AD performed the phenotypic and phylogenetic experiments. AP-C and SB carried out the genome sequencing and analysis. AP-C, AL, and JR performed the bioinformatic work. JR, MF, AP-C, and AD wrote the paper.

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UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Nuria Salas Massó

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02077/full#supplementary-material

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UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
Nuria Salas Massó

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UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY OF Arcobacter-related spp. IN SHELLFISH EXPOSED TO MARINE AND BRACKISH WATER WITH DIFFERENT LEVELS OF FECAL POLLUTION.
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Revisiting the taxonomy of the genus *Arcobacter*: getting order from the chaos.

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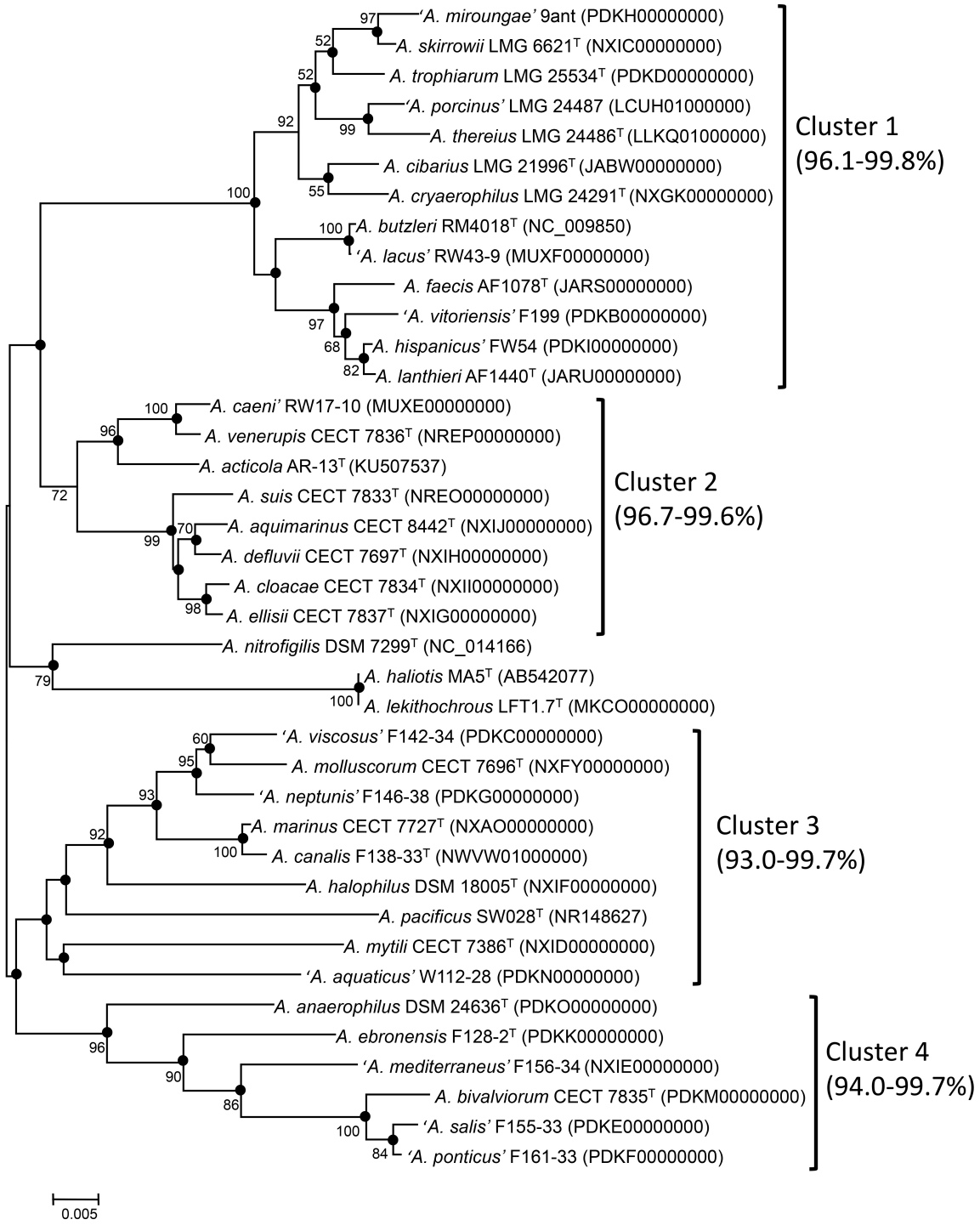
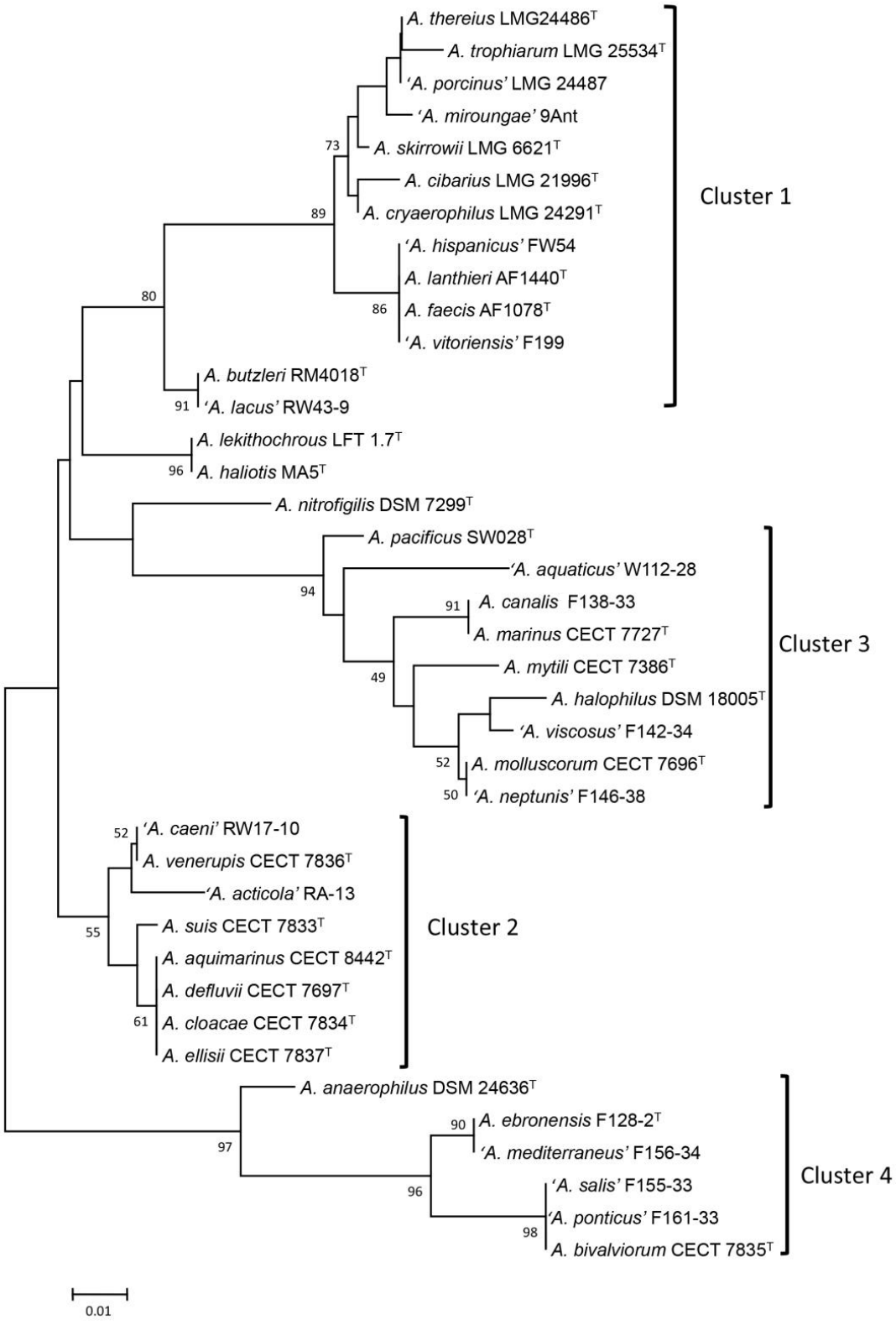
SUPPLEMENTARY MATERIAL

Supplementary Figures S1-S7

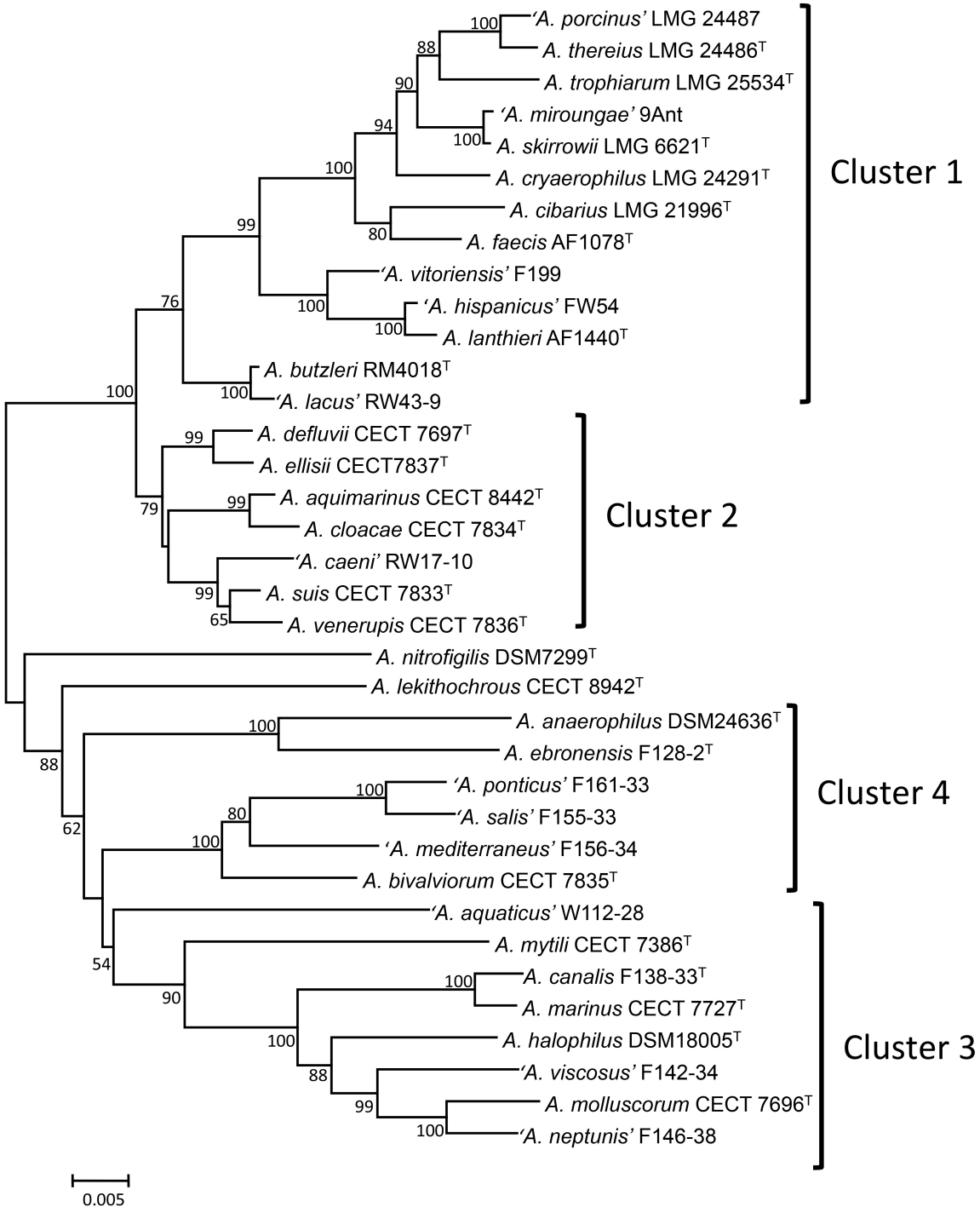
Supplementary Figure S1A.- Phylogenetic tree constructed with the near complete sequences (1450 nt) of the 16S rRNA gene of 36 type and representative strains of *Arcobacter* species by the Maximum-Likelihood algorithm (model GTR+G+I). Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at the nodes. Scale bars indicate the number of substitutions per nucleotide position. Bold circles indicate that corresponding nodes were coincident in the tree generated with Neigbour-Joining algorithm. Brackets indicate the similarity range for 16S rRNA gene sequences. The cluster names in the tree are based in the phylogenetic results obtained from MLSA and core genome analyses. During the preparation of this article *A. haliotis* was confirmed as a later heterotypic synonym of *A. lekithochrous*.



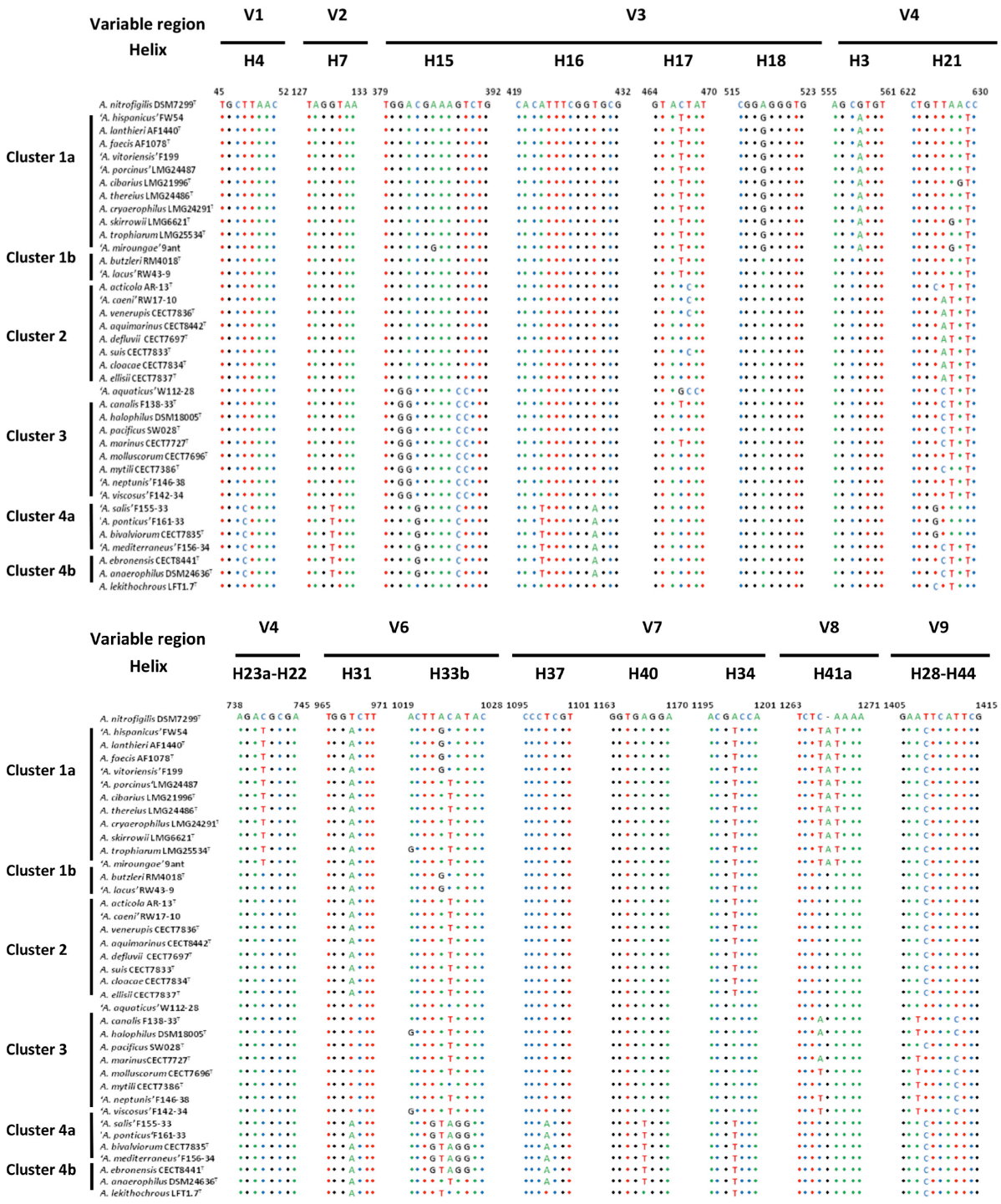
Supplementary Figure S1B.- Tree constructed with the concatenated signature motifs of the 16S rRNA gene for the different clusters. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at the nodes. Scale bars indicate the number of substitutions per nucleotide position. The cluster names in the tree are based in the phylogenetic results obtained from MLSA and core genome analyses. During the preparation of this article *A. haliotis* was confirmed as a later heterotypic synonym of *A. lekithochrous*.



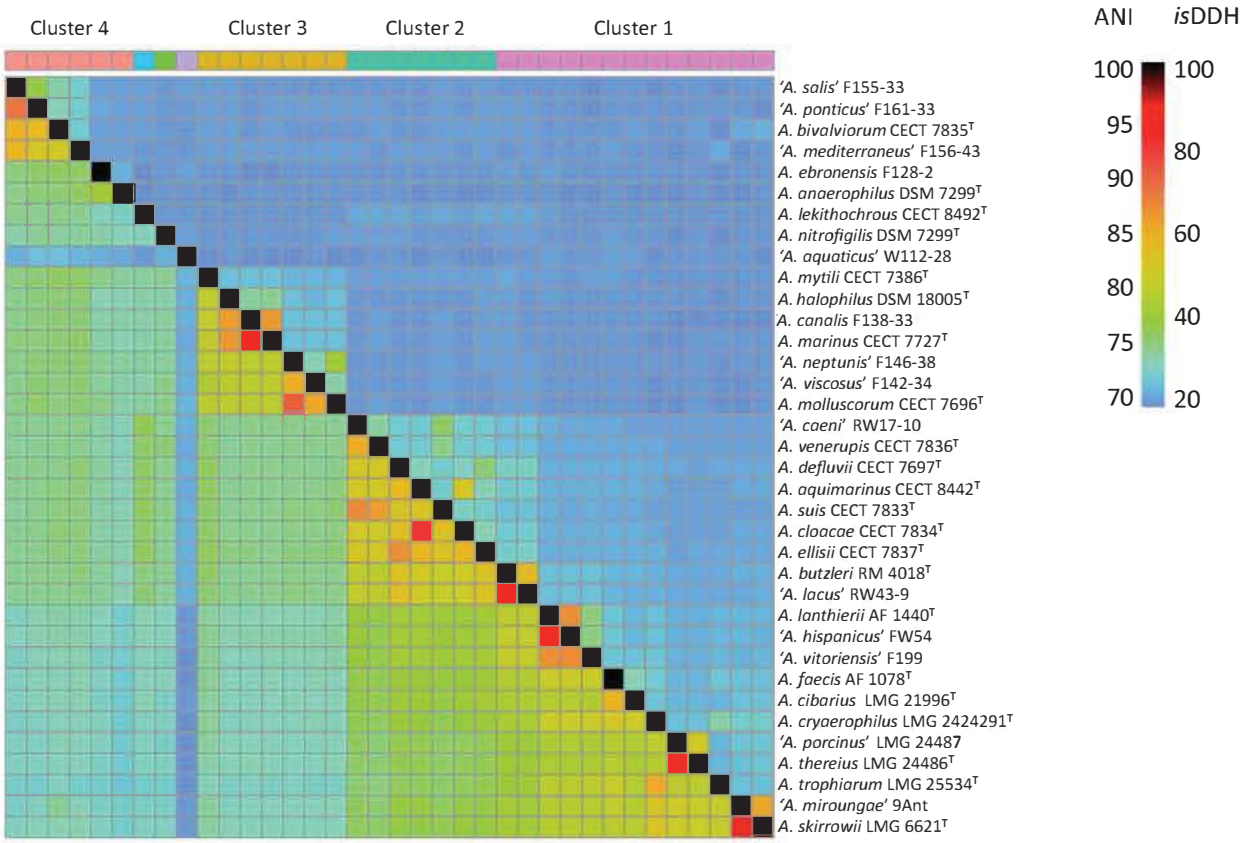
Supplementary Figure S2.- Neighbour joining phylogenetic tree constructed with the 23S rRNA gene sequences (2948 bp) of the type and representative strains of 36 species of *Arcobacter*. Numbers at the nodes indicated bootstrap values >50% obtained by repeating the analysis 1000 times. Scale bar indicates the number of substitutions per nucleotide position. The cluster names in the tree are based in the phylogenetic results obtained from MLSA and core genome analyses.



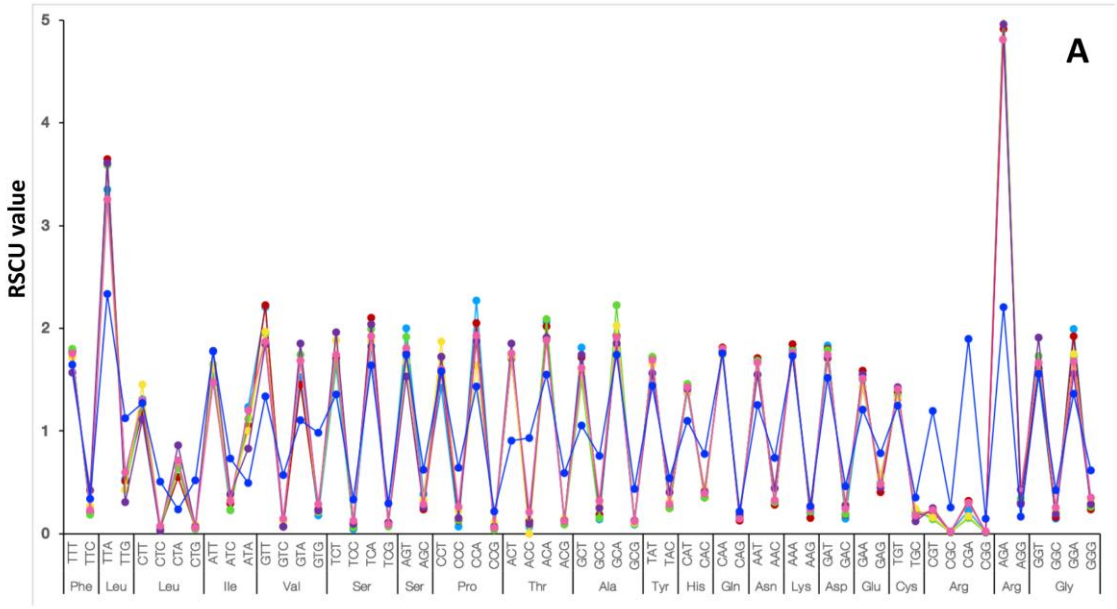
Supplementary Figure S3.- Group specific 16S rRNA gene signatures differentiating the type species of the genus, *Arcobacter nitrofigilis* DSM 7299T and other species of the genus. The numbers at the top give the position in the gene. The location of the signature motifs in the different variable regions and helix of the 16S rRNA gene secondary structure are also indicated. *Escherichia coli* sequence was used as reference to enumerate the nucleotide postitions (Adilakshmi et al., 2008).



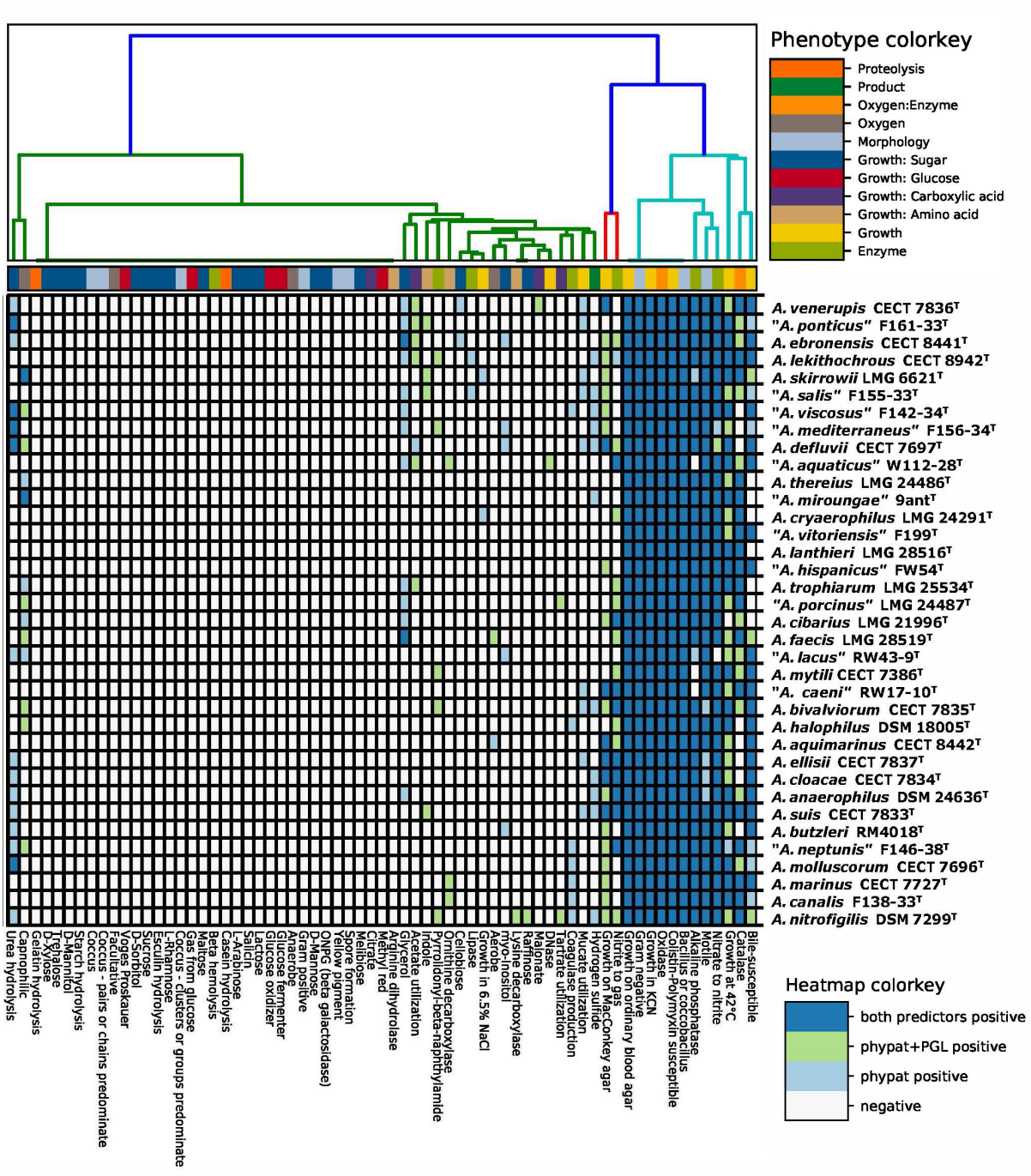
Supplementary Figure S4.- Heatmap representing the similarities (%) among the *Arcobacter* species obtained for ANI (left-down) and isDDH (up-right) indexes.



Supplementary Figure S5.- Comparison of the RSCU data of the 59 synonymous codon among the different clusters and orphan species of *Arcobacter*. Lines: blue, cluster 1; red, cluster 2; green, cluster 3; yellow, cluster 4; purple, *A. lekithochrous*; pink, *A. nitrofigilis*; dark blue, *A. aquaticus*.



Supplementary Figure S6.- Heatmap obtained with the phenotypic predictor tool Traitar for the type and representative strains of *Arcobacter* species. The origin of the phenotypes prediction (Traitar phypat and/or phypat+PGL classifier) determines the color of the heatmap entries.



Supplementary figure S7.- Phylogenetic network of the 57 analyzed genomes based on the concatenated sequences of core genes. Scale bar, base substitutions per site.

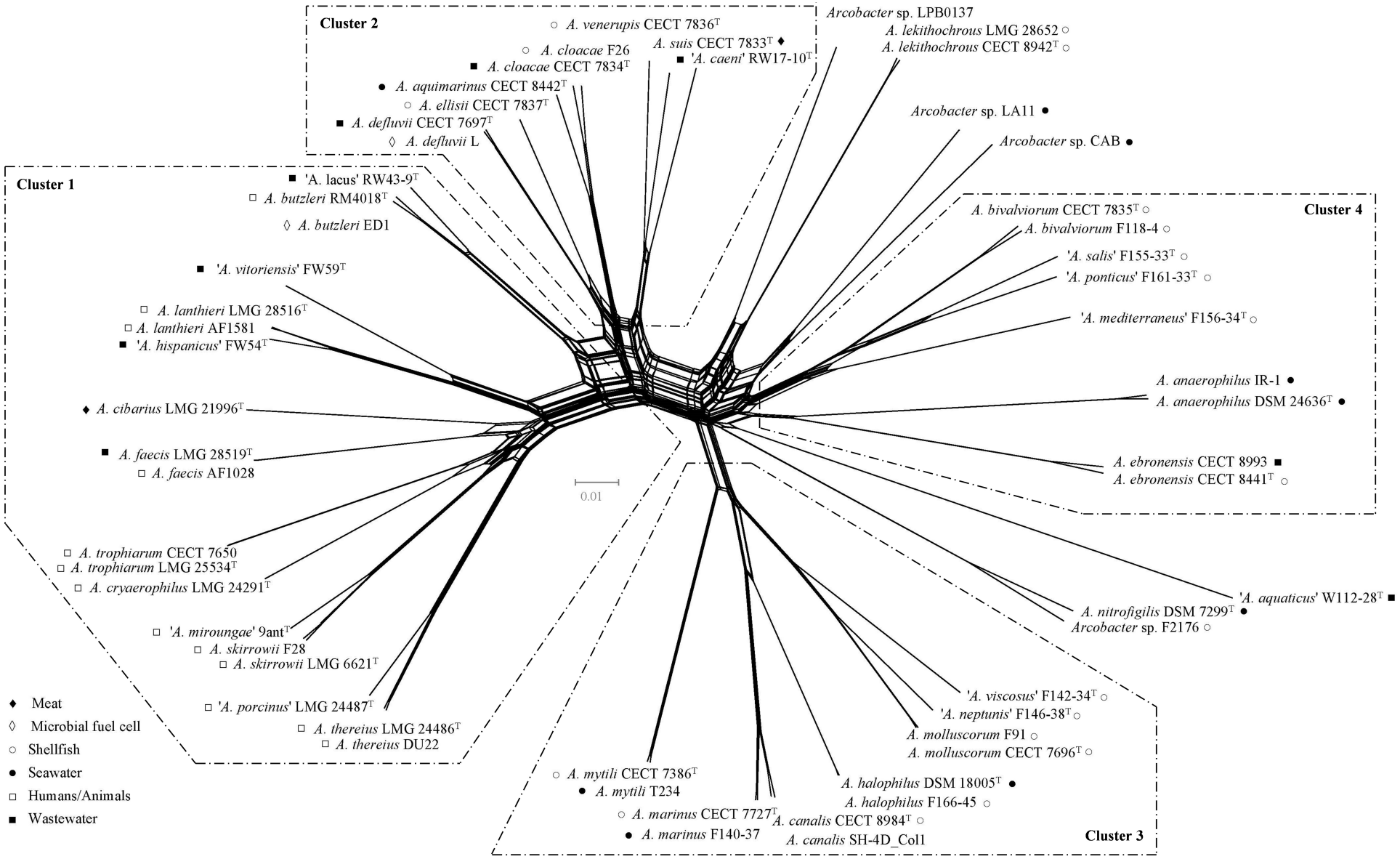


Table S1. Similarities (%) in the 16S rRNA gene (down-left) and 23S rRNA gene (up-right) among type and representative strains of the different *Arcobacter* species

	A. salis ' F155-33T	*A. ponticus* ' F161-33T	*A. bivalviorum* CECT 7835^T	*A. mediterraneus* ' F156-34T	*A. ebronensis* CECT 8441^T	*A. anaerophilus* DSM 24636^T	*A. lekithochrous* CECT 8942^T	*A. nitrofigilis* DSM 7299^T	*A. aquaticus* ' W112-28T	*A. mytili* CECT 7386^T	*A. halophilus* DSM 18005^T	*A. canalis* ' F138-33T	*A. marinus* CECT 7727^T	*A. neptunis* ' F146-38T	A. viscosus' F142-34T	*A. molluscorum* CECT 7696^T	*A. caeni* ' RW17-10T	*A. venerupis* CECT 7836^T	*A. defluvii* CECT 7697^T	*A. aquimarinus* CECT 8442^T	*A. suis* CECT 7833^T	*A. cloacae* CECT 7834^T	*A. ellisii* CECT 7837^T	*A. butzleri* RM4018^T	*A. lacus* ' RW43-9T	*A. lanthierii* LMG 28516^T	*A. hispanicus* ' FW54T	A. vitoriensis' FW59T	*A. faecis* LMG 28519^T	*A. cibarius* LMG 21996^T	*A. cryaerophilus* LMG 24291	*A. porcinus* ' LMG 24487T	*A. thereius* LMG 24486^T	*A. trophiarum* LMG 25534^T	*A. miroungae* ' 9AntT	*A. skirrowii* LMG 6621^T
***A. salis* ' F155-33T**	*	99,0	97.3	97.2	94.8	93.3	94.6	94.3	94.3	94.6	95.4	94,0	93.9	93.8	94.3	94.1	94.2	94.3	94,0	93.9	94.1	94,0	94.2	93.5	93.5	93.2	93,0	92.8	93,0	93,0	92.6	93,0	93,0	92.7	93,0	93.1
***A. ponticus* ' F161-33T**	99.5	*	97.2	97.4	95.1	93.5	94.4	94.1	94.5	94.6	95.6	94.2	94.3	93.9	94.4	94.2	94.1	94.2	94.1	93.9	94.1	93.8	94.3	93.6	93.6	93.1	92.9	92.8	93.1	93,0	92.5	92.9	93,0	92.6	93,0	93.1
***A. bivalviorum* CECT 7835^T**	98.9	98.6	*	97.3	95.7	94.1	95.1	94.1	95,0	94.5	95.2	93.9	93.9	94.4	94.4	93.9	95.2	94.8	95.2	94.9	94.9	94.8	95.1	94.4	94.4	93.8	93.7	93.6	93.4	93.7	93.7	93.8	93.9	93.7	93.7	93.8
***A. mediterraneus* ' F156-34T**	96.8	96.9	96.6	*	95.5	93.6	95.5	94,0	95.1	94.4	95.3	94.3	94.4	94.7	95,0	94.2	94.9	94.7	95.1	94.6	94.8	94.5	94.8	94.3	94.1	93.6	93.7	93.5	93.5	93.4	93.2	93.3	93.4	93.2	93.2	93.3
***A. ebronensis* CECT 8441^T**	96.4	96.3	96.7	95.8	*	96.9	94.6	93.6	93.7	93.7	94.3	93.6	93.5	94.2	94.2	93.8	94.5	94.6	95,0	94.6	94.7	94.5	94.9	94.8	95,0	94.2	93.8	94,0	94.4	94.5	94.1	93.9	94,0	93.7	94.2	94.3
***A. anaerophilus* DSM 24636^T**	94,0	94.1	94.6	94.8	96.6	*	93.5	93.3	93.1	92.4	93.5	92.8	92.5	93.5	93.1	93.3	93.1	93.1	93.1	92.8	93.2	92.9	93,0	92.9	92.9	92.1	92.3	92.3	92,0	92.1	92.5	92.5	92.7	92.3	92.3	92.4
***A. lekithochrous* CECT 8942^T**	93,0	93.4	92.9	93.5	92.8	93.8	*	94.2	94.6	93.2	94.5	94.3	94.2	93.6	93.9	93.6	95,0	95.2	94.9	94.8	95.2	94.6	94.9	94.5	94.4	93.7	94.1	93.8	93.1	93.2	93.7	93.9	93.9	93.6	93.8	93.8
***A. nitrofigilis* DSM 7299^T**	93.3	93.4	93.4	93.8	93.8	94.6	95.1	*	95.1	94.4	94.8	95,0	94.9	95.2	95.2	94.9	95.1	94.3	94.8	94.6	94.7	94.8	94.6	94.4	94.3	94.2	94.5	93.9	93.8	93.7	94,0	93.9	94,0	93.8	94.1	94.1
***A. aquaticus* ' W112-28T**	92.4	92.7	93,0	93,0	93.8	95.1	94,0	94.9	*	94.9	95.1	94.3	94.3	94.9	94.7	94.8	94.1	94.1	94.5	93.7	94.4	93.7	94.6	94,0	93.9	93.3	93.3	93.1	93,0	93.2	93,0	93.2	93.4	93.1	92.9	93,0
***A. mytili* CECT 7386^T**	92.8	92.8	92.8	92.6	93.6	93.5	93,0	94,0	94.7	*	95.2	94.6	94.9	94.6	94.2	95,0	93.8	94,0	93.9	93.6	94.2	93.5	94.4	93.9	93.9	92.9	92.9	93.3	92.7	92.5	92.6	92.3	92.2	92.2	92.9	92.9
***A. halophilus* DSM 18005^T**	93.3	93.3	93.6	93.4	93.8	94.6	92.9	94.5	94.2	94.7	*	96.8	96.8	96.9	97,0	97.3	93.5	94,0	93.7	93.4	93.9	93.1	94,0	93.5	93.5	92.4	92.4	92.6	92.4	92.4	92.5	92.7	92.8	92.6	92.7	92.8
***A. canalis* ' F138-33T**	92.8	92.9	93,0	93.1	94,0	95.6	93,0	94.6	95.4	94.4	96.4	*	99.2	95.9	96.2	96.3	93.1	93.7	93.6	93.5	93.7	93.1	93.8	93.6	93.5	92.4	92.6	92.9	92.1	91.7	92.2	92.6	92.7	92.8	92.4	92.4
***A. marinus* CECT 7727^T**	93.3	93.1	93,0	93.7	94,0	95.6	92.7	94.6	95.4	94.2	96,0	99.1	*	95.8	96,0	96.2	93.4	93.7	93.7	93.5	94,0	93,0	93.9	93.6	93.5	92.4	92.7	92.9	92.2	91.8	92.4	92.5	92.7	92.8	92.5	92.6
***A. neptunis* ' F146-38T**	93,0	93,0	93.2	93.2	94.2	95.6	93.2	95,0	95.8	95,0	96.3	97.7	97.5	*	97.8	98.6	93.5	93.6	94.1	93.5	93.7	93.2	93.7	93.4	93.4	92.5	92.6	92.6	92.4	92.1	92.4	92.5	92.5	92.7	92.6	92.7
***A. viscosus* ' F142-34T**	92.4	92.6	92.8	93.2	93.6	95.2	93,0	94.8	95.8	94.4	96,0	97.1	97,0	98.6	*	97.4	93.5	93.5	93.9	93.7	93.6	93.4	93.6	93.5	93.5	92.3	92.3	92.6	92.3	92.3	92.4	92.6	92.7	92.8	92.6	92.7
***A. molluscorum* CECT 7696^T**	92.8	92.8	93.1	93,0	93.7	95.2	93.1	94.6	95.8	94.8	95.8	97.5	97.3	98.4	98.5	*	92.8	93.4	93.2	93.1	93.5	92.8	93.4	93,0	93.1	92.4	92.4	92.4	92.4	92.1	92.3	92.5	92.5	92.8	92.6	92.7
***A. caeni* ' RW17-10T**	92.8	93,0	93.2	93.8	94.3	95.5	94.6	95.9	95.8	93.4	94,0	95.1	95,0	95.6	95.4	95.4	*	98.7	98.2	97.9	99.1	98.3	97.9	97.1	97,0	96,0	96.3	96.3	95.7	95.6	96.1	95.2	95.2	95.2	95.4	95.5
***A. venerupis* CECT 7836^T**	93,0	93.2	93.3	93.9	94.2	95.4	94.4	96,0	95.8	93.5	93.9	94.9	94.8	95.6	95.5	95.5	99.3	*	98.4	98.3	99.3	98,0	98.6	97.7	97.5	96,0	96.3	96.7	95.8	95.4	96,0	95.2	95.2	95.2	95.4	95.4
***A. defluvii* CECT 7697^T**	94.7	95.1	95,0	94.6	96,0	95.6	94.2	95.8	94.8	93.8	94.6	94.9	94.7	95.2	94.9	95,0	97,0	97,0	*	98.5	98.5	98.1	99.4	98.3	98.1	96.4	96.7	97,0	95.9	95.6	95.7	95.3	95.4	95.4	95.5	95.5
***A. aquimarinus* CECT 8442^T**	95,0	95.3	95.3	94.9	95.9	95.6	94.4	95.7	94.6	94,0	94.4	94.6	94.4	95.2	94.7	94.8	96.8	96.7	99.4	*	98.3	99.4	98.3	98.3	98.1	96.4	96.7	97.3	96.2	95.7	95.9	95.6	95.6	95.5	95.7	95.7
***A. suis* CECT 7833^T**	94.4	94.6	94.5	94.6	95.7	95.2	94,0	95.3	95,0	93.6	94.4	94.7	94.5	95.1	94.7	94.8	97.7	97.7	99,0	98.7	*	98.1	98.6	97.8	97.7	96.3	96.6	97,0	96,0	95.7	96.2	95.4	95.5	95.4	95.5	95.6
***A. cloacae* CECT 7834^T**	94.4	94.8	94.6	94.4	95.8	95.2	94.3	95.6	94.5	94.2	94.4	94.8	94.6	95,0	94.6	94.7	96.8	97,0	99.2	98.9	98.9	*	97.9	97.8	97.6	96.4	96.7	96.8	96.1	95.9	95.9	95.6	95.6	95.5	95.7	95.7
***A. ellisii* CECT 7837^T**	94.6	95,0	94.7	94.6	95.8	95.3	94.4	95.8	94.6	94.1	94.6	94.4	94.2	95.2	94.8	94.8	96.8	97,0	99.2	98.9	98.9	99.6	*	98.6	98.5	96.7	97,0	97.3	96.2	95.8	96,0	95.6	95.7	95.7	95.9	96,0
***A. butzleri* RM4018^T**	91.6	91.9	91.6	92.6	93,0	93.8	93.6	94.4	93,0	94,0	93.2	93.1	93,0	93.4	93,0	92.8	94.8	94.9	95,0	95.2	94.6	95.1	95.4	*	99.7	97.1	97.4	98.1	97,0	96.3	96.4	96.4	96.3	96.1	96.5	96.5
***A. lacus* ' RW43-9T**	91.7	92,0	91.6	92.7	93.1	93.9	93.6	94.4	93,0	94,0	93.3	93.2	93,0	93.5	93,0	92.9	94.9	95,0	95.1	95.2	94.6	95.1	95.4	99.9	*	97.3	97.4	98,0	97.2	96.5	96.3	96.3	96.2	96,0	96.6	96.6
***A. lanthierii* LMG 28516^T**	91.5	91.8	91.8	92.4	93,0	93.7	92.9	94.1	92.4	94,0	93.2	93.1	93,0	93.1	92.8	92.6	94.4	94.2	94.9	94.8	94.3	94.6	94.5	97.9	98,0	*	99.6	98.7	97.7	96.9	96.5	96.5	96.5	96.4	97.1	97.1
***A. hispanicus* ' FW54T**	91.6	91.9	92,0	92.4	93,0	93.6	92.9	94.1	92.4	93.9	93.1	93,0	92.9	93,0	92.9	92.5	94.6	94.4	94.9	94.8	94.4	94.6	94.5	97.9	97.9	99.8	*	99.1	97.4	96.5	96.8	96.7	96.7	96.7	97.1	97.1
***A. vitoriensis* ' FW59T**	91.6	91.9	91.4	92.4	92.8	93.9	93.1	94.3	92.4	93.8	93.1	93.2	93.1	93,0	92.8	92.5	94.2	94,0	94.7	94.6	94.1	94.3	94.3	97.7	97.8	99.3	99.1	*	97.4	96.5	96.6	96.5	96.5	96.4	96.9	96.9
***A. faecis* LMG 28519^T**	91.1	91.4	91.6	91.9	92.6	93.5	92.7	94,0	92.2	93.7	92.9	93,0	92.9	92.9	92.7	92.4	94.5	94.2	94.6	94.6	94.2	94.3	94.3	97.9	98,0	99.1	99.1	98.6	*	98.5	97.4	97.5	97.2	97.6	98,0	98,0
***A. cibarius* LMG 21996^T**	91.5	91.9	91.5	92.2	92.5	93.2	92.5	93.9	92.4	93.4	92.5	92.4	92.2	93,0	92.7	92.6	94.7	94.8	94.8	95,0	94.6	95,0	95.2	97.7	97.7	98.1	98.2	97.6	98.1	*	98,0	97.6	97.8	97.3	98,0	97.9
***A. cryaerophilus* LMG 24291^T**	91.2	91.4	91.2	92,0	92.1	93,0	92,0	93.8	92.6	93.2	92.5	92.3	92.1	92.8	92.6	92.5	95,0	94.8	94.5	94.7	94.5	94.5	94.6	97.3	97.3	97.6	97.7	97.1	97.8	98.8	*	98,0	98.2	98.3	98.5	98.5
***A. porcinus* ' LMG 24487T**	91.7	92.1	91.8	92.3	92.6	93.1	92.3	93.8	92.7	93.1	92.5	92.8	92.7	92.9	92.6	92.7	94.7	94.4	94.7	94.6	94.4	94.7	94.6	96.5	96.6	97.5	97.5	96.9	97.1	97.8	98.1	*	99.5	98.5	98.6	98.5
***A. thereius* LMG 24486^T**	91.7	91.8	91.7	92.2	92.6	92.6	92.1	93.4	92.5	93.2	92.2	92.8	92.5	92.7	92.5	92.5	94.3	94,0	94.4	94.4	94.2	94.5	94.4	96.1	96.1	97.3	97.5	96.8	96.9	97.5	97.6	99,0	*	98.2	98.3	98.3
***A. trophiarum* LMG 25534^T**	91.6	91.9	91.7	92.2	92.8	93.1	92.1	94,0	93,0	93.2	92.9	92.7	92.6	93.1	93.1	93,0	94.9	94.7	94.9	95,0	94.4	94.8	94.8	96.9	97,0	97.5	97.7	97.2	97.3	97.9	98.5	98.4	98.2	*	98.5	98.5
***A. miroungae* ' 9AntT**	91,0	91.4	91.4	91.6	92.4	92.6	91.8	93.8	92.7	93.1	92.4	92.6	92.4	92.8	92.6	92.6	94.4	94.2	94.5	94.7	94.2	94.6	94.5	96.6	96.6	97.5	97.7	96.8	97.3	98,0	98.3	97.9	98.1	98.7	*	99.9
***A. skirrowii* LMG 6621^T**	91.2	91.4	91.5	91.8	92.6	93.2	92,0	94.1	92.8	93.1	92.6	92.7	92.6	93.1	92.8	92.8	94.6	94.5	94.6	94.8	94.2	94.8	94.6	96.9	96.9	97.9	97.9	97.2	97.7	98.2	98.5	98.1	97.7	98.7	99.4	*

Table S2. Values (%) of ANI (down-left) and *is* DDH (up-right) among type and representative strains of the different *Arcobacter* species

	A. salis ' F155-33T	*A. ponticus* ' F161-33T	*A. bivalviorum* CECT 7835^T	*A. mediterraneus* ' F156-34T	*A. ebronensis* CECT 8441^T	*A. anaerophilus* DSM 24636	*A. lekithochrous* CECT 894	*A. nitrofigilis* DSM 7299^T	*A. aquaticus* ' W112-28T	*A. mytili* CECT 7386^T	*A. halophilus* DSM 18005^T	*A. canalis* ' F138-33T	*A. marinus* CECT 7727^T	*A. neptunis* ' F146-38T	A. viscosus' F142-34T	*A. molluscorum* CECT 7696	*A. caeni* ' RW17-10T	*A. venerupis* CECT 7836^T	*A. defluvii* CECT 7697^T	*A. aquimarinus* CECT 8442	*A. suis* CECT 7833^T	*A. cloacae* CECT 7834^T	*A. ellisii* CECT 7837^T	*A. butzleri* RM4018^T	*A. lacus* ' RW43-9T	*A. lanthierii* LMG 28516^T	*A. hispanicus* ' FW54T	A. vitoriensis' FW59T	*A. faecis* LMG 28519^T	*A. cibarius* LMG 21996^T	*A. cryaerophilus* LMG 2429	*A. porcinus* ' LMG 24487T	*A. thereius* LMG 24486^T	*A. trophiarum* LMG 25534^T	*A. miroungae* ' 9AntT	*A. skirrowii* LMG 6621^T
***A. salis* ' F155-33T**	*	37.4	29.4	25.3	19.7	19.5	19.5	19.0	19.6	19.5	19.7	19.1	19.2	19.3	19.3	19.4	19.4	19.3	19.1	19.1	19.5	19.2	19.6	19.4	19.3	19,0	18.9	18.5	19.1	18.8	18.3	18.8	18.8	18.6	18.8	18.7
***A. ponticus* ' F161-33T**	89.0	*	28.2	25.6	20.3	19.6	19.7	19.4	19.3	19.8	19.8	19.4	19.6	19.6	19.9	20.0	19.7	19.5	19.2	19.2	19.7	19.3	19.5	19.6	19.6	19.1	18.9	18.5	19.0	19.2	18.8	19.0	19.0	18.7	18.9	18.6
***A. bivalviorum* CECT 7835^T**	84.9	84.2	*	24.8	20.0	19.7	19.8	19.2	18.8	20.1	20.0	19.4	19.7	19.4	19.2	19.4	19.4	19.7	19.4	19.4	19.9	20,0	19.6	19.7	19.7	19.1	19.1	19.0	19.2	19.8	19.4	19.2	19.5	18.6	20.5	21.6
***A. mediterraneus* ' F156-34T**	84.8	82.2	81.8	*	19.9	20.0	19.4	19.3	18.7	19.5	19.9	19.7	20.1	19.5	19.7	19.5	19.5	19.4	19.6	19.5	19.8	19.4	19.5	19.4	19.6	19.2	18.8	18.8	19.6	19.3	18.7	18.5	19.0	20.7	18.6	18.9
***A. ebronensis* CECT 8441^T**	75.6	76.2	76.5	76.1	*	21.3	18.3	18.8	19.8	19.2	18.9	18.4	18.5	19.2	18.9	19.2	18.9	18.9	19.0	18.9	19.1	18.9	18.9	19.5	19.2	19.6	18.8	18.7	19.0	18.7	18.7	18.7	18.8	18.3	18.8	18.8
***A. anaerophilus* DSM 24636^T**	75.2	75.7	75.7	76.4	78.6	*	18.4	19.0	18.7	18.9	18.6	18.8	18.7	19.1	18.7	18.8	18.8	19.1	18.9	18.5	18.8	18.4	18.5	19.3	19.3	18.8	18.4	18.6	18.8	18.6	18.5	18.5	18.9	18.5	18.2	19.0
***A. lekithochrous* CECT 8942^T**	75.2	74.9	75.2	74.8	73.5	73.2	*	19.0	19.3	19.1	19.0	18.8	18.9	19.0	18.9	18.8	20.6	20.5	20.3	20.1	20.6	20.2	20.5	20.5	20.3	19.8	19.2	19.3	19.3	19.3	18.9	18.8	19.4	19.2	18.9	18.9
***A. nitrofigilis* DSM 7299^T**	74.4	74.8	74.6	74.6	74.4	73.7	73.7	*	19.8	19.3	19.2	19.2	19.3	18.9	19.3	19.5	19.5	20.0	19.5	19.3	19.4	19.4	19.4	19.7	19.5	19.2	18.8	19.0	19.5	19.4	18.9	18.6	18.8	18.6	19.2	19.2
***A. aquaticus* ' W112-28T**	71.3	71.8	71.9	71.6	71.4	71.1	70.4	71.5	*	17.8	18.9	18.6	18.6	18.5	18.7	18.5	18.6	18.8	18.8	18.3	19.4	18.1	18.3	19.3	19.2	19.6	18.7	19.0	20.0	19.6	18.6	18.7	19.3	19.4	18.8	18.4
***A. mytili* CECT 7386^T**	75.4	75.9	76.3	76.1	75.1	74.6	74.8	74.9	71.6	*	23.0	23.0	22.8	22.7	22.2	22.7	19.6	19.6	19.8	19.8	20.0	20.1	19.7	20.3	20.2	19.8	19.3	19.6	19.5	19.3	19.1	19.1	19.3	19.1	19.3	19.4
***A. halophilus* DSM 18005^T**	75.2	75.6	75.9	75.8	74.1	74.0	74.2	74.2	71.3	80.7	*	30.4	31.3	22.9	22.7	22.8	19.2	19.3	19.3	19.4	19.2	19.4	19.5	19.6	19.4	19.7	19.3	19.2	19.1	19.1	18.7	18.7	19.2	18.5	18.7	18.8
***A. canalis* ' F138-33T**	75.1	75.5	75.9	75.7	74.3	74.2	74.3	74.6	71.2	80.5	86.3	*	63.6	22.8	22.8	22.8	19.4	19.7	19.1	19.1	19.5	19.2	19.5	19.5	19.3	19.1	19,0	19.3	18.7	19.1	18.8	18.6	18.5	18.6	18.6	18.6
***A. marinus* CECT 7727^T**	75.4	75.6	75.7	76.1	74.2	73.9	74.3	74.6	70.9	80.3	86.6	95.4	*	22.8	22.9	22.9	19.3	19.5	19.0	19.4	19.5	19.3	19.5	19.8	19.4	19.2	18.9	19,0	18.7	19.1	18.3	18.6	18.7	19.1	18.7	18.8
***A. neptunis* ' F146-38T**	75.3	75.8	75.8	75.9	74.9	74.5	74.6	74.7	71.3	80.1	80.6	80.5	80.3	*	29.1	40.4	19.2	19.2	19.4	19.4	19.6	19.4	19.6	19.7	19.6	19.1	19,0	18.9	18.8	19,0	18.7	19.1	19.0	18.4	18.7	18.7
***A. viscosus* ' F142-34T**	75.2	75.7	75.5	75.9	74.7	74.3	74.4	75.2	71.4	79.4	79.9	80.4	80.3	85.3	*	29.4	19.3	19.2	19.2	19.2	19.5	19.2	19.1	20.1	19.9	19.1	18.7	19,0	19.1	19.3	18.6	18.7	19.0	18.4	18.5	18.9
***A. molluscorum* CECT 7696^T**	75.1	76.0	75.7	75.8	75.1	74.3	74.6	75.1	71.5	80.1	80.2	80.4	80.4	90.3	85.6	*	19.2	19.5	19.4	19.4	19.7	19.2	19.3	19.8	19.7	19.1	19.2	18.8	19.0	19.3	18.5	18.6	19.1	18.2	18.6	18.7
***A. caeni* ' RW17-10T**	74.8	75.0	75.2	75.3	74.3	73.6	76.8	74.8	71.4	75.8	74.9	75.2	75.2	75.1	74.9	75.0	*	30.2	24.8	24.9	34.4	25.2	24.9	23.1	23.2	21.1	20.7	20.9	20.5	20.2	20.2	19.9	19.9	19.8	19.8	19.8
***A. venerupis* CECT 7836^T**	74.5	74.9	75.1	75.0	74.2	73.6	76.2	75.7	71.1	75.8	74.6	75.1	74.9	75.1	75.1	75.2	85.3	*	24.9	24.8	31.8	24.8	25.0	23.3	23.2	20.8	20.9	20.9	20.5	20.4	20.6	19.5	20.0	19.7	19.9	19.9
***A. defluvii* CECT 7697^T**	74.8	75.2	75.2	75.7	74.6	73.9	76.2	74.9	71.4	76.5	75.0	75.2	75.2	75.2	74.9	75.3	82.2	81.9	*	27.8	25.3	27.4	33.7	26.1	26.6	21.7	21.6	21.9	21.3	21.1	20.9	20.3	20.2	20.4	20.8	20.7
***A. aquimarinus* CECT 8442^T**	74.8	75.1	75.6	75.5	74.5	73.6	76.1	74.8	71.1	76.3	75.0	75.3	75.2	75.4	75.3	75.5	82.1	81.8	84.3	*	25.7	50.9	28.2	24.8	24.6	21.7	21.5	21.6	21.1	21.2	20.7	20.2	20.1	20.1	20.6	20.7
***A. suis* CECT 7833^T**	74.7	75.1	75.5	75.5	74.3	74.0	76.3	75.2	71.3	76.1	74.9	75.2	75.1	75.5	75.4	75.8	87.5	86.3	82.6	82.7	*	26.1	25.7	23.4	23.5	21.1	21.0	21.1	20.5	20.4	20.2	19.7	19.8	19.6	20.0	20.0
***A. cloacae* CECT 7834^T**	74.8	75.1	75.7	75.5	74.2	74.0	76.0	74.8	71.3	76.2	75.1	75.3	75.3	75.3	75.3	75.6	82.2	81.6	83.7	92.5	83.0	*	28.8	24.5	24.6	21.5	21.5	21.5	21.4	21.3	20.9	20.0	20.1	20.0	20.7	20.9
***A. ellisii* CECT 7837^T**	74.9	75.3	75.6	75.5	74.5	74.1	76.3	74.9	71.2	76.5	75.4	75.5	75.4	75.6	75.4	75.7	82.2	82.1	87.3	84.5	83.0	84.6	*	26.0	26.4	21.8	21.5	21.7	21.2	21.6	21.2	20.2	20.3	20.3	20.7	20.9
***A. butzleri* RM4018^T**	74.9	75.1	75.1	75.4	74.6	73.8	75.6	74.9	71.4	76.3	74.8	75.0	75.1	75.2	75.3	75.2	80.4	80.0	82.8	81.7	80.8	81.4	82.7	*	55.7	22.8	22.8	23.3	22.4	22.0	21.6	20.7	20.6	20.7	21.3	21.5
***A. lacus* ' RW43-9T**	75.0	74.9	75.3	75.2	74.6	74.0	75.7	74.7	71.2	75.9	75.2	75.0	75.1	75.6	75.2	75.3	80.5	80.0	83.2	81.5	80.9	81.6	82.9	94.2	*	22.9	22.9	23.2	22.3	21.7	21.2	20.6	20.5	20.6	20.8	21.2
***A. lanthierii* LMG 28516^T**	73.5	73.9	74.2	74.2	73.6	72.8	74.0	73.8	70.5	74.8	74.0	74.0	74.0	74.1	74.1	74.3	77.7	77.0	78.6	78.2	77.9	78.0	78.5	80.4	80.4	*	65.4	33.2	23.7	22.5	23.4	21.7	21.9	22.6	22.1	22.0
***A. hispanicus* ' FW54T**	73.6	74.0	74.4	74.3	73.6	73.2	73.9	73.8	70.4	75.2	74.1	74.2	74.2	74.3	74.3	74.6	77.7	77.1	78.7	78.4	77.7	78.2	78.5	80.6	80.5	95.4	*	33.0	23.8	22.7	23.4	21.7	21.8	22.6	22.0	21.9
***A. vitoriensis* ' FW59T**	73.2	73.7	74.1	73.9	73.2	72.7	73.7	73.5	70.2	74.9	73.7	73.8	73.9	74.0	74.1	74.3	77.6	77.0	78.5	78.3	77.8	78.1	78.5	80.7	80.7	87.1	87.4	*	23.7	22.8	23.6	21.6	21.4	22.4	21.9	22.3
***A. faecis* LMG 28519^T**	73.4	73.8	73.7	74.0	73.4	72.4	73.7	73.7	70.2	75.0	73.6	73.9	74.0	73.8	74.1	73.8	77.1	76.6	77.7	77.7	77.3	77.7	78.0	79.4	79.4	81.1	81.2	81.0	*	28.5	25.1	22.7	22.6	24.0	23.3	23.5
***A. cibarius* LMG 21996^T**	73.3	73.8	73.9	74.1	73.0	72.6	73.9	73.2	70.3	74.5	73.6	73.7	73.6	73.9	73.7	73.9	77.1	76.6	77.9	77.9	77.1	78.1	78.4	79.1	79.0	79.6	79.7	79.5	84.6	*	24.1	22.3	22.3	22.7	23.2	23.2
***A. cryaerophilus* LMG 24291^T**	73.1	73.5	73.6	73.6	73.3	72.5	73.4	73.4	70.0	74.8	73.5	73.8	73.8	73.9	73.9	73.7	76.7	76.3	77.6	77.3	76.7	77.5	77.7	78.7	78.9	81.0	80.9	80.9	82.0	81.2	*	22.9	22.8	29.8	25.2	26.0
***A. porcinus* ' LMG 24487T**	73.3	73.5	73.9	73.6	73.5	72.6	73.5	73.2	70.2	74.5	73.6	73.7	73.6	73.8	73.7	73.8	76.4	75.8	76.7	76.7	76.2	76.4	76.6	77.9	77.9	79.2	79.3	79.1	79.8	79.3	80.3	*	51.1	22.6	23.7	23.9
***A. thereius* LMG 24486^T**	73.3	73.5	73.7	73.6	73.1	72.6	73.4	73.4	70.1	74.4	73.7	73.9	73.7	73.8	73.8	73.9	76.1	75.7	76.8	76.8	76.0	76.3	76.6	78.0	77.8	79.2	79.2	79.1	79.6	78.9	79.9	93.3	*	22.5	23.5	23.6
***A. trophiarum* LMG 25534^T**	72.7	72.9	73.3	72.9	72.9	72.2	72.9	72.8	70.0	73.8	73.3	73.3	73.2	73.4	73.1	73.1	75.8	75.5	76.5	76.3	76.0	76.3	76.6	77.7	77.9	79.9	80.1	80.2	80.9	79.5	85.5	80.1	79.9	*	23.0	23.2
***A. miroungae* ' 9AntT**	73.1	73.4	74.8	73.6	73.3	72.8	73.2	73.3	70.3	74.6	73.5	73.8	73.8	73.8	73.7	73.9	76.4	76.0	77.3	77.2	76.5	77.0	77.1	78.4	78.3	79.5	79.4	79.6	80.5	79.8	82.4	81.5	81.3	80.6	*	61.0
***A. skirrowii* LMG 6621^T**	73.48	73.7	73.9	73.9	73.39	72.7	73.5	73.3	70.3	74.7	73.7	73.8	73.8	73.7	73.8	73.6	76.4	76.1	77.0	77.1	76.6	77.2	77.4	78.5	78.6	79.6	79.6	79.7	80.2	80.1	82.8	81.5	81.2	80.6	94.9	*

Table S3. Values (%) of AAI (down-left) and POCP (up-right) among type and representative strains of the different *Arcobacter* species

	A. salis ' F155-33T	*A. ponticus* ' F161-33T	*A. bivalviorum* CECT 7835^T	*A. mediterraneus* ' F156-34T	*A. ebronensis* CECT 8441^T	*A. anaerophilus* DSM 24636^T	*A. lekithochrous* CECT 8942^T	*A. nitrofigilis* DSM 7299^T	*A. aquaticus* ' W112-28T	*A. mytili* CECT 7386^T	*A. halophilus* DSM 18005^T	*A. canalis* ' F138-33T	*A. marinus* CECT 7727^T	*A. neptunis* ' F146-38T	A. viscosus' F142-34T	*A. molluscorum* CECT 7696^T	*A. caeni* ' RW17-10T	*A. venerupis* CECT 7836^T	*A. defluvii* CECT 7697^T	*A. aquimarinus* CECT 8442^T	*A. suis* CECT 7833^T	*A. cloacae* CECT 7834^T	*A. ellisii* CECT 7837^T	*A. butzleri* RM4018^T	*A. lacus* ' RW43-9T	*A. lanthieri* LMG 28516^T	*A. hispanicus* ' FW54T	A. vitoriensis' FW59T	*A. faecis* LMG 28519^T	*A. cibarius* LMG 21996^T	*A. cryaerophilus* LMG 24291^T	*A. porcinus* ' LMG 24487T	*A. thereius* LMG 24486^T	*A. trophiarum* LMG 25534^T	*A. mirounga* ' 9AntT	*A. skirrowii* LMG 6621^T
***A. salis* ' F155-33T**	*	90.68	86.76	74.4	71.6	72.16	68.5	65.82	66.94	68.29	69.84	69.88	69.12	69.16	68.85	68.7	64.91	64.12	62.97	68.3	65.93	64.96	64.8	63.97	61.97	60.8	61.15	60.33	60.17	60.32	60.42	56.46	58.79	59.62	57.97	59.22
***A. ponticus* ' F161-33T**	82.86	*	85.07	77.6	72.19	72.16	67.48	68.67	67.58	68.19	69.48	70.08	69.52	69.6	69.54	69.59	67,0	67.4	65.27	68.67	67.8	67.06	66.88	67.32	63.76	63.69	63.17	62.02	60.44	62.56	62.15	57.59	60.58	61.47	60.58	61.76
***A. bivalviorum* CECT 7835^T**	83.38	82.98	*	77.65	71.91	72.09	69.09	67.12	67.47	68.61	69.73	69.97	69.29	69.41	69.12	69.1	69.07	65.93	65.44	70.67	68.11	67.5	67.42	66.4	64.37	63.72	63,0	62.82	61.58	63.79	64.81	59.06	61.61	63.9	61.2	62.08
***A. mediterraneus* ' F156-34T**	80.51	80.63	80.34	*	72.14	73.06	65.07	65.21	66.92	69.16	69.32	69.93	69.86	68.93	69.27	68.87	64.7	65.75	67.06	68.79	64.57	66.57	64.78	64.48	64.34	63.58	64.25	62.86	62.1	61.05	61.43	57.47	60.1	60.8	58.66	59.49
***A. ebronensis* CECT 8441^T**	69.63	73.15	72.39	69.81	*	77.87	62.51	70.29	66.79	67.01	67.85	67.97	67.17	68.44	68.29	68.39	63.11	67.54	65.16	64.72	66.58	62.78	65.55	62.24	59.98	58.38	58.42	58.12	57.72	57.31	57.54	53.71	56.27	57.28	56.05	57.27
***A. anaerophilus* DSM 24636^T**	68.7	71.33	71.37	72.63	78.44	*	62.59	66.72	67.8	68.17	68.2	68.93	67.94	69.42	69.12	68.85	63.9	66.58	68.77	65.79	65.76	63.61	66.29	62.98	63.29	58.45	59.59	58.04	59.68	59.32	58.46	54.84	57.35	58.17	57.36	57.53
***A. lekithochrous* CECT 8942^T**	68.61	67.8	68.14	67.34	66.32	66.71	*	63.13	64.77	67.93	67.52	68.42	67.51	67.17	67.07	67.11	65.36	64.75	62.63	65.93	63.73	64.12	63.82	58.88	56.86	56.1	56.41	55.44	56.32	57.77	56.29	52.02	53.95	56.03	52.98	53.89
***A. nitrofigilis* DSM 7299^T**	67.63	68.08	67.34	67.48	68.24	67.99	66.25	*	66.82	67.01	66.75	67.35	66.95	67.11	68.26	67.6	62.82	67.2	64.5	64.01	65.62	61.39	64.12	60.83	58.7	56.83	56.39	56.58	55.89	56.41	57.21	52.64	54.62	55.54	54.09	55.69
***A. aquaticus* ' W112-28T**	67.64	70,0	72.16	68.61	68.36	66.5	62.41	65.69	*	68.11	66.79	66.79	65.79	66.63	66.85	66.62	66.76	64.1	64.74	66.65	66.29	63.21	65.13	64.97	63.73	61.98	61.37	60.71	59.46	60.2	61.32	56.69	59.87	62.28	59.32	59.72
***A. mytili* CECT 7386^T**	70.14	70.49	73.65	66.26	66.56	65.15	66.16	63.46	68.28	*	77.87	75.4	76.53	77.12	76.06	76.39	65.38	63.11	62.39	66.65	64.17	63.7	64.09	62.73	60.82	59.32	58.76	59.41	58.14	59.61	60.58	54.68	57.37	60.46	56.86	58.06
***A. halophilus* DSM 18005^T**	72.65	75.14	75.68	69.21	69.01	68.6	66.05	66.97	68.82	67.64	*	84.58	88.88	78.16	77.4	78.1	67.53	64.94	65.19	68.89	67.06	66.6	66.81	65.41	62.34	63.02	62.6	61.57	61.24	63,0	63.01	58.02	61.01	61.29	60.82	62.01
***A. canalis* ' F138-33T**	69.88	72.72	72.58	68.36	66.46	66.87	64.46	65.07	66.67	67.93	86.52	*	86.66	78.12	77.94	77.78	64.68	64.65	63.87	68.39	65.68	65.62	64.97	64.56	60.79	61.57	61.26	60.57	60.01	61.62	61.1	57.81	60.5	60.11	60.16	61.41
***A. marinus* CECT 7727^T**	71.98	74.45	74.63	70.05	68.41	68.52	66.56	66.79	68.55	68.65	87.48	95.68	*	77.68	77.32	77.53	66.48	65.16	64.38	68.18	66.53	65.56	65.64	64.79	61.26	62.02	61.46	61.18	60.82	61.71	62.02	57.52	60.89	60.8	60.57	62.12
***A. neptunis* ' F146-38T**	70.03	73.24	72.27	67.79	71.72	71.53	65.33	68.4	68.34	74.13	80.22	77.06	78.3	*	85.04	91.36	66.33	68.31	67.36	67.92	68.38	65.9	69.38	65.43	63.44	61.01	61.23	60.1	60.39	62.3	61.79	57.32	60.4	60.44	59.84	61.49
***A. viscosus* ' F142-34T**	70.07	73.17	71.95	69.46	71.24	70.22	64.47	70.64	68.52	72.45	78.76	78.94	78.46	85.62	*	85.44	64.61	67.4	66.19	67.13	67.17	64.28	66.15	64.48	61.84	59.78	59.7	59.4	58.56	60.36	60.8	55.82	58.62	59.31	58.75	60.45
***A. molluscorum* CECT 7696^T**	68.28	73.06	71.3	67.76	71.64	70.78	63.8	68.94	68.01	72.31	79.15	77.71	79.15	87.12	87.6	*	65.45	67.9	66.72	67.22	67.76	65.44	67.1	65.64	62.56	60.92	61.16	59.79	59.73	61.35	60.56	56.72	59.45	59.29	58.9	61.03
***A. caeni* ' RW17-10T**	66.92	67.23	67.69	67.6	67.11	67.11	70.35	67.04	66.19	67.64	66.68	67.72	66.79	67.62	67.19	66.97	*	84.55	72.22	75.57	87.53	73.39	75.63	73.09	71.06	68.1	67.99	66.17	68.61	71.24	69.69	63.75	66.85	70.32	66.63	66.61
***A. venerupis* CECT 7836^T**	67.21	67.22	67.41	67.13	66.83	67.36	70.53	68.31	65.7	68.11	67.1	68.12	67.33	67.51	67.69	67.42	73.14	*	76.15	72.08	86.21	71.7	77.26	70.46	67.1	64.02	63.6	63.68	63.24	64.33	63.06	58.06	60.49	61.17	59.31	60.84
***A. defluvii* CECT 7697^T**	67.63	67.53	67.99	68.24	66.99	67.45	70.14	66.39	66.38	68.15	67.21	67.53	67.37	67.38	67.29	67.01	79.61	79.61	*	82.81	80.68	82.85	78.93	70.41	65.59	65.69	67.33	64.16	66.45	69.24	64.65	60.2	63.02	65.3	62.79	63.73
***A. aquimarinus* CECT 8442^T**	68.09	67.84	68.34	68.55	66.98	67.21	70.78	66.52	65.99	68.17	67.75	67.77	67.79	67.55	68.17	67.3	79.64	79.23	77,0	*	80.41	82.33	77.73	75.48	72.13	70.2	70.81	70.12	70.37	73.44	71.36	64.44	67.35	70.18	67.42	68.71
***A. suis* CECT 7833^T**	67.1	67.45	67.36	67.71	68.02	67.42	70.42	67.41	66.31	68.29	67.28	67.84	66.83	67.85	67.51	67.83	79.9	78.32	76.49	76.76	*	73.61	78.68	76.07	73.34	69.03	68.5	68.37	68.28	70.25	68.73	62.54	65.98	67.44	65.34	66.58
***A. cloacae* CECT 7834^T**	67.66	67.75	68.02	68.23	66.9	67.14	70.51	66.55	65.89	68.61	67.35	67.93	67.5	67.18	67.24	67.16	79.58	79.23	75.45	93.53	81.01	*	79.22	72.02	68.58	67.62	68.23	66.84	68.47	72.29	68.42	62.42	65.69	66.51	64.9	66.48
***A. ellisii* CECT 7837^T**	67.55	67.81	68.02	67.81	67.32	67.63	70.63	66.82	66.3	68.5	67.43	67.72	67.19	68.05	67.56	67.42	80.01	80.1	87.03	83.23	81.19	83.47	*	71.99	69.21	65.9	66.01	64.57	67.23	71.2	67.56	62.15	64.01	65.68	63.13	65.14
***A. butzleri* RM4018^T**	66.93	67.15	66.9	67.03	66.37	66.55	67.91	66.01	65.75	67.23	66.22	66.52	66.36	66.76	66.49	66.8	76.18	75.76	79.54	77.74	77.03	77.6	79.28	*	84.28	78.65	78.12	78.79	73.53	75,0	75.96	68.44	73.1	74.71	71.46	73.16
***A. lacus* ' RW43-9T**	66.72	67.05	67.31	67.67	66.62	66.96	68.05	65.93	65.55	66.97	66.41	66.72	66.14	66.96	66.77	66.57	75.89	75.68	79.97	77.74	77.12	72.72	79.49	93.72	*	76.48	76.7	75.5	72.45	72.91	73.93	67,0	71.01	74.17	69.36	70.34
***A. lanthierii* LMG 28516^T**	64.88	64.68	65.5	65.43	64.92	64.67	65.63	64.47	63.89	65.2	64.46	65.08	64.68	64.93	64.47	64.48	71.98	70.75	72.44	72.9	71.98	72.21	73.16	76.39	76.33	*	95.57	84,0	76.77	73.79	76.61	71.26	76.48	78.09	72.96	74.02
***A. hispanicus* ' FW54T**	65,0	64.49	65.53	65.28	65.09	64.86	66.02	64.28	63.7	65.19	64.77	65.05	64.63	64.87	64.55	64.72	71.99	71.04	72.82	73.02	72.19	72.83	73.15	76.62	76.65	88.62	*	83.93	77.05	75.49	76.68	71.21	76.52	79.88	73.65	74.73
***A. vitoriensis* ' FW59T**	64.65	64.92	65.5	65.14	64.53	64.35	65.42	63.9	63.49	65.01	64.73	64.87	64.64	64.43	64.57	64.58	71.58	71.07	72.52	72.27	72.3	72.03	72.55	76.87	77.14	87.23	86.97	*	74.94	71.7	76.09	69.89	74.12	75.47	71.58	72.66
***A. faecis* LMG 28519^T**	64.82	64.76	65.06	65.23	64.2	64.05	66.27	63.52	63.22	65.37	64.44	65.17	64.5	64.06	63.91	64.13	70.84	70.67	72.42	72.66	71.72	72.28	72.5	74.94	74.8	77.52	77.61	77.67	*	84.34	73.6	69.4	71.73	73.52	68.15	71.56
***A. cibarius* LMG 21996^T**	64.96	64.93	65.45	65.19	64.58	65.03	66.56	63.95	64.1	65.59	65.15	65.24	64.77	64.9	64.53	64.61	70.95	70.33	72.89	73.01	71.83	73.02	73.13	74.88	74.74	76.19	76.14	75.98	75.76	*	76.44	68.46	71.48	76.55	72.83	74.27
***A. cryaerophilus* LMG 24291^T**	64.21	65.01	64.93	64.76	64.01	64.25	65.42	63.47	63.35	65.2	64.41	64.7	64.31	64.39	64.28	64.46	69.73	69.81	71.77	71.63	70.93	72.35	71.72	74.62	74.06	78.47	78.29	78.32	78.18	78.2	*	71.45	75.54	81.81	78.92	79.37
***A. porcinus* ' LMG 24487T**	64.64	64.1	64.9	64.48	63.85	63.91	64.79	63.53	62.83	64.4	64.17	64.36	64.25	63.54	63.67	63.78	68.74	67.9	68.95	69.74	69.22	68.91	69.43	72.01	71.72	74.64	74.47	75.03	74.58	73.73	76.24	*	84.46	76.51	79.14	79.19
***A. thereius* LMG 24486^T**	64.47	63.66	64.88	64.35	63.86	63.93	64.86	63.54	62.68	64.48	63.94	64.71	64.13	64.11	63.97	64.04	68.93	68.07	68.97	69.69	69.43	68.8	69.4	71.87	71.47	74.5	74.74	75.04	74.35	74.14	76.25	93.15	*	76.4	78.41	78.6
***A. trophiarum* LMG 25534^T**	64.35	64.69	65.18	64.65	64.07	64.28	65.44	63.95	63.16	64.58	64.26	64.48	64.15	64.11	64.01	63.99	69.87	69.42	71.21	71.15	70.55	71.06	71.3	73.67	73.46	77.92	77.6	77.95	77.43	76.62	85,0	72.45	77.37	*	77.28	77.1
***A. mirounga* ' 9AntT**	64.68	64.65	65.55	65.15	64.82	64.56	65.4	64.2	63.57	65.25	64.89	65.05	65.05	64.62	64.54	64.27	69.8	69.44	71.37	74.39	70.1	71.35	71.13	73.57	73.22	75.49	75.45	76.13	75.68	75.89	76.87	76.51	81.36	77.15	*	85.35
***A. skirrowii* LMG 6621^T**	65.49	65.48	65.77	65.25	64.69	64.64	66,0	64.03	63.64	65.5	64.78	65.33	64.55	64.35	64.5	64.31	69.74	69.28	70.84	71.14	70.27	71.19	71.3	74.06	73.95	75.7	75.59	76.51	75.6	76.25	77.05	77.38	81.44	75.96	94.99	*

Table S4. Similarities (%) in the 16S rRNA gene among type species of each new described genus and the other genera of the family *Campylobacteraceae*.

		1	2	3	4	5	6	7	8	9	10	11	12	13
1	***Arcobacter nitrofigilis* DSM 7299^T**	*												
2	***Aliiarcobacter cryaerophilus* LMG 24291^T**	93.8	*											
3	***Pseudoarcobacter defluvii* CECT 7697^T**	95.8	94.5	*										
4	***Haloarcobacter bivalviorum* CECT 7835^T**	93.4	91.2	95.0	*									
5	***Malacobacter halophilus* DSM 18005^T**	94.5	92.5	94.6	93.6	*								
6	***Poseidonibacter lekithochrous CECT 8942 ^{T}***	95.1	92.0	94.2	92.9	92.9	*							
7	***Arcomarinus aquaticus'* W112-28**	94.9	92.6	94.8	93.0	94.2	94.0	*						
8	***Campylobacter fetus* subsp. *fetus* ATCC 2737**	85.4	85.9	85.7	86.1	85.2	85.7	86.9	*					
9	***Sulfurospirillum deleyianum* DSM 6946^T**	87.8	87.7	88.3	87.1	86.7	88.3	88.7	88.9	*				
10	***Thiovulum* sp.**	84.2	84.6	83.9	84.3	84.1	84.5	83.9	83.9	84.4	*			
11	***Sulfuricurvum kujiense* DSM 16994^T**	85.1	85.1	85.7	84.8	85.1	85.3	85.4	86.1	87.9	87.8	*		
12	***Sulfurimonas autotrophica* DSM 16294^T**	84.7	85.7	85.2	84.7	85.9	84.6	85.9	85.5	86.9	86.7	89.7	*	
13	***Helicobacter pylori* NCTC 11637^T**	83.8	85.1	84.6	84.3	84.7	84.1	84.1	85.1	84.2	85.1	86.7	85.6	*

Supplemetary Table S5: Genes used in the core genome analysis and their function.

Gene	Annotation
tap_1	Methyl-accepting chemotaxis protein II
asd	Aspartate-semialdehyde dehydrogenase
gatB	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B
tadA	Riboflavin biosynthesis protein RibD
rnj	Ribonuclease J
ubiA	4-hydroxybenzoate octaprenyltransferase
rpsT	30S ribosomal protein S20
ppaC	Manganese-dependent inorganic pyrophosphatase
acnB	Aconitate hydratase B
fabH	3-oxoacyl-[acyl-carrier-protein] synthase 3
nikB	Nickel transport system permease protein NikB
mutS	DNA mismatch repair protein MutS
tyrS	Tyrosine--tRNA ligase
guaB_1	hypothetical protein
mtaB	Threonylcarbamoyladenosine tRNA methylthiotransferase MtaB
prfB	Peptide chain release factor 2
aspS	Aspartate--tRNA(Asp/Asn) ligase
pyrE	Orotate phosphoribosyltransferase
qseB_1	Transcriptional regulatory protein QseB
htpG	Chaperone protein HtpG
clpY	ATP-dependent protease ATPase subunit ClpY
rpe	Ribulose-phosphate 3-epimerase
ftsK	DNA translocase FtsK
yajQ	Cyclic di-GMP-binding protein
hisF	Imidazole glycerol phosphate synthase subunit HisF
purA	Adenylosuccinate synthetase
purS	Phosphoribosylformylglycinamidine synthase subunit PurS
lysA	Diaminopimelate decarboxylase
mqnC	Cyclic dehypoxanthine futalosine synthase
luxS	S-ribosylhomocysteine lyase
lysC	Aspartate kinase Ask_LysC
hpf	Ribosome hibernation promotion factor
atpE	ATP synthase subunit c
flhF	Flagellar biosynthesis protein FlhF
ffh	Signal recognition particle protein
ydcP	putative protease YdcP
rplJ	50S ribosomal protein L10
leuA_2	2-isopropylmalate synthase
lepA	Elongation factor 4
ackA_1	Acetate kinase
dapB	4-hydroxy-tetrahydrodipicolinate reductase
ccoN1	Cbb3-type cytochrome c oxidase subunit CcoN1
serA	D-3-phosphoglycerate dehydrogenase
pheS	Phenylalanine--tRNA ligase alpha subunit
accA	Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha
lysS	Lysine--tRNA ligase
mdeA	L-methionine gamma-lyase

dnaE	DNA polymerase III subunit alpha
ileS	Isoleucine--tRNA ligase
pgsA	CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase
glcD	putative FAD-linked oxidoreductase
gyrB	DNA gyrase subunit B
accC	Biotin carboxylase
pgk	Phosphoglycerate kinase
group_169	hypothetical protein
ftsY	Signal recognition particle receptor FtsY
group_170	hypothetical protein
guaB_1	Inosine-5'-monophosphate dehydrogenase
rplN	50S ribosomal protein L14
rpsJ	30S ribosomal protein S10
alsS	Acetolactate synthase
acnD	2-methylcitrate dehydratase (2-methyl-trans-aconitate forming)
fliS	Flagellar protein FliS
rplA	50S ribosomal protein L1
rplK	50S ribosomal protein L11
def1	Peptide deformylase 1
group_181	Carboxynorspermidine synthase
group_182	hypothetical protein
dus	putative tRNA-dihydrouridine synthase
hisA	1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino] imidazo
ilvD	Dihydroxy-acid dehydratase
greA	Transcription elongation factor GreA
pcrA	ATP-dependent DNA helicase PcrA
lpxC	UDP-3-O-acyl-N-acetylglucosamine deacetylase
fabF	3-oxoacyl-[acyl-carrier-protein] synthase 2
fabG_1	3-oxoacyl-[acyl-carrier-protein] reductase FabG
petA	Ubiquinol-cytochrome c reductase iron-sulfur subunit
rplO	50S ribosomal protein L15
ychF	Ribosome-binding ATPase YchF
dnaB	Replicative DNA helicase
ybiT	putative ABC transporter ATP-binding protein YbiT
fliP	Flagellar biosynthetic protein FliP
engB	putative GTP-binding protein EngB
rny	Ribonuclease Y
korB	2-oxoglutarate oxidoreductase subunit KorB
nrdA	Ribonucleoside-diphosphate reductase 1 subunit alpha
rlmN	putative dual-specificity RNA methyltransferase RlmN
purM	Phosphoribosylformylglycinamidine cyclo-ligase
thrZ	Threonine--tRNA ligase 2
pheA	P-protein
lptB	Lipopolysaccharide export system ATP-binding protein LptB
pckA	Phosphoenolpyruvate carboxykinase (ATP)
ttcA	tRNA 2-thiocytidine biosynthesis protein TtcA
group_216	putative transcriptional regulatory protein
carB	Carbamoyl-phosphate synthase large chain
nuoF	NADH-quinone oxidoreductase subunit F
nuoI_1	NADH-quinone oxidoreductase subunit I

group_22	hypothetical protein
frdA	Fumarate reductase flavoprotein subunit
miaB	tRNA-2-methylthio-N(6)-dimethylallyladenosine synthase
ruvB	Holliday junction ATP-dependent DNA helicase RuvB
fliI	Flagellum-specific ATP synthase
glnA	Glutamine synthetase
atpG_1	ATP synthase gamma chain
oppC	Putative peptide transport permease protein
hisD	Histidinol dehydrogenase
accD	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta
yfbR	5'-deoxynucleotidase YfbR
lon	Lon protease
purF	Amidophosphoribosyltransferase
map	Methionine aminopeptidase 1
group_237	hypothetical protein
group_24	hypothetical protein
pyrG	CTP synthase
purU	Formyltetrahydrofolate deformylase
gyrA	DNA gyrase subunit A
nrdB	Ribonucleoside-diphosphate reductase subunit beta
alaC	Glutamate-pyruvate aminotransferase AlaC
dmdA	2,3-dimethylmalate dehydratase large subunit
cmoB	tRNA U34 carboxymethyltransferase
fabI	Enoyl-[acyl-carrier-protein] reductase [NADH] FabI
rppH	RNA pyrophosphohydrolase
group_253	hypothetical protein
aroH	Phospho-2-dehydro-3-deoxyheptonate aldolase
fliG	Flagellar motor switch protein FliG
glyQ	Glycine--tRNA ligase alpha subunit
fusA	Elongation factor G
rpoBC_1	Bifunctional DNA-directed RNA polymerase subunit beta-beta'
purH	Bifunctional purine biosynthesis protein PurH
cheB	Chemotaxis response regulator protein-glutamate methylesterase
era	GTPase Era
panD	Aspartate 1-decarboxylase
groL	60 kDa chaperonin
rpoZ	DNA-directed RNA polymerase subunit omega
uvrB	UvrABC system protein B
argB	Acetylglutamate kinase
atpB	ATP synthase subunit a
secG	Protein-export membrane protein SecG
guaA	GMP synthase [glutamine-hydrolyzing]
fabL	Enoyl-[acyl-carrier-protein] reductase [NADPH] FabL
ruvC	Crossover junction endodeoxyribonuclease RuvC
trmD	tRNA (guanine-N(1)-)-methyltransferase
purB	Adenylosuccinate lyase
mcl1	L-malyl-CoA/beta-methylmalyl-CoA lyase
gltA	Citrate synthase
ilvC	Ketol-acid reductoisomerase (NADP(+))
rpoBC_2	Bifunctional DNA-directed RNA polymerase subunit beta-beta'

trpS	Tryptophan--tRNA ligase
rnc	Ribonuclease 3
folE	GTP cyclohydrolase 1
group_293	Nucleoid-associated protein
eno	Enolase
dnaK	Chaperone protein DnaK
cysL	HTH-type transcriptional regulator CysL
nadC_2	putative nicotinate-nucleotide pyrophosphorylase [carboxylating]
rplF	50S ribosomal protein L6
flaA_2	Flagellar filament 33 kDa core protein
icd	Isocitrate dehydrogenase [NADP]
radA	DNA repair protein RadA
thiC	Phosphomethylpyrimidine synthase
prfA	Peptide chain release factor RF1
hisB	Imidazoleglycerol-phosphate dehydratase
rpoD	RNA polymerase sigma factor RpoD
plsX	Phosphate acyltransferase
recA	Protein RecA
rpsA_1	30S ribosomal protein S1
group_32	Aspartate aminotransferase
typA	GTP-binding protein TypA/BipA
group_322	putative acyl-CoA thioester hydrolase
hslV	ATP-dependent protease subunit HslV
ribH	6,7-dimethyl-8-ribityllumazine synthase
rpsI	30S ribosomal protein S9
group_343	hypothetical protein
tufA	Elongation factor Tu
aroQ	3-dehydroquinate dehydratase
purQ	Phosphoribosylformylglycinamidine synthase subunit PurQ
petB	Cytochrome b
rplS	50S ribosomal protein L19
rplX	50S ribosomal protein L24
rpmC	50S ribosomal protein L29
rplD	50S ribosomal protein L4
rplC	50S ribosomal protein L3
rpsB	30S ribosomal protein S2
ispG	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase (flavodoxin)
rplT	50S ribosomal protein L20
mcl2	(3S)-malyl-CoA thioesterase
argG	Argininosuccinate synthase
purD	Phosphoribosylamine--glycine ligase
prpB	2-methylisocitrate lyase
ndk	Nucleoside diphosphate kinase
clpP	ATP-dependent Clp protease proteolytic subunit
trpG	Anthranilate synthase component 2
prs	Ribose-phosphate pyrophosphokinase
pyrH	Uridylate kinase
efp	Elongation factor P
secY	Protein translocase subunit SecY
rplE	50S ribosomal protein L5

rpsQ	30S ribosomal protein S17
tsaA_2	putative peroxiredoxin
slyD	FKBP-type peptidyl-prolyl cis-trans isomerase SlyD
ftsH_1	ATP-dependent zinc metalloprotease FtsH
DmdB	2,3-dimethylmalate dehydratase small subunit
rplQ	50S ribosomal protein L17
nqo6_1	NADH-quinone oxidoreductase subunit 6
nuoK	NADH-quinone oxidoreductase subunit K
cheY_3	Chemotaxis protein CheY
nusG	Transcription termination/antitermination protein NusG
rpmA	50S ribosomal protein L27
topA	DNA topoisomerase 1
group_423	hypothetical protein
rpsH	30S ribosomal protein S8
hisG	ATP phosphoribosyltransferase
pdg	Ultraviolet N-glycosylase/AP lyase
argH	Argininosuccinate lyase
fdx	Ferredoxin
dnaA	Chromosomal replication initiator protein DnaA
rpmI	50S ribosomal protein L35
rpsD	30S ribosomal protein S4
rpoA	DNA-directed RNA polymerase subunit alpha
atpC	ATP synthase epsilon chain
atpA	ATP synthase subunit alpha
valS	Valine--tRNA ligase
rpsS	30S ribosomal protein S19
mnmG	tRNA uridine 5-carboxymethylaminomethyl modification enzyme MnmG
rplU	50S ribosomal protein L21
rplB	50S ribosomal protein L2
rplM	50S ribosomal protein L13
cheY_1	Chemotaxis protein CheY
rpsM	30S ribosomal protein S13
yhdN	General stress protein 69
rho	Transcription termination factor Rho
rplL	50S ribosomal protein L7/L12
atpD_1	ATP synthase subunit beta
ygiC	Putative acid--amine ligase YgiC
kdsA	2-dehydro-3-deoxyphosphooctonate aldolase
rpsR	30S ribosomal protein S18
rhpA	DEAD-box ATP-dependent RNA helicase RhpA
group_55	Serine-pyruvate aminotransferase
metH	Methionine synthase
rpsG	30S ribosomal protein S7
soj	Sporulation initiation inhibitor protein Soj
hemN_2	Oxygen-independent coproporphyrinogen III oxidase
rplW	50S ribosomal protein L23
argF	Ornithine carbamoyltransferase
rpmB	50S ribosomal protein L28
rplP	50S ribosomal protein L16
rpsC	30S ribosomal protein S3

hemL	Glutamate-1-semialdehyde 2,1-aminomutase
mreB	Rod shape-determining protein MreB
ubiD	3-octaprenyl-4-hydroxybenzoate carboxy-lyase
mqnE	Aminodeoxyfutalosine synthase
mqnD	1,4-dihydroxy-6-naphtoate synthase
trpE	Anthranilate synthase component 1
aroA1	3-phosphoshikimate 1-carboxyvinyltransferase 1
tatC	Sec-independent protein translocase protein TatC
rpsK	30S ribosomal protein S11
hup	DNA-binding protein HU
coaD	Phosphopantetheine adenylyltransferase
padE	hypothetical protein
hemE	Uroporphyrinogen decarboxylase
rpsP	30S ribosomal protein S16
acpP	Acyl carrier protein
rplV	50S ribosomal protein L22
infA	Translation initiation factor IF-1
dapH	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-acetyltransferase
hemB	Delta-aminolevulinic acid dehydratase
ribBA	Riboflavin biosynthesis protein RibBA
metG	Methionine--tRNA ligase
rpsL	30S ribosomal protein S12
tgt	Queuine tRNA-ribosyltransferase
purL	Phosphoribosylformylglycinamidine synthase subunit PurL
fba	Fructose-bisphosphate aldolase
rpmF	50S ribosomal protein L32
rpmG2	50S ribosomal protein L33 2
rpmH	50S ribosomal protein L34
ribD	Riboflavin biosynthesis protein RibD
glmS	Glutamine--fructose-6-phosphate aminotransferase [isomerizing]
sufB	FeS cluster assembly protein SufB
aroB	3-dehydroquinate synthase
trpB_1	Tryptophan synthase beta chain
serS	Serine--tRNA ligase
tcrA	Transcriptional regulatory protein TcrA
rpsE	30S ribosomal protein S5
rpsZ	30S ribosomal protein S14 type Z
rpsO	30S ribosomal protein S15
uvrA	UvrABC system protein A

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