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EXPRESSIÓ GÈNICA EN MICROORGANISMES MARINS

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RESUM

Ara que es comença a treure l'entrellat -o si més no a tenir una nova perspectiva- de la diversitat de microorganismes present als oceans gràcies a la biologia molecular i a la metagenòmica, el següent pas és esbrinar quines són les noves funcions que aquesta amaga i com són utilitzades i per qui a l'oceà. La regulació de l'expressió gènica és la base de la versatilitat i l'adaptabilitat de qualsevol organisme al medi on viu. De l'estudi dels gens expressats d'un organisme es poden deduir correctament moltes de les característiques del medi on la seva vida es desenvolupa habitualment. En cada situació els organismes expressen solament una part del seus gens, responent tant a factors interns (per exemple el cicle cel·lular) com a factors externs (temperatura, llum, aport de nutrients...).

Les tecnologies de seqüenciació massiva també s'han aplicat en l'estudi de l'expressió de les comunitats microbianes marines (metatranscriptòmica). Tanmateix, aquestes tecnologies encara no estan prou optimitzades i sovint proporcionen seqüències que no poden ser assignades a cap gen conegut. En aquesta tesi ens hem plantejat l'estudi de l'expressió gènica dels microorganismes marins a tres escales diferents: a escala de comunitat, de genoma, i de gen.

L'esforç més gran ha estat estudiar l'expressió gènica a escala de comunitat, on el nostre repte ha estat desenvolupar una tècnica equivalent als mètodes "d'empremta dactilar" (*fingerprinting*) del DNA que s'usen de forma rutinària –com la DGGE o l'ARISA- per tal d'explorar la dinàmica dels patrons d'expressió gènica de les comunitats de microbis marins, permetent la comparació d'un gran nombre de mostres a un preu assequible i sense la necessitat prèvia de saber les seqüències dels RNA missatgers. Aquesta tècnica, batejada com a TFA (de "*Transcriptome Fingerprinting Analysis*"), ens ha permès estudiar I) les variacions estacionals en els patrons d'expressió gènica dels picoeucariotes marins de l'Observatori Microbià de la Badia de Blanes durant 4 anys, i II) les variacions dels patrons d'expressió al llarg d'un gradient espacial horitzontal i vertical i d'un gradient temporal. En ambdós casos, els canvis d'expressió s'han comparat amb els canvis en l'estructura de la comunitat (mitjançant l'ARISA).

A escala genòmica hem estudiat la resposta transcripcional global d'un microorganisme heteròtrof a la llum. La llum és responsable d'una gran quantitat de respostes fisiològiques. Una gran part dels microorganismes del mar que utilitzen la llum ho fan mitjançant la fotosíntesis, però existeixen d'altres microorganismes que utilitzen la llum de manera diferent, com els fotoheteròtrofs, que la utilitzen per generar energia però no fixen CO_2. En un dels estudis de genòmica ambiental es va descobrir la presència d'una proteïna fotoactiva, la proteorodopsina, associada a un grup de bacteris marins no cultivats. Les proteorodopsines són responsables d'un nou mecanisme de fototrofia als oceans; funcionen com a bombes de protons accionades per la llum que generen un gradient de protons a la membrana per tal de sintetitzar ATP.

A escala de gen, en aquesta tesi hem estudiat mitjançant RT-PCR l'expressió del gen de la proteorodopsina en un cultiu d'una flavobacteria marina i hem vist que la llum augmentava els seus nivells d'expressió.

SUMMARY

Recent advances have been crucial to understand, or at least to have a new perspective, on the diversity of microorganisms present in the oceans through molecular biology and metagenomics. The next step is to find out what functions are hidden within this diversity and how and when are they used. The regulation of gene expression is the basis of the versatility and adaptability of any living organism to the environment. The study of the genes expressed in an organism can help to deduce many of the characteristics of the environment. Usually, organisms express only a portion of their genes in response to both internal factors (e.g. cell cycle) and external factors (temperature, light, nutrients, etc.).

Massive sequencing technologies have also been applied to the study of the expression of genes in marine microbial communities (metatranscriptomics). However, these technologies are not yet sufficiently optimized and often provide sequences that cannot be assigned to known genes.

In this PhD thesis I have studied gene expression of marine organisms at three different levels: at the community level, at the genome level, and at the gene level.

The major effort was dedicated to gene expression at the community level, where the challenge was to develop a technique equivalent to DNA fingerprinting methods that are routinely used -such as ARISA or DGGE- in order to explore the dynamics of gene expression patterns in marine microbial communities, allowing the comparison of a large number of samples at an affordable price and without the need for prior knowledge of the messenger RNA sequences. This technique, called TFA (from "Transcriptome Fingerprinting Analysis"), has then been used to study I) seasonal variations in gene expression patterns of marine picoeukaryotes at the Blanes Bay Microbial Observatory during 4 years, and II) changes in expression patterns along spatial horizontal and vertical gradients and diel cycles. In both cases, expression changes were compared with changes in community structure (by ARISA).

At the genomic level I have studied the global transcriptional response to light of a heterotrophic microorganism. Light is responsible for a large number of physiological responses. A large fraction of marine microorganisms that use light perform photosynthesis, but there are other organisms as photo-heterotrophs, who use light to generate energy but do not fix CO_2. At the gene level, we have studied the proteorhodopsin gene expression by RT-PCR in a culture of a marine flavobacterium. In a study of environmental genomics, the presence of this photoactive protein was found to be associated with a group of uncultivated marine bacteria. Proteorhodopsins are responsible of a new mechanism of phototrophy in the oceans; they act as proton pumps powered by light that generate a membrane proton gradient in order to synthesize ATP. In the present study it was found that light increased the expression levels of the proteorhodopsin gene.

Introducció

Els microorganismes són els éssers vius més abundants i diversos que hi ha a la Terra (Baldauf, 2008; Whitman, Coleman, & Wiebe, 1998) i abarquen tots els processos dels quals les cèl·lules vives són capaces. Solament els microbis, especialment els bacteris, conserven la major part, si no totes, de les diverses potencialitats de la vida. La diversitat procariota, bacteris i arqueus, es manifesta sobretot en el seu metabolisme, en canvi, tot i que els eucariotes també tenen certa diversitat metabòlica, manifesten un ventall molt més gran de diversitat morfològica i de comportament.

ELS MICROORGANISMES MARINS

Més del 90% de la biomassa d'éssers vius a l'oceà són microorganismes (Whitman et al., 1998). L'activitat d'aquests microorganismes és essencial pel metabolisme del sistema marí, tant pel cicle biogeoquímic del carboni (producció i respiració) com pels altres cicles. Són responsables del 50% de la producció primària del planeta (Field, Behrenfeld, Randerson, & Falkowski, 1998) i la major part de la respiració a les aigües marines és també deguda a ells (del Giorgio & Duarte, 2002). Aital producció pot fluir a través de la cadena d'herbívors (zooplàncton i peixos), circular per la xarxa tròfica microbiana (bacteris i protistes) o sedimentar cap a les profunditats. La importància relativa de cadascuna d'aquestes tres vies té grans repercussions per als recursos pesquers, la bomba biològica del carboni i la regulació del clima. Per tant, són un component essencial de les xarxes tròfiques dels ecosistemes marins.

La majoria d'aquests microorganismes encara no són cultivables però gràcies a les tècniques moleculars i a les últimes tecnologies de seqüenciació podem dir que tenim una idea aproximada de quins són els membres que formen les comunitats. L'amplificació per PCR de molècules filogenèticament informatives com l'rRNA 16S i el 18S ha proporcionat una nova perspectiva de la biodiversitat (Massana & Pedrós-Alió, 2008; Pace, Stahl, Lane, & Olsen, 1986). Podem dir que l'estat del coneixement de la diversitat microbiana als oceans està en una fase descriptiva força avançada (sobretot respecte pocs anys enrere) i en canvi continu degut a l'aplicació de tècniques cada vegada més potents (Sogin et al., 2006). Però per aquest motiu i també per la controvèrsia a l'hora de definir les "espècies" microbianes encara es fa força difícil fer una estimació concreta de la diversitat (Pedrós-Alió, 2006).

Amb el descobriment de tanta diversitat "inesperada" sorgeix la necessitat paral·lela d'esbrinar el paper que tenen a la comunitat aquests nous microorganismes, explorar el reservori de funcions que aquesta amaga i com aquestes funcions són utilitzades i per qui a l'oceà. Una manera d'aproximar-nos a l'estudi de la funció és a través de l'expressió gènica.

EL PROCÉS DE L'EXPRESSIÓ GÈNICA

L'expressió gènica és un procés complex que determina quins gens seran expressats com a proteïnes en un moment determinat, així com els nivells relatius d'abundància d'aquestes proteïnes. El procés d'expressió gènica implica una sèrie d'etapes: (i) síntesi de l'RNA, o transcripció, (ii) processament de l'mRNA (sols en eucariotes generalment. Després del seu processament, l'RNA es considera mRNA), (iii) transport de l'mRNA des del nucli al citoplasma (en eucariotes), (iv) síntesi proteica, o traducció (l'mRNA és usat com a motlle per fer la traducció a proteïnes, la seqüència d'aminoàcids de les quals està relacionada amb la seqüència nucleotídica de l'mRNA), (v) degradació de l'mRNA, i (vi) modificacions post-traduccionals (incloent degradació) de les proteïnes. Hi ha gens que no codifiquen per proteïnes, com per exemple els que codifiquen per l'RNA ribosòmic i l'RNA de transferència.

Els mRNA bacterians generalment no són processats després de la transcripció. Els mRNA d'eucarionts són sintetitzats al nucli i han de ser exportats al citoplasma per tal de ser traduïts a proteïna. La majoria de missatgers eucariotes tenen una seqüència poliadenílica a la zona 3', la cua poliA (aquesta característica dels missatgers eucariotes ens serà molt útil a l'hora del plantejament metodològic). La funció d'aquesta cua encara no és del tot coneguda, però pot tenir a veure amb l'exportació de l'mRNA madur cap al citoplasma o pot actuar com a estabilitzador de les molècules d'mRNA retardant la seva degradació al citoplasma (Dreyfus & Régnier, 2002).

La regulació de l'expressió gènica és la base de la versatilitat i adaptabilitat de qualsevol organisme al medi on viu. En cada situació concreta els organismes expressen solament una part dels seus gens. El conjunt de gens que s'expressen respon a factors interns (per exemple el cicle cel·lular) i a factors externs (temperatura, aport de nutrients, llum...). És evident que per entendre l'ecologia de qualsevol medi seria molt útil saber quins són els gens que s'estan expressant a cada moment.

ESTUDI DE LES FUNCIONS DELS MICROORGANISMES A L'OCEÀ: LA METATRANSCRIPTÒMICA

Els intents per relacionar diversitat i funció han estat presents des dels primers estudis de diversitat microbiana. Anteriorment a la revolució de la genòmica/metatranscriptòmica, la funcionalitat dels organismes que habiten l'oceà ha estat abordada de formes diverses. Per exemple (Brock & Brock, 1996) van introduir la microautoradiografia (MA) com una eina per estudiar l'activitat d'organismes aquatics individuals de mostres naturals. Aquesta tècnica consisteix en incubar la comunitat natural amb un precursor metabòlic marcat radioactivament i després es determina quina població l'incorpora mitjançant el revelat d'una emulsió fotogràfica. A més a més, es pot determinar la identitat de les cèl·lules amb sondes moleculars específiques. Per exemple, amb aquesta tècnica s'ha establert que els alfa-proteobacteris consumeixen aminoàcids mentre que les citòfagues prefereixen les proteïnes (Cottrell & Kirchman, 2000).

Com hem vist, l'expressió gènica és un bon indicador dels processos que s'estan duent a terme, ja que de l'estudi dels gens expressats d'un organisme es poden deduir correctament moltes de les característiques del medi on la seva vida es desenvolupa habitualment. Existeixen diversos mètodes per detectar l'expressió dels gens, per exemple: I) La detecció *in situ* de l'RNA mitjançant sondes (Pernthaler & Amann, 2004), aquesta tècnica però encara està lluny de ser aplicada de forma rutinària, sobretot per la inestabilitat de l'mRNA. II) Fins fa poc, el mètode escollit per detectar l'expressió de gens funcionals ha estat la RT-PCR: la transcripció reversa (RT) dels mRNA amb la seva posterior amplificació per PCR (*he usat aquesta tècnica en el capítol 5 per a la detecció de l'expressió del gen de la proteorodopsina*), i III) un altre mètode força utilitzat fins que va aparèixer la genòmica i els seus derivats han estat els microarrays -o xips de DNA- (Schena, Shalon, Davis, & Brown, 1995). Els microarrays són una col·lecció de fragments de DNA microscòpics ancorats individualment en diferents cel·les per enllaços covalents a una superfície sòlida (com un portaobjectes). Els microarrays permeten monitoritzar els nivells d'expressió de milers de gens de forma simultània (*en el capítol 4 he usat els microarrays per detectar la resposta transcripcional global d'un microorganisme a la llum*).

Seguint l'onada del desenvolupament de la genòmica, l'any 2008 va irrompre la seqüenciació de l'RNA (RNA-seq), que consisteix en la seqüenciació en profunditat del DNA complementari (cDNA) generat a partir de mostres d'RNA (Wang, Gerstein, & Snyder, 2009). Tanmateix, la metatranscriptòmica (igual que la resta d'òmiques) encara no ha assolit un nivell òptim d'estandardització i romanen diversos aspectes per millorar que van adreçant-se mica en mica (Behnke et al., 2011; Huse, Huber, Morrison, Sogin, & Welch, 2007; Levin et al., 2010; Quince et al., 2009; Van Vliet, 2010).

Els metatranscriptomes es poden obtenir a través de diferents metodologies de seqüenciació de nova generació (NextGen): Roche 454, Illumina i ABI SOLiD. L'aplicació d'aquestes tècniques dóna informació detallada de la diversitat taxonòmica i funcional de la comunitat transcripcionalment activa, ja que es caracteritzen tant els RNA missatgers com els ribosòmics.

Per una banda, el fet que també es caracteritzi l'RNA ribosomal és un avantatge ja que així s'obté una idea de la composició taxonòmica de la comunitat, però al mateix temps és un inconvenient ja que, com hem dit abans, s'obté gràcies a una tècnica encara no prou optimitzada incapaç de recuperar només els mRNA. Per exemple, les preparacions d'RNA bacterià normalment contenen fins a un 80% d'rRNA i tRNA (Condon, 2007). A més a més, els mRNA bacterians sovint tenen una vida mitjana molt curta i són altament inestables (Deutscher, 2006). La majoria d'estudis de transcriptòmica eliminen parcialment les seqüències d'rRNA i tRNA (Passalacqua et al., 2009). Existeixen diferents estudis de metatranscriptòmica microbiana on s'adreça el problema de l'eliminació de part de l'RNA ribosòmic (He et al., 2010; Stewart, Ottesen, & DeLong, 2010).

L'altre gran inconvenient de la metatranscriptòmica és que fa diana a seqüències que són altament expressades i que molt sovint són noves. La interpretació d'aquestes dades està subjecte a la informació prèvia continguda a les bases de dades i als microorganismes als quals se'ls destina més estudi. La proporció de seqüències desconegudes pot arribar a ser més que considerable, en molts dels estudis inclús pot arribar a ser fins el 90% de les seqüències obtingudes! (Gilbert et al., 2008). Per tant, elucidar les funcions de famílies senceres de gens és un dels grans reptes actuals.

CORRESPONDÈNCIA DELS GENS EXPRESSATS AMB L'ACTIVITAT. ACOBLAMENT TRANSCRIPCIÓ-FUNCIÓ

S'ha vist que el nivell d'activitat d'una funció bioquímica concreta de la comunitat microbiana pot ser predita a partir de l'abundància del corresponent trànscrit del metatranscriptoma de la comunitat (Helbling, Ackermann, Fenner, Kohler, & Johnson, 2012). Existeixen varis estudis que

demostren que bona part de la regulació gènica tant en eucariotes com en procariotes es produeix a nivell transcripcional (Lu, Vogel, Wang, Yao, & Marcotte, 2007). En aquest estudi van veure que el 73% de la variància en l'abundància de proteïnes d'un llevat i el 47% en *E. coli* s'explicava per l'abundància dels mRNAs. Tanmateix, també hi ha estudis que mostren que no sempre els canvis produïts en el transcriptoma estan en concordància amb els canvis traduccionals (Le Roch et al., 2004; Smith et al., 2010).

LES TÈCNIQUES D'EMPREMTES DACTILARS (*FINGERPRINTING*)

En molts dels estudis de metatranscriptòmica, sobretot en els inicials, la majoria de gens seqüenciats corresponen a maquinària bàsica que la cèl·lula utilitza per sobreviure, la qual cosa fa difícil veure quins són els gens rellevants donats per una situació ambiental. Per això, els estudis on es comparen situacions diferents són més informatius. Una manera de detectar fàcilment com són de diferents dues o més mostres entre sí abans de començar a fer els metatranscriptomes de tot plegat és aplicant tècniques de *fingerprinting* d'RNA o DNA. Les tècniques de *fingerprinting*, com la DGGE o l'ARISA, tenen l'avantatge que permeten comparar simultàniament moltes mostres diferents de forma ràpida i a un preu relativament barat. A la taula 1 es compara el nombre aproximat de bandes produïdes per algunes d'aquestes tècniques.

Taula 1. Mètodes de *fingerprinting* i número aproximat de bandes obtingudes amb cadascun d'ells.

Tècnica de *fingerprinting*	Nº de bandes
DGGE	~35
T-RFLP	~200
ARISA	~500
TFA	~100-500

En aquesta tesi he desenvolupat una tècnica de fingerprinting d'mRNA que pot ser útil a l'hora de determinar prèviament quines mostres són les més apropiades per l'obtenció dels seus metatranscriptomes (capítol 1).

D'aquí la importància de les tècniques de *fingerprinting,* com la desenvolupada en aquesta tesi, el *Transcriptome Fingerprinting Analysis* (TFA), que ens permeten captar una signatura filogenètica i funcional que farà que poguem separar i identificar diferents transicions espacials i temporals en el medi marí.

Del *Differential Display* al *Transcriptome Fingerprinting Analysis*

L'any 1992 es va descriure un nou mètode per identificar gens expressats diferencialment (Liang & Pardee, 1992); l'anomenat *differential display* (Figura 1). La tècnica del *differential display* consisteix en obtenir patrons d'expressió globals de diferents situacions (posteriorment, amb un notable esforç extra es poden identificar els gens expressats diferencialment). Per això, cal extreure l'RNA present en les mostres d'interès, sintetitzar els cDNAs complementaris amb encebadors oligodT (s'uneixen a la zona 3' dels missatgers dels eucariotes -cua poliA-), i després fer la PCR combinant els encebadors oligodT (en aquest pas marcats amb un fluoròfor) amb diferents encebadors arbitraris.

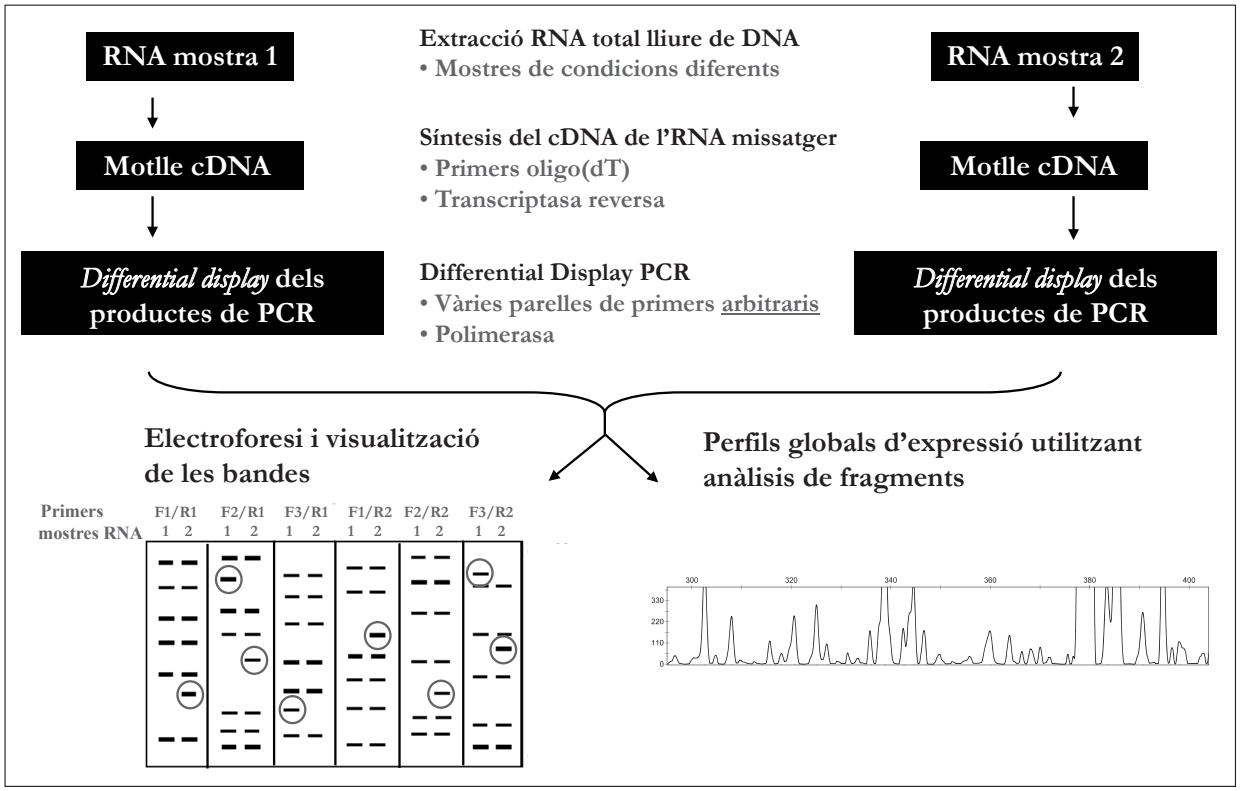

Figura 1. Esquema dels principals passos del protocol de *differential display*.

Els productes de PCR es separen en un analitzador genètic per obtenir un patró de pics, on cada pic correspon a un RNA missatger diferent (també es podrien separar mitjançant electroforesis en gel). Comparant els patrons de bandes de cada mostra es poden identificar les bandes que apareixen en una situació o en una altra, i finalment es podrien tallar i seqüenciar per identificar els gens expressats característics de cada situació.

En aquesta tesi he adaptat aquesta tècnica per tal d'explorar la dinàmica dels patrons d'expressió gènica de les comunitats de microorganismes marins, permetent la comparació d'un gran nombre de mostres a un preu assequible i sense la necessitat prèvia de saber les seqüències dels missatgers.

Un punt bàsic en el TFA és que no requereix el coneixement previ de les seqüències dels mRNAs que s'han de detectar tal com passa en els microarrays, i això és molt útil en sistemes on no hi ha o hi ha poca informació genòmica. Per tant, en el TFA detectem indistintament patrons tant de gens coneguts com de desconeguts.

En el TFA s'ha de tenir en compte una sèrie de punts importants: la quantitat d'RNA inicial, l'absència de DNA genòmic a la mostra d'RNA, la selecció dels encebadors, les condicions de PCR, el mètode de marcatge dels amplicons (radioactivitat, fluorescència), etc. Les decisions que s'han pres al llarg de tot el procés i que determinaran el resultat final són explicades amb detall al capítol 1 d'aquesta tesi.

EL PICOPLÀNCTON EUCARIOTA A LA COSTA DE BLANES

Una gran part d'aquesta tesi s'ha realitzat estudiant els picoeucariotes de la Badia de Blanes. Els picoeucariotes són un component menor -en termes de número de cèl·lules- del picoplàncton (<2-3μm) comparat amb els procariotes, però molt importants en termes de biomassa. Lluny de ser una mena de *background* als oceans, en són un component destacat (Marañón et al., 2001). Aquests organismes, com a mínim aquells que són fotosintètitcs, juguen un paper molt important en la producció primària, sobretot en àrees oligotròfiques, on poden representar fins el 80% de la biomassa autotròfica (Worden, Nolan, & Palenik, 2004). Mentre que els heteròtrofs i mixòtrofs consumeixen i mineralitzen d'altres microbis (Sherr & Sherr, 2002).

Massana (2011) ha defensat que la classificació dels picoeucariotes com a grup igual o més petit de 3μm és una classificació coherent. A l'Observatori Microbià de la Badia de Blanes (http://www.icm.csic.es/bio/projects/icmicrobis/bbmo), d'on es tenen registres de recomptes de picoeucariotes de fa més de 10 anys, s'ha vist que en la superfície els picoeucariotes fototròfics dominen respecte els heterotròfics amb una marcada estacionalitat (Figura 2).

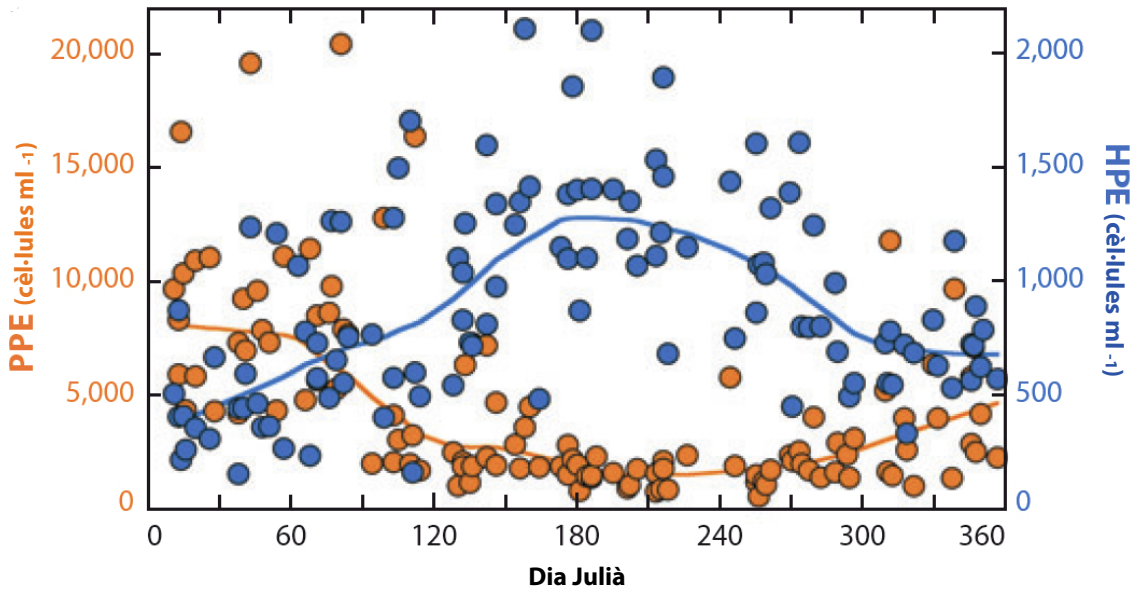

Figura 2. Abundància dels picoeucariotes fototròfics (PPEs) i els picoeucariotes heterotròfics (HPEs) (protistes ≤ 3) a l'Observatori Microbià de la Badia de Blanes durant nou anys de mostrejos mensuals (117 punts). De Massana (2011).

Destaca que durant l'estiu els recomptes de picoeucariotes fototròfics disminueixen però sobretot formen una comunitat molt constant -almenys en recompte-, en comparació a l'hivern, on augmenten però amb una gran variabilitat anual (Figura 2). A la primavera-hivern es produeix un bloom d'un grup de prasinofícies.

ELS MICROORGANISMES I LA LLUM

La llum és responsable d'una gran quantitat de respostes fisiològiques. Una gran part dels microorganismes del mar que utilitzen la llum ho fan mitjançant la fotosíntesis, però existeixen d'altres microorganismes que utilitzen la llum de manera diferent (Figura 3), com els fotoheteròtrofs, que la utilitzen per generar energia però no fixen CO_2 (Schwalbach, Brown, & Fuhrman, 2005).

Dos dels capítols d'aquesta tesi (4 i 5) estan relacionats amb la resposta dels microorganismes a la llum.

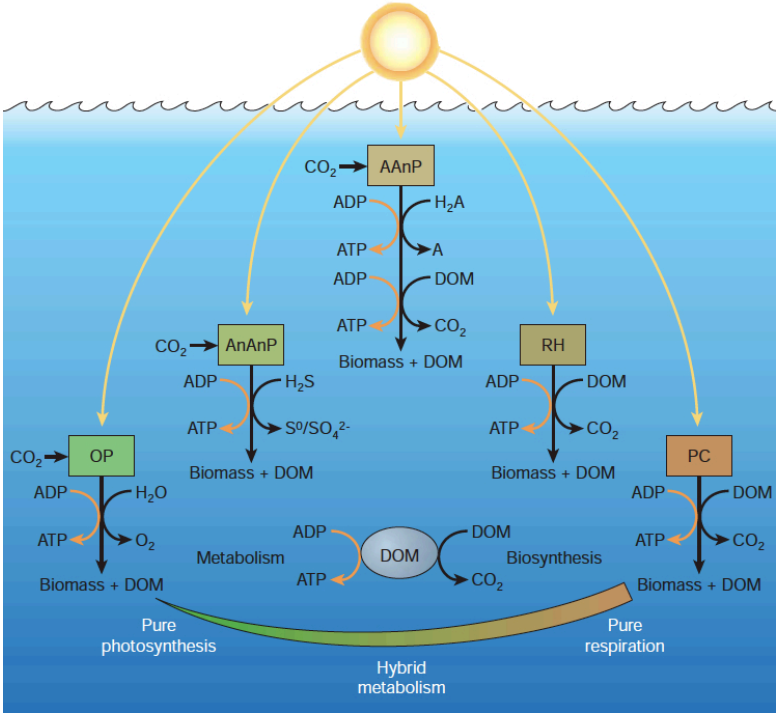

Figura 3. Representació dels metabolismes depenents de la llum de potencial importància en els microorganismes marins. OP: Fotosíntesis oxigènica basada en la clorofil·la *a*. Exemple: *Prochlorococcus*. AnAnP: Fotosíntesis anoxigènica anaeròbica basada en bacterioclorofil·les. Exemple: *Chromatium*. AAnP: Fotosíntesis anoxigènica aeròbica basada en bacterioclorofil·les. Exemple: *Roseobacter*. RH: Fotometabolisme basat en rodopsines. Exemple: SAR86. PC: Metabolisme basat en fitocroms. De Karl (2002).

Els bacteris fotoheteròtrofs anoxigènics aeròbics (AAnP) presenten un metabolisme quimioheterotròfic amb respiració aeròbica i a la vegada són capaços de realitzar fotosíntesis anoxigènica mitjançant complexes fotosintètics basats en bacterioclorofil·les. Els bacteris responsables d'aquesta activitat serien principalment del grup *Roseobacter*, uns alfa-Proteobacteris ben representats en mostres ambientals i de cultius (Giovannoni, 2000).

En aquesta tesi he utilitzat un bacteri d'aquest grup, Ruegeria sp.TM1040 per veure la seva resposta transcripcional global quan és estimulat per la llum (capítol 4). En aquests cas però, Ruegeria sp.TM1040 no té bacterioclorofil·les, però si altres receptors de la llum (com el BLUF de "sensors for Blue-Light Using FAD").

En un dels estudis de genòmica ambiental es va descobrir la presència d'un pigment relacionat amb les rodopsines (proteorodopsines) associat a un grup de bacteris marins no cultivats, el SAR86 dins dels gamma-Proteobacteria (Béjà et al., 2000). Les proteorodopsines funcionen com a bombes de protons fotoactives que generen un gradient de protons a la membrana per tal de generar ATP (Figura 4).

Més tard es va veure que les proteorodopsines estan àmpliament distribuïdes a les aigües marines superficials i que són presents als genomes de moltes espècies no cultivades (de la Torre et al., 2003; Sabehi et al., 2003; Venter et al., 2004).

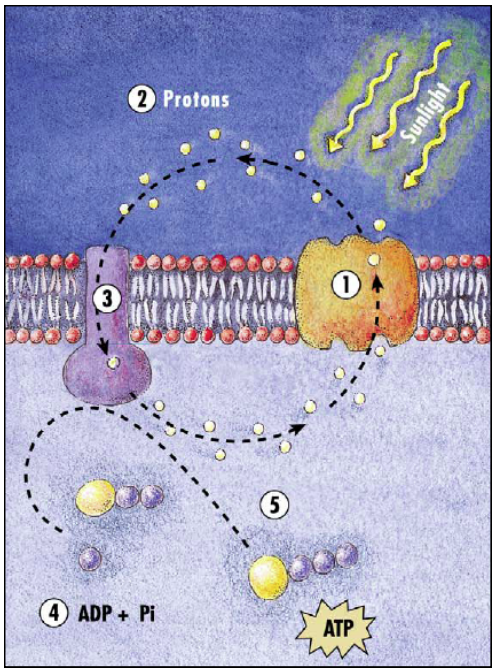

Figura 4. Les proteorodopsines són bombes de protons bacterianes (1) que amb l'energia de la llum generen un gradient de protons a través de la membrana (2). A través d'una ATPasa (3), aquest gradient és utilitzat per generar ATP (5) a partir d'ADP i fósfor (4). La molècula de retinal (cromòfor) canvia de conformació quan absorbeix un fotó, i aquest canvi produeix també un canvi conformacional de la proteïna i llavors és quan es produeix l'acció de bombejar protons cap a l'exterior. De DeLong i Béjà (2010). Il·lustració de Kirsten Carlson.

Hi ha diferents tipus de proteorodopsines que absorbeixen més eficientment segons la longitud d'ona. El fet que absorbeixin a una o altra longitud depèn sols de la identitat d'un aminoàcid de la seva seqüència (es diu que estan sintonitzades per absorbir a determinada longitud d'ona -*spectral tuning*-). N'hi ha de dos tipus: les que absorbeixen en el verd (525nm, i poden tenir l'aminoàcid leucina o metionina) i les que absorbeixen en el blau (490nm, amb l'aminoàcid glutamina). Aquestes variants del gen de la proteorodopsina no estan distribuïdes a l'atzar. De fet, el seu màxim d'absorció té diferents distribucions al llarg de gradients de profunditat i de gradients horitzontals (Man et al., 2003; Sabehi et al., 2003).

El gen de la proteorodopsina va ser detectat en el genoma seqüenciat complet de *Dokdonia* sp. MED134, una Flavobacteria aïllada de l'Observatori Microbià de la Badia de Blanes.

És per això que es van utilitzar cultius d'aquesta soca per veure com podia influenciar el fet de tenir aquesta proteïna en el creixement del bacteri i es va estudiar mitjançant RT-PCR l'expressió del gen de la proteorodopsina, responsable d'un nou mecanisme de fototrofia als oceans (capítol 5).

En resum, en el primer capítol de la tesi descric la posada a punt del mètode de *fingerprinting* de l'mRNA (el *Transcriptome Fingerprinting Analysis*) i en els capítols 2 i 3 aplico aquest mètode per tal de veure les variacions estacionals (capítol 2) i les variacions espacials i temporals (capítol 3) dels patrons d'expressió gènica de les comunitats de picoeucariotes marins. En el capítol 4 utilizo un microarray amb el genoma complet de *Ruegeria* sp. TM1040, un membre del grup dels Roseobacter, per tal de veure la seva resposta transcripcional global a la llum. I en el capítol 5 utilitzo la tècnica de RT-PCR per detectar l'expressió de la proteorodopsina en un cultiu d'una Flavobacteria marina (*Dokdonia* sp. MED134).

Chapter 1

Transcriptome Fingerprinting Analysis: An Approach to Explore Gene Expression Patterns in Marine Microbial Communities

ABSTRACT

Microbial transcriptomics are providing new insights into the functional processes of microbial communities. However, analysis of each sample is still expensive and time consuming. A rapid and low cost method that would allow the identification of the most interesting samples for posterior in depth metatranscriptomics analysis would be extremely useful. Here we present Transcriptome Fingerprinting Analysis (TFA) as an approach to fulfill this objective in microbial ecology studies. We have adapted the differential display technique for mRNA fingerprinting based on the PCR amplification of expressed transcripts to interrogate natural microbial eukaryotic communities. Unlike other techniques, TFA does not require prior knowledge of the mRNA sequences to be detected. We have used a set of arbitrary primers coupled with a fluorescence labeled primer targeting the poly(A) tail of the eukaryotic mRNA, with further detection of the resulting labeled cDNA products in an automated genetic analyzer. The output represented by electropherogram peak patterns allowed the comparison of a set of genes expressed at the time of sampling. TFA has been optimized by testing the sensitivity of the method for different initial RNA amounts, and the repeatability of the gene expression patterns with increasing time after sampling both with cultures and environmental samples. Results show that TFA is a promising approach to explore the dynamics of gene expression patterns in microbial communities.

Transcriptome Fingerprinting Analysis: An Approach to Explore Gene Expression Patterns in Marine Microbial Communities. **Montserrat Coll-Lladó, Silvia G. Acinas, Cristina Pujades and Carles Pedrós-Alió**. 2011. PLoS ONE, 6(8): e22950.

INTRODUCTION

Information about dynamics of the genes expressed by microbial communities is being explored by several approaches. Expression of specific genes can be successfully determined through quantitative RT-PCR, and microarrays are helpful tools to detect the expression level of a set of known genes. In addition, the 454 pyrosequencing technology has been recently applied to analyze marine microbial metatranscriptomes [1-6]. These metatranscriptomics studies of marine microbial communities are very powerful at uncovering active metabolisms and functional processes. However, this technology is still very costly and cannot be applied to a large set of samples. Thus, for example, Hewson et al. [7] analyzed the metatranscriptome of only eight samples: one from station Aloha, four from the Atlantic and three from the Pacific Ocean. These are only eight isolated stations from two huge oceans. If a fingerprinting method had been available, it would have been possible to determine how representative these samples were of the different water masses studied. Therefore alternative high-throughput approaches are needed to systematically compare and detect gene expression profiles with reasonable time and money costs.

Fingerprinting DNA techniques such DGGE [8,9], RFLP [10], t-RFLP [11] or ARISA [12,13] are widely used to compare microbial community composition among different samples. These techniques target the predominant taxa and allow the comparison of an extensive number of samples at a relatively low cost. Thus, studies of the seasonal and spatial distribution of both eukaryotes and prokaryotes have been successfully conducted and a fairly robust view of microbial distribution in the oceans has been obtained [9,14-20]. The next step would be to explore how the activity patterns of such communities change and whether they do so in correlation with taxonomic composition or not. A technique equivalent to DNA fingerprinting, however, is not currently available for patterns of gene expression in microbial communities. We developed an approach that has the advantages of fingerprinting, namely it is relatively cheap and allows processing of a large number of samples.

Here we present an approach to detect gene expression patterns in picoeukaryotic marine microbial communities. Transcriptome Fingerprinting Analysis (TFA) is based on the well-known differential display approach [21,22], but with some modifications to adapt it to marine microbial ecology studies (Figure 1). In this procedure, nucleic acids are extracted from the natural sample and treated with DNAase to leave only RNA. Then, reverse transcription is carried out with anchor primers. In our case, these primers target the poly(A) tail of eukaryotic mRNAs, insuring that rRNA will not be reverse-transcribed. Next, PCR is carried out with the same anchor primers plus a set of random primers. We used fluorochrome labeled anchor primers for this amplification so that the amplicons could be separated in a conventional gene analyzer. In the end, for each sample we had a profile in which every peak corresponded to an expressed gene. The differences between

the expression profiles in two different environments could then be easily explored. Allegedly, each sample should show peaks that were unique to that environment and peaks that were common for a specific set of conditions. The differences are presumably the result of different parameters associated with the specific environment. We determined the sensitivity and repeatability of the method using both cultures of the prasinophyte *Micromonas pusilla,* and natural marine picoeukaryotic communities from the Mediterranean Sea.

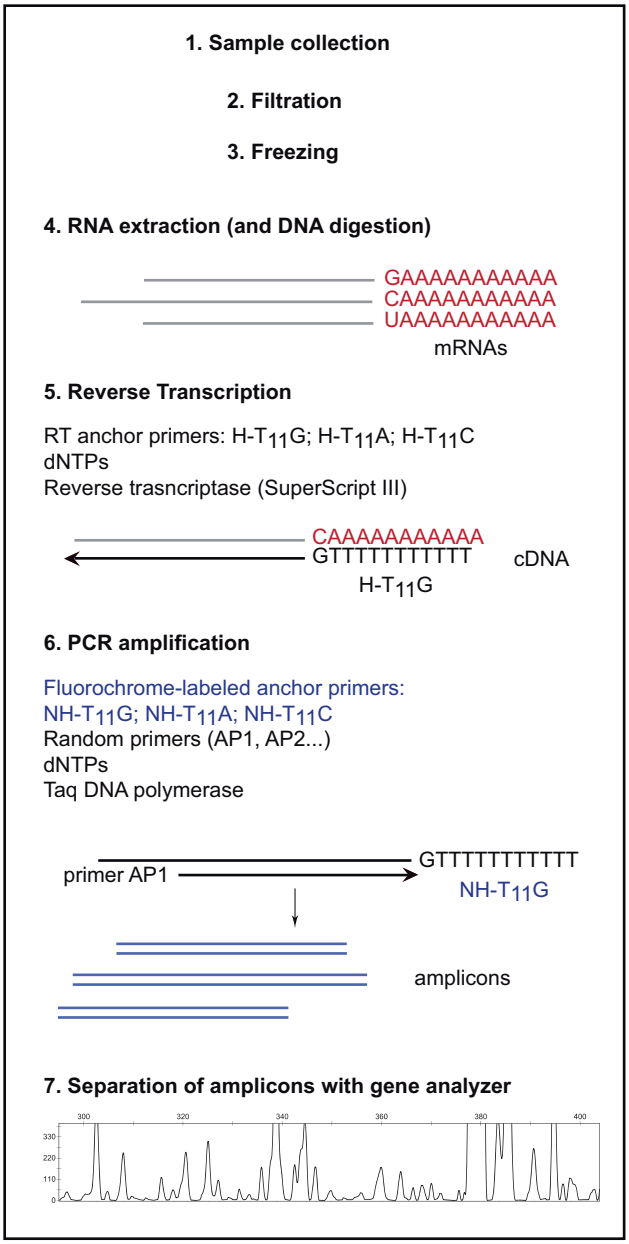

Figure 1. Scheme of the different steps in Transcriptome Fingerprinting Analysis (TFA). Note that in step 4 there is a mixture of ribosomal, transfer, and messenger RNAs. By using primers against the poly(A) tail, step 5 reverse-transcribe mRNAs only.

MATERIALS AND METHODS

Sampling and collection of biomass. Sea surface samples were obtained from the tip of the Gas pier in the Barceloneta beach (Barcelona) in 8 liter carboys. In experiment 1 (see Table 1), carried out on October 3, 2007, samples were kept on ice and in experiment 2 (Table 1), carried out on September 25, 2008, samples were either kept on ice (ICE samples) or at room temperature (RT samples) until the end of the filtration process. Water was prefiltered through a 200-μm mesh net. Additional water samples were collected during the MODIVUS cruise (17-27 September 2007) on board *R/V García del Cid* at three stations from coastal to open sea. Seawater (8 liters) was collected using Niskin bottles and was also prefiltered through 200-μm mesh net. A piece of 20-μm Nylon mesh was attached to the entrance tube cap of the filtration system and all environmental water samples were filtered first through a 3-μm pore-size polycarbonate filter (Poretics) and then through a 0.2-μm polycarbonate filter (Poretics) using a peristaltic pump (MasterFlex 7553-89 with cartridges Easy Load II 77200-62, Cole-Parmer Instrument Company) to collect the bacteria and picoeukaryotes. Filters were flash-frozen in liquid nitrogen and then stored at -80ºC until processed. Total RNA was extracted from the 0.2-μm polycarbonate filters.

***Micromonas* experiments.** Axenic cultures of the prasinophyte *Micromonas pusilla* CCMP 1545 obtained from the Provasoli–Guillard National Center for Culture of Marine Phytoplankton (CCMP; https://ccmp.bigelow.org/) were grown in f/2 medium [23] at 19ºC under a daily regime consisting of 12h of light and 12h of darkness. Growth of *M. pusilla* was followed by flow cytometry (procedure described in [24]) to be sure that the cultures were in exponential growth phase. Experiment 3 (Table 1) was initiated when cultures reached sufficient biomass after 6 days of growth and triplicates of a time zero control were taken. Half of the bottles were kept at 4ºC and half at 19ºC. After temperature equilibration (about five minutes) all bottles were transferred to the dark. 4ºC and 19ºC cultures were sampled in triplicate after 15 min, 30 min, 1h, 2h and 4h of incubation. At each sampling point 10 ml of culture were filtered through 0.8-μm-pore-size Durapore Filters and the filters were flash-frozen in liquid nitrogen and kept at -80º until RNA extraction. In experiment 4 (Table 1), a *M. pusilla* culture was growing at 19ºC under a 12 h light/ 12 h dark cycle also until late exponential phase. Then, part of the culture was incubated separately in the dark for 24 hours while the other remained under the light/dark regime. *M. pusilla* cultures were sampled in triplicate under light and dark conditions 24 hours after splitting conditions.

Table 1. Key to the different experiments showing the sample used and the variables tested in each case.

Experiment	Sample	Variables tested
1	Natural sample	Primer combinations, time since sampling
2	Natural sample	Time since sampling at two temperatures
3	*Micromonas*	Time elapsed at two temperatures
4	*Micromonas*	Light and dark conditions

RNA extraction and purification. The procedure was adapted from [25]. For RNA extraction, filters were transferred to 2 ml screw-cap microcentrifuge tubes containing 200-µl of 0.1-mm-diameter zirconia-silica beads (BioSpec Products, Inc.) and 100-µl of 0.5 mm glass beads (BioSpec Products, Inc.) mixed with 450-µl RLT lysis buffer (provided by the RNeasy® Mini Kit Qiagen, Inc.) plus β-mercaptoethanol (Fluka). Samples were mechanically disrupted in a Mini-beadbeater-8™ cell disrupter (BioSpec Products, Inc., Bartlesville, OK) for 10 min. After disruption, samples were incubated on ice for 5 min and the beads were allowed to settle out of the lysis mixture. Samples underwent centrifugation (in an Eppendorf centrifuge at 2100 rcf 1min). The lysate was transferred to a new tube. 300 µl of lysis solution was added to the vials with beads to increase the final yield. The tubes were shaken vigorously and the supernatant was also recovered. The same volume of 70% ethanol was added to the lysate and samples were purified according to the RNeasy® Mini Kit (Qiagen, Inc.). The isolated total RNA was treated with TurboDNase I (Ambion) to remove contaminating genomic DNA according to the manufacturer's instructions. RNA was aliquoted and quantified by absorbance at 260 nm with a NanoDrop 1000 (Thermo Fisher Scientific Inc., Wilmington, DE).

Reverse transcription and PCR amplification. First-strand cDNA synthesis was conducted with 20 or 40ng of total RNA as starting material. mRNAs were reverse-transcribed to single-stranded complementary DNA using the SuperScript III reverse transcriptase (Invitrogen) and three different primers, H-T_{11}G (5'-AAGCTTTTTTTTTTT**G**-3'), H-T_{11}A (5'-AAGCTTTTTTTTTTT-T**A-3'**) and H-T_{11}C (5'-AAGCTTTTTTTTTTT**C**-3'). To denature any secondary structure, an aliquot of each of the RNA extracts plus the oligo(dT) primer were heated for 5 min at 65 ºC and immediately placed on ice before mixing with the final reaction solution (20µl): 5X First-Strand buffer, 0.1M DTT, 40U RNaseOUT, 10mM dNTPs and 200U SuperScript III RT. Reaction mixtures were incubated at 50ºC during 50 min, and inactivated by heating 70ºC for 15 min. 2 µl of the RT reaction product was used in a subsequent PCR. PCR reactions were carried out using Taq polymerase (Qiagen) in a final reaction volume of 20 µl. Arbitrary primers coupled with the same primers used in the RT reaction but labeled with a fluorescence tag (NED) were used for the amplification of cDNA. Primers targeting the poly(A) tail and arbitrary primers for PCR were

from RNAspectra Yellow kit 1 of GenHunter Corporation. These primer sequences are given in Table 2. The PCR cycle was 40 cycles of 94 ºC for 30 s, annealing at 40 ºC for 2 min, 72 ºC for 60 s followed by 1 cycle of 72 ºC for 5 min in a Techne thermal cycler (Techne, Ltd., Cambridge). A negative control was run for each primer combination to assess the background levels (usually very low, below 20 relative fluorescence units -rfu-) and to ensure that there was no amplification of genomic DNA an aliquot of the RNA extracts was added directly to the PCR.

Table 2. Primers used in the present study (from a commercial primer kit: RNAspectra kit of GenHunter). One anchor primer was used in the RT reaction, and the same primer but fluorescently-labeled was combined with one of the arbitrary primers in the subsequent PCR.

Primer	Sequence (5'-3')
RT anchor primers	
H-T_{11}G	5'-AAGCTTTTTTTTTTG-3'
H-T_{11}A	5'-AAGCTTTTTTTTTTA-3'
H-T_{11}C	5'-AAGCTTTTTTTTTTC-3'
Fluorescently-labeled PCR anchor primers	
NH-T_{11}G	5'-AAGCTTTTTTTTTTG-3'
NH-T_{11}A	5'-AAGCTTTTTTTTTTA-3'
NH-T_{11}C	5'-AAGCTTTTTTTTTTC-3'
Arbitrary primers	
H-AP1	5'-AAGCTTGATTGCC-3'
H-AP2	5'-AAGCTTCGACTGT-3'
H-AP3	5'-AAGCTTTGGTCAG-3'
H-AP4	5'-AAGCTTCTCAACG-3'
H-AP5	5'-AAGCTTAGTAGGC-3'
H-AP6	5'-AAGCTTGCACCAT-3'
H-AP7	5'-AAGCTTAACGAGG-3'
H-AP8	5'-AAGCTTTTACCGC-3'

Detection and analysis of peaks. 1µl of PCR product from each sample was mixed with 9 µl of Hi-Di Formamide (Applied Biosystems). 0.5 µl of size standard (ROX 500, Applied Biosystems) was added to every reaction to define the standard curve between 25 and 500bp. The cDNA peaks obtained ranged in length from 30 to 500 bp, according to the internal size standard used. The mixtures were run on an ABI automated sequencer operating as a fragment analyzer (ABI 3130XL). The sequencer electropherograms were then analyzed using the GeneMarker software, version 1.90 (SoftGenetics, LLC). Raw data were treated with some filters activated according to GeneMarker instructions: baseline substraction, spike removal, auto pull up removal, smooth. The cubic spline algorithm was used to calculate bp lengths of identified fluorescence peaks. The following peak detection thresholds were applied: I) An intensity cutoff of 150 rfu was chosen,

although the use of this cutoff may have reduced the diversity of the communities (some peaks larger than background were present below that cutoff. II) The stutter peak filter with a 5% left and right percentage and a peak score between 5 and 7. In addition, the peaks were visually inspected for sporadic inconsistencies in the binning, basically for those peaks with high intensity (larger than 500 rfu) that could make a big difference in the community pattern. Once the peaks were selected, peak areas were used as output from GeneMarker software and were transferred to Microsoft Excel (Seattle, WA) for subsequent analysis.

Statistical analysis. The peak area data from GeneMarker were standardized (the relative peak heights within a profile were calculated by dividing the height of an individual peak by the total peak height -sum of the heights of all peaks in a pattern-). TFA was evaluated by comparing the number and area of peaks (bands) between electropherograms (profiles). The similarity of TFA profiles derived from different communities was assessed computing Bray-Curtis distances to construct the similarity matrices. Patterns were explored using nonmetric multidimensional scaling (NMDS) and clustering analysis. Primer-E version 6 was used for these analyses [26].

RESULTS

Sensitivity and repeatability of TFA

The amount of total RNA usually obtained from 8 L of seawater from oligotrophic Blanes Bay was approximately 90 ng. Experiments showed that around 10-20 ng were optimal for good quality patterns. Higher RNA concentrations, such as 80 ng, resulted in lower signal (data not shown). Although TFA was found to be very sensitive, it failed to produce repeatable patterns under the initially assayed conditions with the RNAspectra Yellow Kit 1 (GeneHunter). This kit is based on the MMLV reverse transcriptase (operating at 37 °C). Different reverse transcription enzymes were tested and the SuperScript-III enzyme (Invitrogen) produced the desired results. This is an engineered version of the former enzyme active at 50 ºC. With this enzyme the repeatability was very high, as evidenced by the fact that the electropherograms representing four replicates were identical, both with 20 and 40 ng of RNA (Figure 2). Finally, different times for the reverse transcription reaction were tested and no differences were found between 30 and 60 min (data not shown).

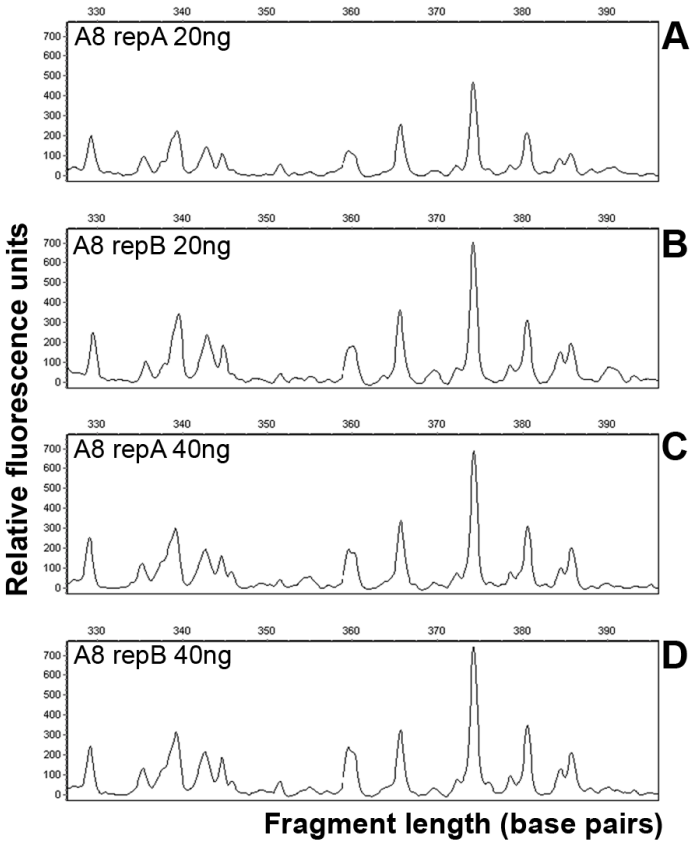

Figure 2. Examples of TFA profiles, showing the sensitivity and repeatability of the technique. All panels are replicates of the same environmental sample. Panels A and B show replicate fingerprints obtained from 20 ng of total RNA and panels C and D from 40 ng of RNA. The horizontal scale goes from 330 to 390 bp from left to right.

Elapsed time between sampling and filtration

Three different experiments were carried out to test the changes in expression patterns with time elapsed since sampling. Experiments 1 and 2 were done with seawater samples and experiment 3 with a culture of *Micromonas pusilla*. The shortest feasible time between sampling and filtration in the experiments with natural seawater was 30 minutes. The volume filtered was 2-4 L of water in 15-30 min for each time point for all the samples. Water was kept on ice until filtration was completed. The dendrogram in Figure 3A corresponds to experiment 1, carried out on 3 October 2007 in which the samples were filtered 30 min, 1h, 2h, 4h, and 8h after collection. The cDNA was amplified with three different arbitrary primer combinations (A8, C7 and A7). The patterns obtained with different primer combinations differed substantially both in the identity and quantity of genes retrieved as could be expected (see virtual gel in Figure 3A). Lines in gray indicate that the differences between branches were not significantly different at the 5% level. Slight differences between samples filtered at 30 min and one hour and the rest of the samples were observed with one of the primer combinations (C7).

Samples treated with the primer combination A8 were run with two different initial amounts of RNA (20 and 40 ng). The similarities among the treatments were explored with a NMDS diagram (Figure 3B). A gradual change in the patterns from 30 min to 8h was observed in both sets of samples. The amount of RNA had a small influence on the resulting pattern. However, all the samples showed a similarity higher than 90% among them. In conclusion, keeping the samples on ice was enough to guarantee that profiles did not differ significantly, even after 8 hours, with two of the three primer combinations tested.

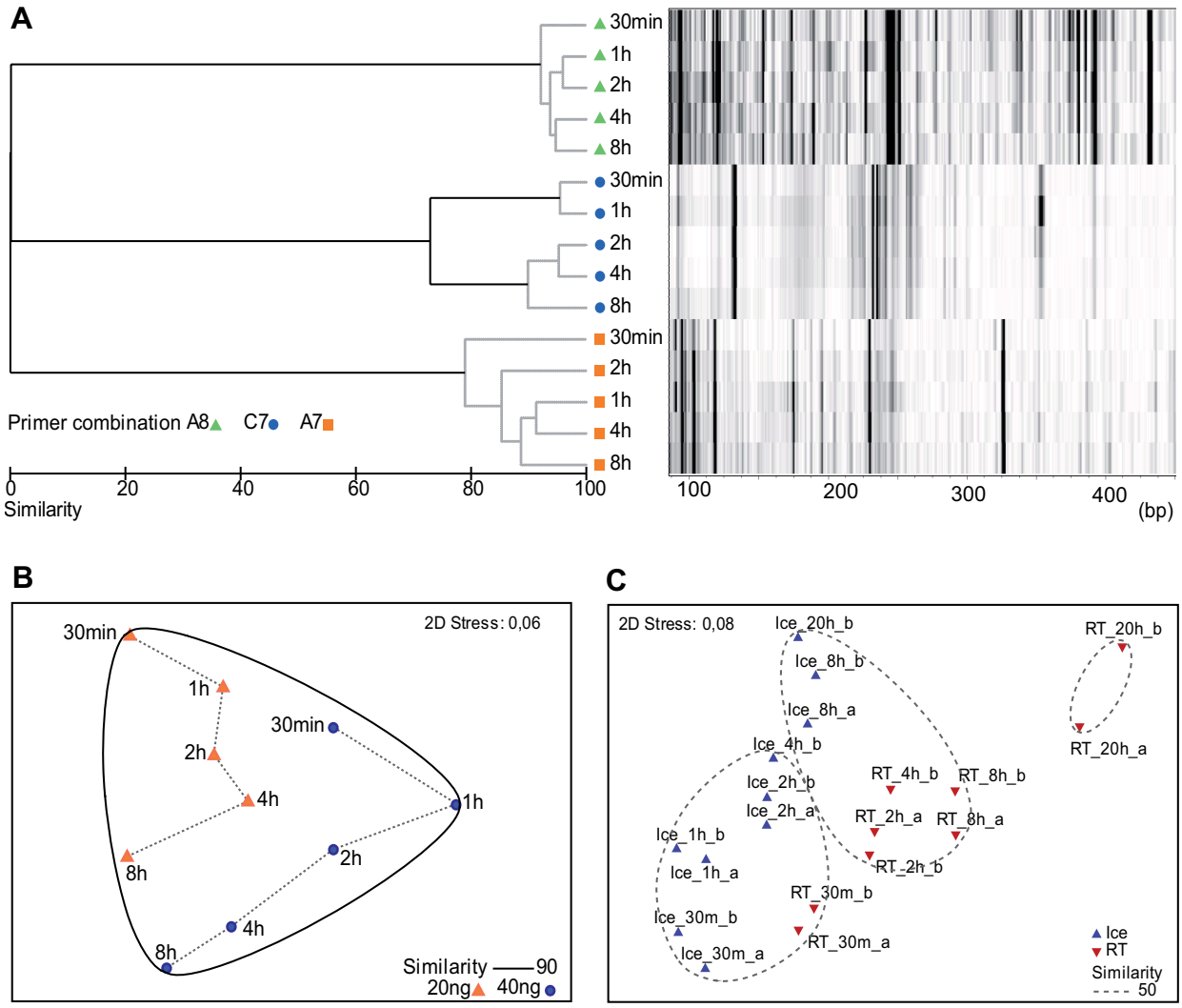

Figure 3. Comparison of TFA profiles from environmental samples filtered at progressively longer times after collection. The shortest practical time was 30 minutes. A) and B) experiment carried out on October 3, 2007 with samples kept on ice; and C) timing experiment carried out on September 25, 2008. A) Cluster analysis from a Bray-Curtis similarity matrix of TFA done with three different primer combinations (A8, C7, and A7) from the same sample filtered at different times between 30 min and 8 h. Gray lines indicate differences were not significant at the 5% level. The lane next to each sample corresponds to the peak pattern (in a base pairs scale) of each sample in a virtual gel. B) NMDS diagram comparing fingerprints obtained using two initial RNA amounts with the A8 primer combination. C) NMDS comparison of samples kept on ice or at room temperature filtered at different times between 30 min and 20 h. Patterns were obtained with the A8 primer combination. Missing replicates are due to low quality electropherograms.

In experiment 2 the effect of keeping samples either on ice or at room temperature was tested (Figure 3C). Duplicates were done for each time point and only one set of primers was used (A8). As expected, samples kept at room temperature during 20 hours differed the most from the initial samples. Samples kept on ice were more similar to the initial ones than their room temperature counterparts for the same sampling times.

Differences with time were tested again with a culture of *Micromonas pusilla* (experiment 3). Triplicates were done for each sampling point, from 15 min to 4 h, maintaining replicates of *M. pusilla* cultures at 4ºC or at 19ºC. Sampling and filtering were practically instantaneous, with no time delay. The primer combination A8 was used to obtain the fingerprints (Figure 4A) and distances among them were represented in a NMDS diagram (Figure 4B). No substantial differences were observed from 15 min to 2h in samples kept at 4ºC (except for one of the triplicates from 2 h that was an outlier). Slightly larger differences were observed at 19ºC, even though all the samples were more than 70% similar to the t=0 ones. At 4 h, however, both samples 4ºC and at 19ºC were significantly different from t=0. In addition, the triplicates of samples kept at 19ºC were very different from each other.

We carried out permutational multiple analysis of variance (PERMANOVA) with the results from experiments 2 and 3. In both cases, time resulted in the largest differences among samples ($r2 = 0.455$ and 0.495 respectively, $p = 0.001$ in both experiments). Temperature was also significant in both experiments although it explained a lower percentage of variability than time ($r2 = 0.232$ and 0.066 respectively, $p = 0.001$ and 0.02).

There were too few replicates in experiment 2 to carry out ANOSIM pairwise tests, but in experiment 3, the R values gradually increased between 15 min and 2 h for samples in ice, while they were high already at 15 min at room temperature. In conclusion, as could be expected, time should be kept as short as practical, but keeping the samples in ice will decrease the problem.

Micromonas pusilla gene expression under dark and light conditions

In experiment 4, a culture of *Micromonas pusilla* was incubated both in the dark and in the light. Different TFA patterns were observed (Figure 5). With the primer combination used, the number of cDNAs retrieved in dark samples was larger than in light samples, but the total number of peaks was very small so that the significance of the differences was low (Figure 5). This particular example shows how the technique can also be used to identify genes with differential regulation under the experimental conditions tested (as is the case in differential display).

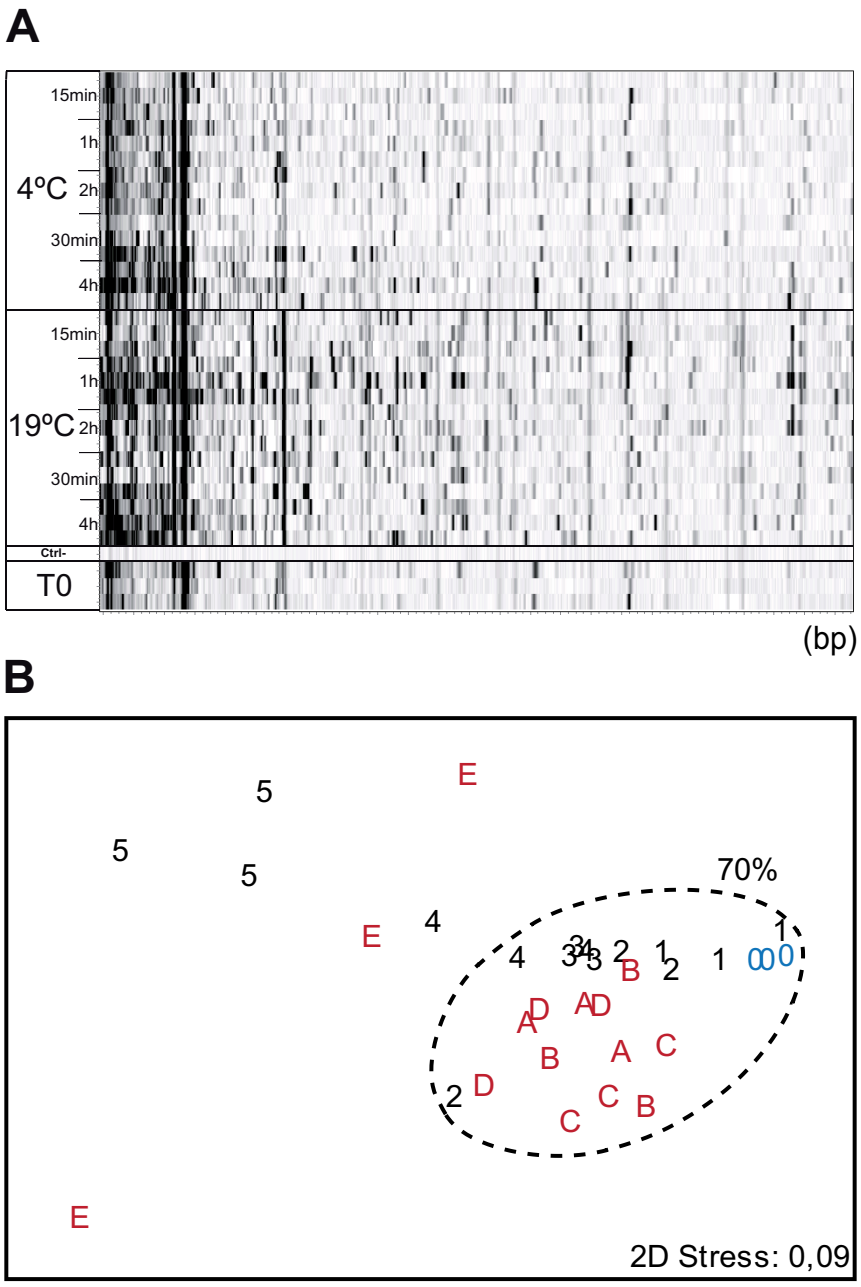

Figure 4. Elapsed time between sampling and filtration in a *Micromonas pusilla* culture. A) Virtual gel of *Micromonas pusilla* fingerprints of triplicate samples filtered at different times between 15 min and 4 h after splitting conditions: 4°C or 19°C samples. Patterns obtained with the A8 primer combination. The horizontal scale shows fragment size, from 80 to 570 bp (left to right).. Each time is represented by three replicates. B) NMDS diagram for the same experiment showing samples kept at 4°C (numbers, following increasing times from 15 min to 4 hours) or at 19°C (letters, following alphabetical order from 15 min to 4 hours). Times correspond to 15, 30 min, 1, 2, or 4 hours.

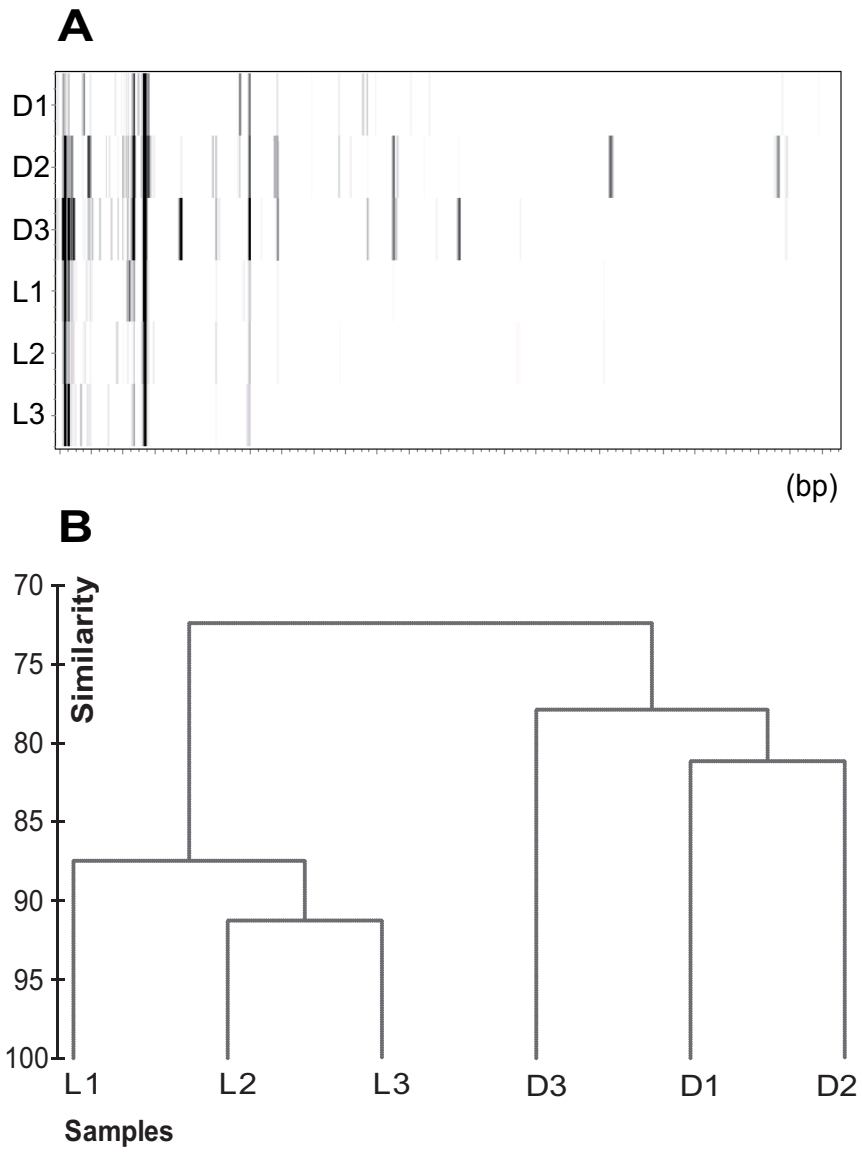

Figure 5. *Micromonas pusilla* gene expression under dark and light conditions. A) Virtual gel of the TFA patterns for experiment 4. The horizontal scale shows fragment size, from 80 to 570 bp (left to right). D: dark samples, L: light samples. Numbers are replicates for each condition. B) Dendrogram showing the clustering of light and dark samples for the same experiment.

Relationship of TFA patterns with different primer combinations

To check whether different primer combinations would cluster samples similarly, samples from three vertical profiles (from stations CM, MD and D) were selected (see location of samples in Table 3) and the procedure was run with three different primer combinations (A8, C6 and G6).

Table 3. Location and depths of samples analyzed in Figure 6.

Station	Latitude N	Longitude E	Depths sampled (m)
CM	41°24'	2°48'	5/30/44
MD	40°54'	2°50'	4 / 140
D	40°39'	2°51'	5 / 65 / 500 / 2000

The NMDS diagram in Figure 6 presents the ordination of TFAs from all these runs. TFAs obtained with A8 showed that samples separated along the depth gradient, the largest distances appearing between intermediate and deep samples. Near-surface samples obtained with the A8 primer combination clustered together. With the C6 combination deep samples were also separated from the rest of the samples but the distance between all the samples was much less compared to the separation obtained with the other combinations. The G6 combination also resolved the vertical profile but the intermediate depths were not as well separated as with the A8 primer combination.

Altogether the surface samples of the horizontal transect clustered together for each primer set and largest differences were observed along the depth profile. This was very clear with primer combinations A8 and G6. In contrast, the C6 primer combination was not as good at resolving the vertical gradient. The A8 primer combination was chosen for all our analyses for its resolution and repeatability.

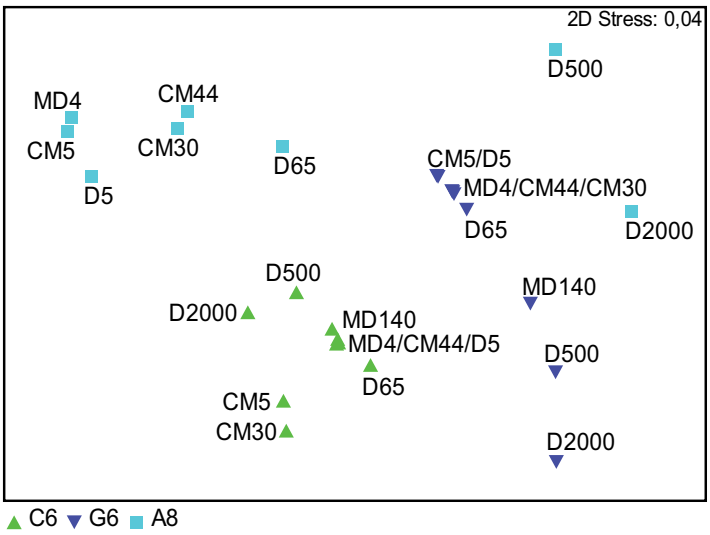

Figure 6. Relationship of TFA patterns with different primer combinations. NMDS diagram comparing fingerprints obtained with three different primer combinations from the MODIVUS transect from the coast to offshore in the NW Mediterranean Sea. Samples are labeled according to station (CM, MD, and D) and depth (4 to 2000m). The number added after the name of the station indicates depth. The primer combinations used were A8, C6 and G6. Sample MD140 analyzed with the A8 primer combination was very distant from all the others and has not been represented for clarity.

DISCUSSION

The aim of this study was to develop a fingerprinting method that could track changes in microbial community gene expression patterns and that was compatible with usual working conditions in oceanographic cruises. The main challenge in a cruise is to obtain sufficient mRNA in as short time as possible. First, samples from several thousand meters deep may take several hours to reach the lab on board. The ideal solution would be to fix the samples at *in situ* depth. However, there is no commercially available sampling bottle able to do this. Besides, fixing *in situ* requires large amounts of fixative making the whole operation impractical and environmentally harmful. And, second, open sea oligotrophic waters have very low concentration of microorganisms and require more filtration time. One possibility is to use mRNA amplification methods. However, these add an additional step that makes the procedure more expensive and complex. We wanted to test whether we could find a method that would provide representative gene expression patterns for a large number of samples despite these difficulties. We chose differential display and modified this technique for faster and easier processing. This technique was developed primarily to identify genes expressed in tumor cells versus normal cells [27-29]. The technique is simple as it is based on PCR and conventional sequencers, it is sensitive and repeatable, and relatively quick and economical. Moreover, TFA does not require prior knowledge of the mRNA sequences to be detected. This last characteristic is especially important for the study of natural communities.

Of course, there are several common difficulties and assumptions when differential display is used. First, a band in gel electrophoresis (or a peak in our case) might be due to several genes. And, conversely, one gene could be represented by more than one fragment. This is also the case with DNA fingerprinting techniques such as DGGE, T-RFLP, or ARISA. Particularly in a mixed natural community, gene fragments of identical length could originate from different microorganisms. As long as these events are repeatable, however, they are of no concern for the fingerprinting objective.

A second concern is the potential bias of the reverse transcription. It is well known that the experimental variation in a RT-PCR process is mainly attributable to the reverse transcription step [30,31]: although PCR is a cyclic reaction that accumulates errors, its repeatability is significantly higher than that of the single-step reverse transcription reaction [30], where there are several factors that could influence the final product. In order to improve the repeatability of the assay we optimized the process by testing several RT enzymes and annealing temperatures. With the thermostable reverse transcriptase chosen we obtained highly repeatable peaks in repeated reactions, not only for the large peaks but also for the small ones (Figure 2). The high annealing temperature during reverse transcription reduced the degree of mRNA secondary structure, which is substantial in the 3'untranslated region (3'UTR) we were targeting by using of oligo(dT) primers. In ad-

dition, the RT might preferentially amplify some mRNAs, thus altering the relative proportions of the genes being expressed. In particular, shorter mRNAs might be preferentially used as targets [32]. We did not find any significant differences in this respect when we tested different reaction times or when we compared peaks corresponding to different sizes.

And third, the final PCR step is subject to the usual PCR biases, and some cDNAs might be differentially amplified. As a result of the two latter caveats, the relative proportions of the expressed genes in the final fingerprint may not be exactly as they were in the natural sample. Because of the clearly delimited purpose of the approach, however, this would not be a problem either as long as the biases were repeatable.

As shown in the Results section, the expression patterns found were always highly repeatable under the conditions used. Moreover, for the approach to be useful as a fingerprint it was not necessary to be able to identify the genes being expressed, to determine how many genes were being expressed at a particular moment, or to quantify the expression of the different genes.

Here, TFA was developed and applied to picoeukaryotic communities taking advantage of the poly(A) tail of mRNA in eukaryotes. However, TFA can be easily modified to be used with prokaryotes by previously removing rRNA and subsequent polyadenilation of the RNA of the bacterial fraction as described in [1].

According to the manufacturer of the kit (www.genhunter.com), the use of three oligo(dT) primers (for the reverse transcription reaction) plus eighty random primers (for the subsequent PCR) will retrieve 96% of the genes in any given eukaryotic cell. Since only one of three oligo(dT)s and only one of eighty random primers were used in the present work, the fingerprints corresponded to a very small fraction of all the genes being expressed at any one time. In effect, when the technique was applied to a pure culture of *M. pusilla* the number of peaks was very low in one of the experiments (Figure 5). This number of peaks would not be enough for a proper classification of samples. However, when the same technique was used with natural samples, in which a mixture of cell populations is present, the number of peaks was sufficient. Economy of resources and reactions being essential for a convenient fingerprinting technique, we decided that the use of one random primer and one anchor primer was the most efficient alternative.

Since the primer combinations are arbitrary, the transcripts retrieved with each set should be a random representation of the genes being expressed at the time of sampling. Therefore, most primer combinations should result in similar clustering of samples. However, the resulting clustering will be more robust if there are more peaks and there is a range of peak heights. Since this will change at random with the primer sets and the particular communities being analyzed, optimization requires testing different primer combinations for each type of environment studied. In the case of

the Mediterranean waters analyzed the primer combination A8 was the best at discriminating samples from the vertical profile (Figure 6) and was, thus, chosen as our preferential combination for subsequent reactions. Likely, the primer combination will have to be optimized for each type of sampling. Once this has been done, the procedure is relatively cheap and quick.

In order to have a positive control, replicate cultures of *Micromonas pusilla* were incubated in the light and in the dark. It is well known that transcripts of algae change dramatically between day and night [33]. Obviously, if the technique is to work in nature it should be able to detect differences between light and dark incubations in a phototrophic protist. The patterns were clearly different, revealing more transcriptional activity in the dark than in the light with the primer combination used. This can be expected since phototrophs tend to concentrate on carrying out photosynthesis during the light hours, while the dark is used for biosynthesis of all the different cell components plus all the regulation involved in nucleus and cell division. As mentioned earlier, the TFA is proposed here only as a fingerprinting technique. Despite this, in some cases it may be of interest to identify some of the genes observed. If a gene turned out to be relevant, the sample could be run in a polyacrylamide gel and the corresponding band could be cloned and sequenced.

The main challenge in a cruise is to obtain sufficient mRNA in as short a time as possible to prevent major changes in the transcript composition from the fresh sample (this is due to the labile nature and relatively short half-lives of mRNAs). Unfortunately this is not always possible: as explained, samples from lower depths take hours to reach the lab on board, and oligotrophic waters have very little material and require more filtering time. Therefore, another important concern was to assess to which extent the time delay between sampling and filtering affected gene expression. The two timing experiments with natural marine communities supported the idea that time did not significantly alter the patterns of gene expression as long as samples were kept on ice, for the picoeukaryotic transcripts retrieved at least up to two hours after sampling with several specific primer combinations.

In summary, TFA is a compromise among the different requirements that provides a repeatable gene expression pattern in a relatively simple and inexpensive way and that will be practical to use in oceanographic cruises. Results suggest that TFA is a useful technique when a large number of conditions or treatments have to be compared side by side, by assessing a portion of the genes expressed by such communities. TFA is an indicator of the extent of changes caused by different environmental conditions. This previous analysis would then help in deciding which samples to use for more powerful, but time-intensive (and costly) methods for estimating gene expression patterns.

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Chapter 2

Comparison of Picoeukaryotic Gene Expression Patterns with Community Composition along the Seasonal Cycle in the NW Mediterranean

ABSTRACT

Coupled analyses of microbial community composition and gene expression are almost inexistent for temporal series and even more so for picoeukaryotic assemblages. In this study, we compared marine picoeukaryotic gene expression patterns (assessed by Transcriptome Fingerprinting Analysis - TFA) with picoeukaryotic community composition (assessed using Automated Ribosomal Intergenic Spacer Analysis -ARISA) over four years in Blanes Bay Microbial Observatory (BBMO), an oligotrophic coastal site of the northwestern Mediterranean Sea. At large scale, regular annual periodicity was detectable with both approaches for the four years analyzed, implying that picoeukaryotic composition and expression dynamics were recurrent. At smaller scale, within a year, the largest differences occurred between winter and summer samples in correlation with distinct environmental and biological variables. During the winter, community composition was less variable than gene expression patterns. During the summer, however, community composition was more variable than gene expression, suggesting functional redundancy in the summer picoeukaryotic assemblage.

Comparison of picoeukaryotic gene expression patterns with community composition along the seasonal cycle in the NW Mediterranean. **Montserrat Coll-Lladó, Guillem Salazar, Hugo Sarmento, Silvia G. Acinas and Carles Pedrós-Alió**. Article submitted to Environmental Microbiology.

INTRODUCTION

Studies on microbial community composition using molecular techniques at different temporal scales has uncovered seasonal changes of bacterioplankton in both freshwater (Casamayor et al., 2002; Newton et al., 2006; Pinhassi & Hagström, 2000; Shade et al., 2007) and marine systems (Fuhrman et al., 2006; Gilbert et al., 2012; Schauer et al., 2003; Treusch et al., 2009). Growing evidence suggest that microbial communities are structured by the environment, following the seasonal cycle, with reoccurring microbial assemblages in specific periods of the year. The number of studies with other members of the picoplankton is extremely small. Chow and Fuhrman (2012) studied viruses. Murray et al. (1999), Auguet et al. (2011) and Robidart et al. (2012) are some of the few seasonal studies of archaea. In studies dealing with this issue, picoeukaryotes have received less attention than other compartments of the microbial food web (McDonald et al., 2007), although, it is reasonable to assume that marine picoeukaryotes will also follow seasonal cycles.

Presumably, changes in composition should also result in changes in the gene expression patterns of the different assemblages. Although many metatranscriptomics studies have been carried out to analyze gene expression *in situ* (Moran et al., 2012; Ottesen et al., 2013), seasonal studies are still lacking. Likely, the expense and effort needed to construct and analyze a metatranscriptome is one of the reasons for this lack of data. Fingerprinting methods provide a snapshot of the entire microbial community at once and allow relatively cheap and fast comparison of a large number of samples. We have recently developed a novel approach, Transcriptome Fingerprinting Analysis (TFA), a fingerprinting technique to analyze gene expression patterns of picoeukaryotic assemblages (Coll-Lladó et al., 2011). The technique is relatively quick and cheap, and allows the analysis of many samples simultaneously. In the present study we combined two molecular fingerprinting techniques: ARISA (Brown et al., 2005; Fisher & Triplett, 1999) to assess picoeukaryotic community structure and TFA to analyze gene expression patterns within the same community. We analyzed these changes in a monthly sampling basis during four consecutive years at the Blanes Bay Microbial Observatory (BBMO).

The seasonal changes in bacterioplankton community composition (Schauer et al., 2003) and picoeukaryotes (Díez, B. Ph.D. Thesis, Autonomous University of Barcelona, 2001) had been followed by DGGE at the BBMO. The seasonal succession of archaea was also analyzed via clone libraries and qPCR (Galand et al., 2010). In all these cases, the community composition showed a clear seasonal pattern (Gasol et al., 2012). In principle, we would expect faster changes in gene expression patterns than in community composition. However, the studies available do not allow discarding two other hypotheses: simultaneous changes in composition and gene expression, and faster changes in composition than in expression patterns (functional redundancy).

The objective of this study was to compare the picoeukaryote community structure at DNA and RNA level based on ARISA and TFA respectively along a 4-year temporal series with monthly sampling and to determine which one of the three hypotheses mentioned above prevails at the different times of the year cycle: 1) simultaneous changes in community composition and in gene expression patterns, 2) faster changes in gene expression patterns than in community composition, or 3) faster changes in community composition than in gene expression patterns.

EXPERIMENTAL PROCEDURES

Blanes Bay Microbial Observatory and sampling

The Blanes Bay Microbial Observatory (BBMO, www.icm.csic.es/bio/projects/icmicrobis/bbmo) is located on the Catalan coast (NW Mediterranean, 41º 40' N, 2º 48' E). The site is 800 m offshore and is a relatively nutrient-poor ecosystem. The site has been studied for several decades and a fairly complete data set exists about its microbial community (Gasol et al., 2012).

Sea sub-surface samples were collected monthly from June 2007 to February 2011 at BBMO in eight liter carboys, one for DNA extraction and another for RNA extraction. Water was prefiltered through a 200-μm mesh net and, once in the lab, prefiltered through 20-μm Nylon mesh. Samples for RNA extraction were kept on ice until the end of the filtration process. Elapsed time between sampling and filtration was less than two hours. In a previous study, we shown that TFA patterns in ice-preserved samples were closer to the patterns of the samples at the time of collection of the samples than samples kept at room temperature (Coll-Lladó et al., 2011).

Temperature, salinity, size-fractionated chlorophyll *a*, and inorganic and organic nutrients were determined in a similar way as described in Alonso-Sáez et al. (2008). Abundance of phototrophic and heterotrophic picoeukaryotes, heterotrophic bacteria and *Synechococcus* like cells was determined by epifluorescence microscopy from samples fixed with 1% paraformaldehyde + 0.05% glutaraldehyde (final concentration) filtered through 0.6 (eukaryotes) or 0.2 μm (prokaryotes) polycarbonate filters, stained with 4,6 diamidino-2-phenylindole (DAPI) at 10 μg mL^{-1} final concentration (Porter & Feig, 1980), and examined with an Olympus BX61 epifluorescence microscope under blue and UV wavelength excitation.

Picoeukaryote community fingerprinting (ARISA)

Microbial biomass was collected by a serial filtration of eight liters of seawater through a 3-μm pore-size polycarbonate prefilter (Poretics) and a 0.2-μm Sterivex filter (Durapore, Millipore) using a peristaltic pump. The Sterivex units were filled with 1.8 mL of lysis buffer (40 mM EDTA pH 8.0, 50 mM Tris-HCl pH 8.3, 0.75 M sucrose) and stored at -20°C. Nucleic acids were extracted as described in (Massana et al., 1997).

Specific primers were used to amplify the internal transcribed spacer 1 (ITS1) (Massana et al., 2008). The sequence of forward primer (EUK RR) was labeled with a fluorescence tag (FAM) and matched the end of the SSU and was: 5'-GTAGGTGAACCTGCAGAAGGATCA-3'. The reverse primer, ITS2, matched the beginning of the 5.8S. The sequence of ITS2 was 5'-GCTGC-GTTCTTCATCGATGC -3'. The PCR mixture (40 μl) contained: 10 ng of DNA template, 250 nM of each primer, 250 nM dNTPs, 2.5 mM Mg Cl_2, 3 U *Taq* DNA polymerase, and the enzyme buffer. PCR cycling, carried out in a DNA Engine® thermal cycler (Bio-Rad Laboratories, Inc.), comprised an initial denaturation step at 94 ˚C for 2 min followed by 32 cycles of denaturation at 94 ˚C for 15 s, annealing at 55 ˚C for 30 s and extension at 72 ˚C for 3 min followed by a final extension step at 72 ˚C for 9 min. A negative control was run for each set of reactions to assess the background levels. We used the same TFA pipeline (see details bellow) to discriminate the ARISA amplicons with gene analyzer, the only difference was that ARISA fragments were detected in a different channel because of the nature of the fluorescent tag.

Transcriptome fingerprinting analysis (TFA)

All environmental water samples for TFA were filtered first through a 3-μm pore-size polycarbonate filter (Poretics) and then through a 0.2-μm polycarbonate filter (Poretics) using a peristaltic pump (MasterFlex 7553-89 with cartridges Easy Load II 77200-62, Cole-Parmer Instrument Company) to collect the bacteria and picoeukaryotes. Filters were flash-frozen in liquid nitrogen and then stored at -80 ˚C until processed. Total RNA was extracted from the 0.2-μm polycarbonate filters.

RNA extraction and purification. The procedure was adapted from (Poretsky et al., 2009). Filters were transferred to 2 ml screw-cap microcentrifuge tubes containing 200-μl of 0.1 mm- diameter zirconia-silica beads (BioSpec Products, Inc.) and 100-μl of 0.5 mm glass beads (BioSpec Products, Inc.) mixed with 450-μl RLT lysis buffer (provided by the RNeasy Mini Kit Qiagen, Inc.) plus ß-mercaptoethanol (Fluka). Samples were mechanically disrupted in a Mini-beadbeater-8TM cell disrupter (BioSpec Products, Inc., Bartlesville, OK) for 10 min. After disruption, samples were incubated on ice for 5 min and the beads were allowed to settle out of the lysis mixture. Samples underwent centrifugation (in an Eppendorf centrifuge at 2100 rcf 1 min). The lysate was trans-

ferred to a new tube. 300 µl of lysis solution was added to the vials with beads to increase the final yield. The tubes were shaken vigorously and the supernatant was also recovered. The same volume of 70% ethanol was added to the lysate and samples were purified according to the RNeasy Mini Kit (Qiagen, Inc.). The isolated total RNA was treated with TurboDNase I (Ambion) to remove contaminating genomic DNA according to the manufacturer's instructions. RNA was aliquoted and quantified by absorbance at 260 nm with a NanoDrop 1000 (Thermo Fisher Scientific Inc., Wilmington, DE).

Reverse transcription and PCR amplification. First-strand cDNA synthesis was conducted with 30 ng of total RNA as starting material. mRNAs were reverse-transcribed to single-stranded complementary DNA using the SuperScript III reverse transcriptase (Invitrogen) and the primer H-T_{11}A (5'-AAGCTTTTTTTTTTTA-3'). To denature any secondary structure, an aliquot of each of the RNA extracts plus the oligo(dT) primer were heated for 5 min at 65ºC and immediately placed on ice before mixing with the final reaction solution (20 µl): 5X First-Strand buffer, 0.1 M DTT, 40 U RNaseOUT, 10 mM dNTPs and 200 U SuperScript III RT. Reaction mixtures were incubated at 50ºC during 50 min, and inactivated by heating 70 ˚C for 15 min. 2 µl of the RT reaction product was used in a subsequent PCR. PCR reactions were carried out using *Taq* polymerase (Qiagen) in a final reaction volume of 20 µl. The arbitrary primer H-AP8 (5'-AAGCTTTTACCGC-3') and the same primer used in the RT reaction but labeled with a fluorescence tag (NED) were used for the amplification of cDNA. The primer targeting the poly(A) tail and the arbitrary primer for PCR were from RNAspectra Yellow kit 1 of GenHunter Corporation. The PCR cycle was 40 cycles of 94 ˚C for 30 s, annealing at 40 ˚C for 2 min, 72 ˚C for 60 s followed by 1 cycle of 72 ˚C for 5 min in a DNA Engine® thermal cycler (Bio-Rad Laboratories, Inc.). A negative control was run for each set of reactions to assess the background levels. To ensure that there was no amplification of genomic DNA a control was run with an aliquot of the RNA extracts added directly to the PCR reaction.

Separation of amplicons with gene analyzer. 1 µl of PCR product from each sample was mixed with 9 µl of Hi-Di Formamide (Applied Biosystems). 0.5 µl of size standard (ROX 500, Applied Biosystems) was added to every reaction to define the standard curve between 25 and 500 bp. The cDNA peaks obtained ranged in length from 30 to 500 bp, according to the internal size standard used. The mixtures were run on an ABI automated sequencer operating as a fragment analyzer (ABI 3130XL). The sequencer electropherograms were then analyzed using the GeneMarker software, version 1.90 (SoftGenetics, LLC). Raw data were treated with some filters activated according to GeneMarker instructions: baseline substraction, spike removal, auto pull up removal, smooth. The cubic spline algorithm was used to calculate bp lengths of identified fluorescence peaks. The different peak detection thresholds applied are detailed below. Once the peaks were

selected, peak areas were used as output from GeneMarker software and were transferred to Microsoft Excel (Seattle, WA) for subsequent analysis.

Peak detection in ARISA and TFA

Two different cutoff thresholds were used to select peaks in both ARISA and TFA electropherograms. In the first case, only the largest peaks were selected (see example in Figure 1).

The threshold for these peaks was set at 200 relative fluorescence units (rfus) for ARISA and at 100 rfus for TFA. These TFA peaks are supposed to correspond to highly expressed genes. From now on we will refer to these as HighP (for high peaks). Settings and filters were applied as previously described (Coll-Lladó et al., 2011). In the second case, all the peaks that could be discerned above the background by the instrument, including the high peaks, were used for the analyses (see example in Figure 1). The threshold for these peaks was set at 30 rfus for ARISA and at 10 rfus for TFA. We will refer to these as AllP (for all peaks). This low intensity was the only criterion to detect these peaks. All the other filters used in the HighP settings were shut off. The fragments considered in the analyses were those between 150 and 500 bp for ARISA and between 90 and 530 bp for TFA. Peaks were visually inspected as a quality control (Coll-Lladó et al., 2011).

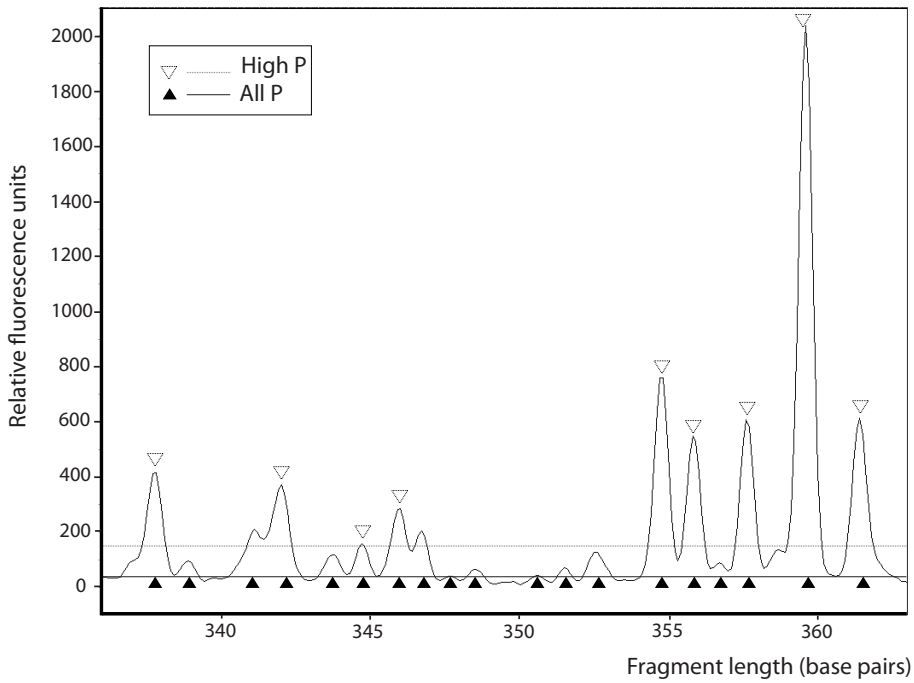

Figure 1. Effect of different cutoffs on the selection of peaks in a TFA electropherogram. The dotted line shows the relative fluorescence units threshold of peak height (intensity) used for detection of highly expressed genes and the empty triangles indicate the resultant peaks (HighP) selected under this corresponding cutoff. Solid black line and filled triangles correspond to the low threshold cutoff where all peaks above background (AllP) were selected.

Statistical analysis

The peak area data from GeneMarker were standardized (the relative peak heights within a profile were calculated by dividing the height of an individual peak by the total peak height (i.e.: sum of the heights of all peaks in an electropherogram). The similarity of patterns derived from different communities was assessed computing Bray-Curtis distances to construct the similarity matrices. Mantel tests between dissimilarity matrices and Mantel correlograms were performed with the Pearson correlation. Patterns were explored using Nonmetric Multidimensional Scaling (NMDS) and Constrained Analysis of Principal Coordinates (CAP). We have used CAP rather than Redundancy Analysis (RDA) because it allows the use of non-Euclidean dissimilarity indices, such as Bray–Curtis distance. Primer-E version 6 (Clarke, 1993) and R software (R development Core Team, 2005) were used for these analyses.

RESULTS

Comparison between HighP and AllP patterns

Electropherograms show a series of peaks with different areas (Figure 1). In principle, large peaks correspond to abundant taxa in the case of ARISA, and to highly expressed genes for TFA. Deciding how many peaks to include in the analysis is a subjective decision and we analyzed the effect on the results of considering two different thresholds. First, in one analysis we considered the largest peaks only (HighP) and secondly, we considered all the peaks above the background (AllP, see Experimental Procedures). We then carried out comparative analyses with both data sets to test the agreement between them.

Sample distance matrices for ARISA (DNA fingerprints) and TFA (mRNA fingerprints) were constructed with both thresholds (HighP and AllP) and were compared with Mantel tests (Figure 2A-B). The distances calculated with the two thresholds were significantly correlated for both DNA (Mantel statistic $r = 0.748$, $p<0.001$) and mRNA ($r = 0.810$, $p<0.001$). However, the slope of the regression lines were significantly lower than 1, indicating that, when distances were short in the AllP matrix, distances in the HighP matrix were longer than expected. On the contrary, when distances in the HighP matrix were long, distances in AllP matrix were shorter. Addition of the small peaks in the electropherograms reduced the range of distance values found with the large peaks only. Subsequent analyses were performed with data generated with the two thresholds.

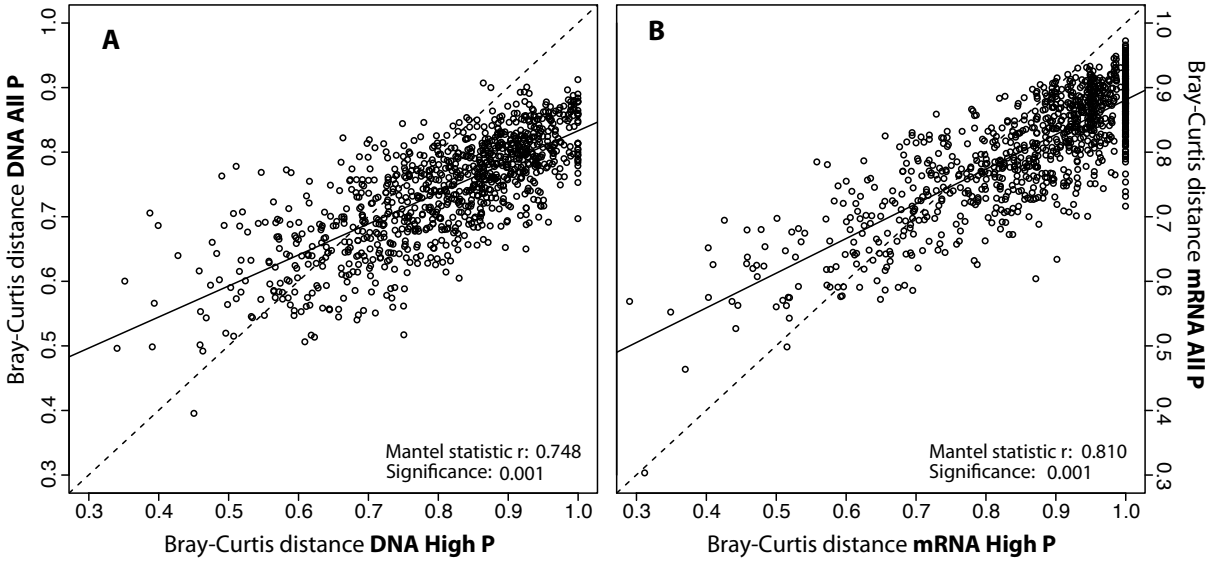

Figure 2. Comparisons among matrices generated with restrictive (HighP) vs. non-restrictive cutoffs (AllP). **A)** Comparison of distance matrices for AllP and HighP of DNA fingerprints (ARISA). **B)** Comparison of distance matrices for HighP and AllP of mRNA fingerprints (TFA). The discontinuous diagonal lines indicate the 1:1 relationship and the continuous lines the linear regressions.

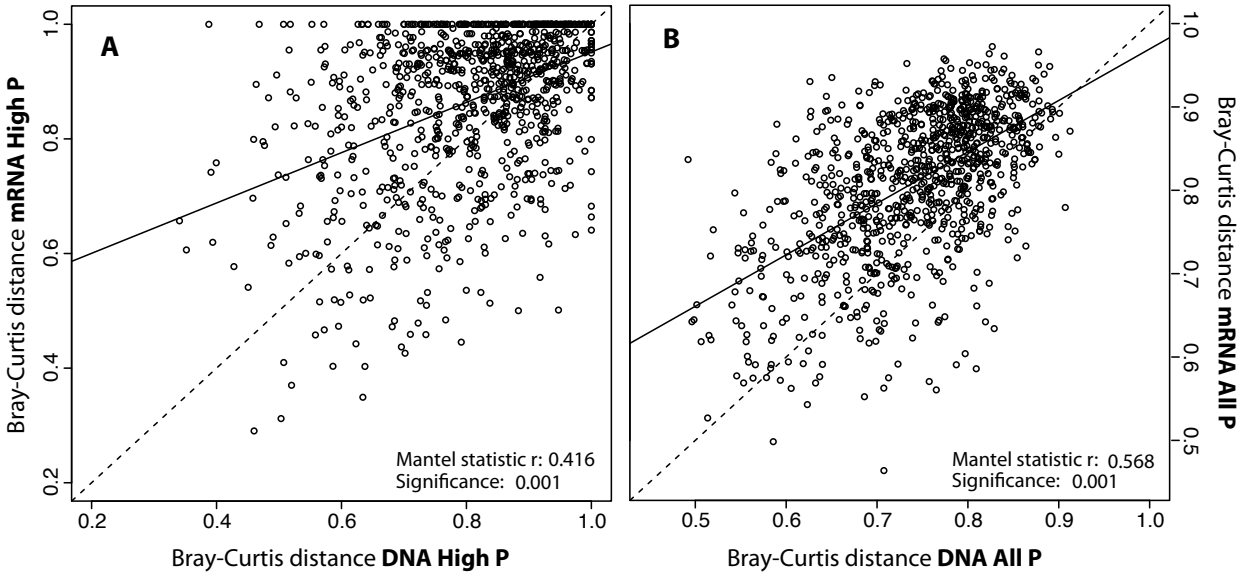

Figure 3. Comparisons among matrices generated with restrictive (HighP) vs. non-restrictive cutoffs (AllP). **A)** Comparison between DNA and mRNA distance matrices for HighP. **B)** Comparison between DNA and mRNA distance matrices for AllP. The discontinuous diagonal lines indicate the 1:1 relationship and the continuous lines the linear regressions.

Comparison between ARISA (DNA) and TFA (mRNA) distances

Distances calculated from DNA and mRNA matrices were significantly correlated according to the Mantel test results (Figure 3A-B). However, the regression coefficients were rather low. The adjusted r^2 values of the regressions were 0.173 and 0.322 for the HighP matrix and the AllP matrix, respectively. This indicates that, as could be expected, similarity in composition was only one of several factors determining the similarities in gene expression patterns.

Seasonal patterns in community composition and expression.

Similar seasonal patterns were observed in ARISA and TFA, as showed by the NMDS ordination (Figures 4A-B and 5A-B). Samples generally clustered into two groups roughly corresponding to winter (cold water, <16ºC) and summer (warm water, >16ºC). This was the case for both ARISA and TFA and for both threshold levels. The composition of picoeukaryotic assemblages in the winter samples were closer among themselves when only the HighP were considered than when AllP were taken into account (Figures 4A and B). This was not the case for the expression patterns (Figures 5A and B). Some "summer" samples were within the "winter" cluster and vice-versa, but in all cases these samples corresponded to the transition months between summer and winter and point to weather variability among years.

Figure 4C shows the distances among picoeukaryotic community composition from different years for each month when only the HighP were considered. Thus, the samples from the winter months January, February and March were relatively similar from one year to the next (Figure 4C). On the other hand, samples from the summer months, particularly July and August, were more variable from year to year (Figure 4C). When AllP were considered, all the months showed similar differences among years (Figure 4D). The equivalent graph for mRNA showed larger variability from year to year for winter months (Figure 5C) and the same smoothing in variability when AllP were included in the analysis (Figure 5D).

Considering the HighP alone, the ARISA patterns were less variable than the TFA patterns in winter (Figures 4C and 5C). In contrast, the summer ARISA patterns were more variable than the TFA patterns. This second situation might point to functional redundancy in the summer. The former situation, on the other hand, suggests a similar community changing its expression patterns from year to year.

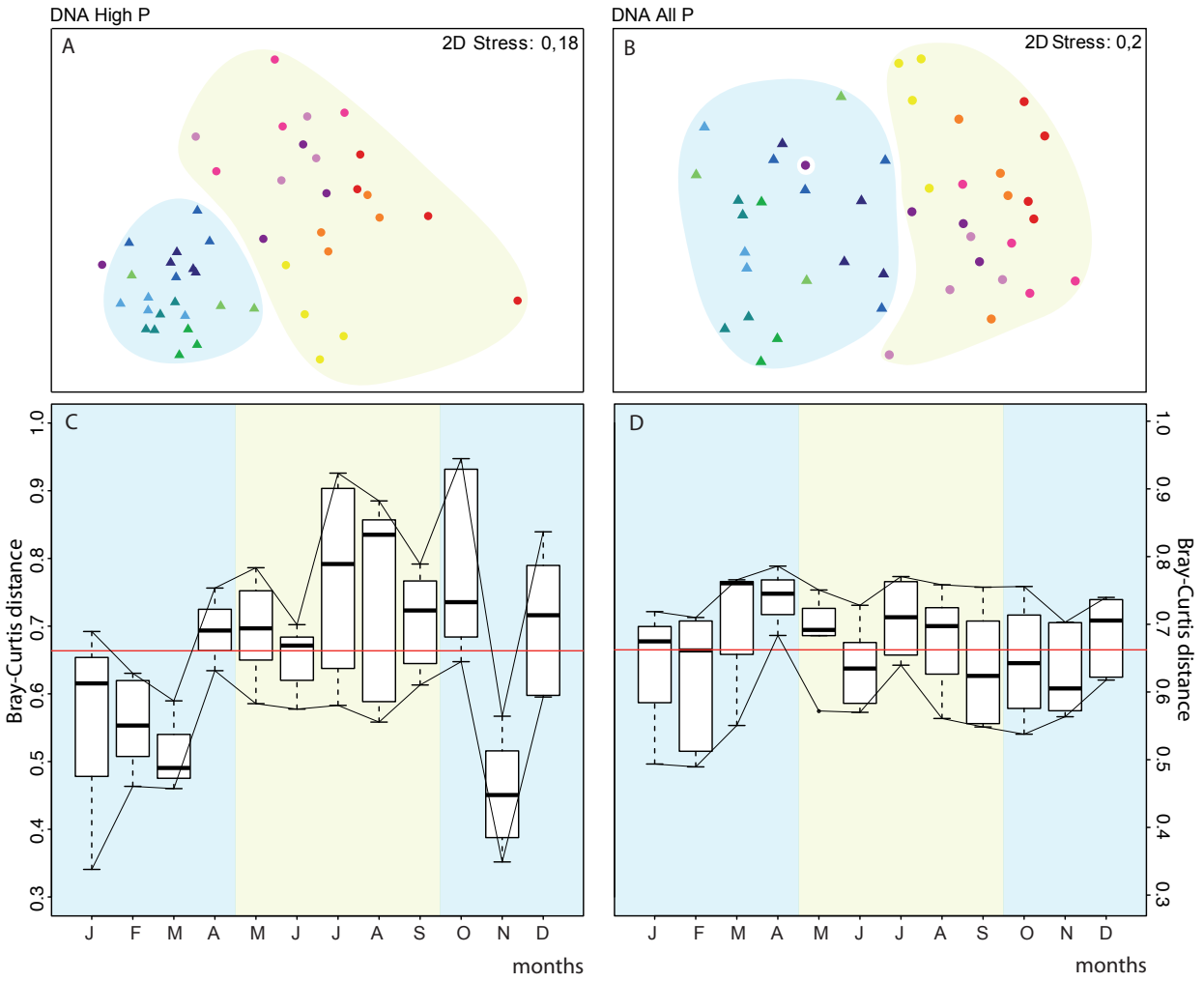

Figure 4. Upper panels: NMDS diagrams comparing DNA fingerprints (ARISA) from samples collected monthly between June 2007 and February 2011 from Blanes Bay Microbial Observatory. Samples are color-labeled according to the month. The light-blue cloud includes the winter assemblage and the light-yellow cloud includes the summer assemblage. Lower panels: Boxplots showing within month variability of ARISA fingerprints (Bray-Curtis dissimilarity) along the year. The red line indicates the mean of within month variability along the year. **A and C**) NMDS and boxplot for HighP. **B and D**) NMDS and boxplot for AllP.

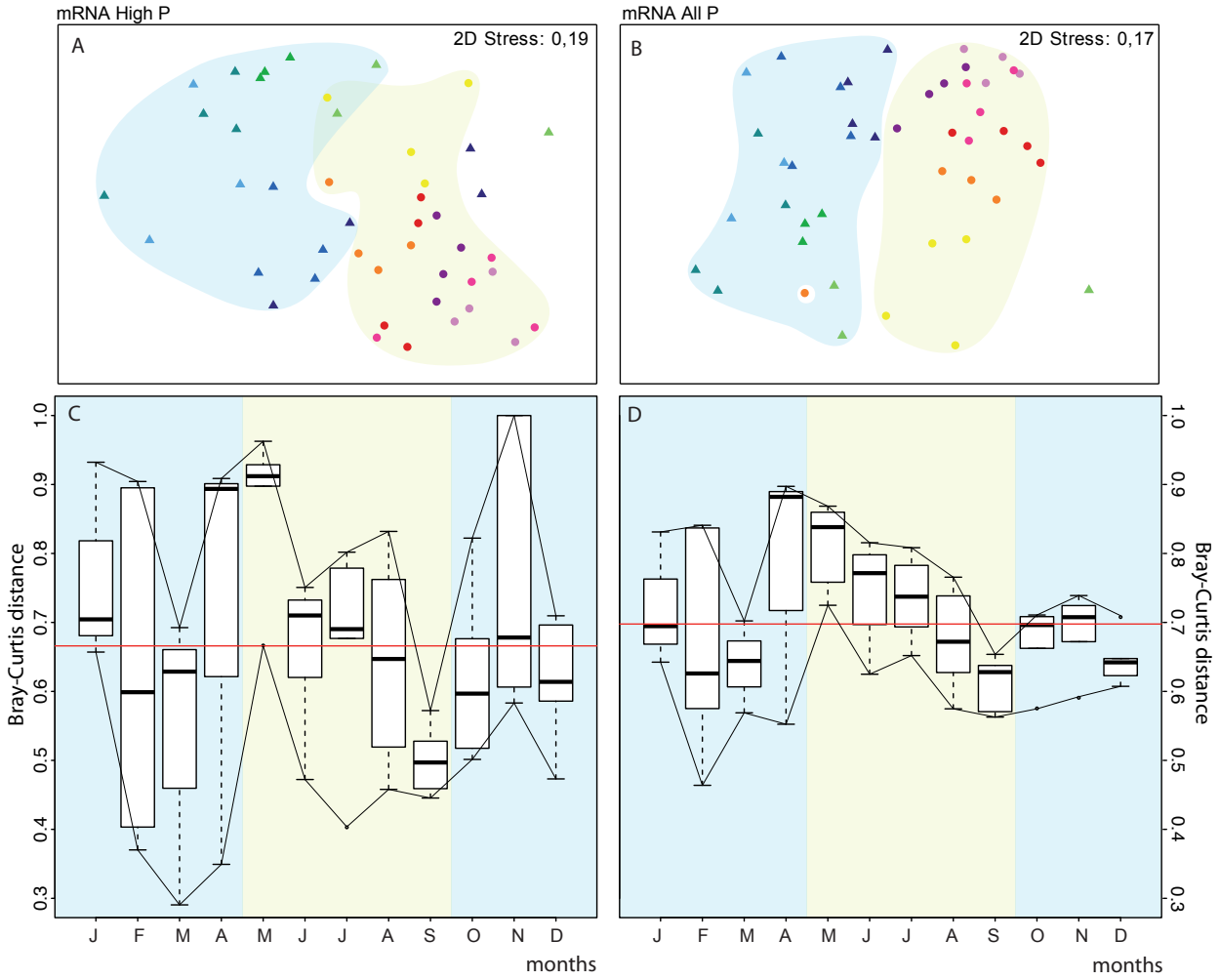

Figure 5. Upper panels: NMDS diagrams comparing RNA fingerprints (TFA) from samples collected monthly between June 2007 and February 2011 from Blanes Bay Microbial Observatory. Samples are color-labeled according to the month. The light-blue cloud includes the winter assemblage and the light-yellow cloud includes the summer assemblage. Lower panels: Boxplots showing within month variability of TFA fingerprints (Bray-Curtis dissimilarity) along the year. The red line indicates the mean of within month variability along the year. **A and C**) NMDS and boxplot for HighP. **B and D**) NMDS and boxplot for AllP.

Despite this within-month variability from year to year, both picoeukaryotic communities and their mRNA expression patterns reoccurred annually (Figure 6). Autocorrelation analysis showed that community composition and gene expression patterns reoccurred with a one-year period. Results with AllP produced a similar pattern (Supplementary Figure 1).

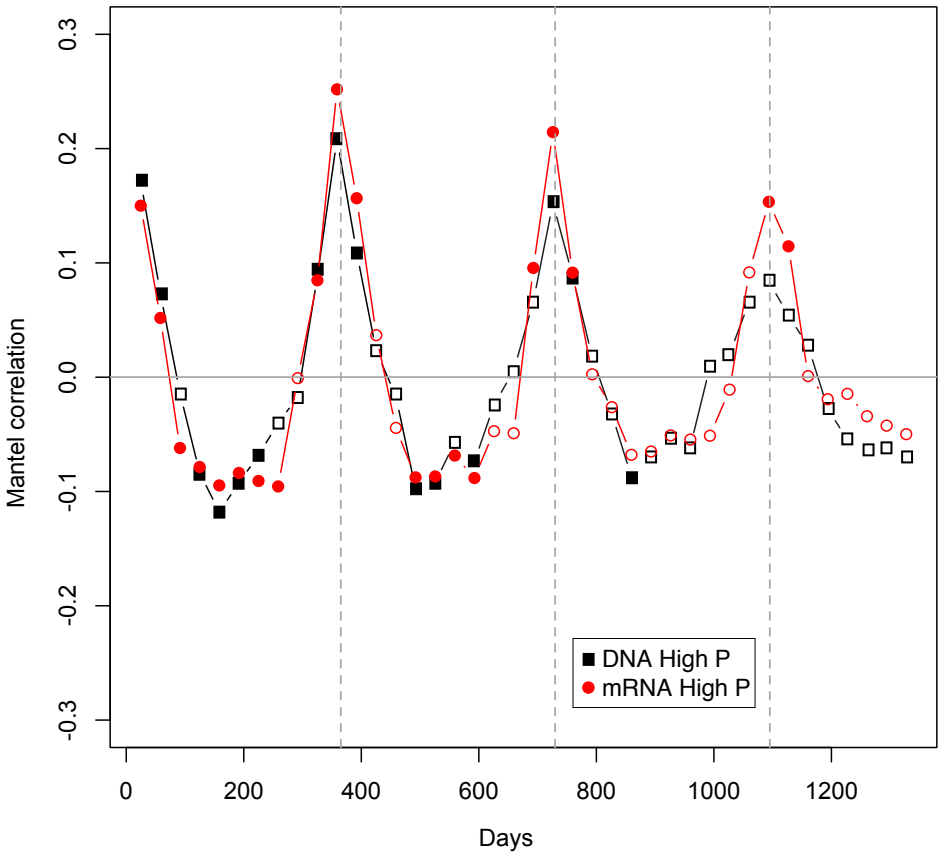

Figure 6. Mantel correlogram. Sea surface was sampled monthly from June 2007 to February 2011 in the Blanes Bay Microbial Observatory. The squares indicate the annual cycles of the picoeukaryotic community composition and the circles the annual cycles of the gene expression patterns. The filled symbols represent correlations with $P<0.05$. Autocorrelations were done with HighP.

When distances from one sample to the next were analyzed, no apparent pattern could be observed (Supplementary Figure 2). However, the average distance between samples was significantly larger for TFA than for ARISA (Figure 7). Finally, richness and evenness were calculated for each of the four datasets. No relationships could be found between any combinations of them (data not shown).

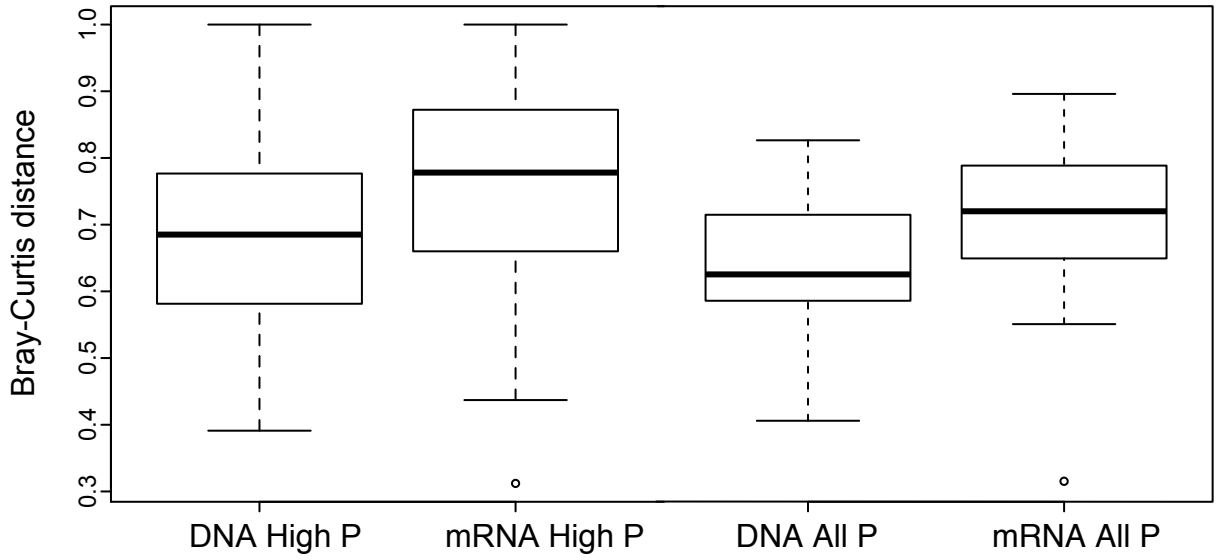

Figure 7. Boxplot showing the average Bray-Curtis distances from one month to the next over 4 years in DNA vs mRNA data for both HighP and AllP.

Relationships between community composition, gene expression patterns, and environmental variables

The relationships between picoeukaryotic ARISA and TFA fingerprints and environmental variables were explored with Constrained Analysis of Principal Coordinates (CAP) to identify potential environmental drivers of variability (Figure 8, 9). Seasonal succession followed a circular clockwise trajectory in all cases. For example, starting in May (yellow dots at bottom center) and moving towards the left, summer months appear in the far left and late summer in the upper left quadrant. Moving towards the right, fall and winter months appear in the upper right quadrant returning to the lower right quadrant. This distribution was found both for ARISA and TFA patterns, both with HighP and AllP matrices (Figures 8, 9 and Supplementary Figure 3A, B).

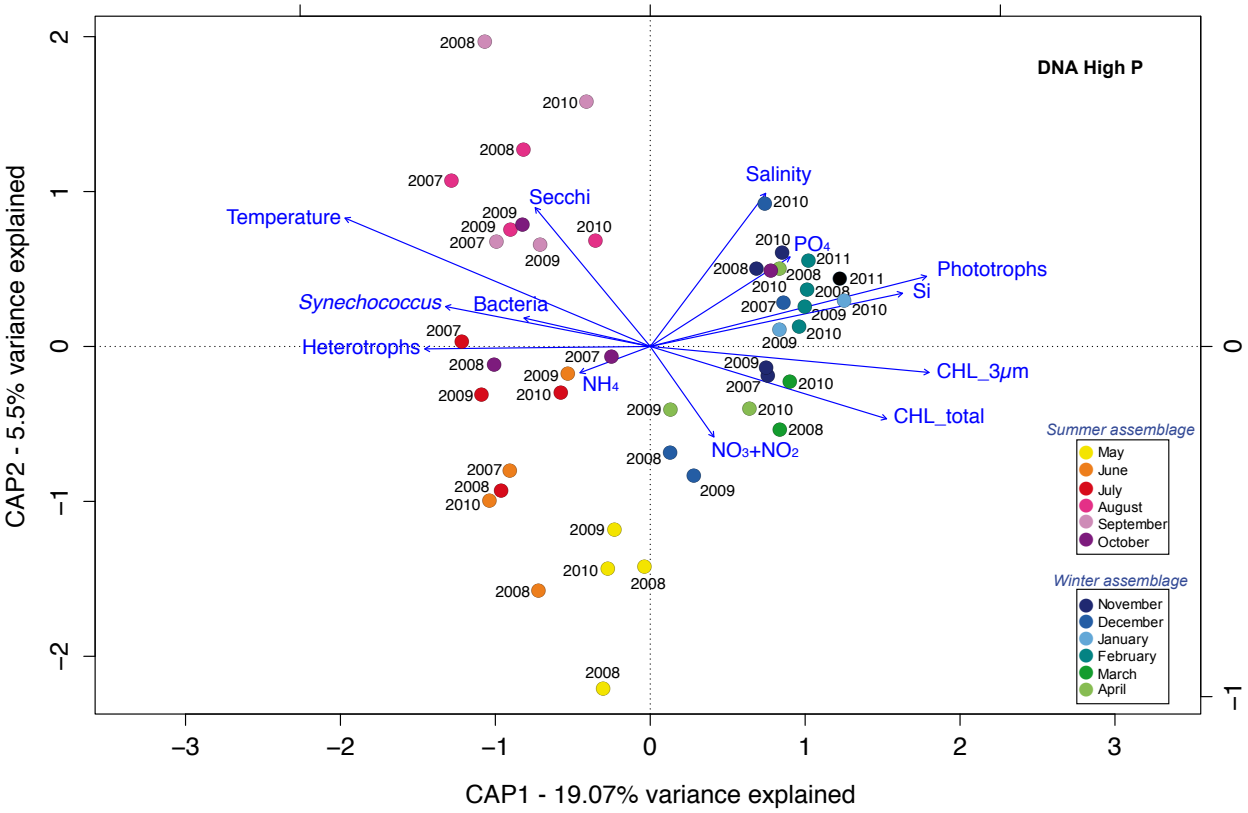

Figure 8. Constrained Analysis of Principal Coordinates (CAP) of the HighP data. Samples are color-labeled according to the month. A progressive pattern of community and gene expression change is observed across all four years. A suite of environmental parameters is included to observe their potential relationships to picoeukaryotic community composition dynamics (constructed from ARISA fingerprints). The length and direction of arrows indicate the degree of correlation with the ordination axes.

The first axis separated winter from summer samples. Summer samples in the left part of the chart correlated with temperature, water transparency, abundance of eukaryotic heterotrophs and *Synechococcus*. On the right side, winter samples correlated with eukaryotic phototrophs and chlorophyll *a*. This confirms that a remarkable difference in both composition and expression was found between the winter and summer assemblages.

The second axis was correlated with inorganic nitrogen forms in the case of ARISA and with phosphate in the case of TFA. Inorganic nitrogen correlated mostly with samples from May and June, which had the most negative values along the second component (Figure 8). On the other hand, September and February assemblages showed the highest positive values. In the case of TFA, this second axis showed a stronger influence of phosphate and salinity (Fig. 9). Winter months (November to February) showed the highest values for this axis, while May to July samples showed the more negative values. Apparently, inorganic nutrients had different impact on community composition and community gene expression.

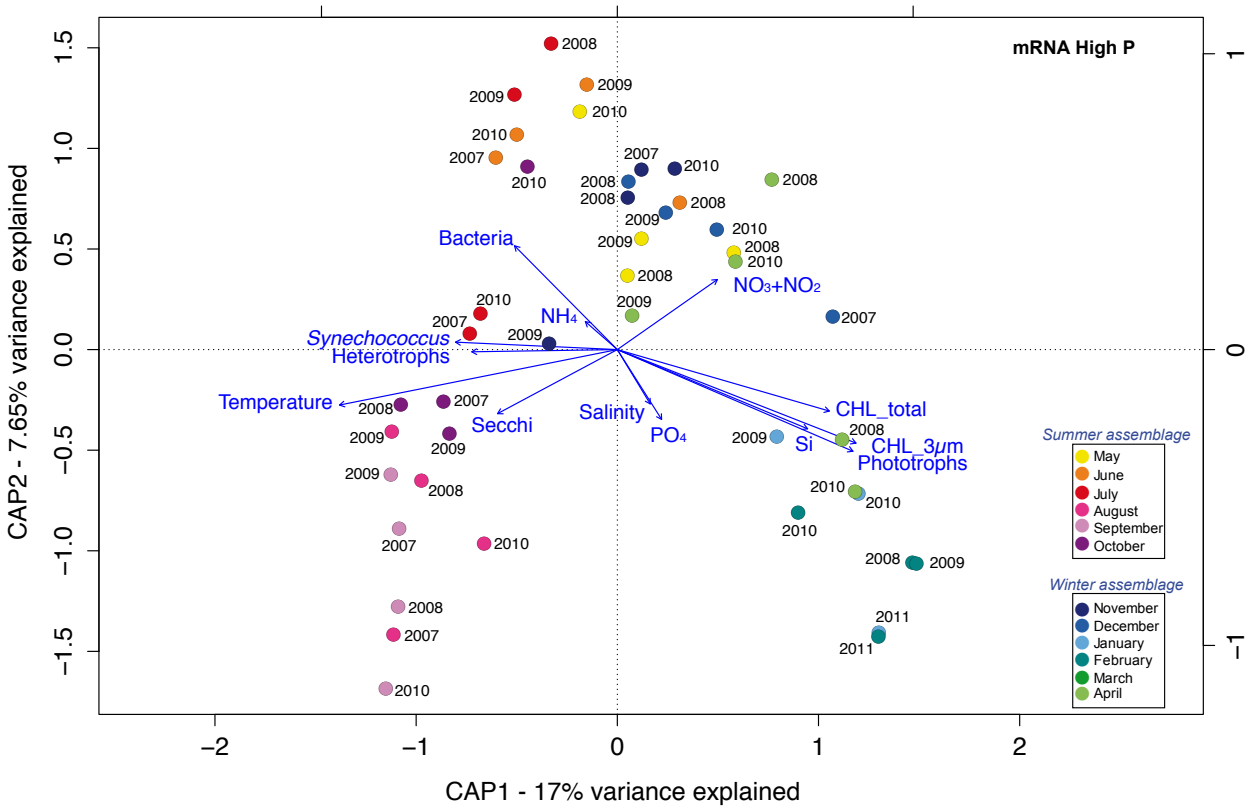

Figure 9. Constrained Analysis of Principal Coordinates (CAP) of the HighP data. Samples are color-labeled according to the month. A progressive pattern of community and gene expression change is observed across all four years. A suite of environmental parameters is included to observe their potential relationships to picoeukaryotic gene expression dynamics (constructed from TFA fingerprints). The length and direction of arrows indicate the degree of correlation with the ordination axes.

DISCUSSION

A very important technical issue for all studies exploring changes in microbial gene expression in environmental samples is to take into account the mRNA longevity. It is known that mRNAs are generally unstable and that gene expression may change in minutes (Moran et al., 2012). Further, a recent study comparing bacteria collected with a normal CTD with bacteria fixed *in situ* (at the depth of collection), revealed that some of the highly expressed genes in the unfixed bacteria were stress response genes, while expression of important genes *in situ*, for example the genes for ammonia oxidation, was severely reduced in the unfixed samples (Feike et al., 2012). It is true that the ecosystem sampled in that study was the anaerobic section of the Baltic Sea and that the anaerobic or microaerophylic bacteria living at those depths would be particularly prone to stress by being pulled through the aerobic water column. However, this study brings attention to one of the main problems in analyzing mRNAs from natural communities. In a previous study (Coll-Lladó et al., 2011), we showed that despite these caveats, with our technique we could obtain repeatable gene expression patterns. Moreover, the patterns were consistent with the spatial distribution of the samples. In the present study we also obtained a robust seasonal pattern of gene expression in BBMO. This indicates that the seasonal signal was strong enough to be retrieved despite the short lifetimes of many mRNAs.

Another interesting comparison was checking how the inclusion of all peaks of the electropherogram in the analysis would affect the results. In principle, large peaks correspond to highly expressed genes only, while small peaks added in the AllP analysis include a variety of expression intensities. Comparing the distance matrices from both methods (Figure 2A-B) it seems that addition of AllP "buffers" the differences among samples. Thus, the highly expressed genes are the ones generating the largest differences among samples, while the other peaks form a background of genes that are almost always being expressed at lower intensities. This same behavior could be observed in the DNA dataset.

Three hypotheses arise when comparing community composition and gene expression patterns. First, both patterns could change in unison. This would mean that every particular assemblage had its own specific gene expression pattern. Second, gene expression patterns could change faster than community composition. This is actually what we would expect *a priory*, since gene expression is very dynamic and has the potential to change in few seconds/minutes. Community composition, on the other hand, requires cell division and death of some taxa and under natural conditions this may take days to weeks. The interpretation of this possibility is that a certain species assemblage is capable to adapt partially to environmental changes by expressing a different set of genes. However, if environmental changes were sufficiently strong that some species would no longer be within their survival range (temperature, nutrients, etc…), the composition would also change.

Third, changes in composition could be faster than changes in gene expression. This possibility seems counterintuitive at a first glance. However, it could be possible that the main genes expressed are the same in all situations. Core metabolism genes, such as certain cytochromes, ATPase or ribulose biphosphate carboxylase should be always abundantly expressed, regardless of the species present. Since our fingerprinting technique only retrieves the highly expressed genes, this functional redundancy is certainly a conceivable possibility. It has to be remembered that all our samples were collected at approximately the same time of the day (between 10-12am) and, thus, the obvious diel changes in gene expression patterns were not an issue.

Both community composition and gene expression patterns followed a very clear seasonal cycle as shown in Figure 6. These changes were driven by temperature and other environmental factors, which explained around 45% of the variability (Figure 8). The main differences were found between summer and winter. This pattern had also been found in previous studies of community composition (Shade et al., 2007). In general, gene expression patterns showed the same contrast between summer and winter.

Picoeukaryotes in Blanes Bay follow a cycle in which phototrophic organisms are more abundant in winter than in summer. Heterotrophic flagellates, on the other hand, show very little changes throughout the year (Massana, 2011). Thus, the relative importance of phototrophs is larger in winter and that of heterotrophs in summer. This can be seen in Figure 8, where phototrophs correlate with chlorophyll and low temperatures, while heterotrophs correlate with high temperature, water transparency, and *Synechococcus* abundance (also more abundant in summer). Thus, the big picture is what we could expect: gene expression patterns change along seasons and the more contrasted differences occur between summer and winter, roughly following changes in community composition. These observations support hypothesis 1: each community shows its own gene expression pattern and, therefore, community composition and gene expression change in parallel.

If individual months are considered, however, subtler relationships appear. In the case of community composition, winter months tended to be rather similar to each other when different years were compared. Summer months, on the other hand, tended to be less similar to each other from one year to another (Figure 4C). In the case of gene expression, both winter and summer months were rather different from year to year (Figure 5C). Thus, in winter, gene expression patterns were more variable than community composition. This corresponds to hypothesis 2: gene expression patterns are more variable than community composition. During summer months, the variability was higher for community composition than for gene expression, suggesting that hypothesis 3 prevailed: there was functional redundancy. Thus, the three hypotheses envisioned initially occurred at some time of the year or at some time scale.

In summary, the TFA technique proved adequate to follow gene expression patterns in a large number of samples in a relatively cheap and quick way. As expected, seasonal changes clearly appeared and these were concomitant with changes in community composition. At shorter time scales, however, both community adaptation and gene redundancy were seen at particular times of the year, when the most abundant OTUs and the highly expressed genes were considered. Inclusion of all the peaks in the ARISA and TFA electropherograms reduced substantially the differentiation between samples, thus acting as a buffer of both composition and gene expression patterns.

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We thank Ramon Massana for epifluorescence microscope counts, Vanessa Balagué for the DNA extractions and Josep M. Gasol for curation of the Blanes Bay Microbial Observatory dataset. This work was supported by grant GEMMA (CTM2007-63753-C02-01/MAR) and PANGENOMICS (CGL2011-26848/BOS) from the Spanish Ministry of Science and Innovation. HS benefited from fellowships from the Spanish Ministry of Science and Innovation (JCI-2008-2727) and Brazilian "Ciências sem Fronteiras" Program from CAPES (BJT 013/2012).

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SUPPLEMENTARY INFORMATION

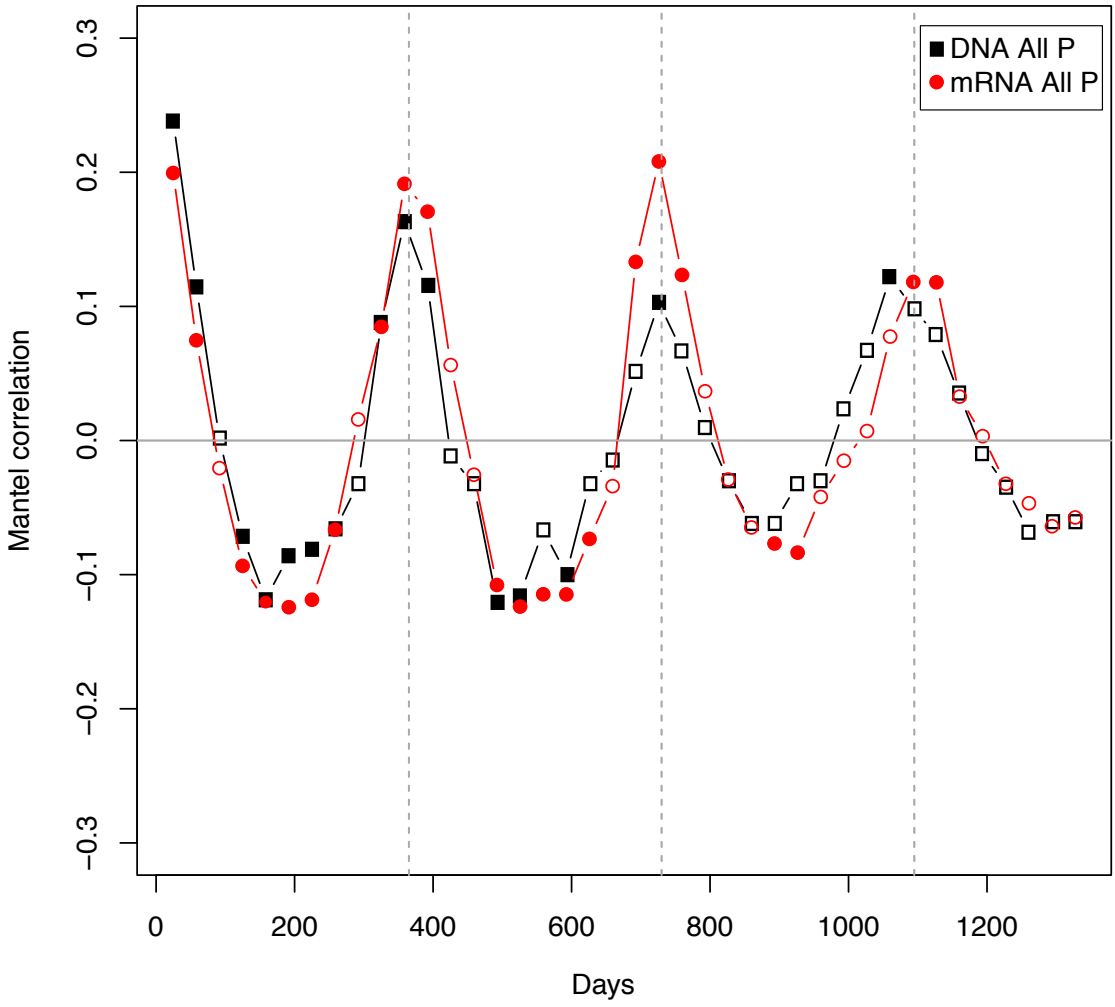


Figure 1. Mantel correlogram. Sea surface was sampled monthly from June 2007 to February 2011 in the Blanes Bay Microbial Observatory. The blue line indicates the annual repeating patterns of the picoeukaryotic community composition and the red line the annual cycle of the gene expression patterns. The filled symbols indicate correlations with $P<0.05$. Autocorrelations were carried out with AllP.

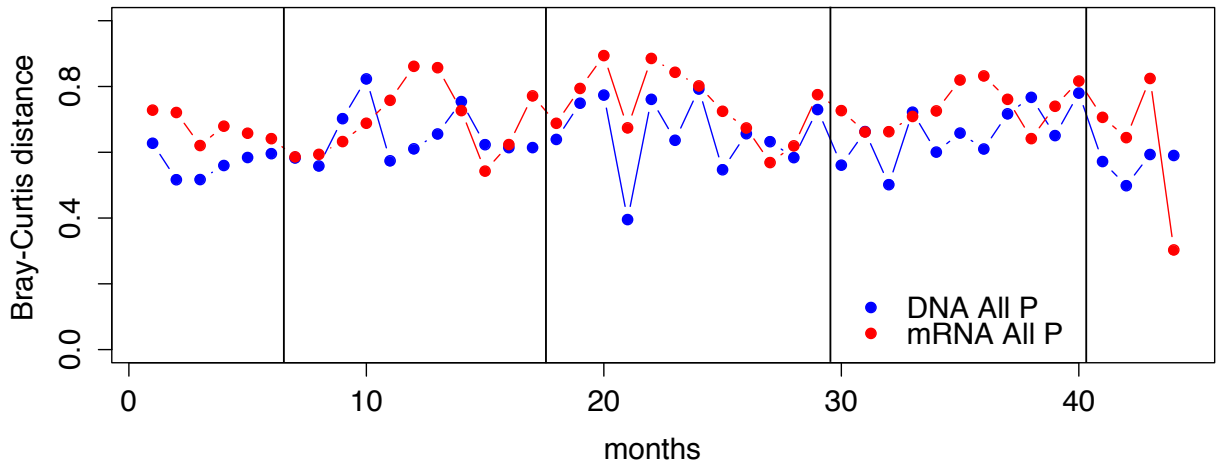
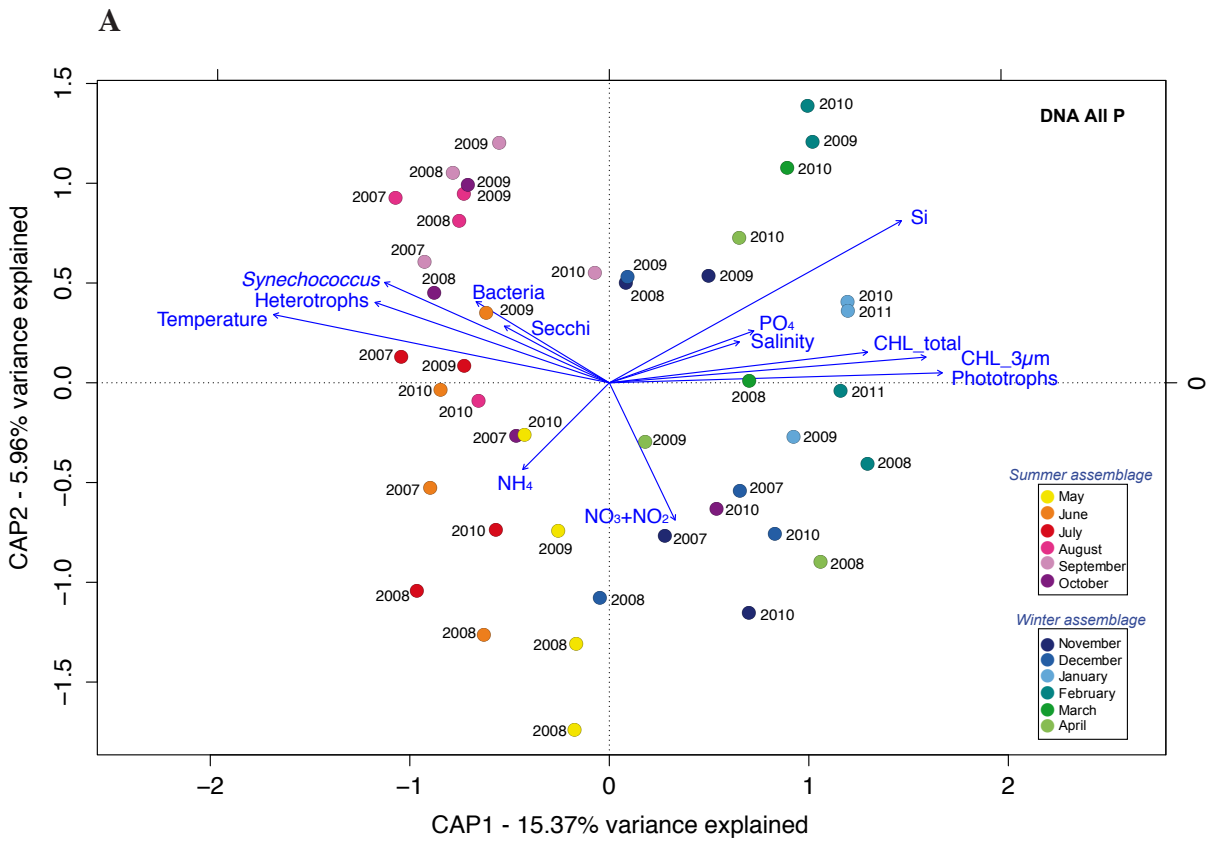


Figure 2. Bray-Curtis distances from one month to the next over 4 years. The blue line are distances in DNA fingerprints and the red line in RNA fingerprints (analysis performed from low treshold settings).

Figure 3. Constrained Analysis of Principal Coordinates (CAP) of the AllP data. Samples are color-labeled according to the month. A suite of environmental parameters is included to observe their potential relationships to **A**) picoeukaryotic community composition dynamics (constructed from ARISA fingerprints, and **B**) picoeukaryotic gene expression dynamics (constructed from TFA fingerprints). The length and direction of arrows indicate the degree of correlation with the ordination axes.



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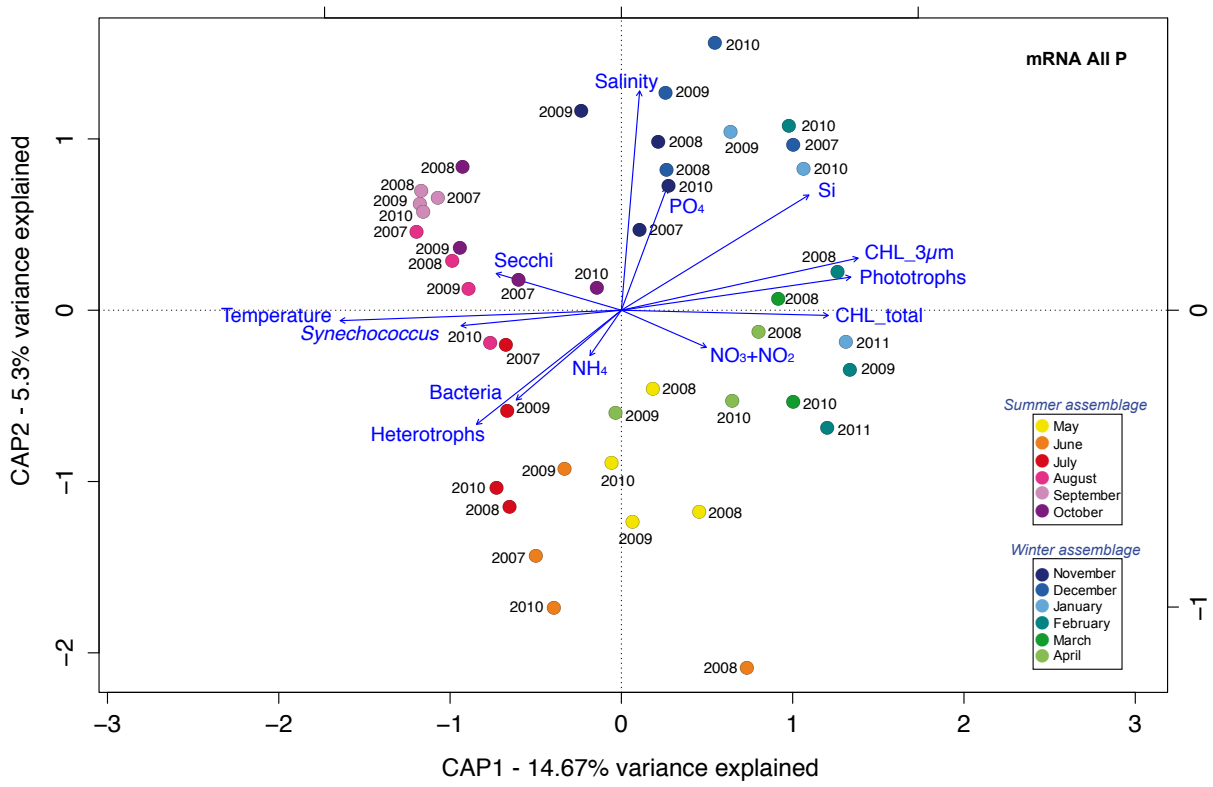
mRNA All P
Salinity
PO4
Si
Secchi
Temperature
Synechococcus
Bacteria
Heterotrophs
NH4
NO3+NO2
CHL_3µm
Phototrophs
CHL_total
Summer assemblage
May
June
July
August
September
October
Winter assemblage
November
December
January
February
March
April
CAP1 - 14.67% variance explained
CAP2 - 5.3% variance explained

Chapter 3

Gene Expression Patterns and Community Composition of Marine Picoeukaryotes: Changes with Depth, Distance from Shore, and along Diel Cycles

ABSTRACT

Understanding structure-function relationships of microbial communities is a central topic of microbial ecology. In fact, studies on microbial composition and expression changes in marine environments across oceanographic features, at distinct depths or along temporal changes are scarce. In this study, the picoeukaryotic gene expression patterns (TFA) were compared with community DNA composition (ARISA) along a horizontal transect and a depth profile in the northwestern Mediterranean Sea. TFA was also used during two diel cycles at two depths both in coastal and open sea waters. Similarities of the picoeukaryotic communities across a 100 km transect were observed in the surface waters using both mRNA and DNA-based fingerprints, the community composition being more stable than its expression patterns. The stable communities on the surface could indicate the influence of the hydrography on the picoeukaryotic assemblages. Analyses of community structure composition and gene expression provided comprehensive relationships to disentangle structure-function relationships of microbial communities.

Coupled gene expression patterns and community composition of marine picoeukaryotes: changes with depth, distance from shore, and along diel cycles. **Montserrat Coll-Lladó, Guillem Salazar, Silvia G. Acinas and Carles Pedrós-Alió**. Manuscript.

INTRODUCTION

Studies analyzing the dynamics of microbial composition with gene expression patterns along environmental gradients, such as depth in the water column, across oceanographic features, or through time, are scarce (Hewson *et al.*, 2010; Ottesen *et al.,* 2013; Poretsky *et al.,* 2009b; Coll-Lladó *et al.,* 2013 submitted). Two of the former studies addressed day-night cycles in bacterial communities. Many microbial processes are expected to be differentially active over a day/night cycle, such as photosynthesis or oxidative phosphorylation (Poretsky *et al.,* 2009b). For example, Poretsky *et al.* (2009b) examined the transcriptional profiles over a pair of day/night cycle samples of a bacterial community in the oligotrophic North Pacific Ocean and found that, as expected, gene transcripts of photosynthetic eukaryotes were more abundant during the day than during the night. Among the most highly expressed genes detected from phototrophic organisms were those encoding chlorophyll binding proteins, light harvesting reactions and photosynthetic machinery.

Overall these authors showed that bacterial community investment was skewed towards energy acquisition and metabolism during the day, while biosynthesis (specifically of membranes, amino acids and vitamins) received relatively greater investments at night (Poretsky *et al.,* 2009b). Ottesen *et al.* analyzed surface Pacific Ocean waters in a Lagrangian study for two days and found diel cycles in the expression patterns of *Prochlorococcus* and *Synechococcus*, but not in those of chemotrophic organisms such as SAR11, SAR86 and marine archaea group II (Ottesen *et al.,* 2013).

Hewson *et al.* (2010) compared bacterial community composition and expression patterns in eight samples from different oceanic regions. In addition, they compared expression patterns at night and day. They found that gene expression patterns were very similar across space and between day and night, despite differences in community composition but that subtle differences could be observed.

Obviously, disentangling patterns of gene expression at different time and space scales in microbial communities remains difficult and, thus, there are very few studies available. Moreover, no studies have addressed these questions for picoeukaryotic plankton. In a previous work (Coll-Lladó *et al.,* 2013 submitted) we analyzed seasonal changes at one coastal site with a fingerprinting technique to detect picoeukaryotic gene expression patterns. We found a yearly repetition of both composition and expression patterns. However, at some times of the year changes were faster in community composition than in gene expression while the opposite was true at other times.

In the present work we examined the spatial variability of the picoeukaryotic community structure and its gene expression patterns across a transect in the Northwestern (NW) Mediterranean Sea.

Spatial changes were examined in surface samples collected over a 100 km transect from shore to open sea. Changes were also analyzed through a vertical profile at the deepest station (2000 m). Finally, diel cycles were studied at the two stations at both ends of the transect.

Marine picoeukaryotes (which are between 0.2 and 2 to 3 μm in diameter) are probably the most abundant eukaryotes on Earth (Marañón *et al.,* 2001; Massana, 2011) and are found throughout the water column. While autotrophic picoeukaryotes are restricted to the photic zone (upper 100–200 m), heterotrophic groups are found at greater depths (Brown *et al.,* 2009; Edgcomb *et al.*, 2002; López-García *et al.*, 2001; Moreira & López-García, 2002) even in the abyssal sea floor at depths near 6,000 m in the southern Atlantic Ocean (Scheckenbach *et al.*, 2010).

Members of the picoplankton are important contributors to both photosynthetic biomass and production (Marañón *et al.,* 2001), particularly in oligotrophic regions such as around Hawaii in which 80% of the chlorophyll *a* biomass could be due to cells <3 μm in waters (Bienfang *et al.*, 1984). The waters from the northwestern Mediterranean Sea are also oligotrophic and their picoeukaryotic diversity has been studied extensively using molecular surveys of the 18S ribosomal DNA gene revealing a large diversity (Massana *et al.*, 2004). Also, fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) have been applied to quickly compare the composition of planktonic eukaryotic assemblages (Díez *et al.*, 2001).

Here, picoeukaryotic communitiy composition was followed by ITS1 ribosomal RNA gene-based fingerprints using Automated Ribosomal Intergenic Spacer Analysis (ARISA) and gene expression patterns were examined through Transcriptome Fingerprinting Analysis (TFA) as described in (Coll-Lladó *et al.*, 2011). The main goal was to study the relationship between changes in composition (ARISA) and expression patterns (TFA) of picoeukaryotes along environmental gradients and day-night cycles.

MATERIALS AND METHODS

Sampling and collection of biomass

Samples were collected during cruise MODIVUS (17-27th September 2007) on board *R/V García del Cid* at five stations from coastal to open sea in the NW Mediterranean Sea (Table 1A). This transect has been studied repeatedly from different perspectives including microbial processes (Estrada *et al.*, 1993; Pedrós-Alió *et al.*, 1999; Sala *et al.*, 2002) and diversity of microorganisms (Crespo *et al.*, 2013; Pommier *et al.*, 2010). Samples were collected with Niskin bottles mounted on a rosette with a CTD. Five stations were occupied and vertical profiles sampled at each station. Water was prefiltered through a 200 μm and 20 μm Nylon mesh in succession and immediately processed on board. Water for RNA was kept on ice throughout the filtration process.

Table 1. A) Geographical location and description of the sampling points of the MODIVUS oceanographic cruise. Samples are numbered according to the respective sampling depth. B) General description of the diel cycle studies.

A

Station	Date (dd/mm/yy)	Latitude N	Longitude E	Sampling depths (m)	Bottom depth (m)	Surface chlorophyll *a* (μg L^{-1})
C	18-20/09/07	41°39'5.6"	2°48'1.3"	5	30	0.09
CM	21/09/07	41°24'5.9"	2°48'4.9"	5, 30, 44, 60, 100, 600	660	0.07
M	21/09/07	41°9'1.3"	2°49'3.3"	5, 48, 400, 1400	1430	0.08
MD	22/09/07	40°54'52.78"	2°50'43.8"	4, 60, 77, 140, 400, 2000	1972	0.10
D	23-25/09/07	40°39'4.7"	2°51'1.6"	5, 25, 44, 65, 500, 2000	2226	0.08

B

Station	Date	Position	Type	Bottom depth (m)	Experiment duration (h)	Sample depth (m)
C_4m	18–20 Sep07	41°39'5.6" N, 2°48'1.3" E	coastal	30	56	4
C_15m	18–20 Sep07	41°39'5.6" N, 2°48'1.3" E	coastal	30	56	15
D_5m	23–25 Sep07	40°39'4.7" N, 2°51'1.6" E	open sea	2226	44	5
D_65m	23–25 Sep07	40°39'4.7" N, 2°51'1.6" E	open sea	2226	44	65

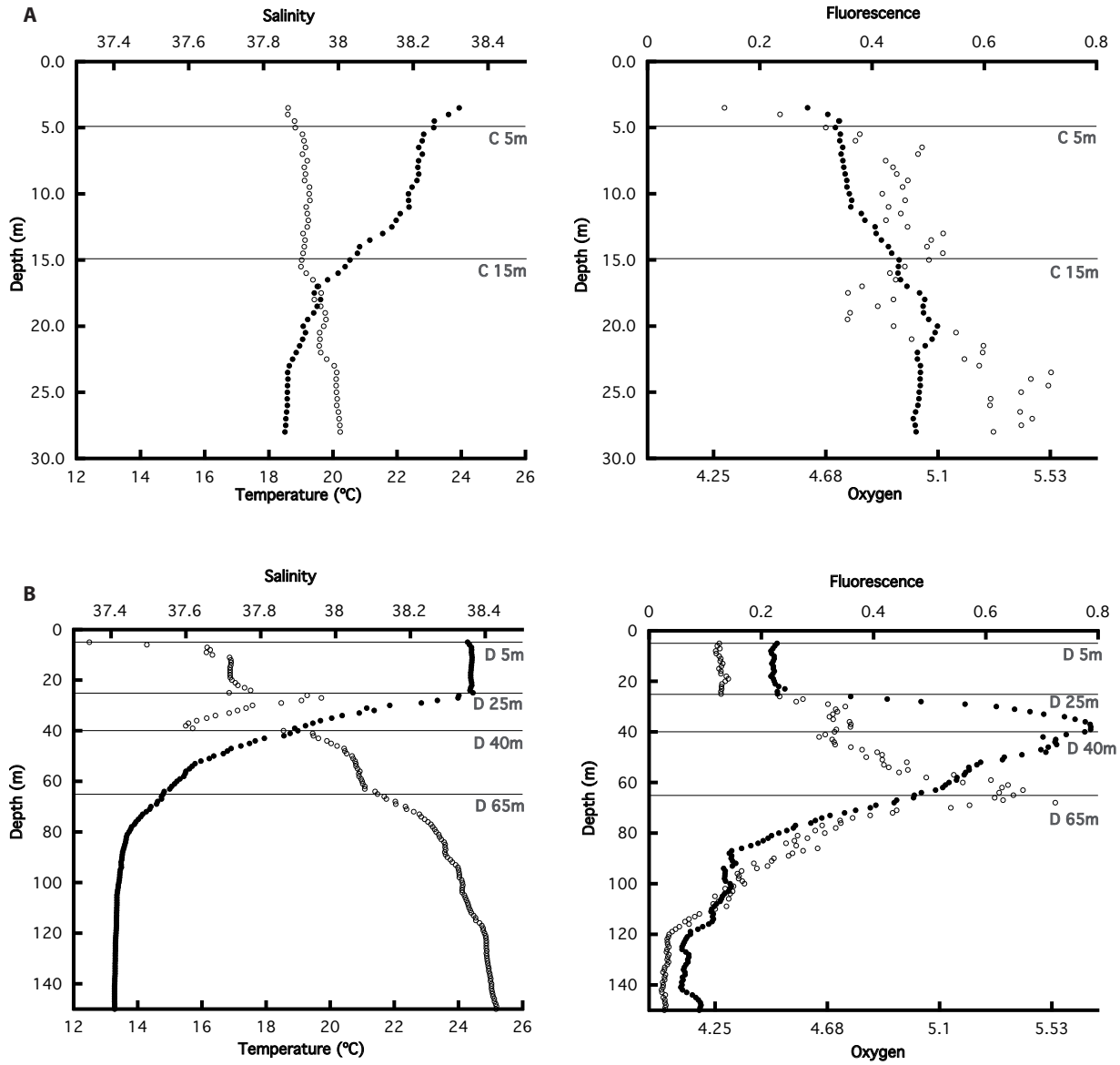

Figure 1. CTD profiles of temperature, salinity, fluorescence and oxygen in the coastal station (A) and the open sea station (B).

Two diel studies were done at stations C and D (Table 1B). Station C was analyzed for 56 hours at both 4 and 15 m depth. Station D was followed for 44 hours at 5 and 65 m depths. During these studies samples were collected every four hours. Vertical profiles of temperature, salinity, fluorescence and oxygen for stations C and D at the beginning of the diel studies are shown in Figure 1. More physicochemical information about the Modivus transect can be found in Pommier *et al.*, (2010) and Crespo *et al.*, (2013).

Picoeukaryote community DNA fingerprinting (ARISA)

Microbial biomass was collected by a serial filtration of eight liters of seawater through a 3-μm pore-size polycarbonate prefilter (Poretics) and a 0.2-μm Sterivex filter (Durapore, Millipore) using a peristaltic pump. The Sterivex units were filled with 1.8 mL of lysis buffer (40 mM EDTA pH 8.0, 50 mM Tris-HCl pH 8.3, 0.75 M sucrose) and stored at -20°C. Nucleic acids were extracted as described in (Massana *et al.*, 1997).

Specific primers were used to amplify the internal transcribed spacer 1 (ITS1) (Massana *et al.*, 2008). The sequence of forward primer (EUK RR) was labeled with a fluorescence tag (FAM) and matched the end of the SSU and was: 5'-GTAGGTGAACCTGCAGAAGGATCA-3'. The reverse primer, ITS2, matched the beginning of the 5.8S. The sequence of ITS2 was 5'-GCTGC-GTTCTTCATCGATGC -3'. The PCR mixture (40 μl) contained: 10 ng of DNA template, 250 nM of each primer, 250 nM dNTPs, 2.5 mM Mg Cl_2, 3 U *Taq* DNA polymerase, and the enzyme buffer. PCR cycling, carried out in a DNA Engine® thermal cycler (Bio-Rad Laboratories, Inc.), comprised an initial denaturation step at 94 ˚C for 2 min followed by 32 cycles of denaturation at 94 ˚C for 15 s, annealing at 55 ˚C for 30 s and extension at 72 ˚C for 3 min followed by a final extension step at 72 ˚C for 9 min. A negative control was run for each set of reactions to assess the background levels. We used the same TFA pipeline (see details below) to discriminate the ARISA amplicons with gene analyzer, the only difference was that ARISA fragments were detected in a different channel because of the nature of the fluorescent tag.

Transcriptome fingerprinting analysis (TFA)

Seawater samples for TFA were filtered first through a 3-μm pore-size polycarbonate filter (Poretics) and then through a 0.2-μm polycarbonate filter (Poretics) using a peristaltic pump (MasterFlex 7553-89 with cartridges Easy Load II 77200-62, Cole-Parmer Instrument Company) to collect the bacteria and picoeukaryotes. Filters were flash-frozen in liquid nitrogen and keep it on it until processed. Total RNA was extracted from the 0.2-μm polycarbonate filters.

RNA extraction and purification. The procedure was adapted from (Poretsky *et al.*, 2009a). Filters were transferred to 2 ml screw-cap microcentrifuge tubes containing 200-μl of 0.1 mm- diameter zirconia-silica beads (BioSpec Products, Inc.) and 100-μl of 0.5 mm glass beads (BioSpec Products, Inc.) mixed with 450-μl RLT lysis buffer (provided by the RNeasy Mini Kit Qiagen, Inc.) plus ß-mercaptoethanol (Fluka). Samples were mechanically disrupted in a Mini-beadbeater-8TM cell disrupter (BioSpec Products, Inc., Bartlesville, OK) for 10 min. After disruption, samples were incubated on ice for 5 min and the beads were allowed to settle out of the lysis mixture. Samples underwent centrifugation (in an Eppendorf centrifuge at 2100 rcf 1 min). The lysate was trans-

ferred to a new tube. 300 µl of lysis solution was added to the vials with beads to increase the final yield. The tubes were shaken vigorously and the supernatant was also recovered. The same volume of 70% ethanol was added to the lysate and samples were purified according to the RNeasy Mini Kit (Qiagen, Inc.). The isolated total RNA was treated with TurboDNase I (Ambion) to remove contaminating genomic DNA according to the manufacturer's instructions. RNA was aliquoted and quantified by absorbance at 260 nm with a NanoDrop 1000 (Thermo Fisher Scientific Inc., Wilmington, DE).

Reverse transcription and PCR amplification. First-strand cDNA synthesis was conducted with 30 ng of total RNA as starting material. mRNAs were reverse-transcribed to single-stranded complementary DNA using the SuperScript III reverse transcriptase (Invitrogen) and the primer H-T_{11}A (5'-AAGCTTTTTTTTTTTTA-3'). To denature any secondary structure, an aliquot of each of the RNA extracts plus the oligo(dT) primer were heated for 5 min at 65ºC and immediately placed on ice before mixing with the final reaction solution (20 µl): 5X First-Strand buffer, 0.1 M DTT, 40 U RNaseOUT, 10 mM dNTPs and 200 U SuperScript III RT. Reaction mixtures were incubated at 50ºC during 50 min, and inactivated by heating 70 ˚C for 15 min. 2 µl of the RT reaction product was used in a subsequent PCR. PCR reactions were carried out using *Taq* polymerase (Qiagen) in a final reaction volume of 20 µl. The arbitrary primer H-AP8 (5'-AAGCTTTTACCGC-3') and the same primer used in the RT reaction but labeled with a fluorescence tag (NED) were used for the amplification of cDNA. The primer targeting the poly(A) tail and the arbitrary primer for PCR were from RNAspectra Yellow kit 1 of GenHunter Corporation. The PCR cycle was 40 cycles of 94 ˚C for 30 s, annealing at 40 ˚C for 2 min, 72 ˚C for 60 s followed by 1 cycle of 72 ˚C for 5 min in a DNA Engine® thermal cycler (Bio-Rad Laboratories, Inc.). A negative control was run for each set of reactions to assess the background levels. To ensure that there was no amplification of genomic DNA a control was run with an aliquot of the RNA extracts added directly to the PCR reaction.

Separation of amplicons with gene analyzer. 1 µl of PCR product from each sample was mixed with 9 µl of Hi-Di Formamide (Applied Biosystems). 0.5 µl of size standard (ROX 500, Applied Biosystems) was added to every reaction to define the standard curve between 25 and 500 bp. The cDNA peaks obtained ranged in length from 30 to 500 bp, according to the internal size standard used. The mixtures were run on an ABI automated sequencer operating as a fragment analyzer (ABI 3130XL). The sequencer electropherograms were then analyzed using the GeneMarker software, version 1.90 (SoftGenetics, LLC). Raw data were treated with some filters activated according to GeneMarker instructions: baseline subtraction, spike removal, auto pull up removal, smooth. The cubic spline algorithm was used to calculate bp lengths of identified fluorescence peaks. The different peak detection thresholds applied are detailed below. Once the peaks were

selected, peak areas were used as output from GeneMarker software and were transferred to Microsoft Excel (Seattle, WA) for subsequent analysis.

Peak detection in ARISA and TFA

Two different cutoff thresholds were used to select peaks in both ARISA and TFA electropherograms. In the first case, only the largest peaks were selected. The threshold for these peaks was set at 200 relative fluorescence units (rfus) for ARISA and at 100 rfus for TFA. These TFA peaks are supposed to correspond to highly expressed genes. From now on we will refer to these as HighP (for high peaks). Settings and filters were as previously described (Coll-Lladó *et al.,* 2011). In the second case, all the peaks that could be discerned above the background by the instrument, including the high peaks, were included in the analyses. The threshold for these peaks was set at 30 rfus for ARISA at 10 rfus for TFA. We will refer to these as AllP (for all peaks). This low intensity was the only criterion to detect these peaks. All the other filters used in the HighP settings were shut off. The fragments considered in the analyses were those between 150 and 500 bp for ARISA and between 90 and 530 bp for TFA. Peaks were visually inspected as a quality control (Coll-Lladó *et al.,* 2011).

Statistical analysis

The peak area data retrieved from GeneMarker were standardized (the relative peak heights within a profile were calculated by dividing the height of an individual peak by the total peak height (i.e.: sum of the heights of all peaks in an electropherogram). The similarity of patterns derived from different communities was assessed computing Bray-Curtis distances to construct the similarity matrices. Mantel tests between dissimilarity matrices and Mantel correlograms were performed with the Pearson correlation. Patterns were explored using Nonmetric Multidimensional Scaling (NMDS) and Constrained Analysis of Principal Coordinates (CAP). Primer-E version 6 (Clarke, 1993) and R software (R development Core Team, 2005) were used for these analyses.

RESULTS AND DISCUSSION

Comparison between HighP and AllP patterns

Electropherograms show a series of peaks of different areas. In principle, the large peaks correspond to abundant taxa in the case of ARISA and to highly expressed genes in the case of TFA. Deciding how many peaks to include in the analysis is a subjective decision and we analyzed the effect of considering two different thresholds on the results (Coll-Lladó *et al.,* 2013 submitted). In one option we considered the largest peaks only (HighP) and in the other case we considered all

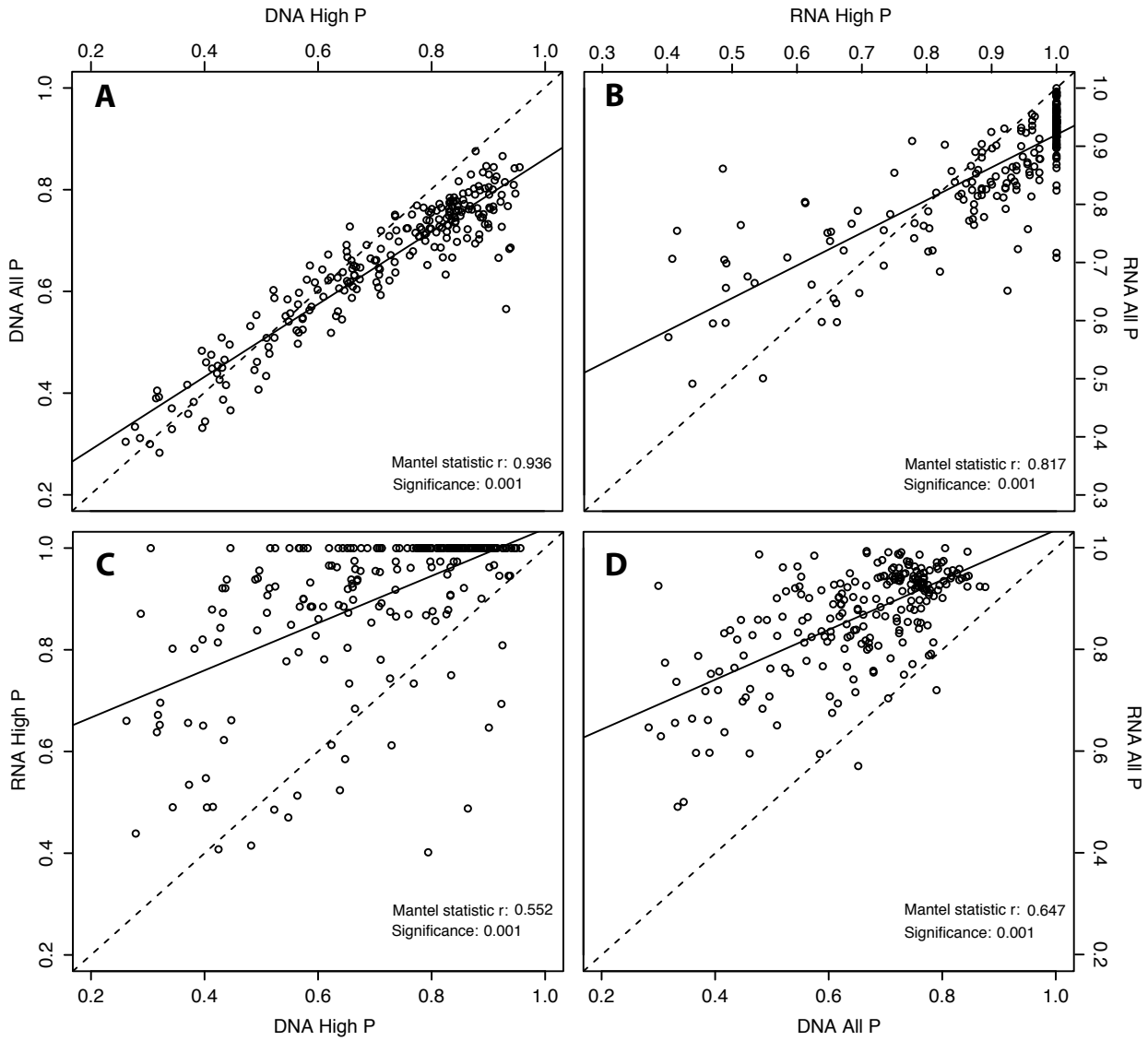

Figure 2. Comparisons among Bray Curtis distance matrices generated with restrictive vs. non-restrictive cutoffs. A) Comparison of distance matrices for AllP and HighP of DNA fingerprints (ARISA) from samples taking along the Modivus transect and vertical profiles. B) Comparison of distance matrices for HighP and AllP of mRNA fingerprints (TFA). C) Comparison between DNA and mRNA distance matrices for HighP. D) Comparison between DNA and mRNA distance matrices for AllP. The discontinuous diagonal lines indicate the 1:1 relationship and the continuous lines the linear regressions.

the peaks above the background (AllP, see Materials and Methods). We then carried out the analyses with both data sets to see whether the results would be different or not. In a recent paper we showed that these alternatives together provided significant information (Coll-LLadó *et al.*, 2013 submitted).

Matrices with distances among spatial samples (surface transect and vertical profile) were constructed with both thresholds and were compared with Mantel tests (Figure 2A-B). The distances calculated with the two thresholds were highly correlated for both DNA (Mantel statistic $r = 0.936$, $p<0.001$) and mRNA ($r = 0.817$, $p<0.001$). The slope of the regression lines, however, were significantly lower than 1. Thus, addition of the small peaks in the electropherograms reduced the range of distance values found with the large peaks only. Subsequent analyses were performed with data generated with the two thresholds.

Distances calculated from DNA and mRNA were significantly correlated according to Mantel tests (Figure 2C-D). Most comparisons showed that mRNA produced longer distances among samples than DNA. As before, considering AllP reduced the distances between samples in mRNA and DNA fingerprint comparisons.

Spatial patterns in community composition and gene expression

NMDS analysis showed similar separation of samples by depth, both in ARISA and TFA fingerprints (Figure 3). In general, the horizontal axis in the diagram separated deep samples from the rest, while the vertical axis separated surface from intermediate depth samples. Deep samples (below 400 m) were clearly separated from all the rest and showed considerable distances among themselves. Surface samples, on the other hand, were closely clustered despite the 120 km between the two ends of the transect. Samples from intermediate depths (between 25 and 140 m) were fairly dispersed and usually occupied a position intermediate between surface and deep samples, although the separation was better resolved with TFA than with ARISA profiles.

There were particular samples that did not follow this general pattern in both ARISA and TFA analyses. In the ARISA (Figure 3A) sample CM44 was very different from all the other samples. This sample coincided with the most marked peak of deep chlorophyll maximum (DCM) of the whole transect. Chlorophyll *a* (Chl*a*) reached 0.6 $\mu g\ L^{-1}$. Although this is a low concentration, it was the highest in the whole transect, where most values were under 0.3 $\mu g\ L^{-1}$ (Lefort *et al.,* in preparation). The Chl*a* peak could be explained by a coincident peak in phototrophic picoeukaryotes that reached a concentration of 3500 cells ml^{-1} compared to the 500 cell ml^{-1} in most of the other samples. Interestingly, the TFA for this sample did not show significant differences with the samples around it. This finding pointed out to the functional redundancy in the gene expression of the picoeukaryotic assemblages.

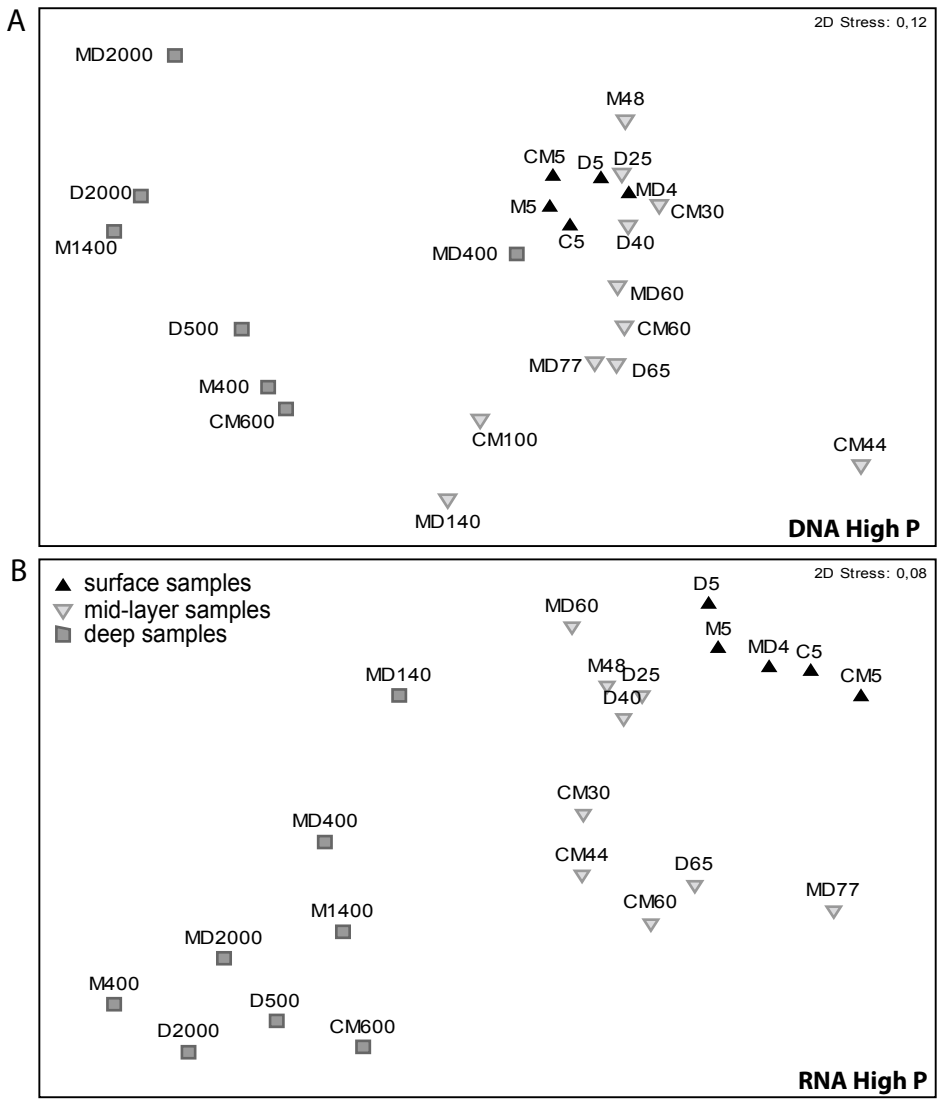

Figure 3. A) NMDS diagrams comparing DNA fingerprints (ARISA) from samples collected during Modivus cruise. B) NMDS diagrams comparing mRNA fingerprints (TFA) from samples collected during Modivus cruise. Samples are labeled according to the depth of the sample. Results with AllP are not shown.

In the TFA (Figure 3B), all stations but MD followed a similar "smooth" trajectory from surface samples in the upper right corner of the NMDS space down to the lower left corner for the bottom samples. Samples MD77 and MD140, however, deviated from this pattern. Station MD was the only one in which an upwelling could be detected: the 27 Kg m^{-3} isopycnal, that generally marked the depth of the DCM in the other stations (at 45 m), was found close to the surface at this station (at 15 m). It appears that similar picoeukaryotic assemblages had reacted to the upwelling by changing their gene expression patterns, but had not had time yet to change the community composition at 77 m, although the composition was certainly different at 140 m.

These patterns can be analyzed more quantitatively looking at the average similarities among samples from the three depth groups (Table 2). If we look at the values for DNA AllP the average similarity among surface samples was 62.6%, and decreased for intermediate (40.6%) and deep samples (37%). These values did not change much when DNA HighP was considered. This shows that surface samples were closer among them than bottom samples in community composition. In the case of the RNA, however, the values of similarity were much lower: 33 – 38%, 15 – 21%, and 25 -27% for surface, intermediate and bottom samples respectively. This shows that samples differed more in gene expression patterns than in community composition, as could be expected although in deeper samples such differences seemed to be attenuated (DNA 37% vs. RNA 27%)

Table 2. Average similarities (Bray-Curtis distance) among samples and number of peaks necessary to explain 50 and 90% of the differences among samples, from the surface, intermediate, and bottom samples.

	Surface samples			**Intermediate depths**			**Deep samples**		
	Average similarity (%)	50%	90%	Average similarity (%)	50%	90%	Average similarity (%)	50%	90%
DNA AllP	62.6	17	60	40.6	15	68	37.0	11	51
DNA High	62.4	10	26	38.3	7	28	32.1	5	17
RNA AllP	32.9	5	33	21.2	18	88	24.7	9	46
RNA High	38.5	3	6	15.4	3	18	27.1	2	5

Following the community structure and gene expression patterns along the horizontal transect and at vertical profile

Bray-Curtis distances between transect samples are plotted in Fig 4A in two different ways. Lines show the distance between each sample and the first sample (sample C5) along the transect, while vertical bars show distance between a sample and the previous sample in the transect. Since the distance between the first sample and itself is zero, sample C5 is not shown. For the next sample (CM5) both the line and the bar have exactly the same value by definition. But, for the remaining samples, we would expect that the distance to the previous sample would be smaller or equal than the distance to the first sample, since samples farther away can be expected to differ more than contiguous samples. Figure 4B shows the same information for the vertical profile at station D from surface down to 2000 m depth. With one exception (sample MD in the transect) the expectation was fulfilled both along the transect and the vertical profile. As explained earlier, there was an upwelling in station MD and this, presumably, increased the distance of this sample with respect to its neighbors relative to the distance to sample C5.

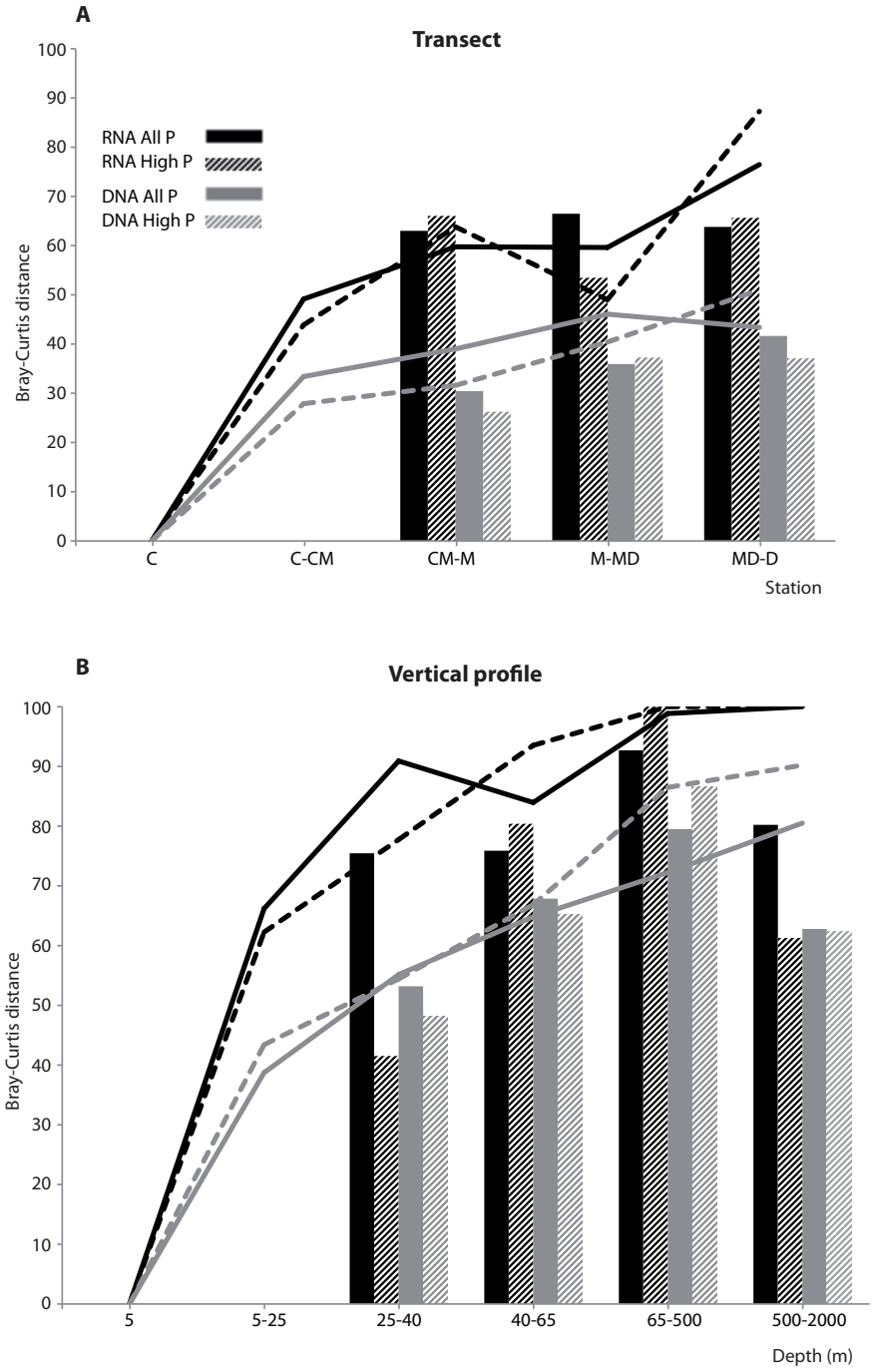

Figure 4. Bray-Curtis distances among samples. Lines show the distance between each sample and the first sample. Histograms show distance between each sample and the previous sample. Black histograms and lines correspond to RNA while the grey ones to DNA. Continuous lines and filled histograms correspond to All P mRNA and DNA distances while striped histograms and discontinuous lines correspond to High P DNA and RNA based distances. A) The Bray-Curtis for the transect. B) The Bray-Curtis for the vertical profile at station D.

Next we will compare RNA and DNA based distances for the horizontal transect (Figure 4A). The two bars for RNA were always higher than those for DNA indicating that, as expected, contiguous communities differed more in their gene expression patterns than in their taxonomic composition. If we look at the lines, again the values for RNA were always higher than those for DNA. Thus, communities differed from C5 more in gene expression patterns (45 to 85% distance) than in composition (30 to 50% distance). This was the case with both HighP and AllP. Finally, the samples differed more along the vertical profile (up to 100%) than along the transect (35% for DNA and 55% for RNA).

It is well known that taxonomic composition of microbial communities change more markedly with depth than with horizontal distance (Acinas *et al.*, 1997; DeLong *et al.,* 2006; Díez *et al.*, 2004). The ARISA data presented here confirm this observation. The spatial scales at which gene expression patterns change, however, have not been analyzed. As could be expected, expression patterns changed more rapidly than community composition both at spatial and the vertical profile. Moreover, changes were much more marked with depth than with horizontal distance from the coast in parallel to changes in composition.

Trends in richness

The number of peaks was considered as a rough estimate of richness of taxa or of expressed genes. In general, DNA peaks richness decreased from the coast to offshore transect (Fig. 1 SM) in accordance with previous observations from the same stations based on 16S tags sequencing (Pommier *et al.,* 2010). However, transcripts richness was higher at station M and decreased both towards the coast and the open sea. These patterns were similar both with HighP and AllP (Fig. 1SM). In the vertical profile, richness of DNA peaks decreased with depth at station D. This was opposite to the trend found for bacteria (Pommier *et al.,* 2010). The latter study analyzed bacterial composition through pyrosequencing and, thus, it included many rare taxa. The fingerprinting method used here, on the other hand, only detects the most abundant taxa. It is known that most species are rare in any environment (Pedrós-Alió, 2012; Sogin *et al.,* 2006) and this may explain the discrepancy. In the case of RNA peaks, there was a maximal richness at intermediate depths (D40 and D65) and lower values were found at the surface and bottom waters.

Diel cycles

The two more distant stations of the transect were selected to analyze the effect of day/night cycles on community composition and gene expression. Specifically, the two diel studies were done at stations C and D at two depths in each station. Station C was analyzed for 56 hours at both 4 and 15 m depth while station D was followed for 44 hours at 5 and 65 m depths (Table 1B). During this time, the ship was allowed to drift with the surface currents. In order to check how close this was to a Lagrangian drift, we plotted salinity and temperature for the different samples (Figure 5). From the close clustering of the data points, it can be concluded that the cycles at 4 m in station C and at 65 m at station D did follow the same water mass. That was not the case for the 15 m data points in station C that corresponded to different water masses. Finally, in the cycle at 5 m in station D, temperature was constant but salinity decreased due to rain. This freshening of the water could have affected gene expression significantly, although probably not at the community structure composition level.

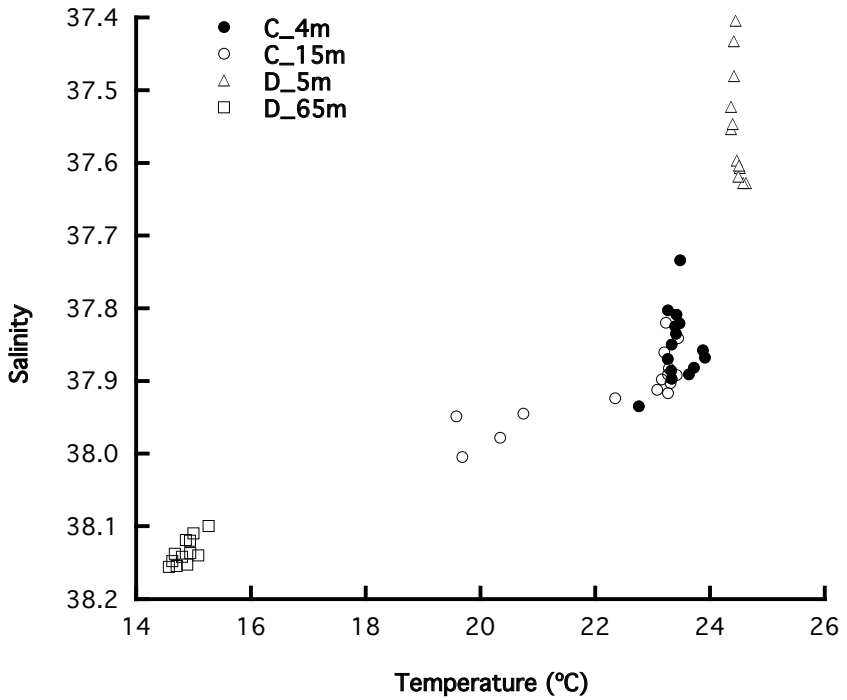

Figure 5. Temperature and salinity values and their changes during the diel cycles in station C and D.

Acinas *et al.* (1997) analyzed the bacterial community structure along the same transect with a 48 hour interval and did not find significant differences. We assumed that this pattern might also hold for picoeukaryotes. Thus, we determined only gene expression patterns for the four diel cycles and distances were plotted in an NMDS diagram separately in Figure 6. The night (solid symbols) and day (empty symbols) samples tended to be in opposite sides of the diagrams. For example, night samples appeared to the right and day samples to the left (Figure 6A).

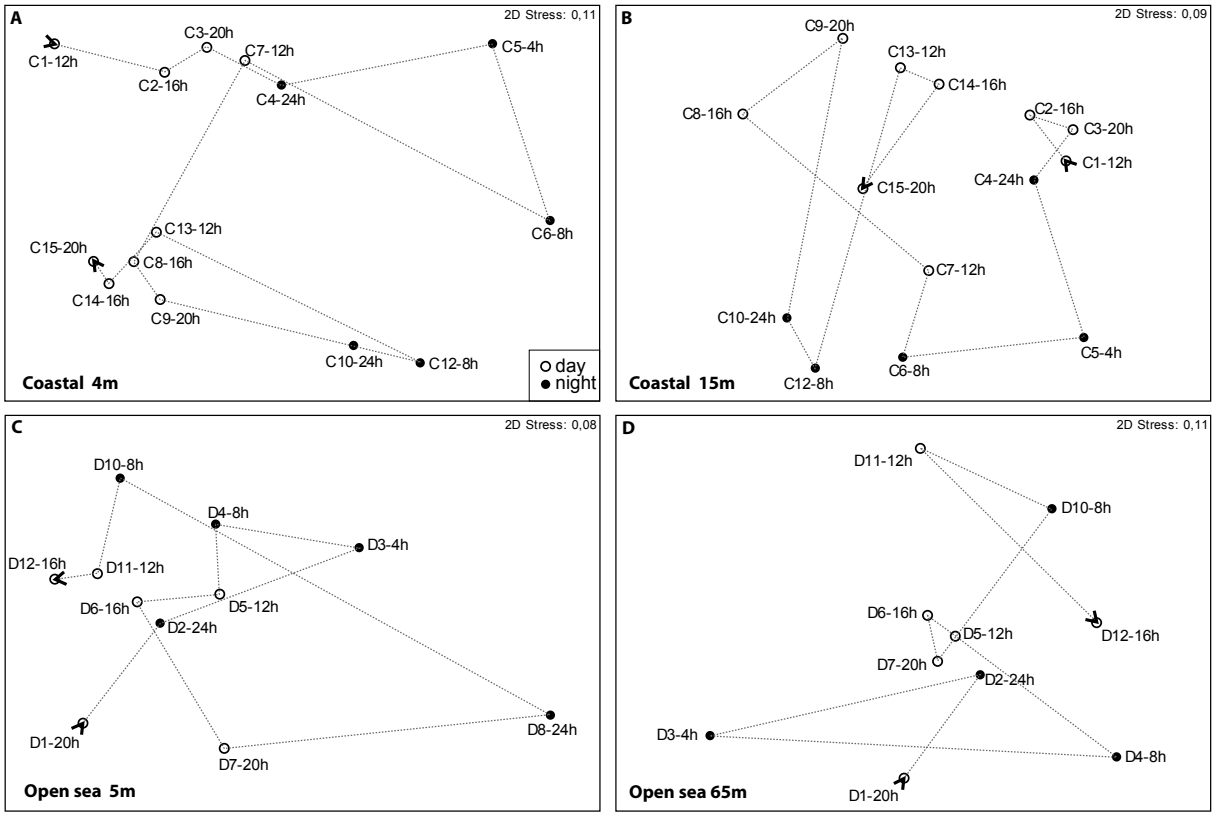

Figure 6. NMDS representations of the relative distances between samples collected along day-night cycles. The four cycles comprise 56 hour periods at the coastal station and 44 hour periods at the open sea station with samples taken every 4 hours. A) Station C surface (4m) samples. B) Station C 15m samples. C) Station D surface (5m) samples. D) Station D deep chlorophyll maximum (65 m) samples. Filled symbols indicate samples taken at night, empty symbols samples taken during light hours.

In order to test the periodicity of these distances, a Mantel correlogram was calculated (Figure 7). A diel pattern was only apparent in the case of C_4m (Figure 7A). However, even in this case, only one of the points was significantly different from zero.

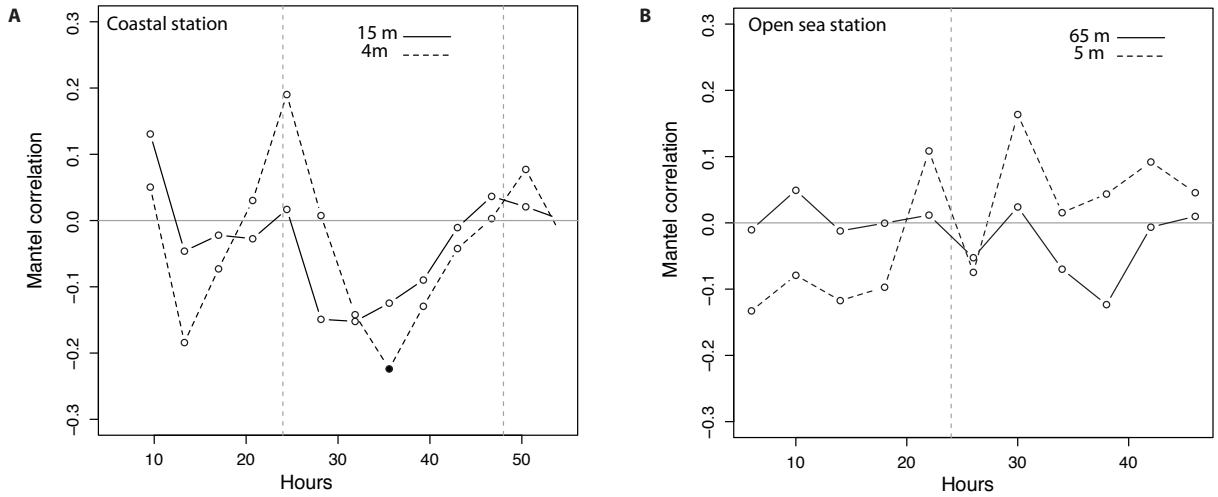

Figure 7. Mantel correlogram of the diel cycles. A) Diel cycle at the coastal site, with two depths 4 and 15m. B) Diel cycle at the open sea station at 5 and 65m.

Obviously, even though some genes did show diel patterns in expression (Figure 8), the diel signal of the whole assemblage was not strong enough to be detected statistically above the background of other changes.

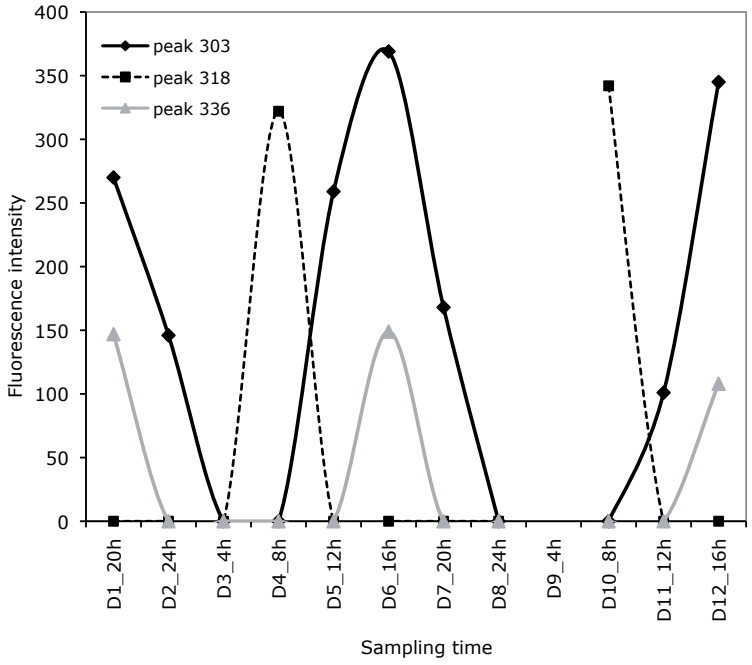

Figure 8. Examples of trancripts that follow circadian rhytms.

A previous study along a very similar transect in which bacterial abundance and heterotrophic production were analyzed at three stations, diel changes in production (but not in abundance) were found at station D, but not at station C (Gasol *et al.,* 1998). Therefore, initially, we expected some changes at least at the offshore station. The first possible explanation for the lack of a diel cycle is that the sampling was not strictly Lagrangian and different water masses could confound the cycles. As already mentioned, this was certainly the case in cycle C_15m, where an initial deep water intrusion was followed by a North wind burst completely altering the conditions of the first 16 hours of the cycle (Galí *et al.,* in preparation). In cycle D_5m, rainfall altered surface salinity at two different time points (Galí *et al.,* in preparation). In this case the lack of periodicity may be attributed to a non-cyclical perturbation in gene expression after the rainfall confounding the regular diel cycle. The two other cycles, however, did not show any signs of lack of Lagrangianity in the T_S diagrams (Figure 5). In cycle C_4m, as explained, there was diel periodicity, although barely strong enough to show significant differences in the Mantel correlogram (Figure 7A) while cycle D_65m did not show any evidence of periodicity (Figure 7B). Periodicity in the expression of the genes of phototrophic microorganisms in the Pacific was detected by metatranscriptomic analyses but this pattern was not found for heterotrophs (Ottesen *et al.,* 2013). The ratio of autotrophs to heterotrophs in Blanes Bay was low in September (Massana, 2011) and this might also have contributed to masking the periodicity at the two coastal studies.

In conclusion, we did not find clear diel cycles in gene expression patterns in the present study. However, in a previous study we found marked seasonal cycles with the same approach and techniques (Coll-Lladó *et al.,* 2013 submitted), so we believe that the lack of periodicity here is not due to the lack of sensitivity of the TFA. However, we cannot discard that such periodicity is contributed by a high diversity of transcripts present at low abundance overlooked by our method and in this case metatranscriptomic analyses would be desirable. More diel studies at different times of the year will have to be carried out to clarify this issue.

ACKNOWLEDGMENTS

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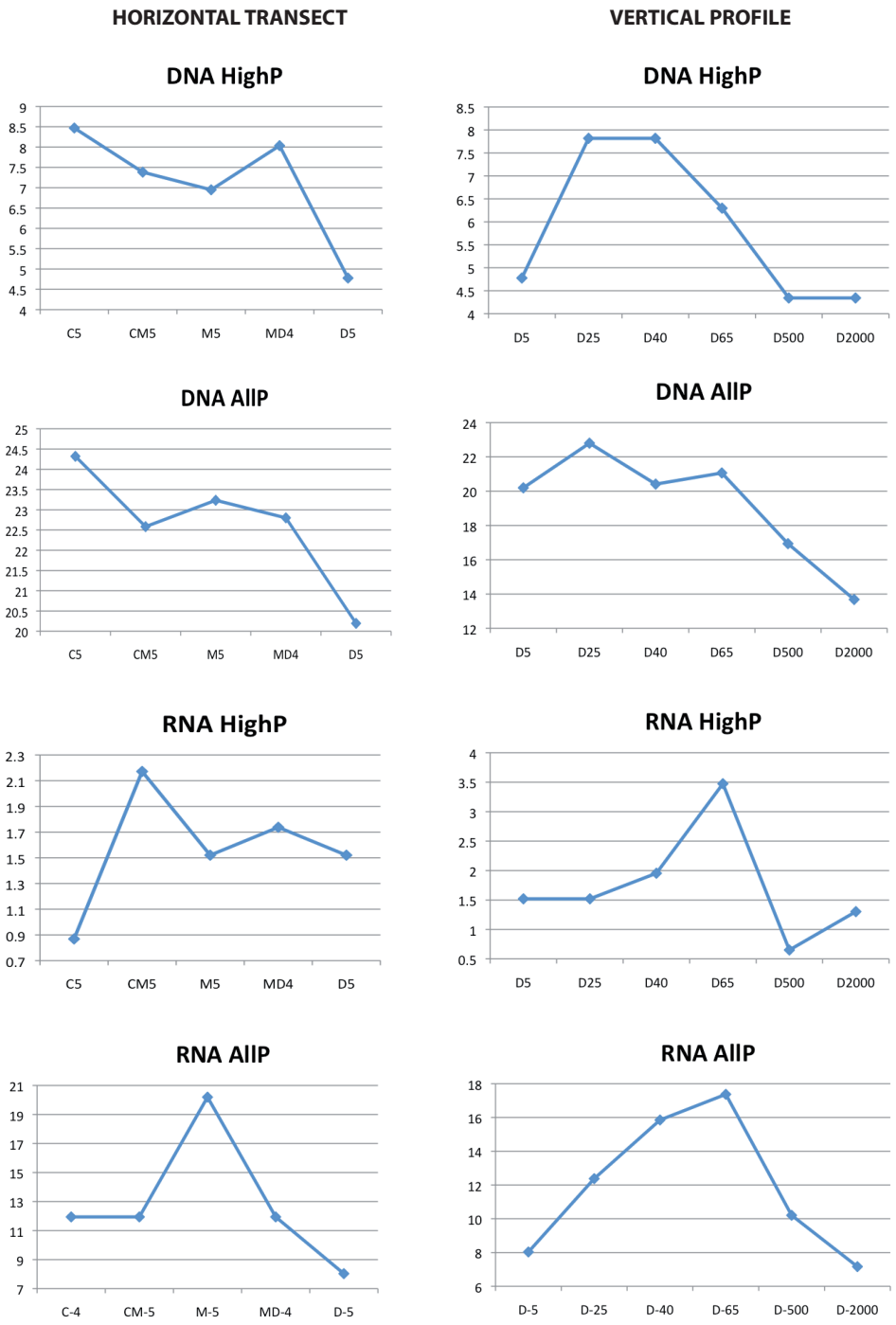
SUPPLEMENTARY INFORMATION



Supplementary Figure I. Peaks richness along the horizontal transect and the vertical profile at station D.

Chapter 4

Transcriptional Response of *Ruegeria* sp. strain TM1040 to Light

ABSTRACT

Microorganisms have evolved a variety of different receptors to sense light, and the availability of the complete genome of many strains now allows access to the physiological and ecological strategies linked to light reception. The main objective of this study was to investigate how marine Roseobacter strain *Ruegeria* sp. TM1040, an organism whose genome contains genes for several putative light receptor proteins, responds to experimental light manipulations. A microarray based on the annotated genome of this strain was used to study the transcriptional response of cultures incubated in the light under two different nutritional conditions. A reverse transcription qPCR with selected genes confirmed and refined the results observed in the microarray study: a stress response was observed upon blue light illumination. A gene encoding a protein with high similarity to fasciclin, a molecule involved in attachment, was also upregulated by blue light and by UV radiation. Overall, the results indicate that light triggers a series of genes involved in protecting the cell from photooxidative damage and in a possible coupling with a dinoflagellate with which TM1040 is usually associated.

Transcriptional response of Ruegeria *sp. strain TM1040 to light.* **Montserrat Coll-Lladó, Ryan J. Newton, Wenying Ye, Shulei Sun, Carles Pedrós-Alió, Mary Ann Moran**. Manuscript.

INTRODUCTION

Members of the Roseobacter group (in the Alphaproteobacteria) account for 10% of bacterial cells in surface waters of the ocean, reaching 20% of the bacterial assemblage in coastal waters (González & Moran, 1997). They can represent a large percentage of the bacteria associated with planktonic algae, mainly dinoflagellates (Buchan et al., 2005).

Light sensing seems a useful property of microorganisms inhabiting surface oceans. In many microorganisms, light triggers photobiological responses such as circadian rhythms and synchronization, phototrophy, bacterial phototaxis, and many others. Indeed, 13 of 32 sequenced Roseobacter genomes have genes for photoheterotrophy (10 aerobic anoxygenic phototrophs -AAnPs-, 3 rhodopsin phototrophs), while another genome contains RuBisCO (Newton et al., 2010). The remaining 18 (including *Ruegeria* sp. TM1040) are strict chemotrophs using organic matter, although some may obtain energy from inorganic compounds such as CO and H_2S.

Ruegeria sp. strain TM1040, formerly *Silicibacter* (Yi et al., 2007), is a member of the Roseobacter clade originally isolated from a culture of the marine dinoflagellate *Pfiesteria piscicida* (Alavi et al., 2001; Miller & Belas, 2006; Miller et al., 2004). Strain TM1040 is found within the phycosphere or physically attached to the surface of the dinoflagellate cell. *P. piscicida* is a heterotrophic dinoflagellate that feeds on algae and other eukaryotic cells. It can also retain chloroplasts from the prey algae and use them to increase growth efficiency in the light, and can act cannibalistically under starvation conditions (Feinstein et al., 2002). The dinoflagellate grows poorly in the absence of the symbiotic *Ruegeria* sp. TM1040, although the exact workings of this association are not well known. TM1040 forms tight physical and physiological associations with its dinoflagellate host cell, in part through behavioral responses to dinoflagellate products (Miller & Belas, 2004).

The genome sequence of *Ruegeria* sp. TM1040 suggested that it can sense light, since it contained several types of photoreceptor genes in its genome (Moran et al., 2007). One of these receptors was BLUF (for "sensor of Blue-Light Using FAD"), which senses blue light based on a conformational change following photon absorption (Gomelsky & Klug, 2002; Jung et al., 2005; Zeller et al., 2005). *Ruegeria* TM1040 also contains a phytochrome gene that is likely involved in detection of far-red light. The objective of the present study was to investigate how *Ruegeria* sp. TM1040 responds to light using an oligonucleotide-based microarray designed from the annotated genome of this strain (Moran et al., 2007). The array was used to study the transcriptional response of *Ruegeria* cultures incubated in the light under two different nutritional conditions.

MATERIALS AND METHODS

A. Microarray study

Culture conditions

Ruegeria sp. TM1040 was grown in 250 ml Erlenmeyer flasks containing 100 ml of marine basal medium (Baumann & Baumann, 1981) supplemented with a vitamin solution. Cultures were inoculated with 1 ml of bacterial culture (OD_{600} = 0.7) previously grown in liquid YTSS medium (4 g yeast extract, 2.5 g tryptone, 20 g sea salts per l). Since it has been shown that members of the Roseobacter clade can use glucose at low substrate concentrations in coastal pelagic environments (Alonso & Pernthaler, 2006), glucose was used as the carbon source.

Two different nutritional conditions were tested. In the lower organic matter (lowOM) conditions, cultures were amended with 5 mM glucose, and in the higher OM (highOM) conditions, they were amended with 10 mM glucose. In batch cultures, this should not make any difference at the beginning of the experiments, since substrate concentrations are above saturation in both cases. However, our intention was to sample both cultures at the time when the lowOM cultures entered the stationary phase while the high OM culture would still be growing exponentially. At this point, both cultures would have approximately the same biomass but different physiological conditions. Six parallel cultures were run for each condition.

Bacteria were incubated at 30 ºC in Erlenmeyer flasks covered with aluminum foil to eliminate light, shaken horizontally at 200 rpm (Innova Shaker, New Brunswick Scientific Inc.). After 18.5 h of incubation, three cultures from each physiological condition were exposed to light (Figure 1). Cells from both light and dark treatments were sampled after another 50 min. The time of response was chosen based on previous studies with related Roseobacter strains that assessed transcriptional activities (Braatsch et al., 2004; Bürgmann et al., 2007).

10,000 K fluorescent lamps (Coralife) that emit blue-white light with high-intensity lumen output were used to simulate marine conditions under midday sun. Intensity was measured at different levels of the incubator in order to place the cultures in the position that approximated annual average light intensity at 32˚ North latitude. Layers of Poly(methyl methacrylate) were used in order to block UV radiation.

Dissolved oxygen was measured with a portable YSI 50B dissolved oxygen meter (YSI incorporated) using the YSI 5739 sensor. A 0% dissolved oxygen value was set by sparging with nitrogen gas, and a 100% dissolved oxygen value was set by sparging in the liquid-air interface.

Array design

The array design was based on the complete genome sequence of *Ruegeria* sp. strain TM1040 [NCBI reference NC_008044.1 (chromosome), NC_008043.1 (megaplasmid), and NC_008042.1 (small plasmid)]. Probes were designed for all predicted genes by Combimatrix Inc. using proprietary software. The final microarray contained 12,000 spots representing two independent probes for each TM1040 gene except for 109 genes for which only one probe or no probe could be designed. For three genes, both probes were replicated 5 times on the microarray for quality checking, and probes for 16S and 23S rRNA genes were included as reference spots.

Total RNA extraction, amplification and array hybridization

Bacteria were harvested by pipetting 9 ml of liquid culture into sterile 15 ml tubes containing 1 ml of stop solution (95% absolute ethanol plus 5% saturated phenol pH 6.6 ± 0.2). Samples were mixed and centrifuged (5000 *g*, 10 min, 4 °C). Supernatant was discarded and cell pellets were stored at –80 °C until processing. Samples were thawed on ice and cell pellets were resuspended with 1.4 ml of TRI reagent (Ambion, Austin, TX) and transferred to Eppendorf tubes. Samples were bead-beaten with 0.5 ml of 0.1 mm zirconia-silica beads for 10 min at top speed on a vortex adapter (Vortex-Genie 2, MoBio Laboratories, Inc., Carlsbad, CA) in order to mechanically disrupt the cells. After disruption, the lysate was clarified by centrifugation and the aqueous phase was transferred to a new tube. Total RNA extraction was carried out using phase separation and precipitated with ethanol as explained in (Bürgmann et al., 2007).

Digestion of DNA was carried out with the DNA-*free* kit (Ambion). RNA was purified using the MEGAclear kit (Ambion) and ribosomal RNA was removed enzymatically using the mRNA-ONLY kit (Epicentre, Madison, WI). After each step RNA quantity and quality were checked with a NanoDrop spectrophotometer (NanoDrop technologies, Wilmington, DE) and with an Experion chip (Bio-Rad Laboratories, Inc., Hercules, CA).

The mRNA was amplified using the MessageAmp Bacteria kit (Ambion), which uses polyadenylation followed by T7 *in vitro* transcription to amplify RNA. The protocol was modified to accommodate indirect labeling by substituting one quarter of the UTP in the T7 reaction with amino–allyl labeled UTP (Ambion).

A competitive hybridization scheme was used where light and dark treatments were labeled with different dyes and hybridized to the same array (for example one array would include samples from both light and dark conditions from the lowOM cultures). Each light replicate was randomly paired with one of the three dark replicates. The dyes used for light and dark samples were alternated with each sample to minimize potential dye bias in the dataset. AlexaFluor dyes 555 and

647 (Invitrogen, Carlsbad, CA) were used to directly label amino allyl-antisense RNA (aa-aRNA). Labelling of the aa-aRNA samples was done as described by Bürgmann et al. (2007). Labeled aa-aRNA was purified using the MEGAclear kit and concentrated by precipitation with ethanol. Resuspension was done in 10 µl of RNAse free water. A 1 µl subsample was used to determine the concentration and dye incorporation using the NanoDrop.

Prior to hybridization, the labeled and purified aa-aRNA was fragmented using fragmentation reagents (Ambion) according to the manufacturer's instructions. A 10 µl aliquot of each labeled and fractionated aa-aRNA sample was hybridized to the *Ruegeria* CombiMatrix Custom Array for 16 h at 45°C following instructions provided by the manufacturer. Chips were stripped for re-hybridization after analysis according to CombiMatrix.

Image acquisition and microarray analysis

The arrays were scanned using an Axon GenePix 4000B microarray scanner (Molecular Devices Corporation, Sunnyvale, CA), and the images were analyzed using the GenePix Pro 6.0 software. Background correction was achieved by subtracting the mean of pixel intensities of the five closest empty spots on the array. Bad spots were located by inspecting the images visually as well as using the criteria specified by Bürgmann et al. (2007). Data processing and analysis were carried out with Acuity 4.0 software. The detection limit per array and changes in transcription criteria were defined as in Bürgmann et al. (2007). The results were interpreted based on the fluorescence ratio calculated for each probe by dividing the mean fluorescence of replicated probes from one condition by the mean fluorescence of replicated probes from the other. Lists of genes fulfilling fold change criteria based on Bürgmann et al. (2007) were created, indicating a two-fold change relative to the control sample. Data for all microarrays was exported to Excel and Significance Analysis of Microarrays (SAM) was done with the program SAM version 2.11 (Tusher et al., 2001).

B. Reverse transcription quantitative PCR (RT qPCR) study

Light Setup

A solar simulator was used to reproduce average wavelength intensities in the surface ocean approximately at 32° N (the latitude of Georgia). The light conditions are shown in Supplementary Figure 1 together with average radiation conditions in July and January for comparison. To exclude specific wavelengths, glass filters were fit to an opening in the bottom of the solar simulator.

Growth conditions

Strain TM1040 was streaked from a frozen stock on 0.5x YTSS medium. A single colony from this streake plate was inoculated into Marine Basal Medium supplemented with 5 mM glucose (the lowOM condition in the microarray experiments). Three colonies were inoculated into separate flasks containing liquid medium and incubated with shaking until each reached an OD of 0.2.

To initiate the light experiment, these three flasks were emptied into a shallow sterile dish containing a stir bar. The cells in each dish were stirred throughout the experiment. Oxygen concentration was monitored, and found to remain relatively high (30-70% saturation, with the lower values at the beginning of the experiment). Separate experiments were run for each of the different time points collected (5 min, 15 min, 50 min). A fan was placed in the solar simulator to reduce the temperature to acceptable levels (never higher than 30 ºC, during the red light experiment; typically 24-28 ºC during the other light experiments).

After the appropriate light exposure, stop solution was added and the samples were stored at -80 ºC until RNA extraction. The RNA was extracted and the DNA was digested enzymatically. Samples were then used in RT qPCR using the Bio-Rad one-step reaction. Genes selected for reverse transcription qPCR, and the primers used, are listed in Table 2.

RESULTS AND DISCUSSION

Microarray experiment

After 50 min of exposure to light, highOM samples were in mid-exponential phase while lowOM samples were at the beginning of stationary phase (Figure 1). In accordance with the cell abundance data, the gene encoding the chromosomal replication initiator protein (dnaA; TM1040_0001) was downregulated in lowOM samples (Supplementary Table 2).

Differences in substrate concentration resulted in some differences in the growth of *Ruegeria*. Cultures growing in the 5 mM glucose treatment grew faster, reached a maximal OD_{600} of 0.6, and stabilized in stationary phase at an OD of 0.4-0.5. Cultures growing at 10 mM glucose reached a maximal OD_{600} of ~1 and stabilized at an OD_{600} of 0.7-0.8 (Figure 1). The slower initial growth rate of the highOM samples at the beginning of the time course was surprising, and suggests the 10 mM glucose was slightly inhibitory to TM1040. In effect, oxygen consumption was faster at the lowOM than at the highOM at 15 hours (Supplementary Figure 2).

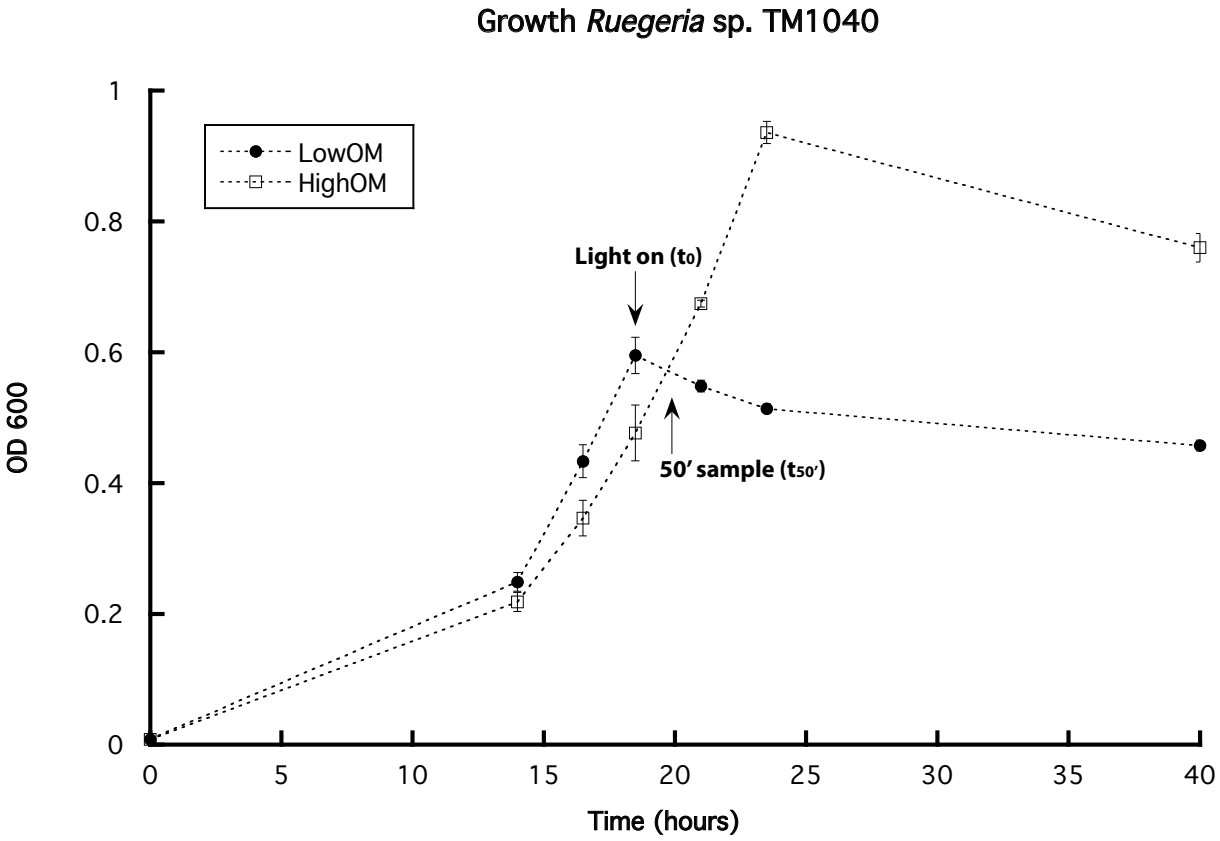


Figure 1. Growth of *Ruegeria* TM1040 in batch cultures with two different glucose concentrations. The times of exposure to light and of sample collections are shown by arrows. Error bars show standard error of triplicates.

After this time the highOM cultures increased their growth rate and reached, as expected, much higher yields. When the light was turned on, the lowOM cultures were entering stationary phase, while the highOM cultures were continuing to grow at the same rate, showing that the light conditions used were not impairing growth.

Dissolved oxygen decreased as bacteria grew (see Supplementary Figure 2). When lights were initially turned on, the oxygen level for the lowOM samples was 18% of saturation while that for the highOM samples was 4.9% (microarray time point T_0). At the next time point (microarray time point $T_{50'}$), oxygen saturation was 34% for the lowOM and 3% for the highOM. Thus, oxygen concentrations were well below saturation and particularly low in the highOM treatments when samples for microarray analysis were collected. This may have been a second factor affecting the expression of genes during light manipulations.

Ruegeria sp. TM1040 has 3,863 potential coding sequences in its genome. A total of 109 ORFs were differentially regulated during light exposure. Of these, 79 were upregulated and 30 were downregulated (see lists in Supplementary Tables 1 and 2, respectively), considering both lowOM and highOM conditions. The number of genes with significant expression changes in response to light was lower in the highOM condition (only 14 genes) than in the lowOM condition (97 genes), although many of the same genes differentially regulated in the lowOM cultures just missed the significance threshold in the highOM cultures, suggesting that the responses were more similar than gene numbers suggest.

For example, the higher growth in highOM cultures at the T_{50} collection point could have resulted in higher transcript levels for transcription, translation, and other growth-related processes that masked light-driven transcript changes. Some genes, however, clearly showed different responses in the lowOM and highOM conditions. For example, *ureE* (urease accessory-like gene) was substantially upregulated under light only in highOM samples, while carbon-monoxide dehydrogenase was downregulated under the light only in lowOM samples.

Neither the BLUF nor phytochrome genes were differentially expressed after exposure to light. However, two ORFs in the neighborhood of the two BLUF domain proteins were upregulated in the lowOM cultures (Figure 2). These encoded a hypothetical protein (TM1040_2023) and an FMN-dependent alpha-hydroxy acid dehydrogenase (TM1040_2026). Another gene that might be involved in light response was TM1040_0414, a FAD dependent oxidoreductase that was upregulated under the light at lowOM, although this protein could be involved in other metabolic functions as well.

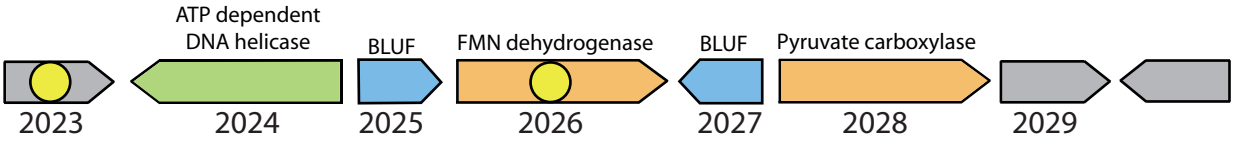


Figure 2. Gene context for the two BLUF domains in *Ruegeria* TM1040. Gray arrows indicate hypothetical proteins. The two genes upregulated under light are indicated by yellow circles.

Several genes involved in maintaining integrity of the cell envelope during stress (Ades, 2004) were upregulated in the lowOM condition, including regulatory factors, proteases and chaperones (Table 1). Genes potentially involved in a response to photo-oxidation were also upregulated: TM1040_0976 (superoxide dismutase), TM1040_2803 (protein-methionine-S-oxide reductase), and TM1040_1013 (osmC-like protein, involved in stress response). Additionally, three redox proteins were upregulated under the light: TM1040_3733 (redoxin), TM1040_2847 (thioredoxin) and TM1040_2583 (putative glutathione S-transferase). Protein transporters that were upregulated included: TM1040_2276 (twin-arginine translocation pathway signal), TM1040_0133 (twin-arginine translocation pathway signal) and TM1040_2270 (ABC transporter related).

Table 1. Genes involved in maintaining the outer envelope and other functions under stress that were upregulated in the light.

Regulatory factors:
TM1040_0416 Anti-ECFsigma factor
TM1040_0415 RNA polymerase sigma-24 subunit ECF subfamily
Proteases:
TM1040_1953 Peptidase S1C (2 PDZ domains)
TM1040_0094/0095 Peptidase M16-like
TM1040_0569 Peptidase M3B oligoendopeptidase-related clade 3
TM1040_0706 Peptidase M1 alanyl aminopeptidase
Chaperones:
TM1040_0596 Chaperonin Cpn10
TM1040_0597 Chaperonin GroEl
TM1040_0010 Chaperone DnaK
TM1040_0412 Cyclopropane-fatty-acyl-phospholipid synthase

Unexpectedly, one of the most upregulated genes was a protein with significant homology to fasciclin (TM1040_2326, annotated as beta-Ig-H3/fasciclin). Moreover, this was the only gene upregulated under both highOM and lowOM conditions. There are two copies of this protein in *Ruegeria* sp. TM1040, and the copy in the plasmid remained unchanged while the copy in the chromosome was urpregulated.

Finally, *Ruegeria* sp. TM1040 has one operon encoding carbon monoxide dehydrogenase for the oxidation of CO to CO_2. This operon was downregulated in the lowOM cultures (TM1040_1765, carbon-monoxide dehydrogenase; TM1040_1764, molybdopterin dehydrogenase).

Reverse transcription quantitative PCR

In the previous experiment, there turned out to be two factors that varied at the same time in the design of the microarray experiment. One treatment ('lowOM') had higher oxygen levels and was in early stationary phase. The other ('highOM') had almost no oxygen and was in exponential phase. Therefore we could not tell if the differences between the effect of light on gene expression in these treatments was related to carbon sufficiency or to oxygen, the latter mediated through reactive oxygen species (ROS), especially since several of the upregulated genes in the lowOM looked like ROS protection genes. Therefore, we addressed the ROS stress concern.

Under plenty of oxygen, and while eliminating any starvation factors, we looked at wavelength-specific effects on gene expression. The question was whether UV radiation, which is responsible for ROS generation, or blue light, which is implicated in BLUF expression, or red light, which is implicated in neither, had different effects on gene expression. We focused on genes that we thought were most likely to be controlled by ROS/UV and genes that we thought might be controlled by BLUF (such as fasciclin and the gene right next to the BLUF domain protein) to see if we could distinguish between these two explanations. The key issue was to first see if we were actually observing a direct effect of light on gene regulation or if instead we were seeing an indirect effect of light on gene expression that was mediated through ROS formation.

We wanted to make sure that the different oxygen levels in the highOM vs lowOM cultures was not what was actually affecting gene regulation, in which case carbon levels had nothing to do with it. So we took away the starvation factor, and addressed the oxygen/ROS issue. If the gene expression effects were due to ROS, then we would expect to see the microarray gene expression pattern in the UV treatment, independent of starvation status. If the gene expression effects were not due to ROS, then the UV treatment would not affect regulation of any of the stress-like genes upregulated in the microarray experiment and the blue light would catch some of the genes, like fasciclin, that were upregulated regardless of carbon status.

Several genes were targeted for RT qPCR in this second series of light-based experiments with TM1040 designed to follow up on the microarray results (Table 2). In these studies, filters were used to isolate the effects of different wavelength ranges of the solar spectrum (see Supplementary Figure 1). Three genes involved in the oxidative stress response were chosen for an RT qPCR study under the same culture conditions: superoxide dismutase, osmC, and methionine sulfoxide reductase. Superoxide dismutase is an enzyme that catalyses the conversion of superoxide (a free radical) into oxygen and hydrogen peroxide.

Table 2. Genes selected for reverse transcription qPCR, their putative functions, and their fold-change when upregulated under the light in the low organic matter treatments.

Gene number	Gene description	Function	Primers (5'-3')	Increase (x fold)
TM1040_0976_141_175	Superoxide dismutase	Oxidative damage repair	TM_SOD419F" GCCTCAAAGTGACCAAGACC "TM_SOD567R" CAGGTTGTCGAGGAAGTTCG	5.5
TM1040_2803_341_375	Methionine sulfoxide reductase	Oxidative damage repair	"TM_MetSulf212F" GTGCAGGAACTCGAAGATCG "TM_MetSulf390R" CGAGGTGAATAAAGCGCAAGG	3.8
TM1040_1013_509_543	OsmC	Oxidative damage repair	"TM_OsmC408F" GGTCATGTGGCGCATCATTTCG "TM_OsmC554R" GAGATCGCCTCTCCAAGTTCC	3.5
TM1040_0415_41_75	RNA polymerase sigma 24	Regulatory factor	"TM_24Sig33F" CGACAGAGTGACAGGTGAGG "TM_24Sig210R" CACGTCCTGAACGATGTCC	5.9
TM1040_2326_359_393	Beta-Ig-H3/fasciclin	Adhesion	"TM_Fasc132F" TGTGTCTGCTGCTGATCTGG "TM_Fasc298R" CACAACGTGGTAGGTGAGG	9.0
TM1040_2026_691_725	FMN-dependent alpha-hydroxy acid dehydrogenase	Next to BLUF domains	"TM_FMN74F" CCCGATTTGTCTGGGAATACC "TM_FMN204R" GGGTACCAAACAAGGTGGTG	6.4

Osmotically inducible protein C (osmC) is a stress-induced protein. The transcription of the osmC gene in *E. coli* is induced during the late exponential phase when the growth rate slows before entry into stationary phase (Gordia & Gutierrez, 1996). In *Mycobacteria*, osmC protects against organic hydroperoxide stress (Dubbs & Mongkolsuk, 2007). Finally, methionine sulfoxide reductase reduces oxidized methionine to methionine. Oxidized methionine molecules are formed following oxidative damage to peptides and the two latter proteins are involved in restoring the proper sulfur bonds.

The genes assayed by RT qPCR exhibited three different types of responses. The three genes involved in oxidative stress response were clearly upregulated by blue light and only by blue light (Figure 3, A-C). Fasciclin and the sigma 24 factor were stimulated by both blue and UV radiation, but this response was transitory, being intense after 15 min but non-significant by 50 min (Figure 3 D-E). Finally, FMN-dependent alpha-hydroxy acid dehydrogenase did not show a very clear pattern (Figure 3 F), with blue light upregulating expression after 5 min of exposure but not later, while UV radiation upregulated at 15 min but not later.

We showed that the regulation of this response was due to blue light and not to red light or UV radiation, as the three genes tested showed increased upregulation under blue light only. Therefore, we hypothesize that the BLUF protein is responsible for the observed response, even though the BLUF genes are not upregulated themselves. This makes sense for genes acting as sensors, since they need to be turned on all the time at the same level so they can detect photons. Intriguingly, there were two ORFs in the vicinity of the two BLUF domains that were significantly upregulated in connection with the oxidative stress response. One of these codes for a hypothetical protein and the other for a FMN-dependent alpha-hydroxy acid dehydrogenase. The latter enzyme might be involved in the synthesis of the BLUF cofactor itself. But the present experiments cannot clarify the issue. The RT qPCR study of this gene did not show a very clear pattern, since the gene was upregulated after 5 min of exposure to blue light, after which expression levels decreased to those in the dark. On the other hand, there was a slight increase under UV radiation, although the variability among replicates precludes a firm conclusion.

The other clear response to light exposure was the upregulation of fasciclin, which took place under both culture conditions. Fasciclin is involved in attachment to other cells of the same species or to symbionts. *Ruegeria* TM1040 is known to form rosettes of cells when grown without shaking (Bruhn et al., 2007) and its mode of life requires attachment to *Pfiesteria piscicida*. In addition TM1040 would want to regulate its adhesion proteins when phytoplankton were present. Phytoplankton is found primarily in the photic zone, so when TM1040 detected light it might be beneficial to ramp up its adhesion proteins in case that it was near phytoplankton. Obviously, this response would be advantageous under both nutritional conditions tested: exponentially growing cells could access additional resources to continue growth, while stationary phase cells would be able to resume growth. However, fasciclin appeared to be the only gene related to adhesion (or other phytoplankton association related genes) that was affected in these light experiments. Also, why this upregulation was stimulated by both UV radiation and blue light remains intriguing. Maybe the filter cutoffs were such that small amounts of blue light came through, or maybe the BLUF proteins detect a range of wavelengths near blue light, even if most efficient with blue.

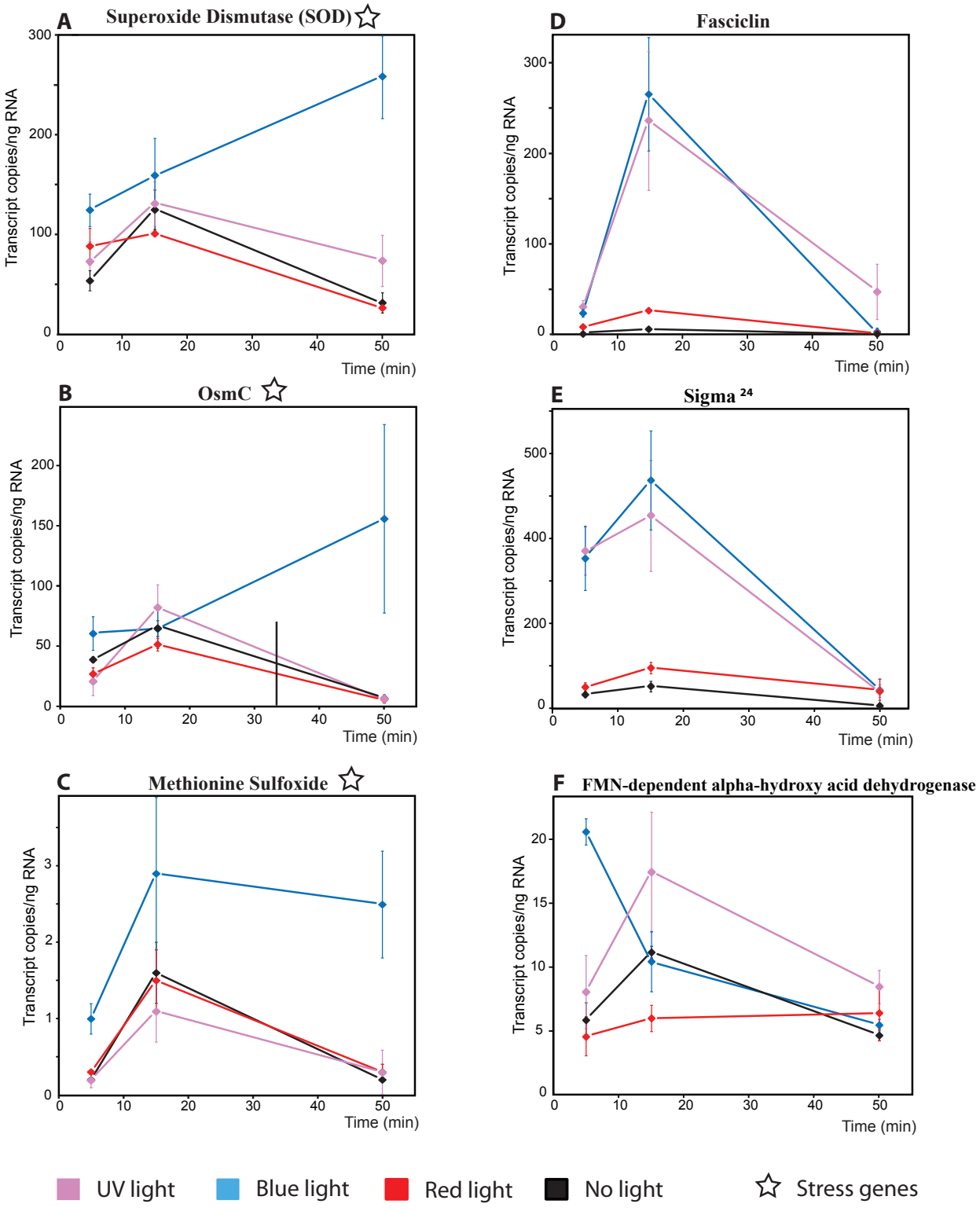


Figure 3. Expression of selected genes after light exposure for different time points (5, 10 and 15 min), determined by reverse transcription qPCR. Cultures were incubated under specific regions of the solar spectrum: UV radiation, blue light, red light, and no light. A) Superoxide dismutase; B) OsmC; C) Methionine sulfoxide reductase; D) Fasciclin; E) Sigma 24; F) FMN-dependent alpha-hydroxy acid dehydrogenase. Error bars show standard deviations of three biological replicates with three technical replicates for each biological replicate.

Another gene that was clearly upregulated under both UV radiation and blue light was RNA polymerase sigma 24. This gene was chosen for a more detailed study because it is known as the "environmental" sigma factor for its frequent role in regulation of environment sensing mechanisms in the cell. Light can be one of the "environmental signals" in which sigma 24 is frequently involved. Further, it was one of the two genes with increased expression in both the low and high organic matter conditions. Since the upregulation pattern was the same as that for fasciclin, the sigma factor is likely related to a response involving attachment.

Overall, the results indicate that the regulation of gene expression in this heterotroph when light is present triggers a series of genes involved in protecting the cell from photooxidative damage and in a possible coupling with a dinoflagellate to access resources produced by the dinoflagellate when light is present.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY INFORMATION

Supplementary Table 1. Genes that were significantly upregulated in the light, under low or high organic matter conditions.

Gene ID	Gene description	Low	High
TM1040_0010_1634_1668	Chaperone DnaK	x	
TM1040_0051_137_171	two component transcriptional regulator	x	
TM1040_0051_137_171_m1	two component transcriptional regulator	x	
TM1040_0051_61_95	two component transcriptional regulator	x	
TM1040_0051_61_95_m1	two component transcriptional regulator	x	
TM1040_0094_603_637	peptidase M16-like	x	
TM1040_0095_975_1009	peptidase M16-like	x	
TM1040_0133_857_891	Twin-arginine translocation pathway signal	x	
TM1040_0136_17_51	response regulator receiver protein	x	
TM1040_0289_1188_1222	multicopper oxidase	x	
TM1040_0310_111_147	hypothetical protein	x	
TM1040_0310_369_403	hypothetical protein	x	
TM1040_0382_109_143	UreE urease accessory-like		x
TM1040_0383_193_227	hypothetical protein	x	
TM1040_0383_81_115	hypothetical protein	x	
TM1040_0412_674_708	Cyclopropane-fatty-acyl-phospholipid synthase	x	
TM1040_0414_997_1031	FAD dependent oxidoreductase	x	
TM1040_0415_184_218	RNA polymerase sigma-24 subunit ECF subfamily	x	
TM1040_0415_41_75	RNA polymerase sigma-24 subunit ECF subfamily	x	
TM1040_0416_433_467	anti-ECFsigma factor	x	
TM1040_0437_1216_1250	Phenylacetic acid degradation protein paaN	x	
TM1040_0569_1126_1160	Peptidase M3B oligoendopeptidase-related clade 3	x	
TM1040_0575_3_37	4Fe-4S ferredoxin	x	
TM1040_0596_5_39	chaperonin Cpn10	x	
TM1040_0597_1437_1471	chaperonin GroEL	x	
TM1040_0609_41_75	hypothetical protein	x	

TM1040_0621_116_150	protein of unknown function DUF526	x
TM1040_0621_37_71	protein of unknown function DUF526	x
TM1040_0706_2166_2200	Peptidase M1 alanyl aminopeptidase	x
TM1040_0761_649_683	Biotin--acetyl-CoA-carboxylase ligase	x
TM1040_0765_233_267	HesB/YadR/YfhF	x
TM1040_0862_391_425	1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase	x
TM1040_0952_161_195	hypothetical protein	x
TM1040_0976_141_175	Superoxide dismutase	x
TM1040_1013_509_543	OsmC-like protein	x
TM1040_1032_683_717	hypothetical protein	x
TM1040_1170_657_691	AMP-dependent synthetase and ligase	x
TM1040_1379_289_323	Homoserine O-succinyltransferase	x
TM1040_1395_189_223	hypothetical protein	x
TM1040_1461_1115_1149	two component sigma54 specific transcriptional regulator Fis family	x
TM1040_1523_113_147	Glyoxalase/bleomycin resistance protein/dioxygenase	x
TM1040_1544_2128_2162	AIR synthase related protein-like	x
TM1040_1702_102_136	hypothetical protein	x
TM1040_1703_241_275	GCN5-related N-acetyltransferase	x
TM1040_1757_93_127	hypothetical protein	x
TM1040_1761_780_814	Ferredoxin--NADP(+) reductase	x
TM1040_1912_124_158	protein of unknown function DUF1127	x
TM1040_1953_645_679	Peptidase S1C	x
TM1040_1953_709_743	Peptidase S1C	x
TM1040_1988_3_37	hypothetical protein	x
TM1040_2023_2_36	protein of unknown function DUF1127	x
TM1040_2026_691_725	L-lactate dehydrogenase (cytochrome)	x
TM1040_2050_293_327	hypothetical protein	x

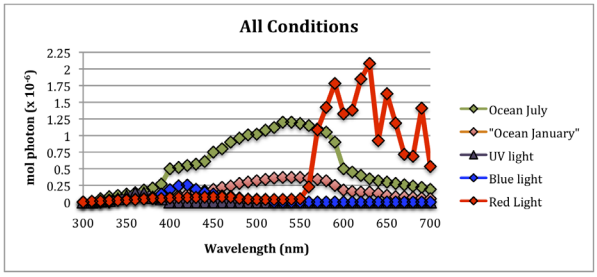
TM1040_2139_57_93	hypothetical protein	x	
TM1040_2177_202_236	RNA polymerase sigma-24 ECF subfamily		x
TM1040_2178_417_451	hypothetical protein		x
TM1040_2178_97_131	hypothetical protein		x
TM1040_2262_315_350	hypothetical protein	x	
TM1040_2270_35_69	ABC transporter related	x	
TM1040_2276_325_359	Twin-arginine translocation pathway signal	x	
TM1040_2276_651_685	Twin-arginine translocation pathway signal	x	
TM1040_2326_359_393	beta-Ig-H3/fasciclin	x	
TM1040_2326_359_393	beta-Ig-H3/fasciclin		x
TM1040_2326_431_465	beta-Ig-H3/fasciclin		x
TM1040_2326_431_465	beta-Ig-H3/fasciclin	x	
TM1040_2439_425_459	HPr kinase	x	
TM1040_2534_29_63	ATP-dependent Clp protease adaptor protein ClpS	x	
TM1040_2583_449_483	putative glutathione S-transferase	x	
TM1040_2720_43_77	protein of unknown function DUF1328	x	
TM1040_2766_225_259	protein of unknown function DUF1285	x	
TM1040_2803_341_375	Protein-methionine-S-oxide reductase	x	
TM1040_2805_2839_m3	23S ribosomal RNA		x
TM1040_2847_233_267	thioredoxin	x	
TM1040_2848_57_91	protein of unknown function DUF1127	x	
TM1040_2848_85_119	protein of unknown function DUF1127	x	
TM1040_2890_57_91	NUDIX hydrolase	x	
TM1040_3065_647_681	secretion protein HlyD	x	
TM1040_3074_193_227	hypothetical protein	x	
TM1040_3458_1008_1042	hypothetical protein	x	
TM1040_3733_110_144	Redoxin	x	
TM1040_3733_357_391	Redoxin	x	
TM1040_3796_455_489	hypothetical protein	x	
TM1040_728_762_m2	16S ribosomal RNA		x

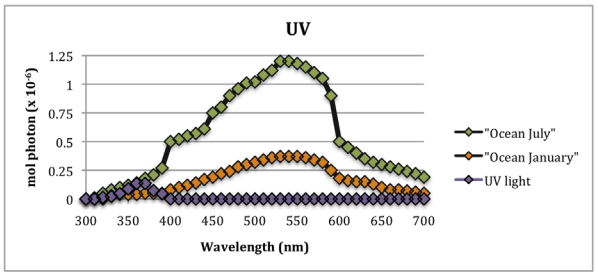
Supplementary Table 2. Genes that were significantly downregulated in the light under low or high organic matter conditions.

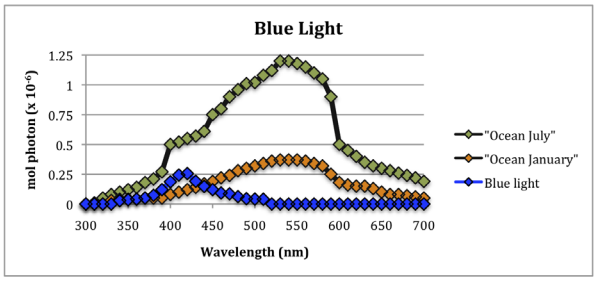
Gene ID	Gene description	Low	High
TM1040_0001_1318_1352	chromosomal replication initiator protein DnaA	x	
TM1040_0001_1318_1352_m1	chromosomal replication initiator protein DnaA	x	
TM1040_0047_321_355	outer membrane porin	x	
TM1040_0100_512_546	ABC-3	x	
TM1040_0115_477_511	inner-membrane translocator	x	
TM1040_0117_157_191	hypothetical protein	x	
TM1040_0119_1069_1103	branched-chain amino acid ABC transporter periplasmic binding protein	x	
TM1040_0119_493_527	branched-chain amino acid ABC transporter	x	
TM1040_0294_498_532	Lysine-arginine-ornithine-binding periplasmic protein	x	
TM1040_0437_1216_1250	Phenylacetic acid degradation protein paaN		x
TM1040_0593_703_737	GumN		x
TM1040_0791_14_48	hypothetical protein		x
TM1040_0961_417_451	oxidoreductase molybdopterin binding		x
TM1040_0981_105_139	Calcium-binding EF-hand	x	
TM1040_1095_323_357	protein of unknown function DUF924	x	
TM1040_1095_33_67	protein of unknown function DUF924	x	
TM1040_1357_792_826	cation transporter		x
TM1040_1493_817_851	ABC transporter	x	
TM1040_1497_490_524	D-hydantoinase	x	
TM1040_1764_732_766	molybdopterin dehydrogenase	x	
TM1040_1765_1892_1926	Carbon-monoxide dehydrogenase	x	
TM1040_1765_1948_1982	Carbon-monoxide dehydrogenase	x	
TM1040_1909_289_323	transcriptional regulator XRE family	x	
TM1040_2444_1585_1619	Isovaleryl-CoA dehydrogenase	x	
TM1040_2965_89_123	flagellar basal-body rod protein; FlgB		x
TM1040_2988_471_505	polyamine ABC transporter	x	

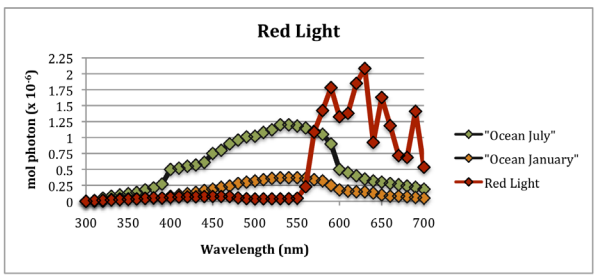
TM1040_3254_200_234	periplasmic binding protein/LacI transcriptional regulator	x	
TM1040_3259_89_123	LamB/YcsF		x
TM1040_3307_1199_1233	extracellular solute-binding protein	x	
TM1040_3598_1295_1329	Gamma-glutamyltransferase		x

Supplementary Figure 1. Characteristics of the radiation spectra obtained with different filters and ideal conditions *in situ* in July and January. We used a solar simulator to simulate annual average wavelength intensities in the surface waters off the coast of Georgia. To generate modified spectra, we fit glass filters to an opening in the bottom of the solar simulator. The light conditions are compared to average ocean conditions for the mixed layer during winter and summer (station GOS014).

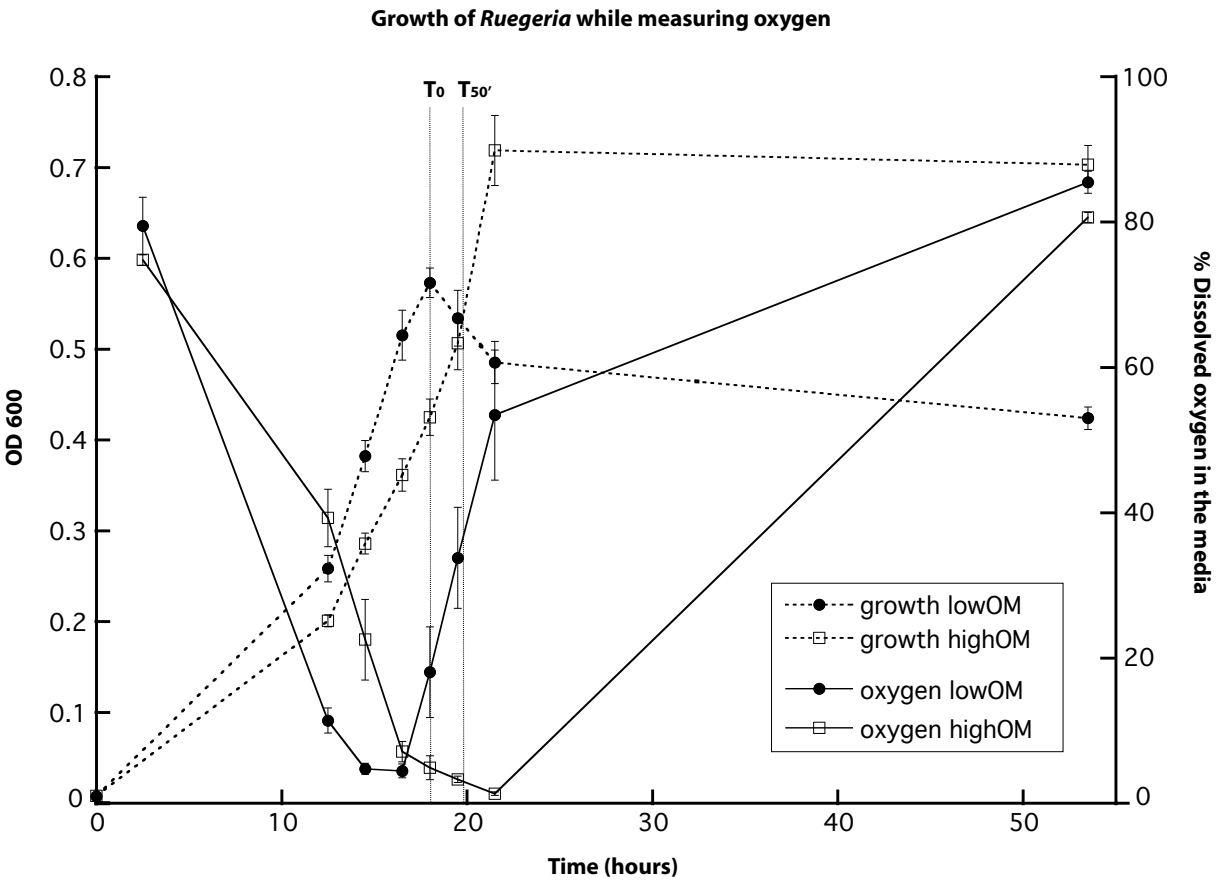








Supplementary Figure 2. Growth of *Ruegeria* TM1040 and changes in dissolved oxygen concentrations in batch cultures with two different glucose concentrations. The times of exposure to light and of sample collection are shown by vertical lines.



Chapter 5

Light Stimulates Growth of Proteorhodopsin-Containing Marine Flavobacteria

ABSTRACT

Proteorhodopsins are bacterial light-dependent proton pumps. Their discovery within genomic material from uncultivated marine bacterioplankton caused considerable excitement because it indicated a potential phototrophic function within these organisms, which had previously been considered strictly chemotrophic[1]. Subsequent studies established that sequences encoding proteorhodopsin are broadly distributed throughout the world's oceans[2–5]. Nevertheless, the role of proteorhodopsins in native marine bacteria is still unknown[6]. Here we show, from an analysis of the complete genomes of three marine Flavobacteria, that cultivated bacteria in the phylum Bacteroidetes, one of the principal components of marine bacterioplankton, contain proteorhodopsin. Moreover, growth experiments in both natural and artificial seawater (low in labile organic matter, which is typical of the world's oceans) establish that exposure to light results in a marked increase in the cell yield of one such bacterium (*Dokdonia* sp. strain MED134) when compared with cells grown in darkness. Thus, our results show that the phototrophy conferred by proteorhodopsin can provide critical amounts of energy, not only for respiration and maintenance but also for active growth of marine bacterioplankton in their natural environment.

Light stimulates growth of proteorhodopsin-containing marine Flavobacteria. **Laura Gómez-Consarnau, José M. González, Montserrat Coll-Lladó*, Pontus Gourdon, Torbjörn Pascher, Richard Neutze, Carlos Pedrós-Alió & Jarone Pinhassi.** 2007. Nature, 445: 210-213.

***** I contributed to this article doing the expression analysis of the proteorhodopsin gene

LETTER

Rhodopsins are found in the domains Archaea, Bacteria and Eukarya. Rhodopsins in Archaea function as energy-transducing light-driven proton or chloride pumps, or as phototactic sensory proteins. In Eukarya, rhodopsins function primarily as sensory proteins and, for example, account for colour vision in the human retina. The recent discovery of rhodopsins in Bacteria (proteorhodopsin; PR) came after the sequence analysis of a cloned genome region from a marine bacterium of the uncultivated SAR86 clade[1]. Subsequent screening of DNA from different oceans revealed a very large diversity of PR in bacteria belonging to divergent clades of the Alphaproteobacteria and Gammaproteobacteria classes[3–8]. Studies on reconstituted PR overproduced in *Escherichia coli* have established that it functions as a light-driven proton pump with the potential to generate energy for cell growth or maintenance[1,6]. However, the physiological role and benefits of PR in native Bacteria in the marine environment have not been demonstrated.

Alphaproteobacteria and Gammaproteobacteria, together with members of the Bacteroidetes phylum, are the most abundant groups of heterotrophic bacteria in the sea[9–12]. In the present study we examined whole-genome sequences of three bacteria belonging to the class Flavobacteria, phylum Bacteroidetes (*Dokdonia* sp. strain MED134, *Polaribacter* sp. strain MED152 and *Leeuwenhoekiella blandensis* strain MED217^T). These bacteria were isolated from surface water from the Mediterranean Sea and were successfully cultured (Fig. 1).

Genome analysis revealed the presence of genes encoding PR in MED134 and MED152 but not in MED217^T. Furthermore, these bacteria lacked genes for bacteriochlorophyll synthesis, reaction centres or light-harvesting complexes necessary to perform photosynthesis. This indicates that, if present, phototrophy in MED134 and MED152 must be due to the activity of PR.

Proteorhodopsin genes from MED134 and MED152 encoded peptides of 247 and 243 amino acid residues, respectively, sharing a sequence similarity of 83%. They are predicted to be heptahelical integral membrane proteins, as are PRs from other marine bacteria such as the SAR86 clade and '*Pelagibacter ubique*', the first cultivated member of the abundant SAR11 clade[13] (Supplementary Fig. S1). Phylogenetic analysis showed that PRs from MED134 and MED152 formed a distinct cluster with two unpublished PR orthologues from Flavobacteria isolates reported in GenBank and previously unclassified environmental sequences from the North Atlantic and the Sargasso Sea[5,8] (Fig. 2). The similarity between PR sequences within this putative Bacteroidetes cluster ranged from 57% to 85%, whereas the similarity to sequences outside this cluster ranged from 43% to 49%. Our results therefore substantiate a previous suggestion[5] that some PR sequences from uncultivated bacteria belong to the phylum Bacteroidetes.

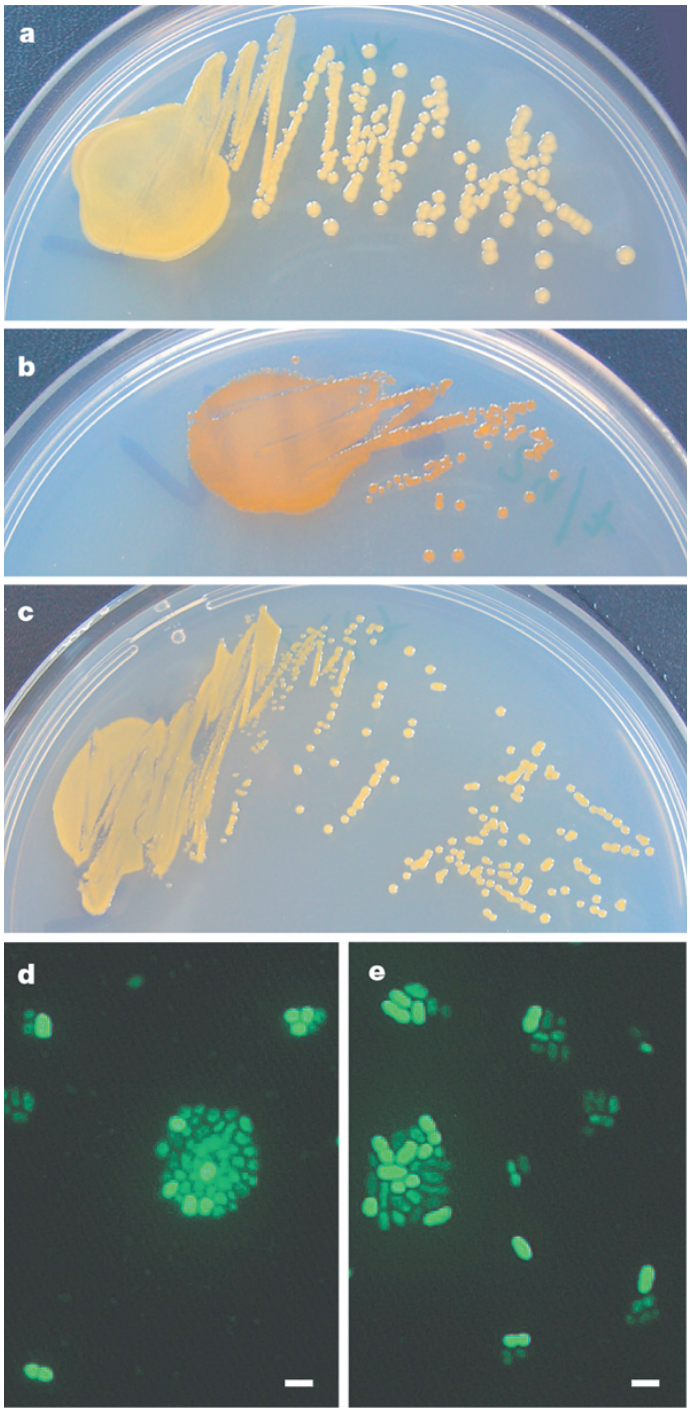


Figure 1. Images of Flavobacteria isolates. **a–c**, Colony morphology of *Dokdonia* sp. MED134 (**a**), *Polaribacter* sp. MED152 (**b**) and *L. blandensis* MED217^{T} (**c**) growing on marine agar (Difco). **d**, **e**, Epifluorescence microscopy images of MED134 growing in the dark (**d**) and in the light (**e**), showing differences in cell morphology. Scale bars, 1 mm.

Several key amino acid positions necessary for energy generation are conserved among rhodopsins functioning as proton or ion pumps (Supplementary Fig. S1). Lys 216, which binds retinal to helix G through a protonated Schiff base in bacteriorhodopsin, was conserved as Lys 230 in MED134 and MED152. Asp 85, the proton acceptor from the Schiff base, was conserved as Asp 97. In PR from marine bacteria Glu 108 replaces Asp 96, which facilitates Schiff-base reprotonation during the latter half of the bacteriorhodopsin photocycle[14,15]. Nevertheless, experiments with SAR86 and *P. ubique* PRs have demonstrated that they also function as proton pumps[1,6].

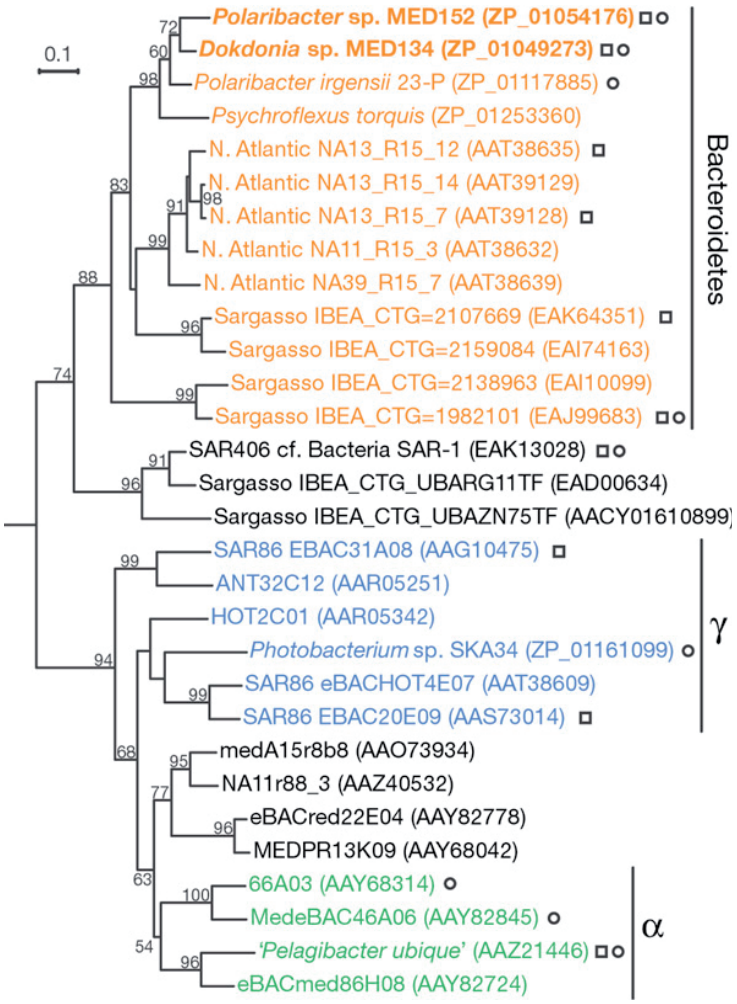


Figure 2. Phylogenetic tree of PR amino acid sequences. The position of MED134 and MED152 (bold type) in relation to selected sequences of tentative Bacteroidetes PRs from the Sargasso Sea and the North Atlantic as well as Alphaproteobacteria (α) and Gammaproteobacteria (γ). Squares and circles denote sequences aligned in Supplementary Fig. S1 and included in the gene arrangement in Supplementary Fig. S3, respectively. The numbers at nodes are bootstrap values more than 50% after 100 replicates. The scale bar represents substitutions per site.

Thus, the essential mechanism of light-driven proton pumping by bacteriorhodopsin[16] seems also to apply for PR. Furthermore, laser flash (532 nm)-induced absorbance changes (probed at 500 nm) in membrane extracts from MED134 showed reaction times less than 50 ms, consistent with the presence of PR functioning as a proton pump (Supplementary Fig. S2).

A phototrophic role for PR implies that its absorption maximum would be tuned to optimize the overlap with environmental light[2,7]. Thus, PRs in near-surface seawater typically have leucine at amino acid position 105 (eBAC31A08 numbering) and show absorption maxima near 530nm (green light), whereas PRs from deeper water have glutamine at this position and absorb near 490 nm (blue light)[2,7,15]. Consistent with these findings is the observation that the near-surface Bacteroidetes PRs contained a hydrophobic side-chain Met 105 (similar to Leu; Supplementary Fig. S1) and showed an absorption maximum at 535nm (Fig. 3b, inset).

Functional PR requires the covalent binding of retinal, which is synthesized from ß-carotene. MED134 and MED152 contained the predicted genes *crtEBIY* (for gene arrangement see Supplementary Fig. S3) encoding enzymes needed to synthesize ß-carotene from farnesyl diphosphate and isopentenyl diphosphate[17]. A gene encoding a candidate for Blh, an enzyme that converts ß-carotene to retinal[8], was found next to the gene encoding PR in the Bacteroidetes, whereas in Proteobacteria the *blh* gene is next to putative genes in the pathway for retinal synthesis[6,8] (Supplementary Fig. S3).

Since its discovery in the sea by cloning environmental DNA, the lack of cultivated bacteria that have and express PR has hampered the determination of its physiological relevance to marine bacteria *in situ*. The gene encoding PR was recently found in cultivated *P. ubique*, and mass spectroscopy indicated that *Pelagibacter* PR is abundant in the surface ocean[6]. Laboratory experiments with *P. ubique*, however, showed no differences in cell yield when growing in light or darkness despite the fact that PR was present in cell membranes. The authors therefore speculated that the benefits of PR might be more pronounced when bacterial growth is limited by organic matter availability. We monitored the growth of MED134 in experiments with natural seawater exposed to light or darkness. Bacteria growing in moderate light reached a maximal abundance of $3x10^5$ cells ml^{-1} after 100 h (Fig. 3a). In contrast, bacteria remained below $0.5x10^5$ cells ml^{-1} in the dark. When dark cultures were exposed to light after 120 h, growth was initiated and bacteria reached $2x10^5$ cells ml^{-1} within 100 h, whereas bacteria in the cultures remaining in darkness slowly decreased in numbers. This is the first confirmation *in vivo* that light has a definite positive impact on the growth of marine bacteria possessing the gene encoding PR.

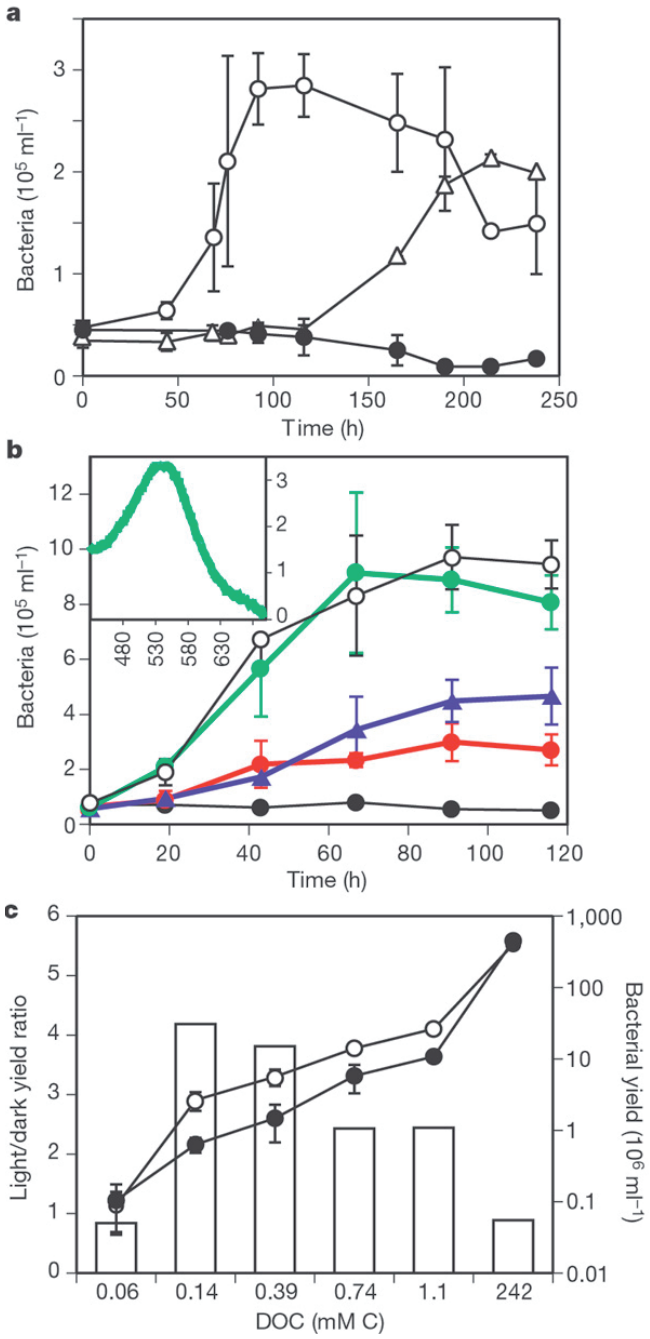


Figure 3. Growth of MED134 in seawater cultures. **a**, Cultures grown in natural sterile filtered and autoclaved seawater, exposed to light (open circles) or in darkness (filled circles). After 120 h, duplicate cultures grown in darkness were exposed to light (open triangles). **b**, Cultures exposed to white light (open circles), blue light (blue triangles), green light (green circles) and red light (red filled circles) and cultures maintained in the dark (black filled circles). Inset: absorption spectrum of MED134 PR purified from *E. coli* membranes (for details see Supplementary methods); the x axis shows wavelength (nm) and the y axis absorbance (10^{-2}). **c**, Cell yields in artificial seawater, incubated in the light (open circles) and in the dark (filled circles), enriched with dissolved organic matter, in comparison with unenriched controls (0.06 mM C) and full-strength medium (242 mM C). Columns represent light/dark ratios of cell yield. Error bars denote s.d. for duplicate or triplicate cultures.

Experiments using light of different wavelengths showed that this enhanced growth was stimulated primarily by green rather than blue or red light (Fig. 3b). This correlated well with the measured absorption spectrum of MED134 PR (Fig. 3b, inset) and substantiates our hypothesis that the observed growth enhancement derives from the direct absorption of light by PR. Furthermore, analysis of the expression of the gene encoding PR with the use of reverse-transcriptase-mediated polymerase chain reaction for detecting the corresponding mRNA showed a significant upregulation of the PR mRNA in light in comparison with dark cultures (Fig. 4). These observations support the notion that PR fulfils a phototrophic function in marine bacteria.

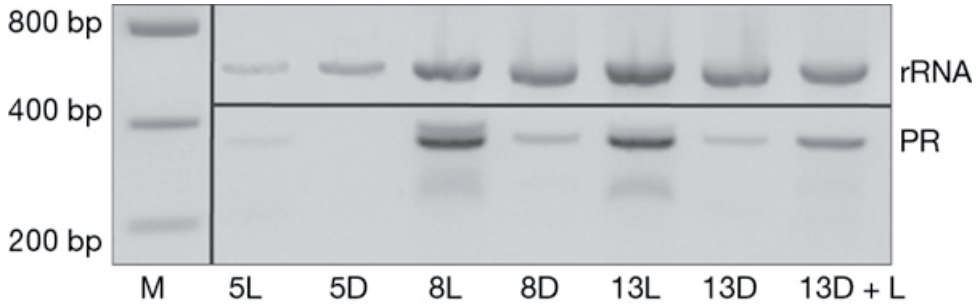


Figure 4. RT–PCR analysis of PR expression in MED134 compared with 16S rRNA levels. PR was expressed preferentially in the light. Bacteria were grown in natural sterile filtered and autoclaved seawater exposed to light or in the dark. Numbers indicate days since inoculation; D, dark incubation; L, light incubation. 13D + L denotes a sample incubated for 10 days in the dark followed by 3 days in the light. M, DNA size marker; bp, base pairs.

We further investigated how different concentrations of dissolved organic matter (DOM, as peptone and yeast extract) affected the growth of MED134 (Fig. 3c). In cultures with artificial seawater without enrichment (0.06 mM C) little growth was observed in either light or darkness, whereas enrichment with DOM corresponding to 0.14 or 0.39 mM C (final concentration) resulted in bacterial cell yields about fourfold higher in light cultures. Greater enrichment (0.74 or 1.10 mM C) gave yields about 2.5-fold higher in the light cultures, whereas in full-strength medium (242 mM C) similar bacterial cell yields were observed in both light and dark conditions. In addition, cells grown in light-exposed cultures seemed larger than those in dark cultures (Fig. 1d, e).

Thus, exposure to light confers a stronger selective advantage when MED134 grows at low or intermediate concentrations of labile organic matter, closer to those found in marine surface waters, than in richer media. It is noteworthy that *Dokdonia* sp. MED134 thrives in conditions under which resources are abundant, whereas *P. ubique*, in which PRs have been suggested to have a subtle function[6], grows only under oligotrophic conditions.

Factors limiting its growth have yet to be identified[6]. These findings emphasize the need to clarify the intricate relationships between a PR-mediated light response, and the availability and composition of organic matter for typically oligotrophic bacteria, in comparison with bacteria adapted to eutrophic environments.

Members of the Bacteroidetes phylum in general, and Flavobacteria in particular, are important in the degradation of organic matter during and after algal blooms in the sea[18–20]. Our findings indicate that phototrophy may allow these organisms to maintain net growth during periods when concentrations of organic matter are declining. If this were true for other PR-bearing bacterioplankton, PR-mediated photoheterotrophy would increase their growth efficiency. An interesting consequence is that, for a given amount of DOM, bacterial assemblages dominated by species expressing genes encoding PR would produce more biomass than assemblages of bacteria lacking PR, making it available to higher trophic levels in the marine microbial food web.

Phototrophism has now been found in all main microbial groups in the sea. These include eukaryotic algae and cyanobacteria performing chlorophyll *a*-driven photosynthesis, Proteobacteria using bacteriochlorophylls for generating energy[21–23], and Proteobacteria[1,3] -and now also Bacteroidetes- using PR. The phylogenetic diversification and wide geographic distribution of genes for PR in the world's oceans indicate that evolution has favoured organisms with the potential to complement their chemotrophic life style by phototrophy[3,24–26]. Surface and near-surface marine microorganisms are bathed in light and it is therefore not surprising that diverse strategies have evolved to exploit such an abundant energy source.

METHODS

Isolation of Flavobacteria strains. Bacteria were isolated from Northwest Mediterranean Sea surface water (0.5 m depth) collected 1 km off the Catalan coast at the Blanes Bay Microbial Observatory (41º 40' N, 2º 48' E, Spain). Strains MED134 and MED152 were isolated from Zo-Bell agar plates inoculated on 20 March 2001, and strain MED217^T on 23 May 2001.

Sequencing, annotation and phylogenetic analysis. Whole-genome sequencing was performed by the J. Craig Venter Institute through the Gordon and Betty Moore Foundation initiative in Marine Microbiology (https://research.venterinstitute.org/moore/). PR sequences were aligned by using ClustalW, and a Neigbour-Joining phylogenetic tree was constructed (see Supplementary Methods).

Seawater culture experiments. For the natural seawater experiment, water from the North Sea was mixed with aged seawater from the North Atlantic. For the DOM gradient experiment, artificial seawater (35 practical salinity units, prepared from Sea Salts; Sigma) was enriched with peptone and yeast extract. All cultures were enriched with N and P to avoid inorganic nutrient limitation. Cultures were incubated under continuous light (180 μmol photons m^{-2} s^{-1}) or in the dark at 20 ºC. Dissolved organic carbon was measured with a high-temperature carbon analyser. Bacteria were counted by epifluorescence microscopy. For details see Supplementary Methods.

Expression analysis. RNA were extracted and purified from cell pellets with RNAqueous-4PCR Kit (Ambion Inc.). mRNA was reverse transcribed with the two-step protocol of RETROscript Kit (Ambion Inc.) and a MED134 PR-specific primer set. For details see Supplementary Methods.

Author Information. The genomes of strains MED134, MED152 and MED217 are deposited in GenBank under accession numbers AAMZ00000000, AANA00000000 and AANC00000000, and their 16S rRNA gene sequences under accession numbers DQ481462, DQ481463 and DQ294290, respectively. The amino acid sequences of MED134 and MED152 PR are deposited in GenBank under accession numbers ZP_01049273 and ZP_01054176.

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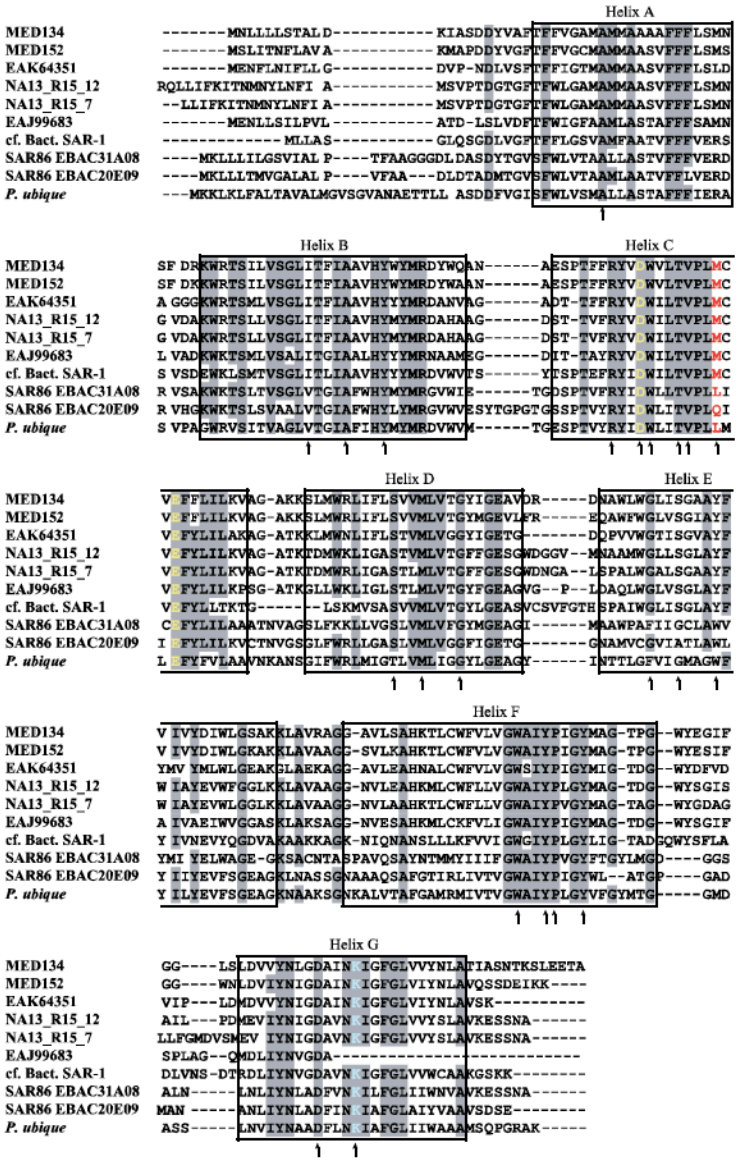
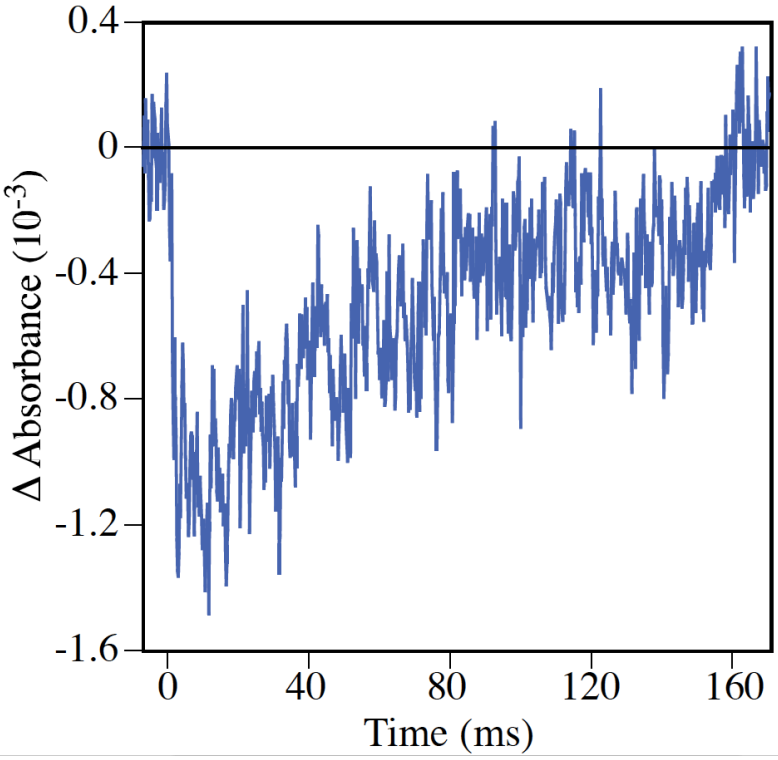
SUPPLEMENTARY INFORMATION

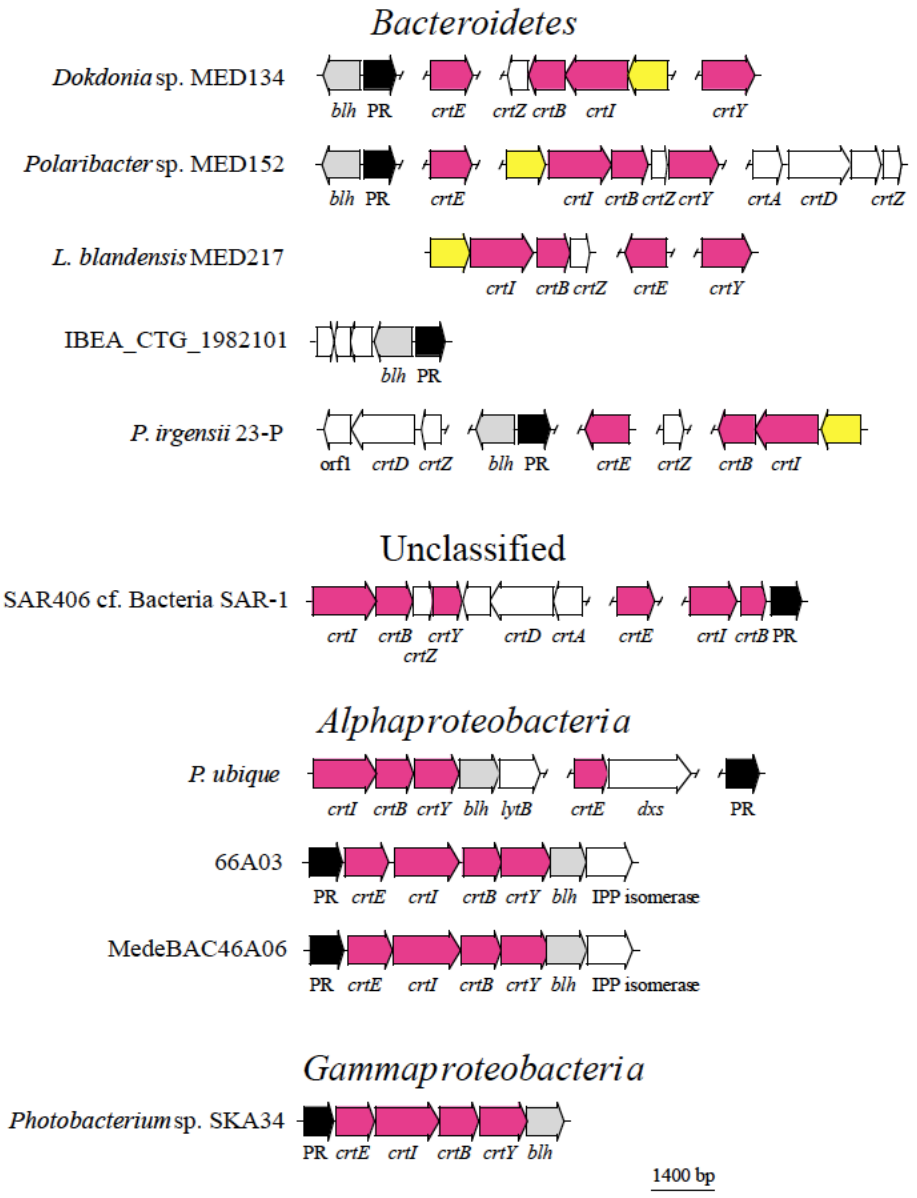
Supplementary figures



Supplementary Figure 1. Proteorhodopsin amino acid sequence alignment. Multiple alignment of the predicted amino acid sequences of PR in *Dokdonia* sp. MED134 and *Polaribacter* sp. MED152 compared to tentative *Bacteroidetes* orthologs and SAR86 and *P. ubique* sequences. Predicted transmembrane regions are marked by boxes with solid lines. Gray shading indicates conserved amino acid positions. Key amino acids for PR functionality are marked by colours: Asp97 and Glu108 in yellow, Met, Leu or Gln at position 105 in red, and Lys230 in light blue (SAR86 eBAC31A08 numbering). Amino acid positions predicted to be part of the retinal binding pocket are marked by arrows. Accession numbers and phylogenetic relationships of the sequences are presented in Fig. 2. Amino acid sequences were aligned using Clustal W. Predicted transmembrane regions were identified using TMpred, based on a statistical analysis of TMbase.



Supplementary Figure 2. Laser-flash induced absorbance changes in membrane preparation of *Dokdonia* sp. MED134. MED134 membrane absorption transient at 500 nm; the laser flash at time 0 was at 532 nm. The short reaction time, on the order of <50 ms, is consistent with the presence of PR functioning as proton pumps in the membranes. Laser flash-induced absorbance changes were measured with a laboratory-constructed flash-photolysis apparatus (Pascher 2001). The extracted cell membrane fractions were added to 1 cm cuvettes fitted with a stirrer. The sample was excited with a 10 mJ, 8 ns laser pulse at 532 nm. The acquisition cycle was: laser excitation (1 pulse), kinetic trace recording, stirring 10 s, resting 10 s, recording of reference kinetic trace (no laser), stirring 10 s and resting 10 s. This procedure was repeated 36 times for averaging.



Supplementary Figure 3. Arrangement of proteorhodopsin, ß-carotene and retinal synthesis genes. Position of proteorhodopsin (PR) genes and the putative *blh* homologs in Flavobacteria strains MED134 and MED152 compared to other marine bacteria. Genes marked in red denote *crtEBIY*, predicted to code for geranylgeranyl diphosphate synthase, phytoene synthase, phytoene desaturase and lycopene β-cyclase, respectively, needed to synthetize β-carotene from farnesyl diphosphate (FPP) and isopentenyl diphosphate (IPP). *crtZ* codes for a putative carotene hydroxylase probably involved in the synthesis of zeaxanthin. Furthermore, MED152 contains *crtD* (methoxyneurosporene dehydrogenase) and *crtA* (spheroidene monooxygenase) homologs. Strains MED134 and MED152 both contain a putative transcriptional regulator (MerR family, marked in yellow) in front of *crtI*. The genes *dxs*, IPP isomerase and *lytB* are involved in the synthesis of FPP and IPP precursors. Strain MED217^{T}, used as control in the present study, also contained the genes *crtEBIYZ*, but lacked PR and *blh* homologs. Interestingly, among the *Bacteroidetes*, the PR and *blh* genes are located next to each other but are transcribed divergently. Among other bacteria these genes are found separately and are transcribed in the same direction. Scale bar denotes 1400 base pairs.

Supplementary methods

Annotation and phylogenetic analysis. Open reading frames were predicted and autoannotated using GenDB (Meyer et al. 2003). In addition, all relevant genes discussed here were manually annotated. For the phylogenetic analysis of PR, amino acid sequences were aligned using Clustal W, and a tree was constructed based on a Kimura's distance matrix and the Neighbour-Joining method using the PHYLIP package (Version 3.2) (Felsenstein 1989). For the phylogenetic tree, the *Halobacterium salinarum* S9 bacteriorhodopsin was used as outgroup (not shown in Fig. 2).

Seawater culture experiments. For the experiment with natural seawater, water collected in the North Sea was mixed with an equal volume of aged seawater from the North Atlantic. For each culture, 1.5 litres of seawater, used as growth medium, was filter-sterilized through a 0.2 μm-pore-size filter (Sterivex; Millipore) and autoclaved. For the DOM gradient experiment, an artificial seawater medium (Sea Salts; Sigma: salinity 35 psu) was filter-sterilized through 0.2 μm-pore-size filters (Sterivex; Millipore) and autoclaved. Cultures of 520 ml each were enriched with 0, 0.23, 0.74, 1.48 and 2.25 ml of full strength medium (5 g peptone [Bacto Peptone; BD] and 1 g yeast extract [Bacto Yeast Extract; Difco] in 1 l artificial seawater). For the coloured light experiment, cultures were grown in artificial seawater at a DOC concentration of 0.14 mM C. Blue (400-500 nm), green (490-570 nm) and red (600-800 nm) light were obtained using coloured filters (HQ Power).

Cultures were maintained in polycarbonate bottles (Nalgene). The natural seawater cultures received final concentrations of 2.1 μM NH_4Cl and 0.3 μM Na_2HPO_4 to avoid inorganic nutrient limitation. In the DOM gradient experiment, N and P were further supplied by the added medium (36-285 μM N and 0.6-3.4 μM P). Cultures were inoculated with bacteria previously grown in seawater. Light cultures were incubated under an artificial light source (180 μmol photons m^{-2} s^{-1}) at 20°C. All material in contact with the samples was rinsed with 1 M hydrochloric acid and extensively washed with MilliQ-water prior to use.

Dissolved organic carbon (DOC) was measured with a high temperature carbon analyzer (Shimadzu TOC 5000) on samples filtered through 0.2 μm-pore-size polycarbonate filters (Supor-200; Gelman Sciences) and acidified with 16 mM HCl (final concentration).

For bacterial counts, samples were fixed with 0.2 μm-pore-size filtered formaldehyde (4%, final concentration), stained with SYBR Gold (1:100 dilution, Molecular Probes), filtered onto black 0.2 μm-pore-size polycarbonate filters (Poretics, Osmonics Inc.) and counted by epifluorescence microscopy within 48 hours.

Cloning, production and determination of the absorption spectrum of proteorhodopsin from *Dokdonia* sp. MED134. The PR gene was cloned from MED134 cells using PCR, incorporating a C-terminal 6xHis-tag. The amplified fragment was inserted into the pBAD-TOPO vector (Invitrogen) and subsequently transformed into the *E. coli* outer membrane protease-deficient strain UT5600. PR was over-produced in cells grown at 37°C and induced at an optical density (600 nm) of 2 with 1.25 g l^{-1} L(+)-arabinose and 1.5 mg l^{-1} all trans-retinal for 16 hours. The cells were disrupted with a French Press (Thermo Spectronic) at 1,500 psi, additional all trans-retinal was added (1 mg retinal per gram of cells), and the membrane fractions were collected by centrifugation at 150,000 g for 90 min. Membranes were then solubilised in n-octyl-β-d-glucopyranoside (Anatrace). Extracted histidine-tagged PR was purified with affinity column chromatography using Chelating Sepharose Fast Flow (GE Healthcare) immobilized with Ni^{2+}. The column with bound PR was washed using 35 mM imidazole and PR was subsequently eluted using 150 mM imidazole. Subsequent ion exchange chromatography resulted in a peak containing PR from which the absorption spectrum could be determined after the fractions had been concentrated in Vivaspin concentration tubes (Sartorius) to 100 µl. The absorption spectrum was measured in a Shimadzu UV-1601 spectrophotometer, using the same buffer as in the last purification step as a baseline.

RNA extraction and purification. Cells were harvested from seawater cultures by pipetting 10 ml samples into 15-ml tubes on ice. Samples were centrifuged and the pellets were stored with 0.5 ml RNAlater® (Ambion Inc., Austin, TX) at –80 °C. For RNA extraction, samples were thawed on ice. The RNAlater was discarded and the pellet was washed in PBS 1X. A total of 500 µl of lysis/binding solution provided by the RNAqueous®-4PCR kit (Ambion, Inc.) were added to the cells. The samples were transferred to 2 ml screw-cap microcentrifuge tubes containing 1.2 g of 100-µm-diameter zirconia-silica beads (BioSpec Products, Inc.). Samples were mechanically disrupted in a Mini-beadbeater-8™ cell disrupter (BioSpec Products Inc., Bartlesville, OK). After disruption, samples were incubated on ice for 5 min and the beads were allowed to settle out of the lysis mixture. The lysate was clarified by centrifugation and the aqueous phase was transferred to a new tube. An equal volume of 64% ethanol was added to the lysate and samples were purified according to the RNAqueous®- 4PCR Kit. The isolated total RNA was treated with DNase I - RNase-free (Ambion Inc.). RNA preparations were checked for DNA contamination with PCR using primers 358F and 907RM for the 16S rRNA gene. Total RNA was quantified by spectrophotometry at 260 nm.

Reverse transcriptase-PCR. A proteorhodopsin primer set was designed to amplify a 349-bp fragment of the *Dokdonia* sp. MED134 PR gene. The sequence of forward primer 238F was 5'-GCAAGCAAATGCTGAGTCAC-3' and the sequence of reverse primer 548R was 5'- AATT-GCCCATCCCACAAGTA-3'. This primer set was designed to amplify a 349-bp fragment of the MED134 proteorhodopsin gene. Total RNA was used to reverse-transcribe proteorhodopsin mRNA into cDNA using primer 548R and the two-step protocol of the RETROscript® Kit (Ambion Inc.). To denature any secondary structures, aliquots of each of the RNA extracts and primer 548R were heated for 3 min at 75 ºC and immediately placed on ice before they were mixed with the final reaction solution (20 μl). 2 μl of the RT reaction product were used in a subsequent PCR. The PCR amplification conditions were as follows: 94 ºC for 2 min, and then 40 cycles of 94 ºC for 30 s, 55 ºC for 30 s, and 72 ºC for 40 s and finally 1 cycle of 72 ºC for 5 min. As positive RNA controls, 16S ribosomal RNA was reverse transcribed and thereafter amplified with bacterial 16S rRNA gene-specific primers 358F (5'-CCTACGGGAGGCAGCAG-3') and 907RM (5'-CCGT-CAATTCA/CTTTGAGTTT-3'). The same amplification protocol as for the PR reaction was used, except that the number of cycles was reduced to 25. PCR products were analyzed by agarose gel electrophoresis (Fig. 4).

Supplementary notes.

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CONSIDERACIONS FINALS

L'eix central d'aquesta tesi és el desenvolupament i aplicació del mètode de *fingerprinting* dels RNA missatgers, el *Transcriptome Fingerprinting Analysis* (TFA) en comunitats de microorganismes marins. L'estudi de l'expressió gènica en comunitats marines es va iniciar encara no fa 10 anys i aquesta tesi s'iniciava en les primeres hores de l'albada de la metatranscriptòmica. En concret, aquesta tesi queda emmarcada pel tret de sortida de l'article de Poretsky *et al.* (Poretsky *et al.*, 2005) publicat l'any 2005 i els nous mètodes de recollida de mostres que suposen un canvi substancial a l'hora de superar el principal repte que ha tingut fins ara qualsevol dels estudis que tractaven amb RNA missatger en comunitats marines.

Poretsky *et al.* va realitzar el primer estudi d'anàlisi de l'expressió gènica del bacterioplàncton marí i d'aigua dolça. Va recuperar els RNA missatgers, els va convertir en el seu corresponent DNA complementari a través de la transcripció reversa, va fer una biblioteca de clons i després els va seqüenciar. Va determinar els gens actius *in situ* sense fer suposicions prèvies sobre quins gens podrien ser els més expressats. A partir de llavors, els avenços en les tecnologies de seqüenciació de nova generació van anar en augment i darrere d'aquests avenços van sorgir un seguit d'estudis a remolc de les últimes novetats tecnològiques.

Els primers estudis de metatranscriptòmica van ser majoritàriament descriptius. Examinaven les diferències en transicions temporals o espacials en diferents tipus d'hàbitats (Frías-López *et al.*, 2008; Gilbert *et al.*, 2010; Hewson *et al.*, 2010; Poretsky *et al.*, 2009; Stewart *et al.*, 2012). Alguns d'aquests estudis van anar més enllà del fet purament descriptiu per analitzar les diferències d'expressió en diverses manipulacions experimentals (Gilbert *et al.*, 2008; Poretsky *et al.*, 2010; Vila-Costa *et al.*, 2010).

El factor clau que marca totes les decisions i resultats posteriors en tot estudi d'expressió gènica d'una comunitat és el temps de mostreig i la biomassa que es recull durant aquest temps. És vital minimitzar el temps entre la recollida de les mostres i la filtració (en el cas que es filtri i no es fixi directament) fins a tenir els filtres congelats o en tampó de lisi, degut a la rapidesa amb què l'RNA missatger es pot degradar o simplement canviar degut a les variacions dels estímuls externs.

Recentment, per tal de solventar aquest problema, s'han enginyat diferents artilugis de mostreig. El primer que es va idear i aplicar va ser el descrit en l'article de Feike *et al.* (Feike *et al.*, 2012). L'aparell permet la fixació *in situ* de les mostres d'aigua al precís moment del seu mostreig. Per una mostra generen un volum de 3L d'aigua contaminada o bé amb fenol o bé amb cloroform.

El problema d'aquest invent és que si es necessiten grans volums d'aigua o s'han de processar moltes mostres, el volum de residus generat és insostenible. Però per masses d'aigua amb abundants biomasses potser sí que el balanç pot sortir a compte.

Un altre aparell que s'ha utilitzat per estudiar l'expressió gènica del bacterioplàncton marí és el Processador de Mostres Ambiental (ESP, de *Environmental Sample Processor*). L'ESP (Scholin *et al.*, 2009) consisteix en un sistema electromecànic/fluídic dissenyat per recollir mostres d'aigua, concentrar els microorganismes, i automatitzar l'aplicació de tecnologies moleculars *in situ*. Ottesen *et al.* (Ottesen *et al.*, 2011) va utilitzar aquest aparell preservant les mostres amb RNA later i va veure que era una bon mètode per caracteritzar els gens expressats. Ottesen *et al.* (Ottesen *et al.*, 2013) també va usar aquesta plataforma robòtica per descriure l'expressió gènica del plàncton microbià durant un període de 2 dies, i van veure que els diferents grups microbians del plàncton coordinaven la seva expressió al llarg del temps. Per una banda, el picoplàncton fotosintètic exhibia forts ritmes circadians coordinant l'expressió de milers de trànscrits expressats en sincronia. D'altra banda, els trànscrits dels organismes heteròtrofs no ciclaven circadiàriament, però també mostraven una expressió sincrònica entre espècies de molts dels seus gens a través del temps, sobretot d'aquells gens associats al creixement i a l'adquisició de nutrients.

Per acabar amb el ventall d'invents de mostreig, el grup de Grossart va idear l'anomenat "*in situ* filtration and fixation sampler" (IFFS) (Wurzbacher *et al.*, 2012). Aquest instrument permet mostrejar a totes les profunditats i disposa d'un sistema ràpid de filtració *in situ* que permet reduir enormement el volum de fixador, així només cal fixar el cartutx del filtre i no cal generar un volum ingent d'aigua contaminada. Aquest aparell, per tant, és ideal per mantenir les condicions *in situ* de la mostra i a més a més no és car i pot ser construït amb els plànols proporcionats pels autors.

Fins i tot, amb aquests nous artilugis s'han arribat a realitzar PCRs i d'altres tècniques de biologia molecular *in situ*. En l'estudi de Preston (Preston *et al.*, 2011) utilitzen l'anteriorment esmentat Processador de Mostres Ambiental (ESP) per assignar abundàncies de gens de forma autonòma, sota l'aigua i gairebé en temps real.

Amb totes aquestes eines a l'abast, en aquests darrers anys els protocols per veure l'expressió gènica de comunitats de microorganismes marins s'han anat perfilant i optimitzant pas a pas i podem dir que actualment disposant de tots els mitjans a l'abast, es pot fer un mostreig i anàlisi tal com Déu mana (cosa que no es podia dir fa escassos anys).

Nosaltres vam iniciar la posada a punt del protocol de *fingerprinting* de l'mRNA en paral·lel a l'adveniment de la metatrascriptòmica. Moltes de les decisions que es van prendre en el seu moment (com quin tipus de filtre utilitzar, temps de filtració, volum filtrat, mètodes per preservar les mostres, protocols d'extracció, trencament de les cèl·lules, tipus de transcriptasa reversa, mètode de detecció dels productes de PCR, i un llarg etc.) per posar a punt l'extracció d'RNA total i el TFA en un camp que tot just s'estava desplegant han acabat resultant majoritàriament encertades i d'aplicació comuna en els protocols d'expressió gènica de les comunitats marines. L'únic que considerem com a francament millorable és el mètode de mostreig, que es podria solventar amb un d'aquests artilugis descrits anteriorment.

Diverses van ser les provatines a l'hora de desenvolupar el TFA abans d'arribar a un protocol estandaritzat. Comentaré breument algunes de les proves metodològiques que no consten en cap dels articles i que poden respondre a alguna de les preguntes de tot aquell que s'inicïi en el món de l'RNA missatger.

Maneres de preservar l'RNA, com, i quina funciona millor

L'obtenció d'RNA de bona qualitat és un dels passos més crítics per tal de realitzar la resta d'experiments. L'RNA missatger és aproximadament un 5% de l'RNA total d'una cèl·lula i és molt sensible als canvis i a la degradació, per això és de vital importància treballar en condicions lliures d'RNases, uns enzims presents a tot arreu que degraden l'RNA.

Es van provar diferents maneres de preservar els filtres un cop filtrades les mostres: I) Amb tampó de lisi de diferents kits, II) Amb TRI reagent/TRIZOL, III) *Flash-freeze* directe amb nitrogen líquid i posterior emmagatzematge a -80ºC i finalment IV) RNA later.

De les diferents proves, la que ha donat més bons resultats en mostres marines és quan es preserva el filtre amb tampó de lisi (el que va donar més rendiment és el tampó de lisi del Qiagen RNeasy Mini kit). L'ús de TRI reagent o TRIZOL funciona molt bé quan disposes de grans biomasses (per exemple de teixits), les quals són gairebé impossibles d'aconseguir en les mostres de picoplàncton marí. Aplicar un protocol d'extracció en fases en aquest tipus de mostra és molt delicat. L'opció de congelació directa també funciona molt bé (tot i que amb un xic menys de rendiment que preservant els filtres amb tampó de lisi) i és més pràctica a l'hora de recollir mostres, sobretot si estàs en una campanya oceanogràfica (en aquesta tesi s'ha acabat optant per aquesta opció en la majoria de mostres). Tot i els grans avantatges que té l'RNA later hem pogut comprovar en vàries ocasions que el rendiment que se n'obté és bastant més baix i és més tediós, ja que la mostra necessita de varis rentats previs abans de procedir amb el protocol d'extracció. Tot i així és una bona opció

per preservar les mostres d'RNA, i si després t'és possible amplificar l'RNA de forma lineal no té perquè ser un problema el rendiment més baix que se n'obté.

La ruptura completa de les cèl·lules per tal d'alliberar els àcids nucleics és un pas fonamental. Es va comprovar experimentalment que la quantitat d'RNA obtinguda és major quan sotmetem les cèl·lules a una ruptura mecànica. Per això afegim una quantitat concreta de boles de zirconi/silica o vidre (depenent del tipus de cèl·lula a lisar) juntament amb un volum de tampó de lisi i s'agita la mescla vigorosament durant uns minuts amb un *Vortex*. Un altre punt bàsic a l'hora de fer RT-PCR és degradar completament el DNA genòmic de la preparació total d'RNA. La presència de petites quantitats de DNA pot generar posteriors falsos positius durant la PCR. Per assegurar-nos que no queda cap traça de DNA genòmic després de cada extracció d'RNA s'ha digerit la mostra amb una DNasa.

Reacció de transcripció reversa

La reacció de transcripció reversa la podem fer de vàries maneres depenent del que ens interessi amplificar després. En el cas del protocol de TFA, com que ens interessa retrotranscriure el màxim d'RNA missatgers possibles, s'han utilitzat encebadors oligo(dT) que s'uneixen a la zona 3' dels missatgers dels eucariotes (cua poliA). En el cas de la detecció específica d'un gen, en aquest cas el de la proteorodopsina, es va dissenyar una parella d'encebadors específics (vegeu el protocol detallat al capítol V) i es va utilitzar l'encebador invers per portar a terme la retrotranscripció de l'RNA missatger del gen de la proteorodopsina.

El pas de la RT afegeix molta més variabilitat que no pas la PCR, és doncs en aquest pas on es va intentar millorar la reproductibilitat del TFA. Un dels factors que vam veure que va ser clau a l'hora de millorar la reproductibilitat dels patrons va ser el tipus de transcriptasa reversa utilitzada. Vam provar tres transcriptases reverses diferents, cadascuna amb unes propietats característiques. De totes les proves fetes, es va veure que utilitzant la transcriptasa reversa termoestable Superscript™, el patró obtingut amb la mateixa mostra a diferents concentracions i la mateixa combinació d'encebadors passava de ser no reproduïble a reproduïble (al portar a terme la reacció a una temperatura més elevada augmenta l'especificitat).

Valoració del *Transcriptome Fingerprinting Analysis*

El TFA és una tècnica sensible, ja que es necessiten quantitats inicials molt petites d'RNA total. És reproduïble, i molt versàtil; permet aconseguir una ampla representació de fragments de gens expressats diferencialment en múltiples condicions.

Ara bé, si el que es vol és identificar els gens expressats diferencialment això ja són figues d'un altre paner. La identificació i confirmació dels gens expressats diferencialment requereix de diferents passos: primer cal córrer un gel desnaturalitzant de poliacrilamida per visualitzar les bandes i després tallar les d'interès. Aquest mateix pas, ja no és tan senzill, de fet es van fer varis intents infructuosos. Després cal reamplificar els amplicons i finalment confirmar els gens expressats diferencialment, preferencialment amb RT qPCR (es podria fer també per Northern blot o reverse Northern).

Un altre inconvenient a l'hora d'identificar els amplicons és el tamany mateix de l'amplicó (fins a 500bp). Com que l'encebador *reverse* usat en el TFA s'uneix al costat de la cua poliA, la majoria de productes corresponen a la regió 3' no traduïda del gen (3'UTRs, de l'anglès "untranslated region"). Aquestes regions normalment no són molt útils a l'hora de comparar seqüències contra gens coneguts de les bases de dades, ja que les bases de dates normalment no contenen la informació de les regions 3'UTR i també perquè els gens homòlegs difereixen força en la seva regió 3'UTR.

Tot això ens porta a la següent conclusió: El TFA com a mètode per identificar gens expressats diferencialment pot arribar a ser força complicat però és una bona eina a l'hora d'obtenir patrons globals d'expressió per comparar múltiples condicions.

La robustesa del mètode es basa en què permet comparar un conjunt força nombrós de gens expressats en situacions molt diverses i això permet delimitar diferents tipus de transicions. Per tant, el TFA pot ser especialment útil en aquests últims temps de febre seqüenciadora per tal de destriar les mostres que més diferències presenten entre elles (un exemple de megaprojecte de sequenciació és el Earth Microbiome Project http://earthmicrobiome.org en el qual estant portant a terme un estudi pilot on hi ha previst seqüenciar els metagenomes de 200.000 mostres ambientals!). Donat l'allau d'informació per cada mostra seqüenciada és de vital importància escollir d'entrada les mostres més representatives.

Donat que en el món microbià és gairebé impossible assignar un microorganisme a una espècie determinada (perquè una espècie tradicional sigui ben definida, cal que els seus gens flueixin verticalment, i aquest no és el cas), a més a més d'investigar la distribució dels components de les comunitats, una bona alternativa a l'hora de classificar els microbis és mirar la distribució en l'espai i el temps dels seus trets funcionals, és a dir, la similitud en el seu estil de vida (Green, Bohannan, & Whitaker, 2008). Les unitats taxonòmiques individuals evolucionen i s'extingeixen, però les màquines responsables de les funcions principals per sobreviure i adaptar-se a un determinat medi romanen sorprenentment impertorbables (Falkowski, Fenchel, & Delong, 2008).

CONCLUSIONS

Les conclusions generals d'aquesta tesi són:

1) Hem demostrat que el mètode de fingerprinting d'RNA missatger desenvolupat en aquesta tesi (el *Transcriptome Fingerprinting Analysis*) és eficient a l'hora d'explorar la dinàmica dels patrons d'expressió gènica en comunitats naturals de microorganismes marins i també en cultius sotmesos a diferents condicions. El mètode és especialment útil quan s'han de comparar un gran nombre de condicions o tractaments.

2) Incloure tots els pics en els electroferogrames de TFA i ARISA redueix substancialment la diferenciació entre les mostres. Els pics de baixa intensitat actuen com un tampó tant en la composició de les comunitats com en els patrons d'expressió gènica.

Amb l'aplicació d'aquest mètode i comparant-lo amb els patrons obtinguts amb ARISA del DNA de la comunitat hem pogut veure que:

3) Les comunitats naturals de picoplàncton eucariota i els seus patrons d'expressió reocorren de forma estacional, produïnt-se les principals diferències entre estiu i hivern.

4) Durant l'hivern, els patrons d'expressió gènica són més variables que la composició de la comunitat. En canvi, durant l'estiu la composició de la comunitat varia més que no pas els patrons d'expressió, la qual cosa suggereix redundància funcional en les comunitats estivals de picoeucariotes.

5) Les comunitats de picoeucariotes de superfície i els seus patrons d'expressió són similars al llarg d'un transecte de 100 km, però canvien radicalment al llarg dels perfils verticals. L'estructura de la comunitat és més estable que els seus patrons d'expressió.

En aquesta tesi també s'ha explorat la manera com la llum interacciona amb els microorganismes de dues maneres diferents: i) veient el canvi transcripcional global d'un microorganisme del grup dels Roseobacter a la llum i ii) monitoritzant l'expressió de proteorodopsina en una Flavobacteria. Hem conclòs que:

6) Els receptors de la llum de l'heteròtrof *Ruegeria* promouen l'activació d'una sèrie de gens involucrats en protegir la cèl·lula del dany oxidatiu en resposta a l'estrés generat per la llum, en concret la llum blava. La llum blava i la UV també activen l'expressió d'un gen involucrat en adhesió cel·lular, segurament important perquè el bacteri pugui associar-se amb el seu dinoflagel·lat preferit i pugui accedir als recursos que aquest genera en presència de llum.

7) L'expressió de proteorodopsina va ser estimulada en condicions de llum i no en condicions de fosca. La transferència d'un cultiu incubat a la fosca cap a condicions de llum va estimular l'expressió de proteorodopsina a nivells similars als dels cultius incubats a la llum. Aquestes dades reforcen la idea que les proteorodopsines medien l'entrada d'energia lumínica a les xarxes tròfiques microbianes que habiten la superfície dels oceans.

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"...La llarga tarda miro, amb pauses d'or i somni..." Salvador Espriu

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