MULTIPLE STRESSOR EFFECTS ON RIVER BIOFILM COMMUNITIES: FROM COMMUNITY COMPOSITION TO ECOSYSTEM PROCESSES USING EXPERIMENTAL MESOCOSMS

Ferran Romero Blanch

Universitat de Girona

ICRA Institut Català de Recerca de l'Aigua

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Multiple stressor effects on river biofilm communities

From community composition to ecosystem processes using experimental mesocosms

Ferran Romero Blanch

Tutored and supervised by

DR. SERGI SABATER I CORTÉS
University of Girona
Catalan Institute for Water Research

Supervised by

DR. VICENÇ ACUÑA I SALAZAR
University of Girona
Catalan Institute for Water Research

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"Man is a part of nature,
and his war against nature is
inevitably a war against himself"

Rachel Carson (1907 - 1964)

A les activistes, les ecologistes, i tots els que utilitzen la ciència per omplir de raons la seva necessària lluita. Guanyarem.

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Ferran Romero,

Girona i Granollers
Octubre de 2019

Derived scientific publications

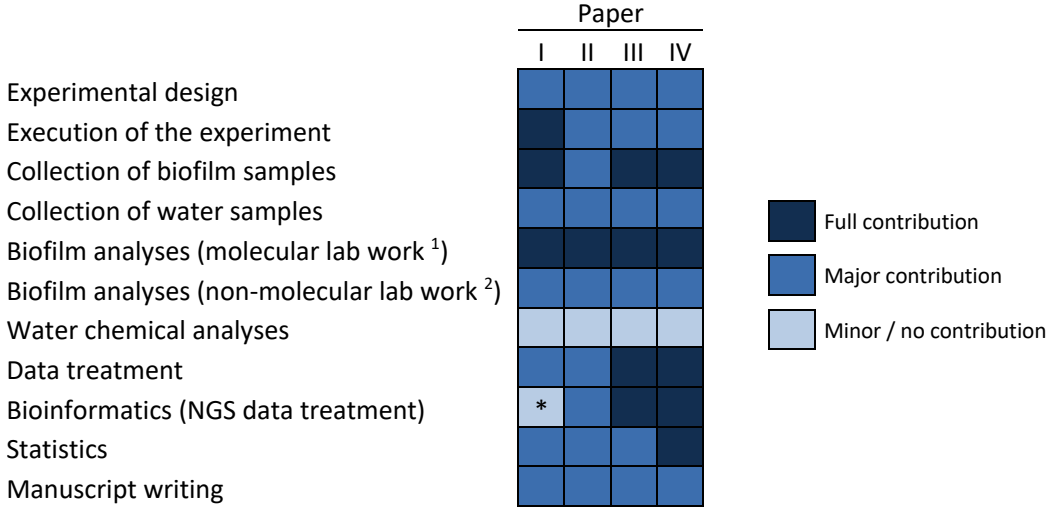
This thesis is based on the following manuscripts, which are referred to in the text by their Roman numerals. My contribution to each paper is depicted in the table below.

I. Romero, F., Sabater, S., Timoner, X., and Acuña, V. (2018) Multistressor effects on river biofilms under global change conditions. *Science of the Total Environment* 627: 1-10
Journal metrics (Scopus): CiteScore 2018 = **5.92**, Highest percentile = 5/117 Environmental Engineering (96th percentile).

II. Romero, F., Sabater, S., Font, C., Balcázar, J.L., and Acuña, V. (2019) Desiccation events change the microbial response to gradients of wastewater effluent pollution. *Water Research* 151: 371-380
Journal metrics (Scopus): CiteScore 2018 = **8.55**, Highest percentile = 1/203 Water Science and Technology (99th percentile).

III. Romero, F., Acuña, V., Font, C., Freixa, A., and Sabater, S. Effects of multiple stressors on river biofilms depend on time scale. Accepted in *Scientific Reports*
Journal metrics (Scopus): CiteScore 2018 = **4.29**, Highest percentile = 6/90 Multidisciplinary (93rd percentile).

IV. Romero, F., Acuña, V., and Sabater, S. Multiple stressors determine structure and estimated function of river biofilm bacterial communities. [Manuscript]



1. Including e.g. nucleic acid extraction, purification and quantification, PCR, RT-PCR and qPCR.
2. Including e.g. photosynthetic, enzymatic, and metabolic measurements.
* Note that Paper I does not include a next-generation sequencing (NGS) technique.

Additional scientific publications

Additionally, I have also co-authored the following paper:

Acuña, V., Casellas, M., Font, C., Romero, F., Sabater, S. (2019) Nutrient dynamics in effluent dominated watercourses. *Water Research* 160: 330-338
Journal metrics (Scopus): CiteScore 2018 = **8.55**, Highest percentile = 1/203 Water Science and Technology (99th percentile).

List of acronyms

16S rRNA	16S ribosomal ribonucleic acid
AFDW	Ash-free dry weight
AMC	Amino methyl coumarin
ANOVA	Analysis of variance
BIOM	Biological observation matrix
CA	Concentration addition
cDNA	Complementary deoxyribonucleic acid
CR (Paper I)	Community respiration
D (Paper I)	Diuron
D (Paper II)	Desiccation
DF	Degrees of freedom
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
dsrA	Dissimilatory sulfite reductase subunit A-encoding gene
E (Paper I)	Erythromycin
EA	Effect addition
EPS	Extracellular polymeric substances
ES	Effect size
ESF	Experimental streams facility
F	F-value
F_0	Basal fluorescence
GLM	Generalized linear model
H (Paper III, IV)	Hydrological stress
ICRA	Catalan institute for water research
IPCC	Intergovernmental panel for climate change
LAPA	Leucine aminopeptidase activity
LOD	Limit of detection
LOQ	Limit of quantification
mcrA	Methyl coenzyme M reductase-encoding gene
MS	Mass spectrometer
MS	Mean of squares
narG	Nitrate reductase-encoding gene
NCBI	National Center for Biotechnology Information
NH_4^+	Ammonium
nMDS	Non-metric multidimensional scaling
NO_3^-	Nitrate

NPQ	Non-photochemical quenching
OTU	Operational taxonomic unit
P (Paper III, IV)	Pesticides
P	P-value
PAR	Photosynthetic active radiation
PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis of variance
PO_4^{3-}	Phosphate
psaA	Photosystem I P700 chlorophyll-a apoprotein A1-encoding gene
psbA	Photosystem II protein D1-encoding gene
PSII	Photosystem II
PVDF	Polyvinylidene fluoride
qPCR	Quantitative (or real-time) polymerase chain reaction
QTRAP	Quadrupole linear ion trap mass spectrometer
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RSE	Relative standard error
S (Paper III, IV)	Substratum type
S	Substratum type
SAM	Stress addition model
SIMPER	Similarity percentages
SPE	Solid-phase extraction
SS	Sum of squares
T (Paper I)	Temperature
T (Paper III)	Time
TITAN	Threshold indicator taxa analysis
UHPLC	Ultra high performance liquid chromatography
W (Paper I)	Desiccation
W (Paper III, IV)	Warming
We	Wastewater treatment plant effluent
WFD	Water framework directive
WWTP	Wastewater treatment plant
Y_{eff}	Photosynthetic efficiency
Y_{max}	Photosynthetic capacity

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Summary

Human activity worldwide exposes aquatic ecosystems to multiple anthropogenic stressors. Freshwater ecosystems (e.g. rivers and streams) are of special concern because of their notable sensitivity to stressors and relevance for global biodiversity and human well-being. Multiple-stressor effects on freshwater ecosystems depend on stressor nature, level and spatial/temporal scale, and their combined effects do not always match the predictions built upon knowledge about individual effects, producing the so-called non-additive responses. Non-additive responses include *synergisms*, which refers to combined effects surpassing the sum of individual effects, and *antagonisms*, when the opposite occurs (i.e. one stressor mitigating the effect of another). Among the many stressors that threat freshwater ecosystems, those derived from land-use change include the release of many pollutants into rivers and streams flowing through urban and agricultural areas. Also, climatic stressors such as warming, and others related to human action such as hydrological stress, affect river ecosystems on a global scale by modifying biodiversity patterns and ecosystem functioning. Among the many organisms exposed to multiple stressors in freshwater ecosystems, those attached to river and stream sediments (i.e. *river biofilms*) play a crucial role in virtually all major ecosystem processes and are frequently used as sentinels when assessing stressor impacts on freshwater ecosystems.

This thesis aims to *identify the single and multiple-stressor effects of warming, hydrological stress and pollutant exposure on river biofilms*. To that purpose, I used several experimental approaches, consisting on glass crystallizers (i.e. microcosms, Paper I) and artificial streams (i.e. mesocosms, Paper II, III and IV) to expose epilithic (i.e. growing on cobbles) and epipsammic (i.e. growing on fine sediments) river biofilms to single and multiple-stressor scenarios under controlled conditions. I included among the stressors individual pollutants (Paper I), as well as complex mixtures (Paper II, III and IV), climatic stressors such as warming (Paper I, III and IV), and hydrological stress (Paper I, II, III and IV). Stressor levels in the experimental designs were generally simplified to two; i.e. presence (treatment) vs. absence (control) of the stressor. I also employed a regressional experimental design to test different stressor levels (Paper II), and search for potential thresholds. In all the above designs, I tested the river biofilm response both at the structural and the functional scale, employing response variables that ranged from photosynthetic and enzymatic activity to gene expression and bacterial community composition.

I detected that hydrological stress was the most influential stressor, specially impairing the biofilm community growing on cobbles (epilithic). Water warming had lesser effects, mostly affecting bacterial activity due to the dependence of metabolic activity on temperature, but showed limited effects on bacterial community composition (Paper IV). Pollutant exposure

had contrasting results depending on the nature of the pollutant used. Single pollutants (Paper I; herbicide, antibiotic) as well as the pesticide mixture (Paper III, IV) shaped biofilm community structure and function according to their mode of action. The antibiotic erythromycin mostly impaired the bacterial community, while the herbicide diuron affected the phototrophs. The complex mixture used in Paper II (i.e. WWTP effluent) induced significant shifts in community structure at WWTP effluent proportions above 50 % of the total stream flow.

I made a main objective of the thesis determining the type of response which might be produced when biofilms are affected by multiple stressors. Additive responses were prevalent in most cases, while non-additive responses accounted between 14.5 % (Paper I) and 29 % (Paper III) of all interactions. Among significant interactions, antagonisms dominated in all cases, representing between 59 % (Paper III) and 89 % (Paper IV) of all biofilm responses, while synergisms were less dominant and relegated to the epilithic biofilm.

The results presented in this thesis show that single and multiple stressors affect both biofilm community structure and function, and emphasize that river biofilms show an adaptive nature when facing multiple-stressor scenarios.

Resum

L'impacte de l'activitat humana a escala global exposa els ecosistemes aquàtics a múltiples estressors d'origen antropogènic. Els ecosistemes d'aigua dolça (p. ex. rius i rierols) són d'especial interès per ser especialment sensibles a l'estrès i per la seva contribució a la biodiversitat del planeta i al benestar de l'ésser humà. Els efectes de múltiples estressors en els ecosistemes d'aigua dolça depenen de la natura de l'estressor en qüestió i del nivell d'exposició a escala espaial/temporal, donant lloc a efectes combinats que no sempre coincideixen amb les prediccions procedents dels efectes individuals, produint els anomenats efectes *no additius*. Els efectes no additius inclouen els sinergismes, que es produeixen quan l'efecte combinat de dos o més estressors supera la suma dels efectes individuals, i els antagonismes, que succeeixen quan s'observa el contrari (és a dir, un estressor atenua l'efecte d'un altre). Dels estressors que afecten els ecosistemes d'aigua dolça, aquells que deriven dels canvis en els usos del sòl inclouen l'entrada de contaminants en rius i rierols que travessen zones urbanes i agrícoles. A més, estressors climàtics com l'increment de temperatura i altres relacionats amb l'activitat humana com l'estrès hídric, afecten rius i rierols a escala global modificant els patrons de biodiversitat i el funcionament dels ecosistemes. Dels organismes exposats a múltiple estressors en ecosistemes d'aigua dolça, aquells adherits als sediments (és a dir, el biofilm de riu) juguen un paper essencial a la majoria de processos ecosistèmics i son freqüentment usats com a sentinelles per avaluar l'impacte dels estressors en ecosistemes d'aigua dolça.

Aquesta tesi té per objectiu identificar els efectes individuals i múltiples de l'increment de temperatura, l'estrès hídric i l'exposició a contaminants en biofilm de riu. Per això, he usat diferents aproximacions experimentals, consistents en cristal·litzadors de vidre (és a dir, microcosmos, Article I) i rius artificials (mesocosmos, Article II, III i IV) per exposar biofilm epilític (és a dir, que es desenvolupa sobre roques) i epipsàmmic (que es desenvolupa sobre sediment fi) a escenaris d'estrès individual i múltiple en condicions controlades. Entre els estressors usats, s'inclouen contaminants individuals (Article I), així com mescles complexes (Article II, III i IV), i estressors climàtics com l'increment de temperatura (Article I, III i IV) i l'estrès hídric (Article I, II, III i IV). Els nivells dels estressors emprats han estat generalment simplificats a dos; és a dir, presència (tractament) vs. absència (control) de l'estressor. També he emprat un disseny regressional per avaluar diferents nivells d'un mateix estressor (Article II), i buscar potencials llindars d'estrès. En tots els dissenys mencionats, he avaluat la resposta del biofilm de riu a nivell tant estructural com funcional, emprant variables resposta que inclouen des de l'activitat fotosintètica i enzimàtica fins a l'expressió gènica i la composició de la comunitat bacteriana.

He observat que l'estrès hídric és l'estressor més influent, alterant especialment la comunitat del biofilm que es desenvolupa sobre les roques (epilítica). L'increment de temperatura de l'aigua té menor efecte, alterant sobretot l'activitat bacteriana degut a la dependència de l'activitat metabòlica en la temperatura, però mostrant efectes limitats a nivell de la composició de la comunitat bacteriana (Article IV). L'exposició a contaminats resulta en efectes oposats depenent del contaminant emprat. Contaminants individuals (Article I, herbicida, antibiòtic), així com la mescla de pesticides (Article III, IV) van modificar l'estructura i la funció del biofilm d'acord al seu mode d'acció. L'antibiòtic eritromicina va afectar essencialment la comunitat bacteriana, mentre que l'herbicida diuró va afectar els fotòtrofs. La mescla complexa usada a l'Article II (és a dir, l'efluent d'EDAR) va provocar canvis significatius en l'estructura de la comunitat a proporcions d'efluent superiors al 50 % del total del cabal del riu artificial.

Determinar el tipus de resposta produïda quan els biofilms es troben afectats per múltiples estressors va ser un dels objectius principals d'aquesta tesi. Les respostes additives van predominar a la majoria de casos, mentre que les no additives van representar entre el 14.5 % (Article I) i el 29 % (Article III) de totes les respostes del biofilm. De les interaccions significatives, els antagonismes van dominar en tots els casos, representant entre el 59 % (Article III) i el 89 % (Article IV) de les interaccions, mentre que els sinergismes van ser menys predominants i relegats al biofilm epilític.

Els resultats presentats en aquesta tesi mostren que estressors individuals així com combinacions dels mateixos afecten tant l'estructura com la funció del biofilm, i emfatitzen la naturalesa adaptativa que presenten els biofilms de riu quan s'enfronten a escenaris de múltiples estressors.

Resumen

El impacto de la actividad humana a escala global expone los ecosistemas acuáticos a múltiples estresores de origen antrópico. Los ecosistemas de agua dulce (p. ej. ríos y arroyos) son de singular interés por ser especialmente sensibles al estrés y por su contribución a la biodiversidad del planeta y al bienestar del ser humano. Los efectos de múltiples estresores en los ecosistemas de agua dulce dependen de la naturaleza del estresor en cuestión y del nivel de exposición a escala espacial/temporal, dando lugar a efectos combinados que no siempre coinciden con las predicciones procedentes de los efectos individuales, produciendo los llamados efectos *no aditivos*. Los efectos no aditivos incluyen los *sinergismos*, que se producen cuando el efecto combinado de dos o más estresores supera la suma de los efectos individuales, y los *antagonismos*, que suceden cuando ocurre el contrario (esto es, un estresor atenúa el efecto de otro). De los estresores que afectan los ecosistemas de agua dulce, aquellos que derivan de los cambios en los usos del suelo incluyen la descarga de contaminantes en ríos y arroyos que traviesan áreas urbanas y agrícolas. Además, estresores climáticos como el incremento de temperatura y otros relacionados con la actividad humana, como el estrés hídrico, afectan ríos y arroyos a escala global modificando los patrones de biodiversidad y el funcionamiento de los ecosistemas. De los organismos expuestos a múltiples estresores en ecosistemas de agua dulce, aquellos adheridos a los sedimentos (esto es, el biofilm de río) juegan un papel esencial en la mayoría de procesos ecosistémicos y son frecuentemente usados como centinelas al evaluar los impactos de los estresores en los ecosistemas de agua dulce.

Esta tesis pretende *identificar los efectos individuales y múltiples del incremento de temperatura, el estrés hídrico y la exposición a contaminantes en biofilm de río*. Para ello, he usado varias aproximaciones experimentales, consistentes en cristalizadores de vidrio (esto es, microcosmos, Artículo I) y ríos artificiales (mesocosmos, Artículo II, III y IV) para exponer biofilm epilítico (esto es, que se desarrolla sobre rocas) y epipsámico (que se desarrolla sobre sedimento fino) a escenarios de estrés individual y múltiple en condiciones controladas. Entre los estresores empleados, he incluido contaminantes individuales (Artículo I), así como en mezclas (Artículo II, III y IV), estresores climáticos como el incremento de temperatura (Artículo I, III y IV) y el estrés hídrico (Artículo I, II, III y IV). Los niveles de los estresores en los diseños experimentales han sido generalmente simplificados a dos; presencia (tratamiento) vs. ausencia (control) del estresor. También he empleado un diseño regresional para evaluar diferentes niveles de un mismo estresor (Artículo II), y buscar potenciales lindares de estrés. En todos los diseños mencionados, he evaluado la respuesta del biofilm de río tanto a nivel estructural como funcional, empleando variables respuesta que cubren desde la actividad

fotosintética y enzimática hasta la expresión génica y la composición de la comunidad bacteriana.

He observado que el estrés hídrico es el estresor más influyente, alterando especialmente la comunidad del biofilm que se desarrolla en las rocas (epilítica). El incremento en la temperatura del agua muestra menor efecto, alterando sobretodo la actividad bacteriana debido a la dependencia de la actividad metabólica en la temperatura, pero mostrando efectos limitados en la composición de la comunidad bacteriana (Artículo IV). La exposición a contaminante(s) resultó en efectos opuestos dependiendo de la naturaleza del contaminante usado. Contaminantes individuales (Artículo I, herbicida, antibiótico), así como la mezcla de pesticidas (Artículo III, IV) modificaron la estructura y la función del biofilm acuerdo a su modo de acción. El antibiótico eritromicina afectó esencialmente la comunidad bacteriana, mientras que el herbicida diurón afectó a los fotótrofos. La mezcla usada en el Artículo II (esto es, el efluente de EDAR) provocó cambios significativos en la estructura de la comunidad a proporciones de efluente superiores al 50 % del total del caudal del río artificial.

Determinar el tipo de respuesta producida cuando el biofilm se ve afectado por múltiples estresores fue uno de los objetivos principales de esta tesis. Las respuestas aditivas predominaron en la mayoría de casos, mientras que las no aditivas representaron entre el 14.5 % (Artículo I) y el 29 % (Artículo III) de todas las interacciones. De las interacciones significativas, los antagonismos dominaron en todos los casos, representando entre el 59 % (Artículo III) y el 89 % (Artículo IV) de todas las respuestas del biofilm, mientras que los sinergismos fueron menos predominantes y relegados al biofilm epilítico.

Los resultados presentados en esta tesis muestran que estresores individuales así como combinaciones de los mismos afectan tanto la estructura como la función del biofilm, y enfatizan la naturaleza adaptativa que presenta el biofilm de río cuando se ve expuesto a escenarios de estrés múltiple.

01

Introduction

Introduction

Overview of freshwater ecosystems

Planet Earth's surface is mainly covered by water, occupying an estimated total volume of 1.38 x 10^9 Km^3, and covering 70 % of the total surface. A 3.9 % of water is continental and include streams and rivers as well as polar ice and groundwater. Streams and rivers represent only 0.02 % of the continental water but are of critical importance to biodiversity and human well-being.

Humanity relies on fresh water for food production, water supply, climate regulation, disease prevention, cultural attributes, and soil formation, to name a few (Millenium Ecosystem Assessment, 2005). Among freshwater ecosystems, large rivers but also small streams (e.g. headwater streams) are known to provide essential ecosystem services such as water provisioning, recharge of ground waters, flood control, trapping of sediments and pollutants, nutrient recycling and support of downstream water quality and productivity (Böck *et al.*, 2018). Also in line with this are temporary streams, which cease flow in some point on a temporal or spatial scale; they support important ecosystem services including local aquifer recharge and energy flow to permanent reaches in arid and semi-arid regions such as the Mediterranean (Acuña *et al.*, 2014).

Many of the ecosystem services provided by rivers and stream rely on local ecosystem processes which, in turn, are highly dependent on the extraordinarily rich biological diversity found in these systems; disproportionate in its relevance according to the volume of water they occupy. The ~125 000 species of freshwater animals that have so far been described represent 9.5 % of all known animal species on the planet, and include 1/3 of all vertebrate species (Strayer and Dudgeon, 2010). Although less represented than animals, freshwaters also contain non-negligible numbers of vegetal species, most of them being aquatic macrophytes (Chambers *et al.*, 2008). Rivers and streams are also extremely rich in terms of microbial organisms. Debroas *et al.*, 2017 estimated to 200 000 the number of microbial eukaryotic species, clustered in at least 1200 different phylogenetic units, most of them within the Fungi kingdom. Zeglin, 2015 synthesized existing research concerning prokaryote diversity across different river compartments and showed that river prokaryotic diversity contains ~10 major groups (i.e. classes), mostly included within the Proteobacteria phylum. Most of these microorganisms (both eukaryotic and prokaryotic) are found in close contact with the sediments at the bottom of rivers and streams, where they are known as *biofilms*.

Freshwater ecosystems and global change

Human use of water resources has led to the over-exploitation and degradation of freshwater ecosystems, with most of the annual global supply of fresh water being derived for human use, with widespread negative consequences for freshwater resources on a global scale (Vörösmarty *et al.*, 2010). This is particularly the case of arid and semi-arid regions, such as the Mediterranean. River biodiversity is currently suffering from changes in river flow, geomorphology and habitat availability, especially as a consequence of five major processes, namely water abstraction, pollution, the modification of flow regimes, the degradation of river habitats and the spread of invasive species (Foley *et al.*, 2005; Dudgeon, 2010; Vörösmarty *et al.*, 2010). In their global scale assessment, Sala *et al.*, 2000 identified climate and land-use change as the main threats for freshwater ecosystems, increasing the uncertainty over the impacts of current and future human pressures on rivers and streams. This translates into most of the ecosystem services being degraded, according to the Millennium Ecosystem Assessment (Millenium Ecosystem Assessment, 2005).

The main anthropogenic threat to freshwater ecosystems and the services they provide is *land-use change*, which is largely accepted to have widespread negative effects on rivers and streams flowing through human-modified lands (Allan, 2004; Cooper *et al.*, 2013). By clearing tropical forests, intensifying farmland production, or expanding urban centers, human actions are changing the world's landscapes to the point that croplands and pastures have become one of the largest biomes on the planet, occupying ~40 % of the land surface (Foley *et al.*, 2005). As a result, excess nutrients and toxic pesticides are currently transported into rivers and streams from adjacent land by rainfall runoff after storm events (Matson *et al.*, 1997). On the other hand, more than 50 % of the global population nowadays lives in urban areas, and this figure is expected to push towards 70 % before 2050 (Leeson, 2018). Urban areas are known to have consistent effects on surrounding water bodies, including decreased water quality, habitat alteration and reduction in biodiversity, due to the significant loads of pollutants from point and diffuse sources (Walsh *et al.*, 2005). One of the main contributors to point-source pollution in urban rivers and streams are wastewater treatment plant (WWTP) effluents. WWTPs are efficient in removing nitrogen and phosphorus from wastewaters, therefore improving water quality parameters such as the biological oxygen demand, however, they are usually not able to cope with emerging pollutants such as pharmaceutical compounds or pesticides, and they end up being transported through their effluents into rivers and streams (Kolpin *et al.*, 2002; Luo *et al.*, 2014; Huerta *et al.*, 2015). WWTP effluents can make up a significant proportion of the flow of the receiving water body, especially under high anthropogenic pressure conditions (e.g. water abstraction). A recent analysis of the

current situation in the US showed that WWTP effluents make up more than 50 % of the river flow for over 900 receiving systems at the point of discharge (Rice and Westerhoff, 2017).

Apart from land modification, *climate change* aggravates many ecological problems within freshwater ecosystems due to the anthropogenic production of energy from fossil sources and the subsequent emission of greenhouse gases to the atmosphere (Döll and Zhang, 2010). Climate change is predicted to alter weather patterns including global temperatures and rainfall/drought events, widely impacting freshwater ecosystems and their associated biodiversity around the globe (Strayer and Dudgeon, 2010; Smith, 2011; Ledger and Milner, 2015). The predictions point towards increases in air temperature and more recurrent extreme weather events, while altered spatial patterns in precipitation and runoff (IPCC, Climate Change, 2014). The Intergovernmental Panel on Climate Change (IPCC) predicts continued increases in greenhouse gases will push temperatures by 2 – 4.5 °C in the next 50 years. Annual average river runoff might increase by 10 – 40 % at higher latitudes and decrease by 10 – 30 % over some dry regions (IPCC, Climate Change, 2014).

Thus, a profound understanding about the response of streams and rivers to global change (i.e. the combination of land use and climate change) is the necessary path to mitigate the current degradation of these ecosystems, assuring the short and long-term access to their ecosystem services and securing current and future human well being.

Multiple stressors and freshwater ecosystems

These land-use related (e.g. chemical pollution) and climatic (e.g. warming, hydrological stress) stressors often occur in the same temporal and spatial scale, impacting ecosystems from individual species to communities (Segner *et al.*, 2014). Recent literature reviews suggest that these so-called multiple stress situations are (and will be) more frequent due to global change (Ormerod *et al.*, 2010; Côté *et al.*, 2016; Jackson *et al.*, 2016; Nõges *et al.*, 2016; Schinegger *et al.*, 2016a; Sabater *et al.*, 2019). A recent review of 75 scientific papers dealing with the effects of multiple stressors in European freshwater bodies found that the most pervasive stressors were nutrient concentration and hydrological alterations, either alone or in combination with additional stressors (Nõges *et al.*, 2016). Schinegger *et al.*, 2012 analyzed multiple stressors on fish and benthic invertebrate communities at 9330 river sites in Europe and found that 47% of the sites were affected by more than one stressor at the same time. In line with this, evaluation of river ecosystem monitoring data by Schäfer *et al.*, 2016 found that multiple-stressor scenarios are the prevailing situation among German rivers. Despite of this, the focus has mostly been placed in the study of single stressors, as shown for urban estuarine ecosystems (O'Brien *et al.*, 2019). In their systematic review of global literature, O'Brien *et al.*, 2019 found that only 7% of the selected 579 studies specifically addressed the interaction

between multiple stressors. In line with this, most of the papers in the literature review by Nõges *et al.*, 2016 dealt with the effects of single stressors or, at almost, with a paired combination of stressors.

We currently address multiple-stressor effects by means of field surveys, manipulative experiments, and mathematical models. Field surveys represent a useful approach to derive general trends from *in-situ* observations (Sabater *et al.*, 2016). However, the complexity of natural settings includes a myriad of confounding factors that may hinder our capacity to derive sound conclusions and link stressor effects to ecosystem responses. On the other hand, manipulative experiments usually rely on factorial experimental designs where the complexity of a natural setting is reduced to only a few factors under highly controlled conditions (Brennan and Collins, 2015; Jeremy J. Piggott, Townsend, *et al.*, 2015a). Under these circumstances, analyses of variance (e.g. ANOVAs) allow identifying interaction effects, defined as responses deviating from simple addition null models. Given that manipulative experiments usually mimic natural conditions only partially, results derived from them should be used to build testable hypotheses under field conditions. Finally, efforts have been done in the recent years to develop mathematical models aimed at providing a predictive understanding of multiple-stressor effects (Schäfer and Piggott, 2018). For chemical stressors, concentration addition (CA) models have classically been used to predict multiple-stressor effects when stressors mode-of-action is similar, whereas effect addition models predict the interaction effects between stressors with dissimilar mode-of-action (Cleuvers, 2003). To move beyond chemical stressors and predict the effects of multiple stressors, the impacts on organisms need to be comparable. To address this, Liess *et al.*, 2016 propose a general stress measure (e.g. mortality) as a "common currency", within the context of the stress addition model (SAM). Provided that stressors generally act at the individual level, a predictive understanding of multiple-stressor effects at the community level depends on the sensitivity distribution across individuals. Moreover, stressor-effect relationships at the community level are highly influenced by biotic interactions and dispersal dynamics. Taken together, these issues impose the simple addition null model as the option of choice when dealing with the effects of multiple stressors at the community level (Schäfer and Piggott, 2018). There is an urgent need to provide data that could be used in the development of new null models, as we currently lack a solid theoretical framework for prediction of multiple-stressor effects at the community level (Kaunisto *et al.*, 2016).

Under the assumptions of the addition null model, multiple stressors can lead to additive outcomes, i.e. the combined effect of two or more stressors equals the sum of the individual effects (Figure I.1). When the combination is non-additive (Folt *et al.*, 1999), it can be depicted into antagonism or synergism, depending on the negative (antagonism) or positive (synergism) effect that one stressor has on the other. In Figure I.1, the conceptual approach

described by Crain *et al.*, 2008 to interpreting interaction types in factorial studies is summarized. Given a control situation (i.e. no stress whatsoever) and two hypothetical stressors (A and B), antagonistic and synergistic interactions are defined according to the response value obtained under exposure to both stressors (A+B) compared to the additive prediction, which implies that the combined effect of stressors A and B is directly the sum of their individual effects. Recent literature reviews have emphasized that interactions in freshwater ecosystems may account for 40% to 69% of all ecological responses (Jackson *et al.*, 2016; Schinegger *et al.*, 2016a), although the prevalence of different types of interactions varies across meta-analyses (Côté *et al.*, 2016). The level of biological organization could partially explain this variability; antagonisms tend to dominate at the community and ecosystem level, whereas synergism is closely related to individual physiological responses (Côté *et al.*, 2016). Despite the indisputable significance of multiple stressors, there is still work to do concerning their interaction effects, especially when dealing with the mechanistic understanding of how multiple stressors influence freshwaters and the ecosystem services they provide (Johnson and Penaluna, 2019).

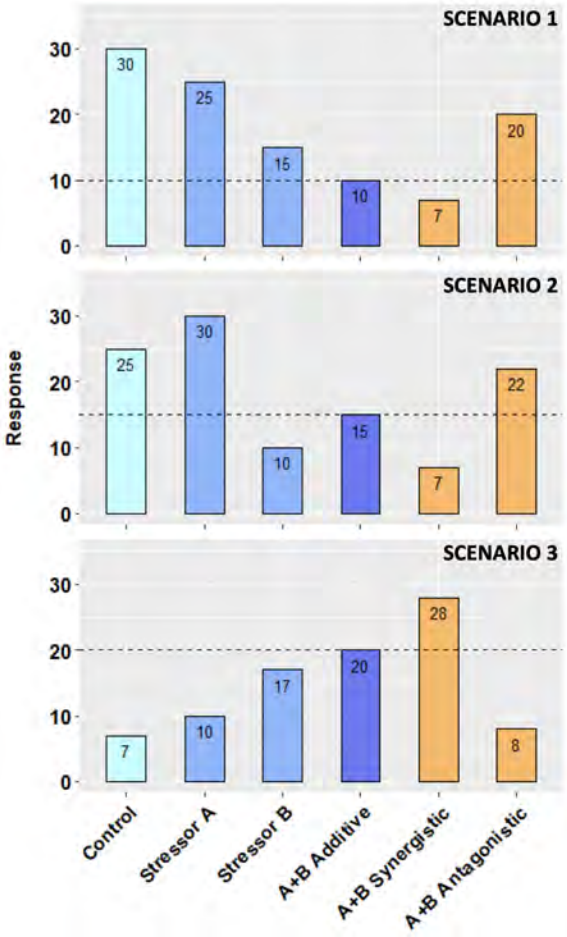


Figure I. 1 Conceptual approach to interpreting interaction types used in this thesis. Treatments in factorial designs include, at least; control, stressor A, stressor B and the combination of both stressors (A+B). The additive prediction implies that the combined effect of A and B equals the sum of individual effects. When at least one of the stressors has a negative impact on a given response variable (scenarios 1 and 2), the interaction is synergistic if the A+B response is inferior to the additive prediction, and antagonistic if the A+B response is superior to the additive prediction. If stressors A and B both have a positive impact on the response variable (scenario 3), the interaction is synergistic if the A+B response is superior to the additive prediction, and antagonistic if the A+B response is inferior to the additive prediction. Re-drawn from Crain et al., 2008.

River biofilms as a model community for the assessment of multiple stressors

Addressing freshwater biodiversity loss requires understanding interactive stressor effects on structure and functioning of affected ecosystems. Freshwater monitoring programs have classically relied on structural changes within the macrobiotic community to assess the ecological status of freshwater bodies, following the guidelines from the EU Water Framework Directive (WFD). Because of their distribution, size and phenotypic variation, the organisms most commonly associated to multiple stressor studies are invertebrates, fish, and macrophytes (Nõges *et al.*, 2016). In line with this, the variables most often analyzed are community composition and total biomass, although some functional variables such as growth or photosynthetic activity are also measured (Corcoll *et al.*, 2015a; Jeremy J. Piggott, Townsend, *et al.*, 2015a; Magbanua *et al.*, 2015; Ponsatí *et al.*, 2016). A rising number of papers in the recent years are demonstrating that integrative measurements such as gross primary production, community respiration and organic matter decomposition can reliably be used to gain mechanistic understanding on the effects of multiple stressors in freshwater ecosystems (von Schiller *et al.*, 2017; Smeti *et al.*, 2019).

Among the many freshwater organisms inhabiting rivers and streams, the ones embedded in biofilms are of special concern because of their diversity, abundance and key ecological features (Besemer *et al.*, 2012; Battin, Besemer, Bengtsson, Romani, *et al.*, 2016). River (or stream) biofilms are assemblages of diverse groups of microorganisms such as bacteria, algae and protozoans growing on surfaces and which are modulated by environmental biotic and abiotic factors (Romani *et al.*, 2017). On the whole, recent estimates indicate that between 40% and 80% of all bacterial cells in Earth reside in biofilms (Flemming and Wuertz, 2019). Key features of biofilms include the production of an extracellular polymeric substances (EPS) matrix, which allows the retention of extracellular enzymes, providing an external digestion system, while acting as a barrier for biocides and other chemical and physical stressors (Flemming and Wingender, 2010). The EPS matrix also retains nutrients, as well as inorganic chemicals, turning biofilms into reliable hotspots for nutrient cycling and pollutant transformation. In rivers and streams, biofilms are key players in many ecosystem processes such as pollution degradation, primary production, retention of organic and inorganic nutrients, and support of food webs (Underwood *et al.*, 2005; Battin *et al.*, 2007; Battin, Besemer, Bengtsson, Romani, *et al.*, 2016).

In aquatic environments, biofilms can be highly diverse depending on the substratum where they develop (Figure I.2); biofilms grow upon inert substrata such as sand, sediment, rocks and cobbles, non-living organic substrata such as wood, leaf litter or particulate organic matter, and living plants such as aquatic macrophytes and macroalgae (Romani *et al.*, 2017). River biofilms attached to rock surfaces (also to gravel and cobbles) are referred to as *epilithic biofilms* and, compared to other biofilms, such as those growing on sand, they have a more complex structure with a higher algal biomass (Romaní, 2009). Data from river epilithic biofilms show a proportion of total carbon of 60 – 90 % for algae, 10 – 40 % for EPS, 1 – 5 % for bacteria and less than 1 % for fungi (Romaní, 2009; Romani *et al.*, 2017). However, in shaded environments (i.e. forested streams), heterotrophic biomass (bacteria, fungi and protozoa) become more important (Romaní *et al.*, 2014; Wagner *et al.*, 2015). On the other hand, river biofilms attached to the particles of fine sediments (e.g. sand) are referred to as *epipsammic biofilms*. In rivers, the biofilm developing on sediment has been defined as playing a key role in organic matter decomposition, also being more heterotrophic (with higher contributions of bacteria and fungi) than the biofilm developing on rocks (Brablcová *et al.*, 2013; Timoner *et al.*, 2014). In an environmental scale, the activities of organisms inhabiting river biofilms range from the microlevel (e.g. localized adsorption of nutrients, surface secretion of exoenzymes) through community dynamics (interactions within planktonic and benthic populations) to large-scale environmental effects. Thus, river biofilms are strongly influenced by their physical and chemical environment, integrating changes at temporal scales that range from hours to months (Sabater, Guasch, Ricart, *et al.*, 2007). This, together with their diverse nature and crucial role in maintaining ecosystem stability make river biofilms good candidates to analyze the interactive effects of multiple stressors on freshwater ecosystems.

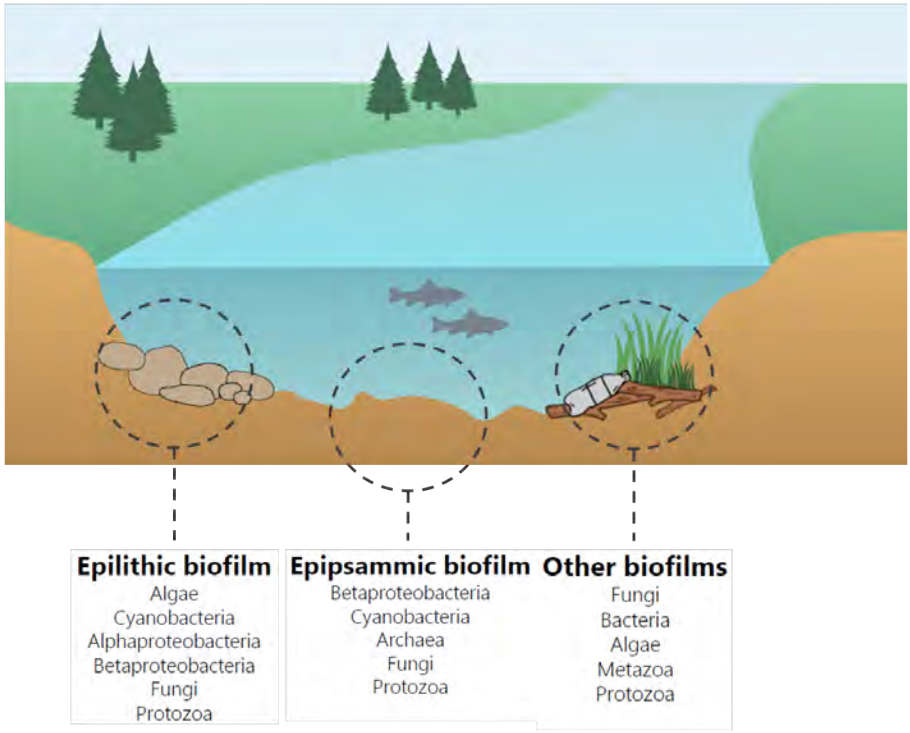


Figure I. 2 Biofilm types according to substratum type and main components. "Other" includes biofilms growing on wood debris (i.e. epixylic), aquatic macrophytes (i.e. epiphytic), and plastic litter (i.e. epiplastic). Own source.

Global change affects river biofilms in several ways; firstly, land-use change profoundly alters river water quality in terms of increased dissolved nitrogen and phosphorus concentrations, which is known to reduce the microbial diversity within river biofilms (Zeglin, 2015). Apart from this, land-use change promotes the accumulation of toxic chemicals (e.g. antibiotics and pesticides) in water, which also contributes to the loss of diversity and functional capacities in river biofilms (Ponsatí *et al.*, 2016). Among climatic stressors, warming is known to alter key trophic interactions within river biofilms, altering ecological rates such as feeding and growth, with potential implications across food webs (Clarke, 2006; Kathol *et al.*, 2009). Hydrological stress, in turn, has been demonstrated to drive river biofilm communities towards those occurring in soils (Pohlon *et al.*, 2013); these structural effects, which translate into altered function of the microbial food web, are especially manifest in temperate rivers and streams, which do not regularly suffer from desiccation events (Ylla *et al.*, 2014a; Pohlon *et al.*, 2018).

Among the microbial organisms defining river biofilms, an often overlooked group is prokaryotes. This could be partially explained because the study of bacteria and archaea has classically relied on cultivation-dependent methods, which are both time-consuming and not sufficiently accurate, as the amount of bacterial/archaeal species able to grow in culture is generally estimated to be < 1 %, although this paradigm has been recently put into doubt (Martiny, 2019). Conveniently, next-generation sequencing technologies, such as the high-throughput sequencing of the 16S rRNA bacterial gene marker, are becoming accessible for researchers and monitoring programs. Thus, large datasets containing valuable information about microbial diversity are becoming increasingly available, which has enabled a more detailed understanding of the composition and diversity patterns of bacterial communities within river biofilms (Besemer *et al.*, 2012; Wilhelm *et al.*, 2015; Zancarini *et al.*, 2017; Simonin *et al.*, 2019). This information suggests that the Proteobacteria phylum generally dominates bacterial communities in river biofilms (Besemer *et al.*, 2012). At the level class, Alpha- and Betaproteobacteria tend to be the numerically dominant group in epilithic and epipsammic biofilms (Figure I.3), possibly because of their ability to degrade major components of DOM in river water (i.e. humic substances) and their tendency to form filamentous, and possibly grazing-resistant, morphologies (Rosenberg, 2013). Cyanobacteria, in turn, are much more associated to epilithic than epipsammic biofilms (Figure I.3), possibly because of their dependency on light and their preference for fixed, non-motile substrata (Cohen and Gurevitz, 2006). Although with lower relative abundance values than Proteobacteria and Cyanobacteria, the phylum Bacteroidetes also characterizes river biofilms, which is certainly related to their capacity to degrade a wide range of biopolymers, such as cellulose and chitin, largely contributing to DOM dynamics in rivers and streams (Wagner *et al.*, 2014; Wilhelm *et al.*, 2015). Other bacteria that are commonly found in river biofilms, but at lower relative abundance, include Gammaproteobacteria, Deltaproteobacteria, Actinobacteria, Firmicutes, Verrucomicrobia, Planctomycetes and Deinococcus–Thermus. Finally, next-generation sequencing data suggests that archaea generally constitute only a minor component of river biofilms, with the exception of biofilms developing on extreme environments, such as highly anoxic or sulfuric rivers (Besemer *et al.*, 2012).

Bacteria inhabiting biofilms play a major role in rivers and streams as they have the ability to break down a variety of chemical substances (Mitra and Mukhopadhyay, 2016). The family *Sphingomonadaceae*, for example, contains several genera which are major components of stream biofilms (e.g. *Erythromicrobium* and *Sphingopyxis*), especially during early stages of biofilm formation, and are known to use oxygenase enzymes to degrade a wide range of organic molecules, from plant-based to recalcitrant aromatic compounds (Aylward *et al.*, 2013). Other abundant taxa within stream biofilms include the genera *Flavobacterium* (phylum *Bacteroidetes*) and *Bacillus* (phylum *Firmicutes*), which degrade pesticide compounds

(e.g. organophosphates and carbamates) mainly via organophosphate-degrading hydrolases, and even use them as a growth substrate (Aislabie and Lloyd-Jones, 1995; Singh and Walker, 2006). A wide range of bacterial taxa is also able to degrade pharmaceutical compounds, among which, antibiotics have classically attracted much attention (Wright, 2005). Within stream biofilms, the families *Microbacteriaceae* (phylum *Actinobacteria*), *Bacillaceae* (phylum *Firmicutes*) and *Burkholderiaceae* (phylum *Proteobacteria*) possess a well-acknowledge capacity to hydrolyze a wide range of antibiotic compounds, from ß-lactams such as penicillin to structurally-complex antibiotic molecules including macrolides and glycopeptides (Topp *et al.*, 2013; Cycoń *et al.*, 2019).

Finally, bacteria have proved to be more sensitive indicators to historical chemical contamination than eukaryotes; in Birrer *et al.*, 2018, bacterial communities were affected at lower metal concentrations than eukaryotes. This could be explained by a lack of specific detoxification pathways, which is present in currently used organisms for biomonitoring, such as invertebrates and macroalgae (Campana *et al.*, 2012; Moenne *et al.*, 2016). The effects that toxicants (e.g. pesticides, antibiotics, metals…) and other stressors (e.g. warming, desiccation…) induce within bacterial communities indicate that they might be useful indicators of stress (Astudillo-García *et al.*, 2019), and therefore could be used to assess the interaction effects of multiple stressors (R K Salis *et al.*, 2017; Nuy *et al.*, 2018).

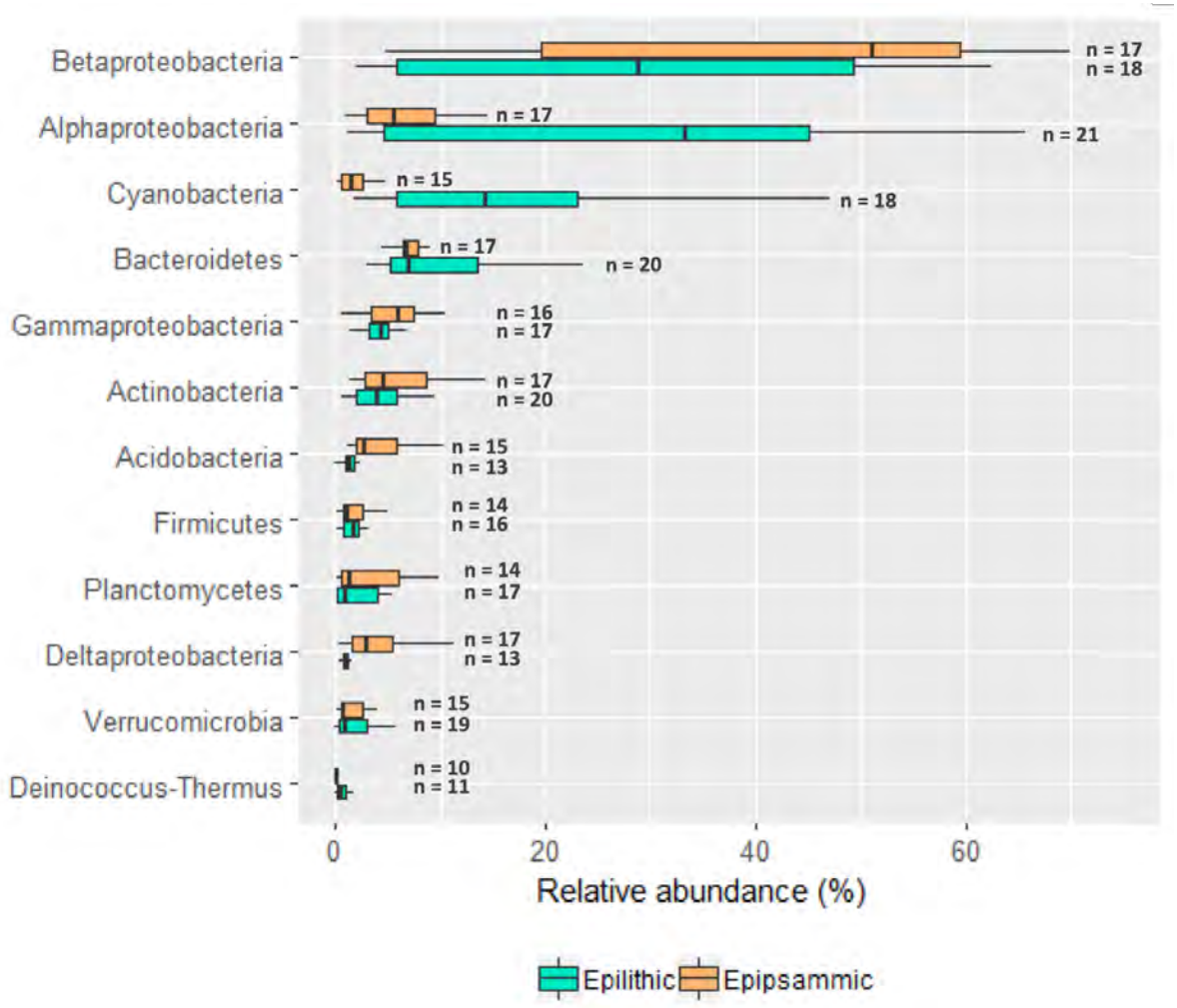


Figure I. 3 Major bacterial phyla (class for Proteobacteria) in epilithic and epipsammic stream biofilms. The values displayed (semi-transparent dots) are derived from a systematic review of 25 studies published between 2010 and 2019 using high-throughput 16S rRNA sequencing (i.e. Illumina MiSeq or 454 pyrosequencing) to assess bacterial community composition in stream biofilms from non-impacted sites. Boxplots represent 25th and 75th percentiles (left and right hinges, respectively), and 50th percentile (black hinge). Own source.

Thesis objectives and research questions

The overall aim of this thesis was to investigate the individual and combined effects of climatic (warming, hydrological stress) and land-use related (chemical pollutants) stressors on river biofilms (Table I.1). Special attention was paid to the bacterial community composition and how it responds to multiple-stressor scenarios. More specifically, I aimed to answer the following questions:

1. Which stressors have the greatest and most pervasive effects on river biofilms?

This question was investigated by means of full-factorial and regressional experimental designs. I hypothesized that climatic stressors (i.e. warming, hydrological stress) would show greater effects than chemical pollutants, due to non-specific alterations and overall metabolic disruption. River biofilms were subjected to combinations of different stressors (i.e. hydrological stress, warming, single pollutants and realistic pollutant mixtures), and the prevalence and effect size of each stressor was assessed. This question is specifically addressed in Papers I, II, III and IV.

2. Are stressor effects maintained at different stressor levels and exposure times?

This question was investigated by means of a regressional replicated-design, where river biofilms were subjected to a gradient of WWTP effluent dilution (Paper II). Here, I hypothesized that stressor effects would depend on stress intensity and exposure time. This question was also investigated in Paper III, where the response of river biofilms to a combination of stressors was assessed both after short and long-term exposure.

3. Are the responses to multiple stressors predictable from knowledge on individual-stressor effects?

This question was investigated by submitting river biofilms to single and multiple stressors following full-factorial and regressional experimental designs. By comparing multiple-stressor responses and individual effects, stressor combinations were classified into additive and non-additive. Non-additive combinations, in turn, were classified into antagonistic and synergistic. I hypothesized that additive responses would be the norm, but interactions would also arise. Among interactions, I expected antagonisms to prevail over synergisms. In Paper I, river biofilms were exposed to a combination of 4 stressors (i.e. antibiotic, herbicide, hydrological stress and warming), in Paper II, they were exposed to 2 stressors (i.e. a gradient of WWTP effluent dilution and hydrological stress), whereas in Paper III (and IV), they were exposed to 3 stressors (i.e. hydrological stress, warming and a mixture of pesticides).

4. Are bacterial communities sensitive organisms to decipher the interaction effects of multiple stressors on river ecosystems?

Special attention was paid to the bacterial community response, as bacteria have been systematically overlooked in previous research dealing with the effects of multiple stressors on aquatic biota (R K Salis *et al.*, 2017). This question was investigated by applying a metabarcoding approach to characterize the responses of bacterial communities after exposure to single and multiple-stressor conditions (Paper II and Paper IV). I hypothesized that single and multiple-stressor scenarios would shift bacterial communities toward increased abundance of stress-tolerant taxa.

STRESSOR		MODE OF ACTION	BIOFILM EXPECTED RESPONSE
HYDROLOGICAL STRESS		Osmotic pressure disruption Generation of free O_2 radicals	↓ Cell density ↓ Algal biomass, Chlorophyll-*a* ↑ Fungi and Gram+ bacteria
WARMING		Increased general reactivity	↑ Heterotrophic processes ↑ Enzymatic activity ↑ Grazing ↑ EPS content
POLLUTANTS	Diuron Simazine	Blockage of D1 protein (Photosystem II)	↓ Algal biomass
	Imazalil Prochloraz	Inhibition of sterol 14α-demethylase	↓ Fungal biomass
	Chlorpyrifos	Inhibition of acetylcholinesterase	↓ Insect biomass
	Erythromycin	Blockage of 23S rRNA (50S subunit)	↓ Bacterial biomass

Table I 1 Stressors used in this thesis (i.e. hydrological stress, warming, and pollutants), together with the corresponding modes-of-action and their expected impacts on stream biofilm components according to target effects.

02

Methods

This section presents analytical methods aimed to study river biofilm community structure and functioning. This section also provides a brief overview of the experimental systems (i.e. micro- and mesocosms) available at the Catalan Institute for Water Research (ICRA), which have been used to perform the experimental studies presented in this thesis.

Methods

Micro- and mesocosms to elucidate multiple stressor effects on river biofilms

Complex interactions among stressors in natural settings hinder our prediction capacity when assessing multiple stressor effects beyond general expectations, such as decline in biodiversity (Vörösmarty *et al.*, 2010). A major issue is therefore how to identify causality under multiple-stressor scenarios, a question than can be only solved through the careful design of studies and appropriate definitions of the spatial and temporal scales of observation (Sabater and Borrego, 2015). In order to evaluate and forecast the patterns of response of the ecosystems to multiple stressors, observational studies must be combined with manipulative experiments, which allow for robust statistical power derived from high replication and highly controlled conditions. Laboratory experiments using artificial systems such as microcosms and mesocosms can provide appropriate evidences of cause-effect relationships between stressor exposure and biofilm response. In this thesis, an ANOVA approach (or equivalent) is used to analyze most of the data sets obtained from factorial studies. Regarding bacterial community composition, multivariate analysis (i.e. MANOVA) is employed. In Papers III and IV, a repeated-measures ANOVA is employed to analyze the effect of exposure time on the response to single and multiple stressors. Finally, experimental observations need to be validated with the corresponding field studies, and the results obtained from these methodologies need to be used with caution.

The size of the artificial system defines the complexity of the experimental approach, including the time during which the experiment can be run. Smaller artificial units (e.g. microcosms) usually allow for manipulative experiments that last between hours and a few days, whereas larger systems (e.g. mesocosms) allow for much longer manipulative experimentation (Sabater and Borrego, 2015). Accordingly, the first experiment of this thesis (Paper I) was performed in 100-mL glass crystallizers (n = 64) and lasted for 96 hours (48 h of stressor exposure and 48 h recovery, Figure M.4A). The second and third experiments (Paper II, III and IV) were performed in artificial streams (n = 24) and lasted several weeks (Figure M.4B). These artificial streams were located in the Experimental Streams Facility (ESF) at the Catalan Institute for Water Research (ICRA). The ESF consists of 24 mesocosms (i.e. artificial streams) made of methacrylate (length-width-depth: 200 cm – 10 cm – 10 cm), connected to 70-L water tanks from which water can be re-circulated. The artificial streams are fed with rainwater filtered through activated carbon filters and can reach up to 100 mL s^{-1} of water flow, operating as an open or closed (i.e. recirculating) system. The ESF has previously been successfully used to assess the effects of chemical pollution and its combination with climatic stressors, such as a simulated low-flow situation (Corcoll *et al.*, 2015b).

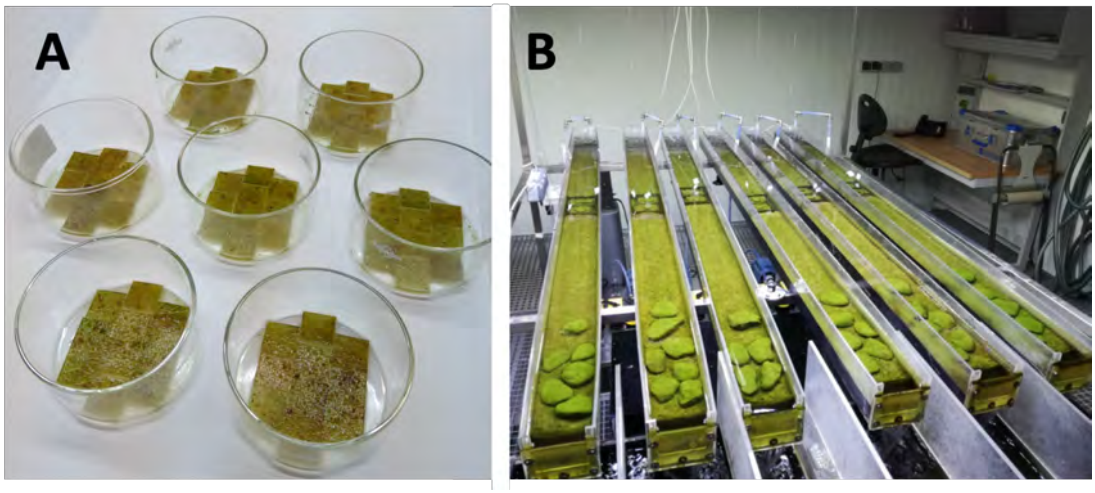


Figure M. 1 Artificial systems employed in this thesis. Microcosms (A) consisted on 100-mL glass crystallizers, whereas mesocosms (B) consisted on 20-L artificial recirculating streams.

General analytical methods

Here I provide an overview of the analytical methods employed in this thesis to characterize the functional and structural responses of river biofilms to multiple stressor conditions (Table M.1). A detailed description of the techniques can be found in the respective papers.

- Ash-free dry weight (AFDW)

AFDW was used as a surrogate of total biofilm biomass. Briefly, river biofilm samples (2.25 cm^2) were dried until constant weight (70 °C), then combusted at 450 °C for 4 h and re-weighted to deduct the ashes weight and estimate the AFDW (Elosegi and Sabater, 2009).

- Chlorophyll-*a* (Chl-*a*) concentration

Chl-*a* concentration was used as a surrogate of the total algal biomass. Epilithic (24.47 – 78.94 cm^2) and epipsammic (19.91 cm^2) biofilm samples were re-suspended in filtered (0.2 µm) stream water. Total Chl-*a* concentration was then estimated spectrophotometrically after a 90 % acetone extraction performed overnight in dark conditions at 4 °C (Elosegi and Sabater, 2009).

- Carbon substrate utilization profile

Biolog Ecoplates (Biolog Inc. Hayward, California, USA) were used to determine carbon substrate utilization profiles. Biofilm samples were diluted using a Ringer solution (1:20) and then inoculated (130 µL) under sterile conditions into the Biolog Ecoplates system, which contains three replicated wells of 31 different carbon sources (and a blank with no substrate). Plates were read every 24 h during 7 days at 590 nm using a microplate reader and data was generated according to Freixa *et al.*, 2016. Briefly, raw absorbance data was control-corrected and Shannon's diversity index and substrate richness were calculated.

- Net primary production (NPP)

Metabolic biofilm rates were assessed through changes in oxygen concentration under light and dark conditions (Figure M.1). Briefly, river biofilms were placed inside cylindrical acrylic chambers (volume 0.96 L) provided with submersible water circulation pumps and oxygen loggers (Colls *et al.*, 2019). The incubations for NPP and community respiration lasted for 45 min, and were performed into an incubator chamber under controlled temperature and light conditions. Dissolved oxygen concentration inside the chambers was measured every 15 s. Net primary production, community respiration and gross primary production were calculated from oxygen data according to Acuña *et al.*, 2008.

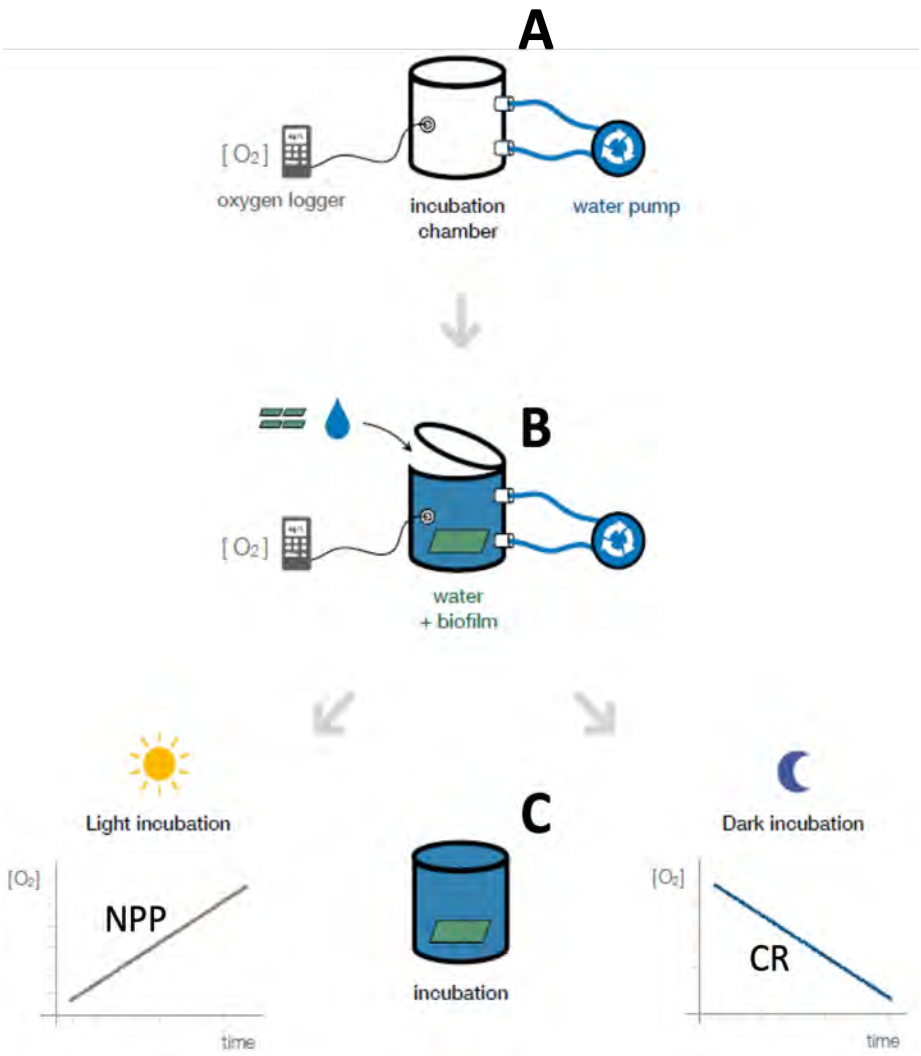


Figure M. 2 Schematic view of the incubation process employed to determine biofilm metabolic rates. The system is composed of an incubation chamber, an oxygen logger and a water recirculating pump (A). Once the biofilm samples (34 cm^2) and the water (0.96 L) are introduced (B), the incubation is performed under light and dark conditions (C), to estimate net primary production (NPP) and community respiration (CR), respectively.

Community respiration (MicroResp™ technique)

In Paper I, community respiration (CR) was used to account for the biofilm capacity to oxidize organic compounds. Briefly, CR assessed by means of the MicroResp™ technique (Campbell *et al.*, 2003; Tlili, Marechal, *et al.*, 2011). Briefly, 500 µL of biofilm suspension were used to fill the MicroResp™ device, consisting on a 96-well microplate containing a carbon source coupled to a second microplate containing the detection gel. The whole device (substrate microplate and detection gel) was incubated after the addition of the biofilm suspension overnight (15 h) at 12.5 °C and read according to Freixa *et al.*, 2018.

- Leucine aminopeptidase activity (LAPA)

LAPA relates to the biofilm capacity to transform dissolved organic nitrogen into inorganic compounds. LAPA was measured after a dark, 1-h incubation of biofilm samples using fluorescent-linked substrata (aminomethyl-coumarin, AMC) at 12.5 °C (Elosegi and Sabater, 2009). Blanks and standards of AMC (0-100 µmol L-1) were also incubated. At the end of the incubation, a glycine buffer (pH 10.4) was added, and fluorescence was measured at 364 / 445 nm excitation/emission for AMC.

- In-vivo chlorophyll-*a* measurements

Chlorophyll-*a* fluorescence was used *in-vivo* to determine photosynthesis-related parameters by means of pulse-amplitude modulated (PAM) fluorometry using a DIVING-PAM fluorometer (Corcoll *et al.*, 2015b; Kim Tiam *et al.*, 2015) (Figure M.2). These measurements included photosynthetic efficiencies (Y_{eff} and Y_{max}), chlorophyll-*a* basal fluorescence (F_0) and non-photochemical quenching (NPQ).

Figure M. 3 Detail of the pulse-amplitude modulated (PAM) fluorometer (DIVING-PAM) used in this thesis to perform in-vivo chlorophyll-a measurements.

Molecular analytical methods

In this thesis, a set of molecular approaches have been applied in order to determine the effects of multiple stressors on gene expression (Paper I), gene abundance (Paper II, III) and bacterial community composition and estimated function (Paper II, IV). This section contains an overview of the molecular methods employed (Figure M.3), which are fully detailed in the respective papers.

- Gene expression

For gene expression measurements, biofilm samples (2.25 cm^2) were collected and immediately stored into an ultra-low temperature (-80°C) freezer. Total RNA extraction took place within 7-10 days after biofilm sample collection. Total RNA was extracted using a standard extraction kit according to manufacturer's instructions (PowerBiofilm™ RNA extraction kit, MO BIO laboratories). Briefly, biofilm samples were lysed by means of a bead-beating technique through vortex mixing, followed by protein and inhibitor removal. Total RNA was captured on a flat bottom silica spin column and eluted on 50 µL of sterile ultrapure water. After quantity check (Qubit 2.0 fluorometer, Life Technologies), RNA was subjected to a DNAse purification treatment (TURBO DNA-free™, Ambition®) and kept at -80 °C until further processing. Reverse transcription of the RNA into complementary DNA (cDNA) was achieved from 1 µL of purified RNA extraction using the SuperScript® III First-strand synthesis system and random hexamers (50 ng μL^{-1}). After quantity (Qubit 2.0 fluorometer) and integrity (agarose gel) check, the cDNA was used for quantitative PCR (qPCR) on an Mx3005P system (Agilent Technologies) following manufacturer's instructions by means of SYBR Green detection chemistry (Marti *et al.*, 2013). Standard curves for qPCR absolute quantification were obtained by cloning of PCR products into *Escherichia coli* competent cells (StrataClone PCR cloning kit, Agilent). Transformant *E. coli* colonies were then subjected to plasmid extraction using a regular commercial kit (PureLink® Quick Plasmid Miniprep, Invitrogen), and the presence of the genes on the final product was verified by Illumina sequencing (MacroGen Inc.). Standard curves for qPCR were obtained by dilution of the plasmid-containing final extract.

- Gene abundance

For gene abundance measurements, biofilm samples (200-300 mg; fresh weight) were collected and immediately stored at -20°C. DNA extraction took place within 7-10 days after sample collection. Total genomic DNA was extracted from biofilm samples using the FastDNA® spin kit for soils (MP Biomedicals) following standard procedures. Briefly, biofilm was lysed using a bead-beating procedure in a FastPrep® homogenizer instrument (MP Biomedicals). The released DNA was purified by a silica-based spin filter method and eluted in 75 µL of

sterile ultrapure water. After quantity (Qubit 2.0 fluorometer, Life Technologies) and integrity (agarose gel) check, DNA was subjected to qPCR using specific primers on an Mx3005P system (Agilent Technologies) following manufacturer's instructions by means of SYBR Green detection chemistry (Marti *et al.*, 2013). Standard curves for absolute quantification were obtained by serial dilutions of PCR products previously purified with the QIAquick PCR purification kit (Qiagen Inc), which were verified by Sanger sequencing (Macrogen).

- Bacterial community composition

High-throughput sequencing of the 16S rRNA gene was performed on independent DNA extractions (FastDNA® spin kit for soils, MP Biomedicals) using an Illumina MiSeq System (2x250 PE) and the prokaryotic V4-specific primer pair 515f/806r (Caporaso *et al.*, 2011), leading to an amplicon size of 250-300 bp complemented with Illumina adapters and sample-specific barcodes at the Research Technology Support Facility of the Michigan State University, USA (Kozich *et al.*, 2013). Data treatment was performed using the Mothur software (Schloss *et al.*, 2009) against the Greengenes reference database (McDonald *et al.*, 2011). A biological information matrix (BIOM) file was also generated and uploaded to the METAGENassist web server to estimate functional community profile from the 16S rRNA dataset (Arndt *et al.*, 2012).

	EXPERIMENT 1	EXPERIMENT 2	EXPERIMENT 3	
	PAPER I	**PAPER II**	**PAPER III**	**PAPER IV**
Ash-free dry weight				
Chlorophyll-*a* concentration				
Carbon substrate utilization profile				
Net primary production				
Community respiration				
Leucine aminopeptidase activity				
In-vivo chlorophyll-*a* measurements				
Gene expression				
Gene abundance				
Bacterial community composition				

Table M. 1 Summary of the different methods employed to characterize biofilm responses across experiments (and papers) in this thesis. Colours indicate whether a given method was used to assess effects on river biofilm structure (blue) or function (orange).

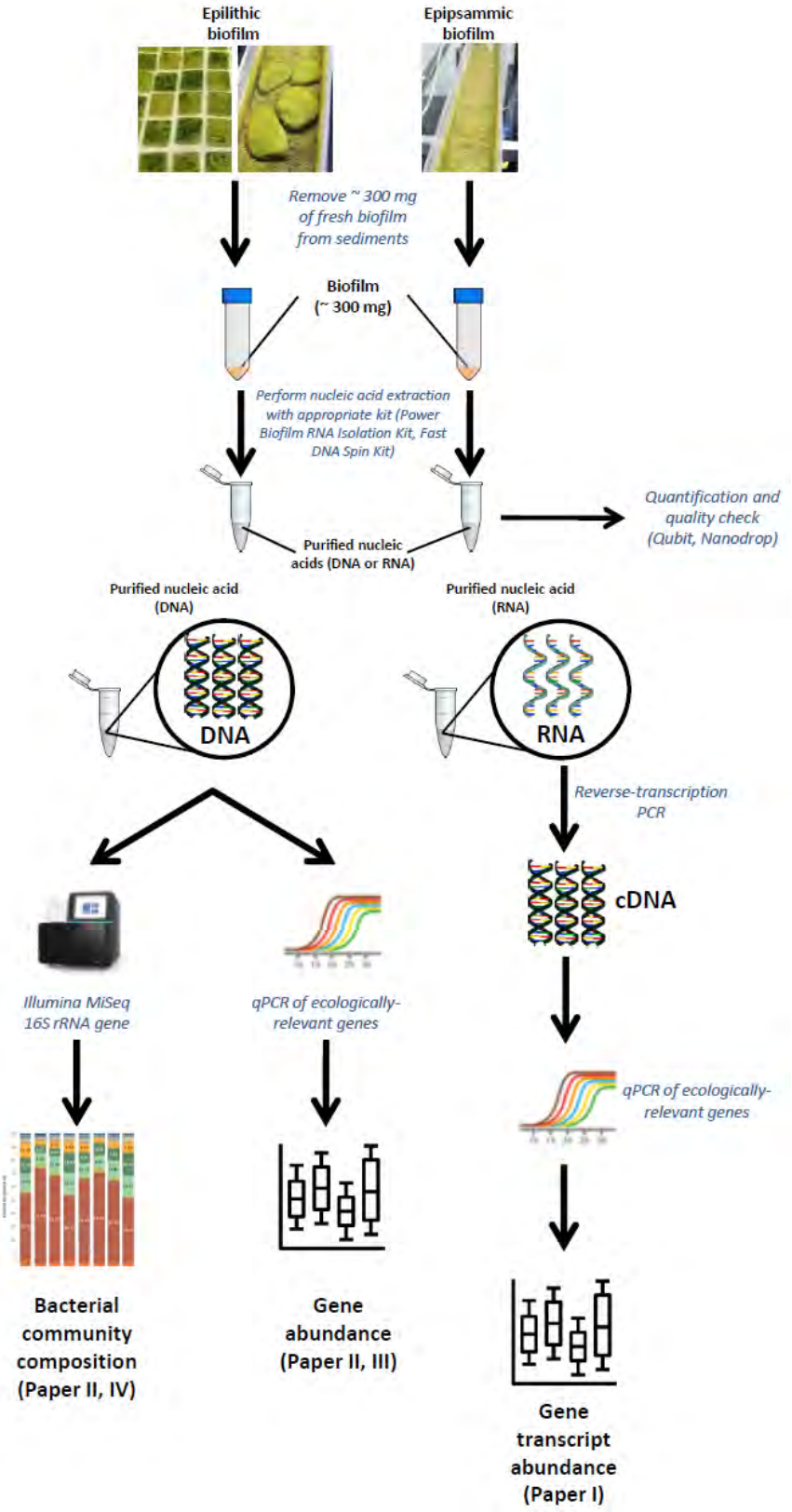


Figure M. 4 Overview of the molecular approaches employed in this thesis including the main steps in each analysis.

Water physical and chemical properties

- Physicochemical parameters

Water temperature was recorded every 10 min using VEMCO Minilog (TR model, AMIRIX System Inc, Halifax, NS, Canada) temperature data loggers (-5 to 35 °C, ±0.2 °C). Dissolved oxygen, pH and electrical conductivity were measured using WTW (Weilheim, Germany) hand-held probes.

- Inorganic nutrients

For nutrient analyses, ~15 mL of water were immediately filtered through 0.2 µm pore size nylon filters intro pre-washed polyethylene containers and kept at -20 °C until further processing. Phosphate ($P\text{-}PO_4^{3-}$) concentration was determined colorimetrically using a spectrophotometer (Alliance-AMS Smartchem 140, AMC, Frépillon, France, Murphy and Riley, 1962. The concentrations of nitrate ($N\text{-}NO_3^-$), sulphate ($S\text{-}SO_4^{2-}$) and ammonium ($N\text{-}NH_4^+$) were determined on a Dionex ICS-5000 ion chromatograph (Dionex, Sunnyvale, US).

- Dissolved organic carbon (DOC)

For DOC analyses, ~200 mL of water were immediately filtered through 0.7 µm glass fiber filters, previously ashed (Whatman GF/G, UK). The concentration of DOC was measured on a Shimadzu TOC-V CSH coupled to a TNM-1 module (Shimadzu Corporation, Kyoto, Japan).

- Pesticides

For pesticides analyses, ~125-mL water samples were filtered through 0.45 µm polyvinylidene fluoride membrane filters (PVDF, Millipore) and kept at -20 °C until further processing. Pesticide concentrations were determined by ultraperformance liquid chromatography (UPLC) coupled to a hybrid quadrupole-linear ion trap mass spectrometer (5500 QTRAP, Applied Biosystems, Foster City, US) following Ricart *et al.*, 2010.

- Erythromycin

For erythromycin analyses, ~125-mL water samples were filtered through 0.45 µm polyvinylidene fluoride membrane filters (PVDF, Millipore) and kept at -20 °C until further processing. Erythromycin concentration was determined on 10-mL water samples by means of on-line solid-phase extraction (SPE) followed by ultra-high-performance-liquid chromatography coupled to a triple quadrupole mass spectrometer (UHPLC-MS-MS), according to Farré *et al.*, 2016.

03

Thesis papers

PAPER I. Multistressor effects on river biofilms under global change conditions

PAPER II. Desiccation events change the microbial response to gradients of wastewater effluent pollution

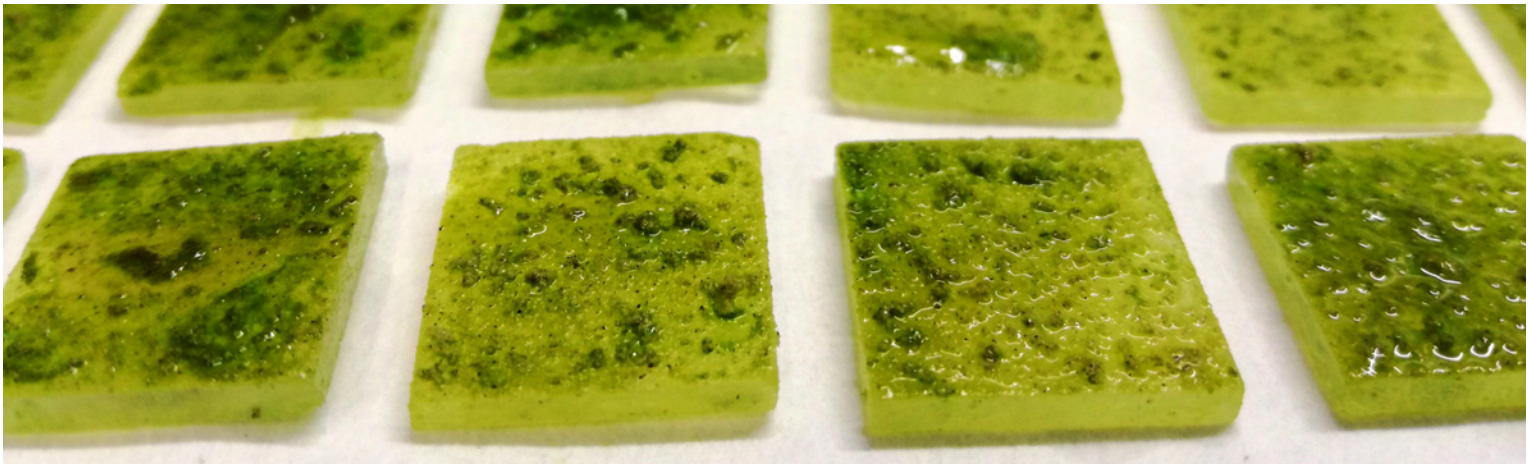
PAPER III. Effects of multiple stressors on river biofilms depend on time scale

PAPER IV. Multiple stressors determine structure and estimated function of river biofilm bacterial communities

Paper I

Multistressor effects on river biofilms under global change conditions

Romero, F., Sabater, S., Timoner, X., and Acuña, V.
Science of the Total Environment 627: 1–10 (2018).



PAPER I. Multistressor effects on river biofilms under global change conditions

Ferran Romero[1], Sergi Sabater[1,2], Xisca Timoner[1], Vicenç Acuña[1]

1. Catalan Institute for Water Research (ICRA), Carrer Emili Grahit 101, 17003 Girona (Spain).
2. Institute of Aquatic Ecology, University of Girona, 17071 Girona (Spain).

Abstract

Freshwater ecosystems are confronted with multiple chemical, biological and physical stressors. Co-occurring stressors commonly result in additive responses, but non-additive interactions may also occur, hindering our predicting capacity. Despite growing interest in multiple stressor research, the response of freshwater communities to co-occurring chemical and climate change-related physical stressors remains largely unexplored. Here, we used a microcosm approach to evaluate the effect of the combined action of chemical and physical stressors on river biofilms. Results showed that additive responses dominated, whereas 14.5% of all responses were non-additive (75% antagonisms and 25% synergisms). Among these non-additive interactions, physical stressors dominated over chemicals and drove the overall responses. Overall, the occurrence of these non-additive interactions, together with the dominance of the climate-change related physical stressors, might lead to unexpected responses as a result of climate change.

Introduction

Unravelling the mechanisms by which aquatic biota respond to global change is still an ongoing major challenge. Current predictions indicate that freshwater communities will face increased physical stress (higher water temperature and desiccation events) (IPCC, 2014), as well as chemical stressors of anthropogenic origin, such as pesticides and pharmaceutical products (Rodriguez-Mozaz et al., 2004; Kuzmanovic et al., 2015). These stressors co-occur in freshwater systems and cause unknown impacts on multiple levels of biological organization, from individual genotypes to communities (Segner et al., 2014). The effects produced by these stressors can be additive, when the effect of the combined action of two or more stressors is equal to the sum of the individual effects, or non-additive (Folt *et al.*, 1999). The latter is further depicted in antagonism or synergism, depending on the negative (antagonism) or positive (synergism) interaction that one stressor has on the other (Crain, 2008; Jeremy J. Piggott, Townsend, *et al.*, 2015b).

Recent analyses have emphasized that interactions in freshwater ecosystems may account for 40% to 69% of all ecological responses (Jackson *et al.*, 2016; Schinegger *et al.*, 2016b) and that additive interactions may be as frequent as non-additive interactions (Nõges *et al.*, 2016). A recent literature review suggested that the observed differences may depend on the ecosystem type and the organization level studied, from individual species to populations and whole ecosystems (Côté *et al.*, 2016). Still, uncertainty persists over the combined impacts of multiple stressors from a climate change perspective (Christensen *et al.*, 2006; Ormerod *et al.*, 2010). Field-based approximations often lead to unclear results, due to the confounding effect of natural variability in freshwater ecosystems (Ponsatí *et al.*, 2016). More solid cause-effect relationships can be established using experimental microcosms. These are simplifications of reality that allow for reduced natural variability and increased replication capacity. Although experimental designs using microcosms often use single-species approaches, more reliable results can be obtained if moving towards community-based analysis (Sabater, Guasch, Ricart, *et al.*, 2007). River biofilms incorporate species with different roles and functions, with autotrophs and heterotrophs co-existing in a highly complex entity. Because of its rapid response to perturbation and major role in nutrient cycling and ecosystem stability, they represent a good candidate to approach the impact of multiple stressors on rivers and streams (Sabater, Guasch, Ricart, *et al.*, 2007).

Here, we experimentally manipulated two physical stressors (water temperature and desiccation) and two chemical stressors (an herbicide and an antibiotic) in a full factorial model using river biofilms as a model community. Physical stressors were applied following scenarios of future climate change (IPCC, 2014), whereas chemical stressors were applied following realistic worst-case current scenarios (Hirsch *et al.*, 1999; Rabiet *et al.*, 2010).

Among chemical stressors, diuron and erythromycin were selected because of their toxicity and occurrence in the environment. Diuron is a phenylurea herbicide widely used to control broadleaf in vineyard areas and flower gardens. Its mode of action is through the blockage of the chloroplast electron transport chain at the photosystem II (PSII) level, ultimately leading to the inhibition of photosynthesis (Moreland, 1980). Its concentration in the environment ranges from < 1 µg L^{-1} to 10 µg L^{-1} during flood events (Rabiet *et al.*, 2010). Erythromycin is a macrolide considered a wide-spectrum antibiotic against gram-positive and some gram-negative bacteria. The mode of action of erythromycin is through binding to the 23S rRNA molecule in the 50S subunit of the bacterial ribosome, which then blocks the elongation in growing peptide chains, thus inhibiting protein synthesis (Prescott *et al.*, 2000). Erythromycin is commonly found in freshwater ecosystems and, although its concentration is on average low, it may be present at > 5 µg L^{-1} at sewage treatment plant effluents (Hirsch *et al.*, 1999).

This study aimed to evaluate the combined impact of 4 stressors on river biofilms. A main question was to determine whether these stressors lead to additive or non-additive responses. We hypothesized that: (i) chemical stressors will have targeted effects on specific biofilm components, consistent with their specific mode of action (ii) physical stressors will mostly have generalized effects, producing non-specific alteration in the selected response variables (iii) desiccation will affect the overall performance of biofilm algae and bacteria, making them sensitive to chemical stress (iv) higher water temperature will oppose the negative effect of chemical stressors by enhancing biofilm metabolism and (v) when occurring, non-additive responses will mostly be antagonistic, given the adaptation of river biofilms to high natural variability (Jackson *et al.*, 2016), therefore showing an inherent capacity to adapt to multiple stressor effects.

Material and methods

Experimental design

We used glass microcosms in an experimental design that followed a full factorial replicated (n = 4) design with four factors and two levels per factor (2^4): erythromycin (E), diuron (D), desiccation (W) and water temperature (T) (Figure PI.1). Both chemical stressors (E and D) were applied at nominal concentrations of 10 µg L^{-1}. These represent environmentally realistic concentrations though in the higher rank (Hirsch *et al.*, 1999; Rabiet *et al.*, 2010). Temperature was increased by 7 °C according to the predictions of short-term climatic extreme events (IPCC, 2014). Desiccation was applied by letting biofilms air-dry for 4 hours. This caused a 70% decrease in photosynthetic efficiency, equivalent to that occurring under field-conditions after 5-6 days of complete desiccation (Timoner *et al.*, 2012; Acuña *et al.*, 2015).

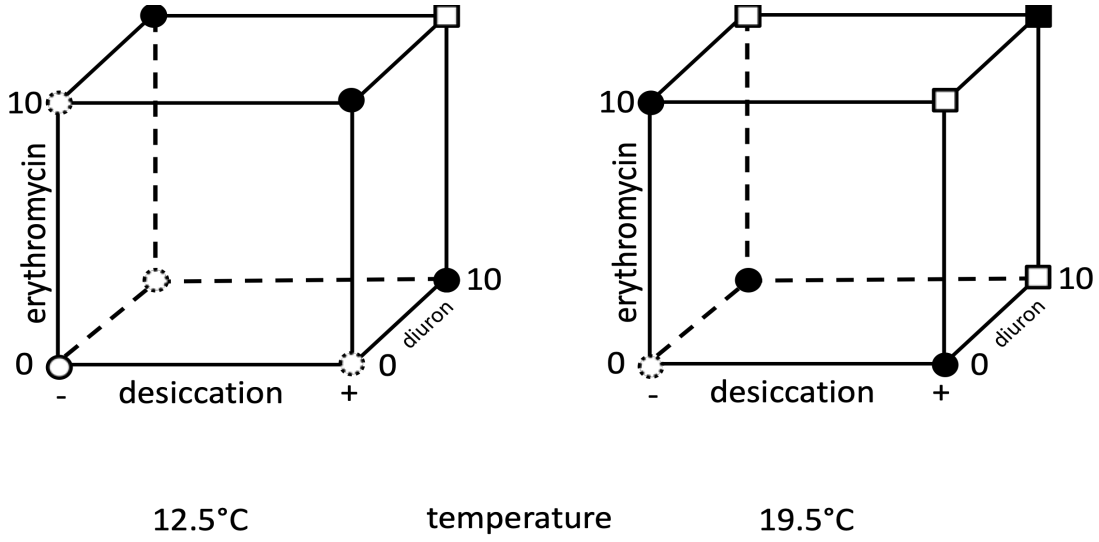


Figure PI. 1 Experimental design used in this study: full-factorial design with 4 factors and 2 levels per factor. The solid white circle represents the control case. Main effects are represented by dotted circles, 2-way interactions by solid black circles, 3-way interactions by solid squares and the 4-way interaction is represented by a solid black square. Concentrations are indicated in µg L^{-1}.

Biofilms were exposed to the stressors over 40 h (impact period), and immediately allowed to recover for 40 h (recovery period) by removing all stressors. Biofilm response to multiple stressors was assessed at the end of the impact and the recovery periods using a variety of structural and functionally-related variables. Basal fluorescence (F_0) and ash-free dry weight (AFDW) were used as a surrogate of algal and total biofilm biomass, respectively (Sabater, Guasch, Ricart, *et al.*, 2007). Photosynthetic efficiency (Y_{eff}), photosynthetic capacity (Y_{max}) and non-photochemical quenching (NPQ) were used as photosynthetic descriptors of the primary producers in the biofilm. Y_{eff} and Y_{max} respectively indicate the effective and optimal photosynthetic activity, whereas NPQ indicates the algal capacity to dissipate light excess during stress conditions (Ponsatí *et al.*, 2016). Leucine aminopeptidase activity (LAPA) relates to the biofilm capacity to transform dissolved organic nitrogen into inorganic compounds (Ylla *et al.*, 2014b). Community respiration (CR) accounts for the capacity of the biofilm community to oxidize organic compounds (Corcoll *et al.*, 2015b). Finally, the abundance of three gene transcripts was assessed by quantitative PCR (Smith and Osborn, 2009). The abundance of 16S rRNA and 18S rRNA gene transcripts was used to determine the status of bacterial and eukaryotic communities, respectively. The abundance of *psbA* gene transcript was used as a surrogate of the activity of the autotrophic compartment, as it codes for the D1 protein, which is the main component of the photosystem II (PSII) (Kim Tiam *et al.*, 2012).

Experimental conditions

Each microcosm consisted on an independent glass crystallizer (diameter = 7 cm, height = 4 cm) filled with 100 mL of water and 10 colonized glass slides. Biofilms were grown in artificial channels (see details at Corcoll., et al 2015) for 4 weeks using an inoculum from a non-impacted reference water body, the Llémena river (Sant Esteve de Llémena, Girona, EU), which is a permanent Mediterranean river draining a calcareous mountainous range (d_{50} = 0.74 mm). After 4 weeks of growth, biofilms were transferred to experimental microcosms. Source water for artificial channels and microcosms was rainwater filtered through activated carbon filters. During the whole experiment (i.e. 4 weeks of growth, 40 hours of exposure and 40 hours of recovery), daily cycles of photosynthetic active radiation (PAR) were defined as 12 h daylight and 12 h darkness, and were simulated by LED lights (Lightech, Girona, EU). PAR was held constant at 173.99 ± 33 µE m^{-2} s^{-1} during the daytime, and was recorded every 10 min using quantum sensors (sensor LI-192SA, LiCOR Inc, Lincoln, USA).

We assessed the similarity between the microbial community structure of the microcosms and that of the Llémena River. Thus, biofilm samples were collected after the 4-week growth period in the laboratory to perform DNA extraction (FastDNA® SPIN kit for soils, MP Biomedicals) and 16S rDNA amplicon sequencing, using the Illumina MiSeq platform available at the Research Technology Support Facility of the Michigan State University, USA (Kozich *et al.*, 2013). A presence-absence analysis based on operational taxonomic units (OTUs) revealed that the community used in this study contained 92% of the OTUs present at the Llémena River. Moreover, a parsimony test revealed that the assessed communities were not statistically different (*p*-value of the maximum parsimony test = 0.306).

Water chemistry

Water samples were taken before and after the exposure period for chemical analyses of nutrients (phosphate, nitrate and ammonium) and contaminants (diuron and erythromycin).

Nutrients

30 mL of water were filtered through 0.7 µm glass fibre filters (Whatman GF/F, Kent, UK) into pre-washed polyethylene containers. The concentration of phosphate was determined colorimetrically using a fully automated discrete analyzer Alliance Instruments Smartchem 140 (AMS, Frépillon, France). The concentrations of nitrate and ammonium were determined on a Dionex ICS-5000 ion chromatograph (Dionex Corpo- ration, Sunnyvale, U.S.A.).

Erythromycin

Erythromycin (CAS 114-07-8, mol. weight 733.93 g mol^{-1}, Sigma Aldrich) analysis was performed on 10 mL water samples by on-line solid-phase extraction (SPE) ultra-high-performance-liquid chromatography coupled to a triple quadrupole mass spectrometer (UHPLC–MS–MS). Fully automated on-line pre-concentration of erythromycin samples,

aqueous standards and operational blanks were analyzed using EQuan MAXTM technology coupled to a TSQ Vantage triple quadrupole mass spectrometer (Thermo Fisher Scientific) (on-line SPE-UHPLC- MS-MS) equipped with an electrospray ionization source (ESI) (Farré *et al.*, 2016).

Diuron

Stock solution of diuron (CAS 330-54-1, mol. weight 233.09 g mol^{-1}, Sigma Aldrich) was prepared on a weight basis, in methanol at 1 mg mL^{-1} and kept frozen at -20 °C. Working standard solution as well as the calibration standard curve was prepared by appropriate dilution in methanol–water (10:90, v/v) of the stock solution of diuron. The samples collected were filtered through 0.45 µm Polyvinylidene fluoride membrane filters (PVDF, Millipore) and analyzed directly by ultraperformance liquid chromatography (UPLC; Waters Corp. Milford, MA, USA) coupled to an hybrid quadrupole-linear ion trap mass spectrometer (5500 QTRAP, Applied Biosystems, Foster City, CA,USA) (LC-MS/MS system) (Ricart *et al.*, 2009).

Biofilm analyses

Leucine Aminopeptidase Activity

The degradation capacity of nitrogen compounds was assessed by means of the extracellular enzyme leucine aminopeptidase activity (LAPA). It was measured by means of fluorescent-linked substrata (aminomethyl-coumarin [AMC]). Colonized glass slides were incubated for 1 h in the dark at 12.5 °C immediately after collection. Blanks and standards of AMC (0-100 µmol L^{-1}) were also incubated. At the end of the incubation, glycine buffer (pH 10.4) was added (1/1 vol/vol), and the fluorescence was measured at 364/445 nm excitation/emission for AMC. Values were expressed as nmol of released AMC cm^{-2} h^{-1}.

Community Respiration

Community Respiration (CR) was assessed to evaluate bacterial community overall performance by means of the MicroResp™ (Tlili, Marechal, *et al.*, 2011). Briefly, 500 µL of biofilm suspension were obtained by scraping biofilm off glass substrata and resuspending it in 0.2 µm Nuclepore-filtered control water. Biofilm suspension was then used to fill the 96 deep-well microplate coupled to a detection gel. Once assembled, the entire system (biofilm-containing microplate and detection gel) was incubated in the dark (to avoid any photosynthesis interference with CO2 release) at control temperature (12.5°C) overnight (15 hours).

Ash-free dry weight

A known surface of biofilm (2.25 cm^2) was scrapped from the glass substrata and filtered through a combusted glass fibre filter (Whatman GF/F, Kent, UK). Filters were dried at 70 °C until constant weight and later combusted at 450 °C for 4 hours to estimate the ash free dry weight (AFDW).

In vivo Fluorescence measurements

Two glass slides in each replicate were used to evaluate maximum chlorophyll-a fluorescence, photosynthetic efficiency, photosynthetic capacity, and non-photochemical quenching (NPQ). Chlorophyll-fluorescence, Effective quantum yield (Y_{eff}) and maximum photosynthetic capacity (Y_{max}) were determined *in vivo* using a Diving PAM (Pulse Amplitude Modulated) underwater fluorometer (Heinz Wlaz, Effeltrich, Germany). Intact glass slides were kept for 30 min in the dark to obtain the maximum chlorophyll-*a* fluorescence (F_0) and Y_{max}. Y_{eff} and Y_{max} were used as indicators of photosynthetic efficiency and maximal photosynthetic capacity, respectively (Timoner *et al.*, 2012). NPQ was used as an indication of the algal capacity to dissipate the excess light during stress conditions and was calculated following NPQ= $(F_m-F_m')/F_m'$ (Bilger and Bjorkman, 1990), where F_m' represents the maximum chlorophyll fluorescence in steady-state conditions and F_m represents the maximum chlorophyll-a fluorescence under dark-adapted conditions.

Molecular analyses

RNA extraction

Total RNA was extracted according to the manufacturer's instructions (PowerBiofilm™ RNA isolation kit, MO BIO laboratories). Briefly, 2.25 cm^2 of biofilm were removed from the colonized glass slides using a razor blade and put in 1.5 mL vials. Vials were then centrifuged at 13000 g during 60 seconds in order to remove excess water. Biofilms were then added to the PowerBiofilm™ Bead Tube then heated to activate lysis components. Lysis was accomplished through vortex mixing using a Vortex Adapter for Vortex-Genie® 2 (MO BIO laboratories), followed by protein and inhibitor removal. Total RNA was then captured on a flat bottom silica spin column and eluted on 50 µL of sterile ultrapure water.

RNA purification (DNAse treatment)

Total RNA (50 µL) was purified using a commercial kit according to the manufacturer's instructions (TURBO DNA-*free*™, Ambion®). Briefly, 5 µL of TURBO DNAse buffer and 1 µL of TURBO DNAse were added to 50 µL of RNA. DNAse activity was performed at 37 °C during 30 min. Purified RNA was kept at -80 °C until further proceeding.

Reverse transcription of RNA

The first strand of complementary DNA (cDNA) was synthesized from 1 µL of total purified RNA using the SuperScript® III First-Strand Synthesis System. The RNA-primer mixture contained 1 µL of total RNA, 1 µL of random hexamers (50 ng μL^{-1}), 1 µL of 10 mM dNTP mix and 7 µL of DEPC-treated water. The RNA-primer mixture was incubated at 65°C for 5min. The synthesis mix contained (per sample): 2 µL of 10X RT buffer, 4 µL of 25 mL $MgCl_2$, 2 µL of 0.1 M DTT, 1 µL of RNAseOUT® (40 U μL^{-1}) and 1 µL of SuperScript® III RT (200U/µL). The synthesis mix was added to the RNA-primer mixture and incubated at 25 °C for 10min, followed by 50 min at 50 °C. The cDNA mixture was conserved at −20 °C until it was used in quantitative real-time PCR.

Quantitative PCR

Quantitative real-time PCR (qPCR) was performed on an Mx3005P system (Agilent Technologies, Santa Clara, CA, USA) following the manufacturer's instructions by means of SYBR Green detection chemistry. Accordingly, 1 activation cycle was performed during 3 min at 95 °C, followed by 50 amplification cycles at 95°C for 15 sec and 60°C for 60 sec. Each 30.4 µL reaction contained 15 µL of SYBR-green mix, 12 µL of DEPC-treated water, 1.2 of forward primer (10 mM), 1.2 of reverse primer (10 mM) and 1 µL of template cDNA. For negative controls, cDNA was replaced by DEPC-treated water. Three genes were quantified from cDNA; (i) 16S rRNA gene, coding for the main component of the small subunit of prokaryotic ribosomes, was here used as a surrogate of bacterial overall performance; (ii) 18S rRNA gene, coding for the main component of the small subunit of eukaryotic ribosomes, was used as a surrogate of eukaryotic (mainly algal) performance; (iii) *psbA* gene, coding for the D1 protein, which is a central component of the photosystem II, was used as a surrogate of overall photosynthetic activity (Table PI.S1). Specificity was determined for each reaction from the dissociation curve of the PCR product. This dissociation curve was obtained by following the SYBR Green fluorescence level during a gradual heating of the PCR products from 60 to 95 °C. Absolute quantification of each gene expression level was performed using a standard curve.

Standard curve set-up for qPCR absolute quantification

16S rDNA, 18S rDNA and *psbA* genes were amplified from biofilm-extracted DNA (PowerSoil™ DNA isolation kit, MO BIO laboratories) by conventional PCR. PCR products were immediately cloned into *Escherichia coli* competent cells using the StrataClone PCR cloning kit (Agilent Technologies). Basically, fresh PCR products were incubated with Topoisomerase I-charged vector arms for 5 minutes and then heat-shock transformed for 60 seconds into competent cells expressing Cre recombinase. Screening of transformants was realized on ampiciline-containing LB medium agar plates. Selected colonies were used for plasmid extraction using a

regular commercial kit (PureLink® Quick Plasmid Miniprep Kit, Invitrogen). The presence of the plasmid on the final product was verified by PCR. The presence of the genes on the final product was verified by Illumina sequencing (MacroGen Inc.) of PCR products. Standard curves for qPCR were obtained by simple dilution of the plasmid-containing final extract.

Statistical analyses

Generalized linear model (GLM) analyses were conducted in R software version 3.3.0 (R Core Team, 2017) using the *glm* default function. Erythromycin (E), diuron (D), desiccation (W) and water temperature (T) were fixed as categorical factors with 2 levels per factor (i.e. presence *vs.* absence of the stressor). Response parameters Y_{eff}, Y_{max}, NPQ, F_0, LAPA, CR, AFDW, 16S, 18S and psbA were fixed as response variables. The model included all interaction terms (up to 4th order). We selected type III sum of squares, as recommended for this type of studies (Garson, 2015) and significance level for all tests was set at $p < 0.05$.

Two types of effects were calculated from the GLM: *Main effects* compared the mean performance in the treatments where a given stressor is present versus the treatments where the stressor is absent. Main effects were classified as positive (+) if the presence of the stressor significantly increased the overall response of the corresponding variable or negative (-) if the stressor significantly decreased the overall response of the corresponding variable. *Interactive effects* were calculated following Crain et al., 2009 and indicated when an interaction between two or more stressors occurred. Interactive effects were classified depending on the combined effect of the interacting stressors: antagonism (A) was assumed when the combined effect was less than predicted additively, whereas synergism (S) was assumed when the combined effect was more pronounced than predicted additively. To evaluate the recovery of the biofilm responses, a classic one-way ANOVA was used to compare the response after the impact and recovery phases.

As GLM is a null hypothesis significance testing approach, it only provides us with evidence against the null hypothesis (i.e. the *p-value*). Null hypothesis significance testing does not provide us with an estimate of the magnitude of the effect of interest. Consequently, we used the *etaSquared* default function in R Software to calculate the effect size (ES), a correlation statistic which estimates the magnitude of an effect as the percentage of variance accounted for by a given treatment (Nakagawa and Cuthill, 2007). Effect size was calculated as the ratio between the type III sum of squares (SS) of a given factor and the type III SS of this factor + the type III SS of the error. Accordingly, an ES of 0 means no relationship whatsoever between a treatment and a response parameter, whereas an ES of 1 means a perfect relationship.

Results

Water chemistry

Diuron and erythromycin were both detected in microcosm water samples at levels near the nominal concentration (Table PI.1). After the exposure period (40 h), the concentration of diuron was significantly lower (4.9 µg L^{-1}) than before the beginning of the experiment. Nitrate and phosphate were detected at lower concentrations after the exposure period, whereas the concentration of ammonium increased (Table PI.1).

Experimental phase	Diuron (µg L^{-1})	Erythromycin (µg L^{-1})	Nitrate (NO_3^-) (mg L^{-1})	Phosphate (PO_4^-) (mg L^{-1})	Ammonium (NH_4^+) (mg L^{-1})
t_{0h}	11.7 ± 1.5	9.2 ± 2.9	1.97 ± 0.48	0.01 ± < 0.01	< 0.01
t_{40h}	4.9* ± 0.8	10.8 ± 3.7	0.37* ± 0.02	< 0.01	0.34* ± 0.02

Table PI. 1 Water chemistry information at the beginning (t_{0h}) and the end (t_{40h}) of the exposure period. For water toxicants (i.e. diuron and erythromycin), concentrations are given in µg L^{-1}, for nutrients (i.e. NO_3^-, PO_4^- and NH_4^+) concentrations are given in mg L^{-1}. The asterisk indicates a significant difference (Tukey's test p-value < 0.05 in a one-way ANOVA) in the toxicant or nutrient concentration detected in microcosm water before and after 40 hours of exposure.

Single stressor effects

Physical stressors desiccation (W) and temperature (T) had the most pervasive effects: the two affected ca. 50% of variables (significant main effects). Desiccation had the strongest effect, with an effect size (ES) between 0.03 - 0.54. The chemical stressors erythromycin (E) and diuron (D) significantly affected 20% and 10% of the variables, respectively. Diuron had the highest effect size of the two (ES = 0.30).

Erythromycin produced a significant positive main effect on community respiration (CR) (Table PI.2), which increased by 64.65% (relative to the control), and negatively affected the abundance of 16S rRNA, which decreased by 67.4% (Figure PI.2). CR recovered significantly and was only 1.6% less than the control after the recovery period (Figure PI.3). The 16S rRNA gene expression also recovered, but remained 29.3% less than the control (Figure PI.3). Diuron had a main negative impact on photosynthetic efficiency (Y_{eff}) (Table PI.2). Exposure to diuron reduced Y_{eff} by 32.9% (Figure PI.2), but it significantly recovered ($p < 0.05$ in one-way ANOVA) after the recovery period (Figure PI.3).

Desiccation had a main negative effect on all photosynthetic parameters, as well as leucine aminopeptidase activity (LAPA) (Table PI.2). Photosynthetic efficiency and photosynthetic capacity (Y_{eff} and Y_{max}) significantly recovered, being 29.3% and 11% higher than the control after the recovery period (Figure PI.3). Temperature had a main negative effect on Y_{max}, non-photochemical quenching (NPQ) and the expression of *psbA*, but it increased the basal fluorescence (F_0) and the LAPA (Table PI.2). Temperature acting individually decreased Y_{max} by 22.7% and NPQ by 68.1%, whereas it increased F_0 by 22.6% and LAPA by 33.4% (Figure PI.2). Y_{max} and NPQ showed significant recovery (10.2% and 39.4% lower than the control after the recovery period, Figure PI.3).

	E	D	W	T	DE	DW	DT	EW	WT	ET	DTW	ETW	DEW	DET	DEWT
Y_{eff}	0.17	< 0.001 (0.30) -	< 0.001 (0.30) -	0.28	0.16	0.58	0.05 (0.08) A	0.22	< 0.001 (0.76) A	0.46	< 0.001 (0.34) A	0.41	0.58	0.26	0.49
Y_{max}	0.14	0.64	0.001 (0.20) -	0.002 (0.18) -	0.43	< 0.001 (0.27) S	0.20	0.53	< 0.001 (0.60) A	0.21	0.76	0.77	0.13	0.55	0.32
NPQ	0.87	0.86	0.001 (0.20) -	< 0.001 (0.23) -	0.36	0.73	0.17	0.38	0.04 (0.089) A	0.98	0.04 (0.087) A	0.61	0.55	0.80	0.19
F_0	0.33	0.14	< 0.001 (0.54) -	0.008 (0.14) +	0.58	0.17	0.10	0.26	0.02 (0.10) S	< 0.001 (0.23) A	0.25	0.01 (0.12) S	0.47	0.11	0.29
LAPA	0.54	0.65	0.008 (0.14) -	0.002 (0.18) +	0.88	0.63	0.33	0.06	0.04 (0.09) S	0.38	0.76	0.13	0.29	0.25	0.94
AFDW	0.29	0.88	0.91	0.66	0.34	0.38	0.52	0.17	0.17	0.12	0.63	0.02 (0.10) A	0.45	0.08	0.12
CR	0.03 (0.09) +	0.07	0.19	0.70	0.35	0.16	0.13	0.14	0.24	0.17	0.40	0.29	0.95	0.22	0.20
16S rRNA	0.04 (0.10) -	0.15	0.37	0.88	0.20	0.71	0.98	0.42	0.86	0.78	0.66	0.02 (0.11) A	0.58	0.05 (0.09) A	0.07
18S rRNA	0.82	0.84	0.55	0.97	0.91	0.71	0.79	0.55	0.91	0.67	0.42	0.33	0.80	0.23	0.21
psbA	0.10	0.39	0.04 (0.03) -	0.04 (0.03) -	0.57	0.80	0.25	0.26	0.16	0.09	0.38	0.25	0.38	< 0.001 (0.14) A	< 0.001 (0.11) A

Table PI. 2 Summary of GLMs (p-values) comparing response variables across experimental treatments. Effect sizes are shown in parentheses for cases where p-value < 0.05. Significant main effects (+, positive, -, negative) and interactions (A, antagonism, S, synergism) are indicated.

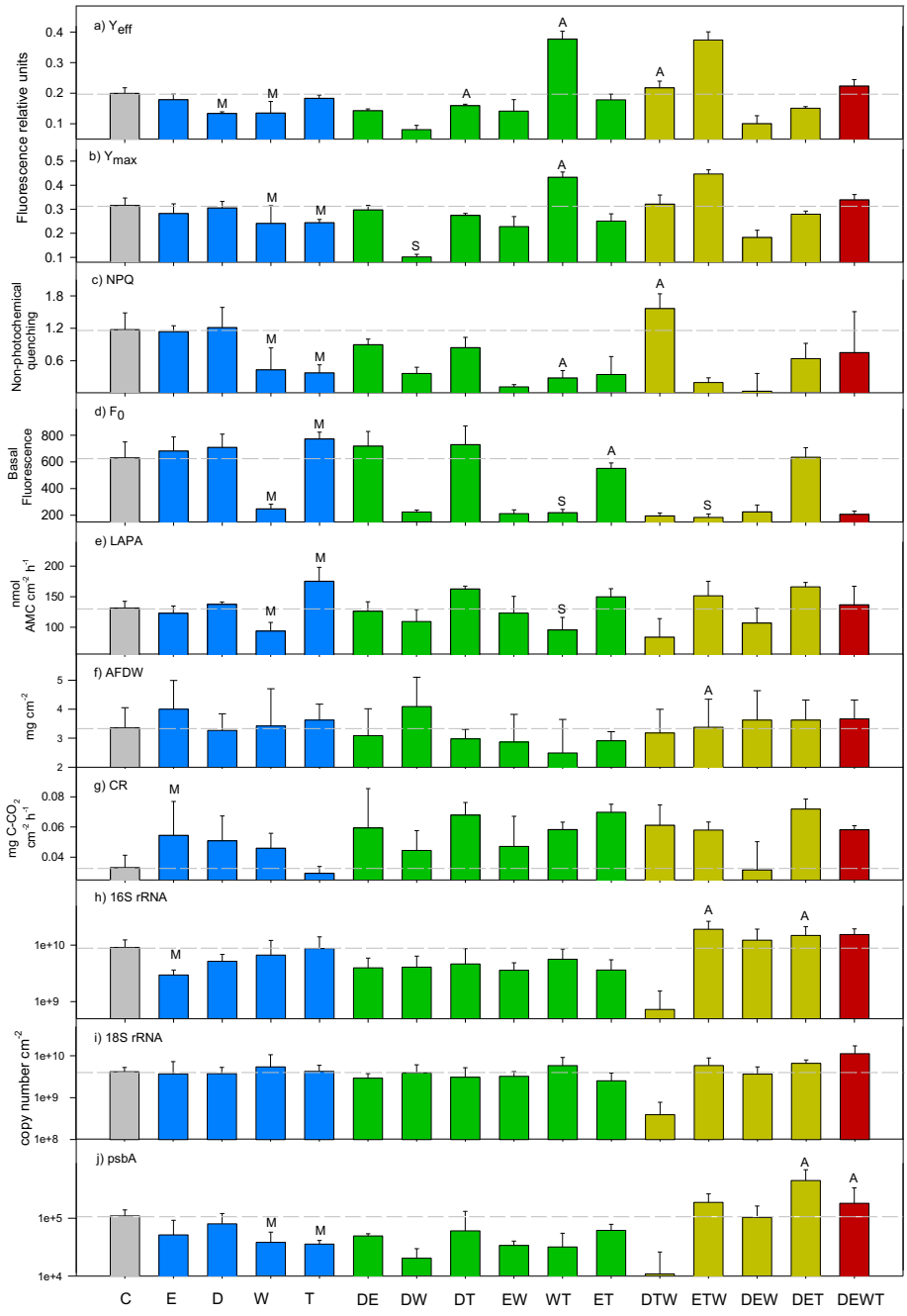


Figure PI. 2 Average of Photosynthetic Efficiency (a), Maximum Photosynthetic Capacity (b), Non-Photochemical Quenching (c), Basal Fluorescence (d), Leucine Amino-Peptidase Activity (e), Ash Free Dry Weight (f), Community Respiration (g), 16S rRNA copy number (h), 18S rRNA copy number (i), and psbA copy number (j). Error bars (SEs) show variation between replicates (n = 4). Grey bar (C) shows control treatment (no stress). Significant main effects (M) are indicated for single stressors (E, erythromycin; D, diuron; W, desiccation and T, temperature). Significant interactions are indicated as A (antagonism) or S (synergism). Dotted dash line allows rapid comparison with control value.

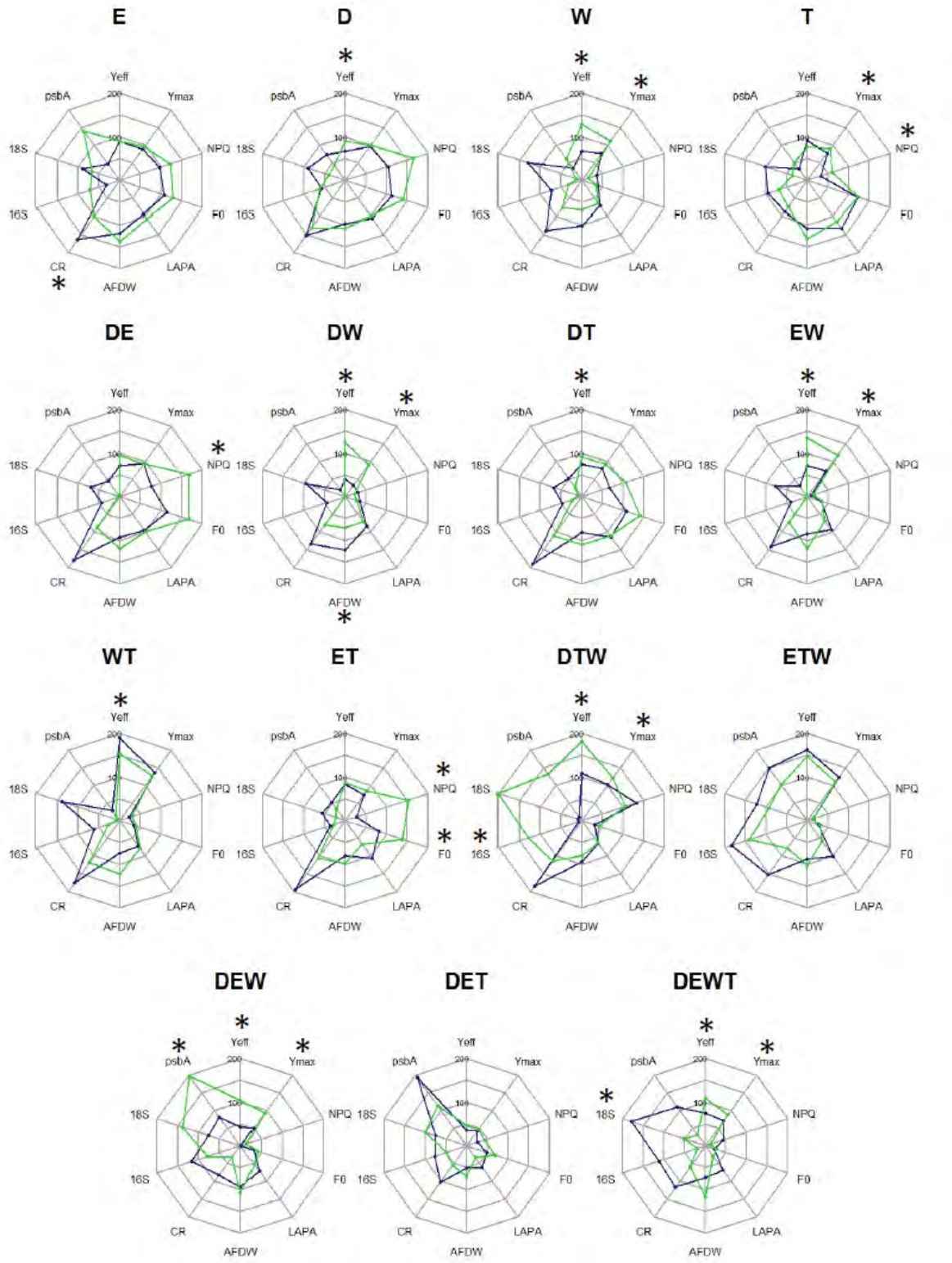


Figure PI. 3 Response variables across treatments. The results are indicated as percentage of the control. Dark blue line indicates percentages obtained after exposure to stressors. Light green line indicates percentages obtained after recovery period under stress-free conditions. Asterisks show significant (p-value < 0.05) difference between exposure and recovery periods.

Multiple stressor effects

The interaction between stressors ranged from additive to significant 2-way and higher-order interactions. Among all combinations, 85.5% produced additive responses, whereas 14.5% resulted in non-additive interactions. Of these, 75% were classified as antagonisms and 25% as synergisms. The most pervasive interactions involved physical stressors. Desiccation and increased temperature (WT) resulted in significant interactions for 50% of the studied variables. This combination also resulted in significant interactions when chemical stressors were considered, both in 3-way (DTW and ETW) and 4-way (DEWT) combinations.

Diuron and desiccation acting jointly synergistically reduced photosynthetic capacity (Y_{max}), which was 67.8% lower than the control after the impact period, and recovered up to 8.8% after the recovery period; Figures PI.2 and PI.3. The interaction between diuron and temperature caused the antagonistic reduction of photosynthetic efficiency, Y_{eff} (19.9% of the control; much less than the predicted additive decrease of 40.4%). Y_{eff} recovered up to 0.6% lower than the control. Erythromycin and temperature antagonistically decreased F_0 by 12.5% (Table PI.2), also diverging from the additive prediction of a 30.7% increase. F_0 increased by 47.9% after recovery. The triple combination of diuron, erythromycin and temperature increased 16S rRNA and *psbA* expression, the interaction being antagonistic in both cases.

Desiccation and temperature resulted in significant interactions for photosynthetic parameters and LAPA. The additive prediction was that Y_{eff} and Y_{max} should decrease by 40.4% and 46.4% in this, but both increased by 89.4% and 37.0% compared to the control, respectively. The combination between desiccation and increased temperature also caused a 76.1% increase in community respiration. The three variables remained higher than the control (54.1%, 26.2% and 18.3%, respectively) after the recovery period. NPQ and F_0 were also reduced by 76.0% and 65.4% compared to the control, respectively, and the LAPA by 27.1% (Figure PI.2). NPQ, F_0 and LAPA remained 71.7%, 59.9% and 21.1% lower than control values after recovery.

Diuron, temperature and desiccation altogether had significant effects on Y_{eff} and NPQ. The additive outcome predicted a decrease in Y_{eff} and NPQ while they increased by 9.3% and 33.2%, respectively. The NPQ approached control values after the recovery period, but Y_{eff} remained 82.2% higher. Erythromycin, temperature and desiccation resulted in a synergistic decrease of basal fluorescence (F_0), whereas the interaction was antagonistic for AFDW and 16S rRNA gene expression. In all cases, the deviation was maintained after recovery. Last, the 4-way interaction between diuron, erythromycin, desiccation and temperature synergistically increased the expression of *psbA* by 65.3% and shifted to 54.5% lower than control after recovery.

Discussion

Considerations on the followed approach

Our microcosms approach has the advantage of combining rigorous control over ecological stressors and sufficient power to detect both main effects and interactions. We were sufficiently able to reproduce the biofilm community growing on the reference site in terms of bacterial diversity (as demonstrated by OTUs analyses, see section 2.2 Experimental conditions). However, the biofilm community used in this study did not contain any consumers (e.g. grazers), so we could not predict any effects across the food web. Importantly, the exposure to selected stressors was short and acute. While chronic stress exposure usually leads to adaptation of the community, as sensitive species are replaced by tolerant species, our approach consisted of an acute exposure to the stressors, likely making adaptation of the community through species succession impossible. This limitation associated with exposure conditions in short-term experiments is in line with the rise of synergisms (D. Vinebrooke *et al.*, 2004). Also, our experimental design did not allow us to assess multiple stressor effects across different levels of stressors. Changing levels of stressors (e.g. applying chemical stressors at other concentrations) could possibly lead to other main effects and interactions. However, the levels of stressors used in this study represented realistic scenarios.

Single stressor effects

Chemical stressors diuron and erythromycin altered biofilm structure and functioning. Here we have shown how a short-term (40 h) exposure to 10 µg L^{-1} of the herbicide diuron significantly reduced the photosynthetic efficiency of a river biofilm. On the other hand, exposure to 10 µg L^{-1} of the antibiotic erythromycin decreased 16S rRNA gene expression and increased community respiration. These results confirm the hypothesis that chemical stressors would have effects according to their mode of action. The herbicide had a short-term effect on photosynthetic performance but did not compromise the integrity of the photosynthetic apparatus. This was evidenced by the significant alteration of photosynthetic efficiency (Y_{eff}) but not the photosynthetic capacity (Y_{max}). Similar results have been observed for the photosynthetic capacity of multispecies biofilms (Larras *et al.*, 2013)**.** The antibiotic showed general toxicity to bacteria whereas it likely promoted respiration activity due to the selection pressure affecting sensitive species and favouring tolerant non-nutrient limited bacteria (Tello *et al.*, 2012).

Physical stressors desiccation and increased temperature (7°C above control) had larger effects than chemical stressors, altering 60% and 50% of the variables, respectively. The altered variables mostly included photosynthesis-related parameters and enzymatic activity

(Table PI.2). These results partially confirm the hypothesis that physical stressors have general effects for algal and bacterial compartments. The prediction was largely supported for desiccation which decreased all photosynthetic parameters (i.e., Y_{eff}, Y_{max}, NPQ, F_0 and the expression of *psbA*), as well as enzymatic activity. Desiccation caused the largest effect size (ES = 0.54). Similar effects were observed in biofilms in field and laboratory experiments (Ylla et al., 2010; Timoner et al., 2012; Proia et al., 2013). The photosynthetic parameters Y_{eff} and Y_{max} were the ones to recover fastest after desiccation, in line with previous studies (Barthès *et al.*, 2014). The prediction that physical stressors will have general effects was only partially fulfilled in the case of temperature stress. Temperature acted as a subsidy for basal fluorescence and enzymatic activity. However, it likely produced a negative effect in the structure of PSII, as shown by a decrease in Y_{max}, NPQ and *psbA* expression.

Multiple stressor effects

Interactive effects were especially pervasive for physical stressors. Desiccation applied as a 4-hour drought made biofilms significantly more sensitive to the herbicide diuron, as shown by a synergistic interaction between these stressors. This result is in line with our third hypothesis, which stated that desiccation would make biofilms sensitive to chemical stress. Desiccation produces direct effects on biofilm cells, which could suffer from stress caused by the lack of water and/or osmotic shock, in many cases leading to cell lysis (Schimel *et al.*, 2007). The biofilm was thin and therefore poorly resistant to desiccation. Certainly, thinner biofilms are known to be more sensitive to chemical stress (Cochran et al., 2000; Ivorra et al., 2000). Beyond these structural causes, physiological mechanisms could also be at play. The allocation of resources on a previously disturbed community would shift towards the maintenance of the cellular machinery, e.g. by producing protective molecules such as heat-shock proteins (Maleki, 2016). Under this situation, less energy is available for detoxifying pathways, ultimately making the community more sensitive to chemical stressors (Schimel et al., 2007). These mechanisms together predict that chemical stressors such as herbicides and antibiotics would be more harmful to river biofilms subjected to desiccation. The poor recovery of structural variables confirms that the biofilm structure was heavily affected by desiccation.

Our fourth hypothesis stated that increased temperature would favour functional variables, counteracting the negative effects of chemical stressors. This indeed was the case for the combination between diuron and increased temperature: the negative effects of diuron on photosynthetic efficiency were compensated by temperature. The detrimental effects of diuron were less pronounced when the biofilm was maintained at higher temperature (Figure PI.2), possibly as a result of the increase in the turnover rate of the *psbA*-encoding protein D1, which could desensitize PSII to the toxic effect of herbicides (Larras *et al.*, 2013). Temperature

preferentially affected functional (av. ES = 0.11 ± 0.09) rather than structural variables (av. ES = 0.04 ± 0.07), in line with the effect that temperature has on metabolic processes (Marcus, Jennifer K. Wey, *et al.*, 2014). Chemical stressors showed a similar effect size on autotrophic and heterotrophic variables, but the effect of physical stressors (especially desiccation) was more pronounced on autotrophic (av. ES = 0.12 ± 0.06) rather than heterotrophic variables (av. ES = 0.06 ± 0.02).

The strongest interaction occurred between physical stressors. The combination of desiccation and temperature resulted in a sharp increase in photosynthetic performance that mitigated the individual effects. This unpredicted nonlinear interaction was among the strongest and more statistically robust, resulting in an ecological surprise (Ormerod *et al.*, 2010). This interaction suggests that the co-occurrence of desiccation and increased temperature mitigated individual effects. A recent meta-analysis of multiple stressor effects in freshwater ecosystems shows that temperature is the stressor most commonly associated with mitigating interactions (Jackson *et al.*, 2016). We argue that increased temperature could activate the metabolism of cells that remain once the negative effect of desiccation has decimated the most sensitive organisms. This activation could favour the per capita use of resources. Increased temperature can rapidly stimulate metabolic activity as long as resources are not limiting, and this might be the case during the re-established early stages of succession after the disturbance (Marcus, Jennifer K. Wey, *et al.*, 2014).

We observed that 50% of non-additive interactions involved at least three stressors. Photosynthetic efficiency (Y_{eff}) was the most affected by diuron, temperature and desiccation, and the resultant Y_{eff} value was higher than its additive prediction. Y_{eff} is reduced by ca. 50% when diuron is added to the interaction between desiccation and temperature. This suggests that while the combination between desiccation and temperature could lead the community back to an early successional stage, the fast-growing communities were more sensitive to herbicides such as diuron. The results, taken together, could be interpreted as an indication that biofilm communities exposed to increased temperature and desiccation due to climate change could be more sensitive to herbicide toxicity.

Our fifth hypothesis stated that antagonism would be the most prevalent response amongst the non-additive interactions. This hypothesis was confirmed but merits further discussion according to the obtained results. Antagonism represented 75% of all non-additive interactions, whereas synergism represented the 25%. However, non-additive interactions represented only 14.5% of all combinations in the full-factorial model, being additive for 85.5% of all combinations. Manipulative studies using higher-order interactions have found results comparable to the ones we observed (Piggott et al., 2012, 2015). Synergism

represented a non-negligible 25% of non-additive interactions, suggesting a role of synergism in a climate change context.

Our study reveals that a complex freshwater community may show non-additive responses to multiple stressors when faced with future global change conditions. These responses, driven primarily by physical stressors, may display unexpected outcomes when increased temperature and water stress occur with chemical stressors such as pharmaceutical products or agricultural pesticides. We exposed river biofilms to acute worst-case conditions, and therefore the effect and direction of these interactions may vary in the long term. However, our results show the path to further studies, assessing the effect of these interactions at a chronic scale or across different levels of stress.

PAPER I. Multistressor effects on river biofilms under global change conditions

Supplementary information

Ferran Romero[1], Sergi Sabater[1,2], Xisca Timoner[1], Vicenç Acuña[1]

1. Catalan Institute for Water Research (ICRA), Carrer Emili Grahit 101, 17003 Girona (Spain).
2. Institute of Aquatic Ecology, University of Girona, 17071 Girona (Spain).

Technique	Target	Primer name	Sequence (5'-3')	Ref
PCR	16S rRNA	27F	AGAGTTTGATCMTGGCTCAG	Jeraldo et al 2014
		1492R	CGGTTACCTTGTTACGACTT	
PCR	18S rRNA	EUK1A	AACCTGGTTGATCCTGCCAGT	Wang et al 2014
		EUK564R	GGCACCAGACTTGCCCTC	
PCR	psbA	psbA3F	GGTATCCGTGAGCCAGTAGCAGGTC	Personal communication from Kim Tiam S
		psbA3R	GCTAAGAAGAAGTGTAAAGCACGAG	
qPCR	16S rRNA	1048F	GTGSTGCAYGGYTGTCGTCA	Maeda et al 2003
		1194R	ACGTCRTCCMCACCTTCCTC	
qPCR	18S rRNA	EUK345F	AAGGAAGGCAGCAGGCG	Zhu et al 2005
		EUK499R	CACCAGACTTGCCCTCYAAT	
qPCR	psbA	psbA3F	GGTATCCGTGAGCCAGTAGCAGGTC	Personal communication from Kim Tiam S
		psbA3R	GCTAAGAAGAAGTGTAAAGCACGAG	

Table PI.S1 Information of primers used in this study.

Target	Primer name	Cycles	qPCR program	R2	Efficiency (%)
16S rRNA	1048F 1194R	35	3 min 95ºC 20 sec 95ºC 60 sec 60ºC	0.998	104.8%
18S rRNA	EUK345F EUK 499R	40	3 min 95ºC 15 sec 95ºC 60 sec 60ºC	0.999	110.1%
psbA	psbA3F psbA3R	50	3 min 95ºC 15 sec 95ºC 60 sec 60ºC	0.999	91.40%

Table PI.S2 Conditions used for genes quantification by qPCR.

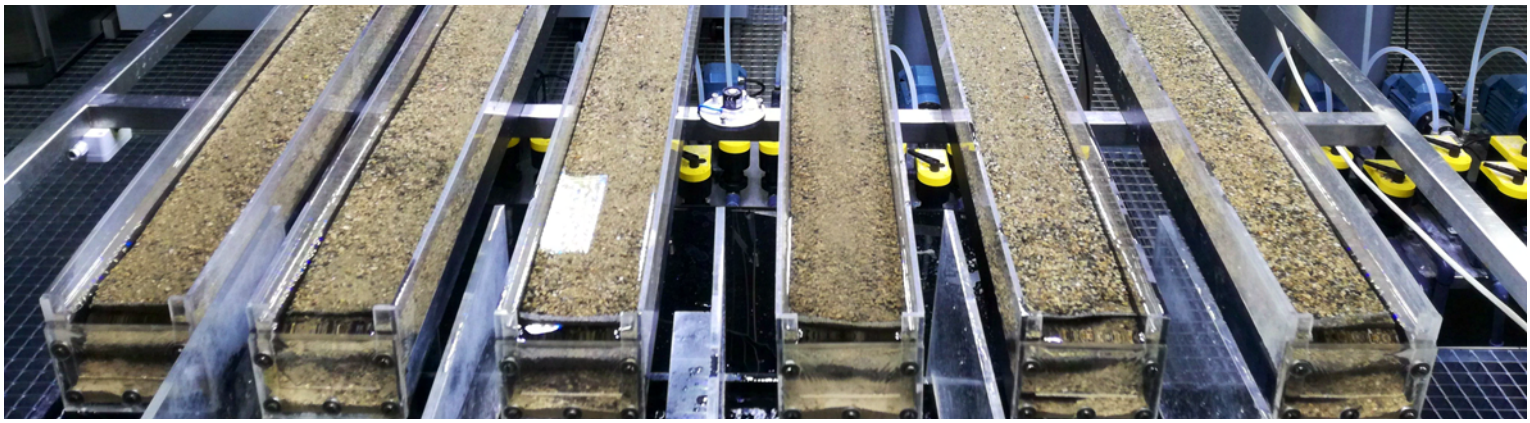
Stressor	Control	Treatment
Erythromycin (µg L^{-1})	0	10
Diuron (µg L^{-1})	0	10
Desiccation	-	+
Temperature (°C)	12.5	19.5

Table PI.S3 A comparison of the control and the treatment conditions used in this study.

Paper II

Desiccation events change the microbial response to gradients of wastewater effluent pollution

Romero, F., Sabater, S., Font, C., Balcázar, J.L., and Acuña, V.
Water Research 151: 371-380 (2019).



PAPER II. Desiccation events change the microbial response to gradients of wastewater effluent pollution

Ferran Romero[1]*, Sergi Sabater[1,2], Carme Font[1], José Luís Balcázar[1] and Vicenç Acuña[1].

1. ICRA, Catalan Institute for Water Research, Emili Grahit 101, University of Girona, 17003 Girona (Spain).
2. Institute of Aquatic Ecology, University of Girona, Campus de Montilivi, Girona 17071 (Spain).

Abstract

While wastewater treatment plant (WWTP) effluents have become increasingly recognized as a stressor for receiving rivers, their effects on river microbial communities remain elusive. Moreover, global change is increasing the frequency and duration of desiccation events in river networks, and we ignore how desiccation might influence the response of microbial communities to WWTP effluents. In this study, we evaluated the interaction between desiccation events and WWTP effluents under different dilution capacities. Specifically, we used artificial streams in a replicated regressional design, exposing first a section of the streams to a 7-day desiccation period and then the full stream to different levels of a realistic WWTP effluent dilution, from 0 % to 100 % of WWTP effluent proportion of the total stream flow. The microbial community response was assessed by means of high-throughput sequencing of 16S rRNA gene amplicons and quantitative PCR targeting ecologically-relevant microbial groups. Threshold Indicator Taxa Analysis (TITAN) was used, together with model fitting, to determine community thresholds and potential indicator taxa. Results show significant interactions between WWTP effluents and desiccation, particularly when sediment type is considered. Indicator taxa included members of Proteobacteria, Actinobacteria and Cyanobacteria, with abrupt changes in community structure at WWTP effluent proportion of the total flow above 50 %, which is related to nutrient levels ranging 4.6 – 5.2 mg $N\text{-}NO_3^-$ L^{-1}, 0.21 – 0.32 mg $P\text{-}PO_4^{3-}$ L^{-1} and 7.09 – 9.00 mg DOC L^{-1}. Our work indicates that situations where WWTP effluents account for > 50 % of the total river flow might risk of dramatic microbial community structure changes and should be avoided.

Introduction

Pollution from point sources such as wastewater treatment plants (WWTPs) is a common impact on freshwater ecosystems (Rice and Westerhoff, 2017). Through their effluents, WWTPs subject rivers to a wide range of chemical stressors including organic matter, inorganic nutrients in excess (Carey and Migliaccio, 2009), and many organic micropollutants such as pesticides, pharmaceuticals or industrial products (Rodriguez-Mozaz *et al.*, 2004; Kuzmanovic *et al.*, 2015). Moreover, rivers may receive other stressors that co-occur with these chemicals. Amongst these stressors, interruption of water flow and higher water temperature threaten the survival of river biota, as they are at the base of local extinctions and the overall decrease of biodiversity (Dudgeon, 2010). These stressors imply environmental filters as they act as additional selective pressures, and condition the composition and relative abundance of species in the riverine biological communities.

At the discharge point, WWTP effluent is mixed and therefore diluted with river discharge. The proportion of WWTP effluent to the total river flow defines the dilution factor. Lower dilution factors indicate a larger fraction of WWTP effluent in the river. The dilution factor ultimately depends on variations of river flow and WWTP effluents, and the balance between them. Exposure time and interaction with environmental factors add even more complexity to the effect that WWTP effluents may have on river ecosystems (Sabater *et al.*, 2016). Under high anthropogenic pressure (e.g. water abstraction), WWTP effluents can make up a significant proportion of the flow of the receiving water body. A recent analysis of the current situation in the US showed that WWTP effluents make up more than 50 % of the river flow for over 900 receiving systems at the point of discharge (i.e. ~6 % of the studied rivers), making these rivers predominantly composed of WWTP effluent (Rice and Westerhoff, 2017). Moreover, global change is expected to change natural variation of rivers flow towards increased low-flow and complete desiccation periods, so the number of WWTP effluent-dominated rivers worldwide is expected to increase (Döll and Schmied, 2012).

The biota inhabiting freshwater ecosystems is the final receptor of these co-occurring pressures. Freshwater communities are modulated by chemical (e.g. dissolved organic matter, nutrients), and physical (e.g. alterations in water flow) stressors, as well as their interactions (Sabater *et al.*, 2016). The response of each group of organisms (e.g. bacteria, algae, invertebrates or fish) to chemical and physical stress is related to their life cycle and environmental requirements. Shorter life-cycle organisms, such as bacterial communities, may respond to rapid changes occurring in the river environment, including physical (e.g. water flow, temperature) and chemical (e.g. nutrient abundance, organic matter availability) stressors. Bacteria inhabiting sediments can be the most responsive to short-term changes

after these pressures, because of their short generation time and intimate contact with the substratum (Sabater *et al.*, 2007; Besemer *et al.*, 2012; Segner et al, 2014).

The overall effect of WWTP effluents on river bacterial communities mostly depends on the resulting effluent proportion to the total flow of the receiving water body. Effluents from WWTP often induce changes in river bacterial diversity (Wakelin *et al.*, 2008; Drury *et al.*, 2013; Price *et al.*, 2018). While some authors have described increases in bacterial diversity as a result of exposure to WWTP effluents (Price *et al.*, 2018), others have associated them to decreases (Wakelin *et al.*, 2008; Drury *et al.*, 2013). The complex nature of bacterial communities, which are extremely diverse, together with the large uncertainty that comes along with field approximations is at the base of these non-consistent responses. Desiccation, in turn, can drive the structure of river bacterial communities from riverine to terrestrial, such as those occurring in soils (Pohlon *et al.*, 2013, 2018). Experimental approximations to the effects of WWTP effluents on bacterial communities are usually focused on the comparison between the community composition upstream and downstream of a WWTP discharge point using composite metrics such as richness and diversity (Marti *et al.*, 2013). Although these approaches are useful to investigate the effects of WWTP effluents at the local scale, they are usually placed in the context of specific hydrological conditions, thus being impossible to derive ecological thresholds from them that could be useful for water managers and policy makers. Moreover, studies assessing how desiccation events modulate the response of bacterial communities to WWTP effluent pollution under different dilution capacities are still lacking. Sophisticated experimental designs under controlled conditions and much more sensitive community analysis methods are required to disentangle the effects of WWTP effluents and desiccation events in river bacterial communities.

This study aimed to evaluate the overall effects of a realistic WWTP effluent and its interaction with desiccation on the bacterial community inhabiting river sediments. We used 24 artificial streams to investigate under controlled conditions how different proportions of WWTP effluent alter sediment bacterial community. Moreover, we applied a simulated 7-day desiccation period to assess how it interacts with the gradient of WWTP effluent dilution. We used non-parametric change point determination together with indicator taxa analysis to determine community thresholds and reliably responding bacterial taxa (Dufrêne and Legendre, 1997; King and Richardson, 2003; Qian *et al.*, 2003). We hypothesize that bacterial community composition will shift across the gradient of WWTP effluent dilution towards increased abundance of pollution-tolerant taxa, and that desiccation will modify the effects of the WWTP effluent by selecting highly tolerant taxa.

Material and Methods

Experimental design

The experiment was performed at the indoor Experimental Streams Facility of the Catalan Institute for Water Research (Girona, EU) between January 19th and March 16th, 2017. The Experimental Streams Facility consists of 24 artificial streams (see details in section 2.2) that have been previously used to explore the effects of contaminants and physical perturbations on stream biofilms (Acuña *et al.*, 2015; Freixa *et al.*, 2017; Subirats *et al.*, 2018).

Each of the 24 artificial streams was assigned to one of eight treatments (0, 14, 29, 43, 58, 72, 86 and 100 % of WWTP effluent water), following a gradient of WWTP effluent dilution. These values span from an unpolluted river to an extreme low-dilution condition, where all the flow (100 %) originates from WWTP discharge. River sediment containing biofilm was transported to the artificial streams and allowed to acclimate in clean water for 2 weeks. At this point, 3 replicated samples were taken to compare the community developed in our artificial streams at the onset of the experiment with the communities present at the source location (i.e. the Llémena River, Girona, EU). After the acclimation period, wire net baskets containing biofilm were placed out of the experimental streams and desiccated for 7 days. The biofilm contained in these baskets was used to explore how a 7-day desiccation event would shape the response of the biofilm to the WWTP effluent gradient. Immediately after wire net baskets were placed back again in the streams and WWTP effluent treatments were implemented during 5 weeks. At the end of the last week, fine and coarse sediment samples were taken for biofilm DNA extraction. Fine sediment was defined as the 5-cm sand column sampled using a cylindrical core, whereas coarse sediment consisted on small flat cobbles placed on top of the sand (see next section).

Experimental conditions

Each artificial stream consisted of an independent methacrylate channel (l – w – d = 200 cm – 10 cm – 10 cm), and a 70 L water tank from which water can be recirculated. Each artificial stream was filled with 5 L of fine sediment (i.e. sand) extracted from an unpolluted segment of the Llémena River (Sant Esteve de Llémena, Girona, EU), which is a permanent river draining a calcareous mountainous range (d_{50} = 0.74 mm). The extracted sand was transported in less than one hour to the artificial streams and evenly distributed to create a plane bed that facilitated the growth of the biofilm. To assess the response of coarse sediment, small flat cobbles (l – w – d = 2 cm – 2 cm – 1 cm) were also extracted from the Llémena River, transported and distributed on the artificial streams. Each stream received a constant flow of 50 mL s^{-1} from the tank, and operated as a closed system for 72 h, as water

from all the channels was renovated every three days. Mean water velocity was 0.71 cm s^{-1}, and water depth over the plane bed ranged 3 - 5 cm.

Water sources for the artificial streams were rainwater, filtered through activated carbon filters, and WWTP effluent water from the Quart WWTP (Girona, EU). During the acclimation period, biofilm was allowed to grow on the artificial streams from the inocula present in the sediments from the Llémena River. Afterwards, water from the WWTP was transported in 200 L tanks and transferred to the artificial streams in less than 2 hours. Desiccation was applied by letting fine and coarse sediment samples contained in wire net baskets air-dry during 7 days, as this has been reported to be the minimum to produce significant effects on biofilm community (Acuña *et al.*, 2015). Daily cycles of photosynthetic active radiation (PAR) were defined as 10 h daylight + 14 h darkness, and were simulated by LED lights (Lightech, Girona, EU). PAR was held constant at 173.99 ± 33 µE m^{-2} s^{-1} during the daytime, and was recorded every 10 min using 4 quantum sensors located across the whole array of streams (sensor LI-192SA, LiCOR Inc, Lincoln, USA). Air temperature was maintained at 15 ° C during the acclimation period and 20 ° C during the exposure period, at an air humidity of 30 %. Water temperature was recorded every 10 min using VEMCO Minilog (TR model, AMIRIX Systems Inc, Halifax, NS, Canada) temperature data loggers (-5 to 35 ° C ± 0.2 ° C). Overall, physico-chemical conditions in the artificial streams (water velocity, temperature and light cycles) emulated those of the Llémena River during early spring.

Water chemistry

Dissolved oxygen, pH and specific conductivity were measured weekly by noon in each artificial stream using WTW (Weilheim, Germany) hand-held probes. Concentrations of nutrients and dissolved organic carbon (DOC) were measured 24 h after the renewal of the artificial streams water from water collected from the channel outlet. Water was filtered immediately through 0.2 µm pore size nylon filters (Whatman, Kent, UK) into pre-washed polyethylene containers for nutrients and through 0.7 µm glass fiber filters, previously ashed (Whatman GF/F, Kent, UK) for DOC. Nutrient and DOC analyses were carried out as described in Corcoll *et al.*, 2015.

Sampling and sample processing

Biofilm was removed from coarse and fine sediment samples by washing them with a sterile Ringer's solution and placed in centrifuge sterile vials. Vials were centrifuged at 13000 *g* during 60 sec to remove excess water and then 300 mg of biofilm were used for total DNA extraction following manufacturer's recommendations (FastDNA™ Spin Kit isolation kit, MP Biomedicals).

Quantification of ecologically-relevant marker genes

Quantitative real-time PCR (qPCR) assays were used to quantify six ecologically-relevant genes, including 16S rRNA (marker for total bacteria), *psaA* and *psbA* (markers for total photosynthesizers), *dsrA* (marker for sulphate-reducing bacteria), *narG* (marker for denitrifying bacteria) and *mcrA* (marker for methanogenic archaea). All qPCR assays were performed in triplicate using SYBR Green detection chemistry on an Mx3005P system (Agilent Technologies; Santa Clara, CA, USA), as previously described (Marti *et al.*, 2013). Briefly, an initial denaturation was performed at 95 °C for 3 min, followed by 40 cycles at 95 °C for 15 sec and 60 °C for 60 sec. Standard curves were obtained by serial dilutions of PCR products previously purified with QIAquick PCR purification kit (Qiagen Inc., Valencia, CA, USA), which were verified by Sanger sequencing (Macrogen: Amsterdam, the Netherlands). All genes were quantified using specific primers (Table PII.S1).

High-throughput sequencing and sequence processing

For community analysis, independent DNA extractions from coarse and fine sediment samples were subjected to high-throughput sequencing of 16S rRNA genes with the Illumina MiSeq System (2 x 250 PE) using the primer pair 515f/806r (Caporaso *et al.*, 2011) complemented with Illumina adapters and sample-specific barcodes at the Research Technology Support Facility, Michigan State University, USA (Kozich *et al.*, 2013). Pair merging, quality filtering, chimera checking, clustering into Operational Taxonomic Units (OTU) and construction of OTU table were carried out against the Greengenes database (McDonald *et al.*, 2011) with a 0.97 similarity cutoff using default parameters in Mothur (Schloss *et al.*, 2009). Sequencing depth was 54652 ± 1676 sequences per sample. OTUs affiliated to eukaryotes, algal chloroplasts, archaea and unclassified were filtered from the original OTU table using appropriate scripts in Mothur. Additionally, a Biological Information Matrix (BIOM) file was generated with Mothur and was uploaded to METAGENassist web served to predict the functional community profile from the 16S rRNA dataset (Arndt *et al.*, 2012).

Statistical approaches

We applied a series of statistical methods to study the response of the microbial community to the WWTP effluent dilution gradient and its interaction with desiccation. The overall single and interactive effects of WWTP effluents and desiccation on bacterial community structure were investigated by permutational multivariate analysis of variance (PERMANOVA). The determination of community thresholds and indicator taxa was performed by means of Threshold Indicator Taxa Analysis (TITAN). In order to understand the shape of the relationship between the proportion of WWTP effluent to total flow and the abundance of gene markers related to ecologically-relevant microbial groups, we fitted a series of regression

models commonly used in environmental sciences to explain the relationship between environmental gradients and response variables, such as the exponential or the Monod model.

Overall effects on bacterial community structure

We constructed a similarity matrix using the Bray-Curtis distance (Legendre and Gallagher, 2001) based on the relative abundance of each bacterial OTU. Analysis of similarity between the taxonomic composition of communities was done after grouping samples by stressor, that is, WWTP effluent (We) and desiccation (D), which were used as factors together with sediment type (S) in PERMANOVA. The interaction terms (i.e. We * D, We * S, D * S and We * D * S) were also included in the analyses. These analyses were run in R.3.4.3 using the *adonis* function in the *vegan* package (Oksanen *et al*., 2018).

Determination of community thresholds and indicator taxa

Threshold Indicator Taxa Analysis (TITAN 2.1) was performed in R.3.4.3 combining indicator taxa analysis (Dufrêne and Legendre, 1997) with nonparametric change-point analysis (King and Richardson, 2003; Qian *et al.*, 2003) in order to identify change points in the frequency and abundance of individual taxon and examine if multiple taxa had synchronous responses over changes in the proportion of WWTP effluent to total stream flow. TITAN allows identifying the optimum value of a continuous variable (here, the proportion of WWTP effluent in each treatment) that partitions sampling units while maximizing taxon-specific scores. Association is measured by indicator values (IndVal), calculated for all taxa and possible change points along the WWTP effluent dilution gradient, with permutation tests to assess the uncertainty in these scores. Permuted IndVal scores are standardized as *z* scores and summed for positive [sum (z+)] and negative [sum (z-)] response values for each possible change point. Sum (z) peaks highlight values of WWTP effluent proportion to the total stream flow around which many taxa exhibit strong changes in relative abundance, thus representing potential community thresholds. Bootstrapping (500 repetitions) was here used to estimate indicator reliability (proportion of bootstrap replicates with maximum IndVal reaching a *p*-value < 0.05) and purity (proportion of bootstrap replicates matching group assignment in the original data) as well as uncertainty around the location of individual taxon and community change points. The following criteria were used to judge evidence for community thresholds from calculated change points and indicator taxa: synchronous shifts in many reliably responding taxa and strong responses of individual taxa (i.e. narrow bootstrapped quantile intervals). More details of TITAN method can be found elsewhere (Baker and King, 2010).

Regression model

The relationship between the copy number of ecologically-relevant gene markers and the proportion of WWTP effluent to total flow in the treatments was searched after application of a series of models fitted to the quantitative PCR data (Table PII.S5).

Polynomial regressions included the linear, quadratic and cubic relationships. The linear relationship implied that the gene copy number increased linearly with increasing proportion of WWTP effluent in the treatment. The exponential and power models implied that the magnitude of the response increased either exponentially or potentially to increasing proportion of WWTP effluent. The logistic and its inverse function (i.e. logit) implied that the increase in the copy number for increasing proportion of WWTP effluent was minimal at low and high concentrations but maximum at intermediate concentrations. The Monod fitting implied that copy numbers increased, but approached an asymptote at high WWTP effluent proportions. The R functions *gnls* (for all equations except the polynomials) and *lm* (for the polynomial equations), both from the R package *nlme* (Pinheiro *et al.*, 2017), were used to determine the parameters in the models that best match the experimental data sets. These functions fit a nonlinear model using generalized least squares (Pinheiro *et al.*, 2017). Once each of the models was run, the corresponding residual standard error (RSE) and R^2 were computed. Thus, the models could be ranked according to their RSE, being the lowest the one that better explains the data.

Results

Experimental conditions

Temperature was held constant during the entire experiment, air temperature averaging 21.0 ± 2.2 °C, and water temperature in the artificial streams averaging 20.13 ± 0.31 °C in all treatments. PAR cycles as well as hydraulics were also steady throughout the experiment. Dissolved oxygen, pH and specific conductivity showed minimal differences between treatments before the start of the experimental manipulation (data not shown).

Water chemistry analyses

The implementation of the treatments (i.e. the exposure to the dilution gradient of WWTP effluent water) involved changes in nutrient concentration, dissolved organic carbon (DOC), dissolved oxygen (DO), specific electrical conductivity (SpCond) and pH between treatments, but were similar between replicates (Table PII.1). The highest values of nutrients, dissolved organic carbon (DOC) and conductivity were measured in the artificial streams containing 100% of WWTP effluent, and decreased progressively up to the 0 % treatment.

% We	$N-NO_3^-$	$P-PO_4^{3-}$	$S-SO_4^{2-}$	$N-NH_4^+$	DOC	SpCond	DO	pH
0	1.2 ± 0.1	0.04 ± 0.04	4.47 ± 0.33	0.05 ± 0.03	1.20 ± 0.07	220 ± 3.54	8.8 ± 0.11	8.3 ± 0.07
14	3.0 ± 0.5	0.03 ± 0.00	6.67 ± 0.23	2.60 ± 0.62	3.03 ± 0.13	400 ± 10.3	9.0 ± 0.07	8.3 ± 0.07
29	3.0 ± 0.7	0.07 ± 0.01	9.30 ± 0.27	6.60 ± 1.10	5.02 ± 0.17	583 ± 24.7	9.2 ± 0.14	8.3 ± 0.07
43	4.6 ± 1.3	0.21 ± 0.06	12.4 ± 0.42	9.50 ± 1.63	7.09 ± 0.27	764 ± 34.6	8.8 ± 0.14	8.2 ± 0.07
58	5.2 ± 1.7	0.32 ± 0.09	14.5 ± 1.20	11.5 ± 2.23	9.00 ± 0.32	930 ± 46.7	8.4 ± 0.25	8.1 ± 0.11
72	7.3 ± 2.6	0.46 ± 0.15	17.6 ± 0.74	14.4 ± 2.62	11.2 ± 0.48	1103 ± 56.2	7.8 ± 0.35	8.0 ± 0.11
86	7.4 ± 2.9	0.55 ± 0.15	19.3 ± 0.81	16.3 ± 2.97	12.3 ± 0.54	1204 ± 64.0	7.4 ± 0.39	8.0 ± 0.14
100	10.1 ± 3.5	0.72 ± 0.16	21.2 ± 0.95	19.2 ± 3.36	14.3 ± 0.60	1343 ± 73.9	6.9 ± 0.42	8.0 ± 0.11

Table PII. 1 Mean ± standard error for nutrients, dissolved organic carbon (DOC), specific electrical conductivity (SpCond), dissolved oxygen (DO) and pH in each treatment during the exposure (n = 24, resulting from 3 replicates per treatment and 8 sampling times). For nutrients, DOC and DO results are given in mg L^{-1}, for SpCond results are given in µS cm^{-1}. The first column (i.e. % We) refers to the proportion of pure WWTP effluent to the total stream flow in each treatment.

Bacterial community response to WWTP effluent and desiccation

Overview of bacterial community structure and functional potential

A total of 5246620 16S rRNA sequences were generated through MiSeq Illumina sequencing after removing short and low quality sequences, with an average read length of 250 bp. A total of 29645 OTUs (defined at the 97% sequence similarity level) were found in sediment samples from the artificial streams. The bacterial communities derived from the investigated treatments were dominated by Proteobacteria, which showed an overall relative abundance of 49.47 % ± 5.42 % of total reads. The second and third most abundant phyla were Bacteroidetes and Planctomycetes. The relative abundances of the main bacterial classes across treatments can be found in Figure PII.1. A table summarizing the overall bacterial community structure, indicating the most abundant genera and their relative abundance can be found in Table PII.S7. Raw sequences are available at the Sequence Read Archive (SRA) of the National Center for Biotechnology Information under accession number SRP155490 (https://www.ncbi.nlm.nih.gov/sra/SRP155490).

The taxonomic functional profiles derived from METAGENassist revealed the existence of 22 metabolic phenotypes in our data set. Among them, ammonia oxidation, dehalogenation, aromatic hydrocarbon degradation, nitrite reduction, sulfate reduction, sulfide oxidation, xylan degradation and sporulation were the 8 most abundant phenotypic features (Figure PII.S1). A detailed version of the phenotypic profile across all treatments can be found in Table PII.S2.

Comparison between the bacterial community in the artificial streams and the Llémena River

Even though the main goal of this study was not to compare the communities present at the Llémena River and the communities in our artificial streams, we used Illumina sequencing to assess the “filtering” effect. The filtering effect is considered here as the amount of OTUs that we might have lost when placing a natural bacterial community into an experimental system. We were able to detect 26 OTUs that were present in Llémena samples but were not present in sediment from our artificial streams. These 26 OTUs represent 8.38 % of the total OTUs obtained in this parallel experiment. This means that the 91.62 % of the OTUs present in the Llémena River were also detected in our experimental streams. The community in the Llémena River shared 72.66 % of similarity with the community in our artificial streams (Bray-Curtis similarity percentage, Figure PII.S2).

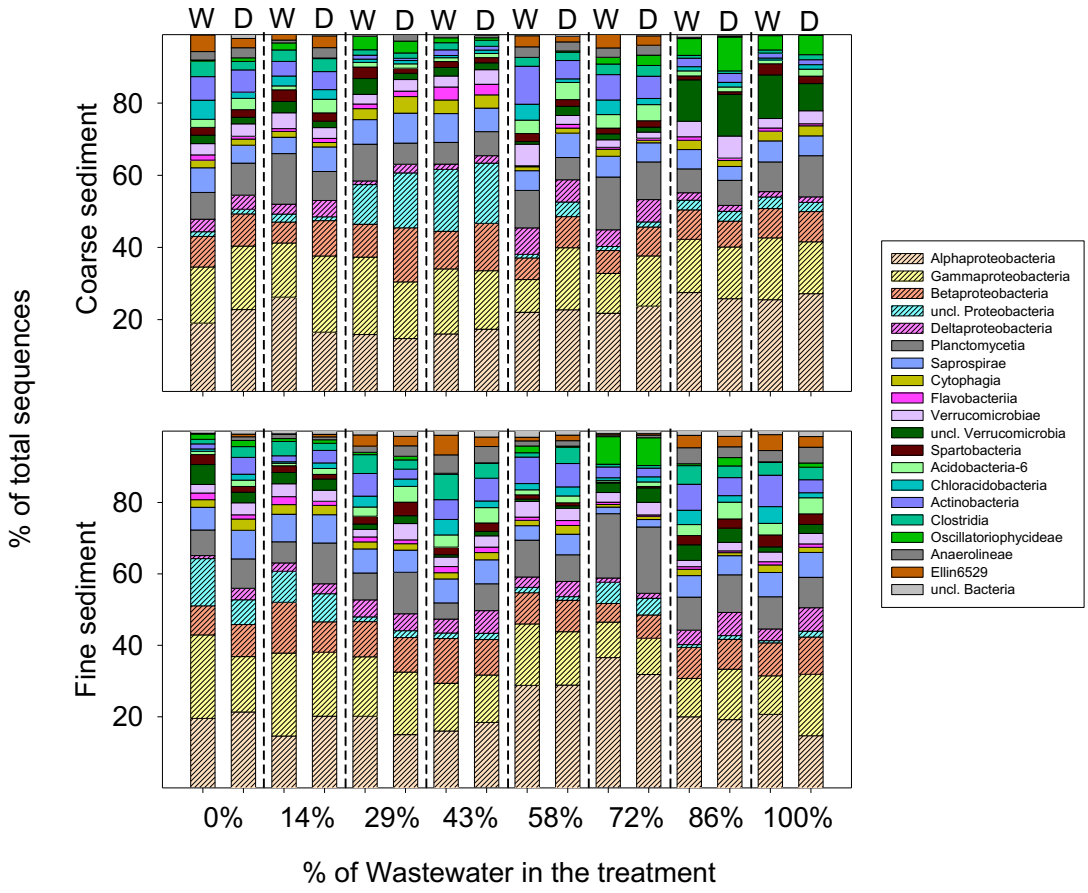


Figure PII. 1 Bacterial community structure across treatments. Composition of sediment bacterial communities at the Class level collected from coarse (top) and fine (bottom) sediment samples across the gradient of WWTP effluent proportion to the total stream flow. Results are shown for non-desiccated (wet, W) and desiccated (dry, D) samples.

Overall effects of WWTP effluents and desiccation on bacterial community

We assessed the overall effect of WWTP effluent (We), desiccation (D), sediment type (S) and their interactions (i.e. We * D, We * S, D * S and We * D * S) on bacterial community composition and functional potential by means of permutational multivariate analysis of variance (PERMANOVA). Bacterial community composition was studied at the phylum, class, order, family and genus level of taxonomic resolution.

WWTP effluent showed a significant effect on bacterial community structure across all taxonomic levels (p-value < 0.05, F-value ranging 5.19 – 8.11), as well as bacterial community functional potential (F-value = 4.54, Table PII.2). Desiccation significantly affected bacterial community structure at low taxonomic levels (F-values ranging 1.42 – 5.44, Table PII.2). Sediment type showed a significant effect on bacterial community structure across all taxonomic levels (F-value ranging 2.96 – 4.36), as well as bacterial community functional potential (F-value = 3.52, Table PII.2). Among the 2-factor interaction terms (We * D, We * S and D * S), only the interaction between WWTP effluent and sediment type was significant (F-value ranging 9.62 – 15.88, Table PII.2). The triple interaction (We * D * S) was significant for bacterial community structure at all taxonomic levels (F-values ranging 1.74 – 2.25, Table PII.2).

Overall responses of bacterial community structure to WWTP effluents and potential community thresholds

The Threshold Indicator Taxa Analysis (TITAN 2.1 for R 3.4.3) was used to evaluate variation in taxonomic composition of the bacterial community in response to a WWTP effluent dilution gradient. Taking into account all the possible sediment-stressor combinations (i.e. fine sediment vs. coarse sediment, or the use of non-desiccated vs. desiccated sediments), an effluent proportion of 21.5 – 72 % to the total stream flow resulted in the greatest changes (Table PII.3). For coarse sediment, WWTP effluent proportions around 50.5 – 72 % of the total flow were strongly associated to abrupt changes in bacterial community structure. The determination of potential community thresholds in fine sediments was complicated by the occurrence of larger confidence intervals in individual taxa. However, the overall trend pointed towards lower WWTP effluent proportions in fine sediment than in coarse sediment (Table PII.3).

Factor		SS	MS	*F*-value	*p*-value
We	Phylum	0.34	0.05	5.19	< 0.001
	Order	1.60	0.22	6.07	< 0.001
	Genus	2.19	0.31	5.29	< 0.001
	Function	0.16	0.02	4.54	< 0.001
D	Phylum	0.01	0.01	1.42	0.220
	Order	0.15	0.15	3.92	< 0.001
	Genus	0.32	0.32	5.44	< 0.001
	Function	< 0.01	< 0.01	0.85	0.370
S	Phylum	0.04	0.04	4.36	0.010
	Order	0.14	0.14	3.73	0.010
	Genus	0.22	0.22	3.79	0.010
	Function	0.02	0.02	3.52	0.050
We * D	Phylum	0.08	0.01	1.17	0.290
	Order	0.31	0.04	1.17	0.210
	Genus	0.45	0.07	1.09	0.310
	Function	0.04	0.01	1.19	0.290
We * S	Phylum	1.04	0.15	15.88	< 0.001
	Order	2.55	0.36	9.65	< 0.001
	Genus	4.07	0.58	9.81	< 0.001
	Function	0.49	0.07	14.05	< 0.001
D * S	Phylum	0.03	0.03	3.06	0.040
	Order	0.04	0.04	1.18	0.270
	Genus	0.06	0.06	1.06	0.330
	Function	0.02	0.02	3.07	0.070
We * D * S	Phylum	0.13	0.02	2.01	0.020
	Order	0.46	0.01	1.74	0.010
	Genus	0.87	0.12	2.10	< 0.010
	Function	0.04	0.01	1.10	0.360

Table PII. 2 Overall effects of WWTP effluent (We), desiccation (D), sediment type (S) and their interactions on bacterial community structure (at the phylum, order and genus level) and functional potential (as predicted by METAGENassist). Resulting p-values and F-values from PERMANOVA are indicated. An extended version of this table (including results at the class and family levels) can be found in supplementary material Table PII.S4.

	Coarse		Fine	
	Wet	**Dry**	**Wet**	**Dry**
Fsum(z+)	50.5 (50.5-50.5)	50.5 (50.5-79)	36 (29 - 58)	65 (50.5-65)
Fsum(z-)	50.5 (50.5-65)	72 (50.5-72)	21.5 (21.5-79)	21.5 (21.5-79)

Table PII. 3 Fsum (z) calculated change point for positive (z+) and negative (z-) taxa and associated confidence intervals across sediment types (coarse vs. fine) and desiccation conditions (non-desiccated, wet; desiccated, dry).

Potential indicator taxa

In coarse sediment samples, 65 taxa responded significantly to the WWTP effluent dilution gradient in the non-desiccated treatments (Figure PII.2, A). Of these, 17 taxa increased their relative abundance at some point across the WWTP effluent dilution gradient (z+ responders). Other 48 taxa decreased their relative abundance (z- responders). On desiccated coarse sediments, 16 taxa were classified as positive responders and 40 as negative responders (Figure PII.2, B). Positive responders (z+) included members of Actinobacteria, Proteobacteria and Bacteroidetes (Figure PII.2, A, B). The negative (z-) responders included members of Firmicutes and Gammaproteobacteria. Among these, members affiliated to the class Gammaproteobacteria (especially the genera *Allochromatium*, *Citrobacter*, *Hahella, Rheinheimera* and *Thermomonas*) were the best indicator taxa, as they showed narrow confidence intervals around ~50 % of WWTP effluent proportion to the total flow (Figure PII.2, A). Only for desiccated coarse samples, members of soil-related Alphaproteobacteria (i.e. *Aminobacter*, *Sphingobium*) were also good indicator taxa (Figure PII.2, B).

In fine sediment samples, 89 taxa responded significantly to the WWTP effluent dilution gradient in non-desiccated treatments. Of these, 51 were classified as positive responders and 38 as negative (Figure PII.2, C). On desiccated fine sediments, a total of 18 taxa responded with high purity and reliability. Of these, 6 taxa were classified as positive responders and 12 as negative responders (Figure PII.2, D). Positive responders (z+) included members of Actinobacteria, Acidobacteria, Bacteroidetes and Proteobacteria. We found two indicator taxa that presented overall abundances > 0.5 % of total reads in our data set, suggesting a strong response to the WWTP effluent dilution gradient. These taxa were the genus *Gemmata* (relative abundance = 2.39 %) and an unclassified member of Cyanobacteria (relative

abundance = 0.79 %) (Figure PII.2, C). In fine sediments, the negative (z-) responders included mainly members of Proteobacteria as well as two members of Firmicutes and Bacteroidetes (Figure PII.2, C, D). Indicator taxa were mainly affiliated to Alphaproteobacteria (*Aminobacter*, *Lutibacterium*) and Gammaproteobacteria (*Hahella*, *Citrobacter*, *Dokdonella*).

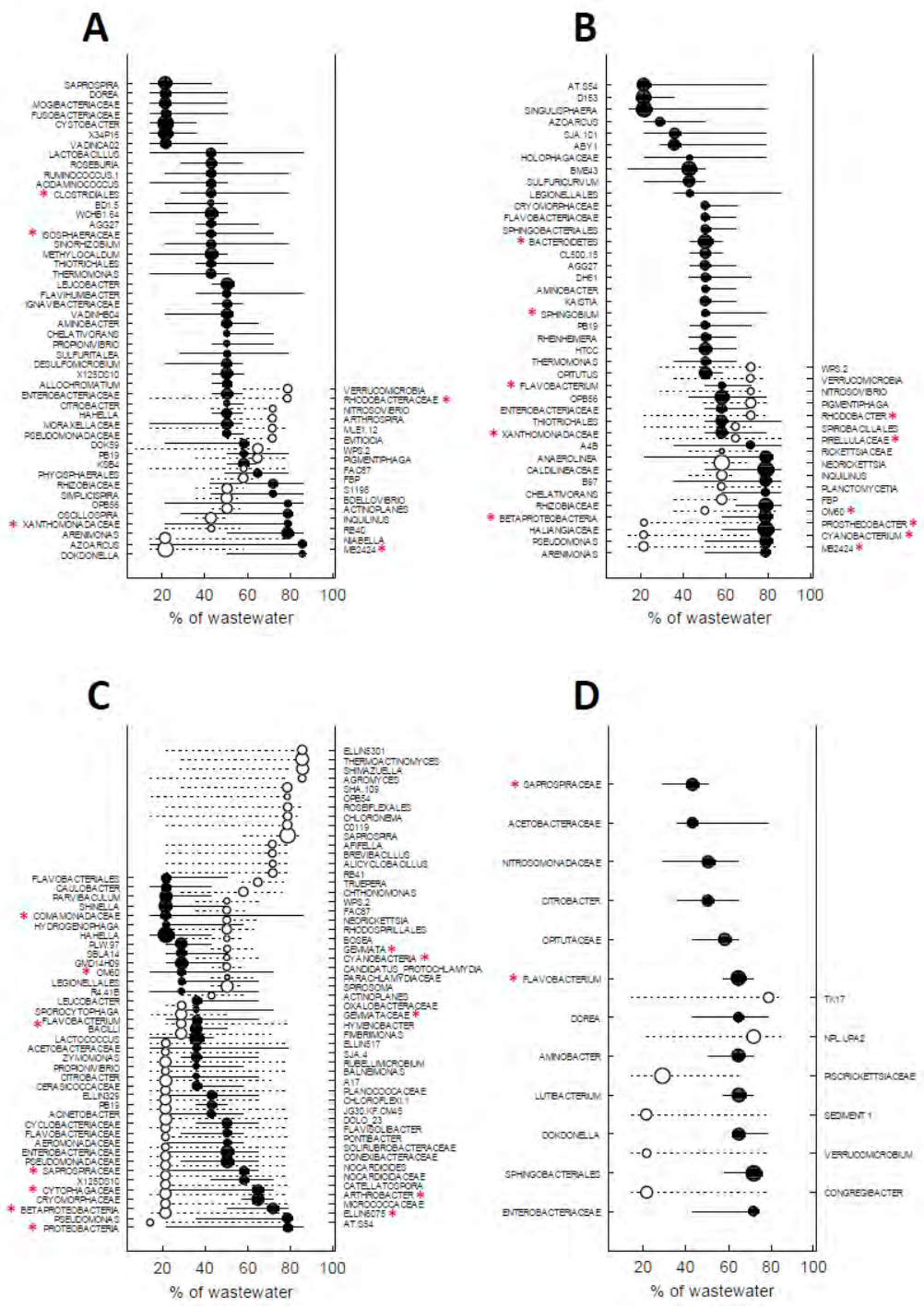


Figure PII. 2 Threshold Indicator Taxa Analysis (TITAN) and change-point analysis (Bray-Curtis distance) of bacterial community response to wastewater gradient. TITAN results for coarse (A: non-desiccated, B: desiccated) and fine (C: non-desiccated, D: desiccated) sediment types are shown, as well as desiccation conditions: wet (left) and dry (right). Significant (purity ≥ 0.95, reliability ≥ 0.95, p-value ≤ 0.05) bacterial indicator taxa are plotted. OTUs are classified according to the Greengenes database to the lowest possible taxonomic level of resolution. Within each plot, negative responders (z-) are indicated on the left side, and their associated calculated change-point is indicated by a black circle. Positive (z+) responders are indicated on the right, and their associated calculated change point is indicated by a white circle. Circles sizes are proportional to calculated z-scores. Asterisks indicate OTUs representing > 0.5 % of total abundance.

Abundance of ecologically-relevant microbial groups

We examined the relationship between the abundance of six ecologically relevant gene markers (i.e. 16S rRNA, *narG*, *mcrA*, *dsrA*, *psaA* and *psbA*) and the WWTP effluent dilution gradient by fitting a series of models and comparing the outputs in terms of residual standard error (RSE) and R^2 (Figure PII.3, Table PII.4). For non-desiccated coarse sediment, the abundance of the studied genes followed a cubic response in all cases except the *dsrA* gene, which showed a quadratic negative response, with R^2 ranging 0.21 – 0.45. The best adjustment to the cubic response was observed for 16S rRNA (R^2 = 0.44), *psaA* (R^2 =0.45) and *psbA* (R^2 = 0.39). Desiccation of coarse sediments shifted gene abundance relationship with WWTP effluent towards a logit-type model (*narG*, *psaA* and *psbA* genes). For non-desiccated fine sediment, half of the studied genes (*narG*, *psaA* and *psbA*) showed a logit-type response, whereas the abundance of 16S rRNA followed an exponential growth. Desiccation of fine sediment resulted in no change for *narG* and *dsrA* genes response, whereas the rest of the genes (16S rRNA, *mcrA*, *psaA* and *psbA*) shifted towards a cubic-type response pattern (Figure PII.3, Table PII.4).

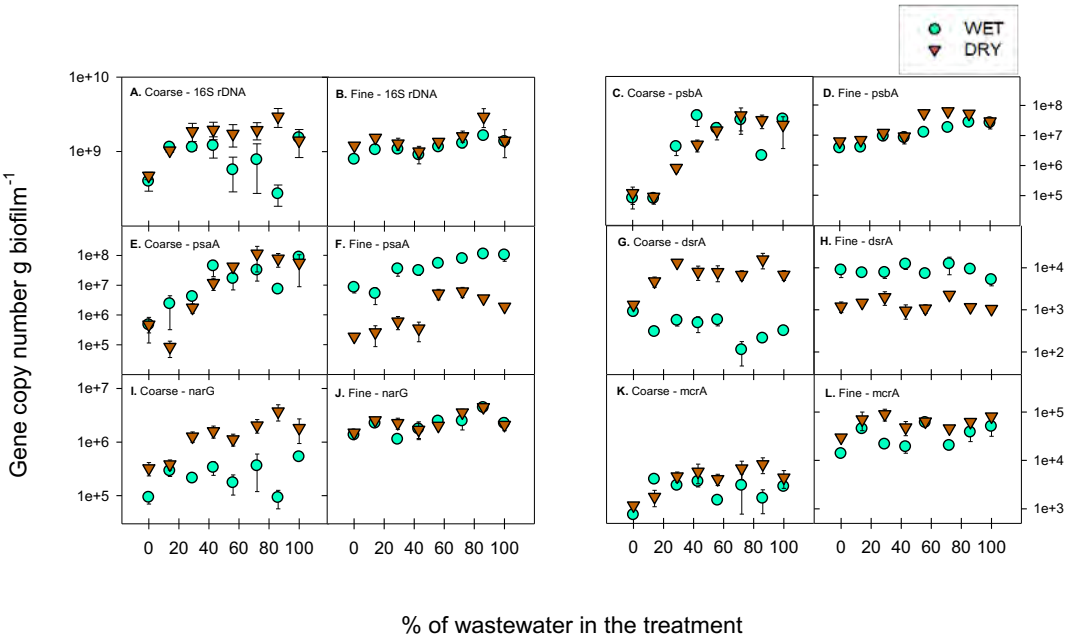


Figure PII. 3 Gene abundance results to percent of WWTP effluent in the treatments. The following genes were quantified: 16S rRNA (A, B), psbA (C, D), psaA (E, F), dsrA (G, H), narG (I, J) and mcrA (K, L). Coarse and fine sediment results are showed. Response for the non-desiccated (WET) treatments is displayed in the plots by light blue dots, while response of the desiccated (DRY) treatments is displayed by brown inverted triangles.

				Best fit			
				COARSE SEDIMENT		FINE SEDIMENT	
Biological process	**Specific function**	**Target group**	**Gene name**	**WET**	**DRY**	**WET**	**DRY**
Ribosome component	30S small subunit-binding	Bacteria	*16S rRNA*	Cubic (R^2 = 0.44)	Quadratic (R^2 = 0.31)	Exp. growth	Cubic (R^2 = 0.44)
Nitrogen metabolism	Nitrate reduction	Nitrate reducers	*narG*	Cubic (R^2 = 0.26)	Logit	Logit	Logit
Methanogenesis	Methyl coenzyme M reduction	Methanogenic archaea	*mcrA*	Cubic (R^2 = 0.21)	Quadratic (R^2 = 0.30)	Linear (R^2 = 0.14)	Cubic (R^2 = 0.32)
Sulphate reduction	Dissimilatory sulphite reduction	Sulphate-reducing bacteria	*dsrA*	Quadratic (R^2 = 0.34)	Quadratic (R^2 = 0.24)	Linear (R^2 < 0.01)	Linear (R^2 = 0.02)
Photosynthesis	PSI structural apoprotein A1	Photosynthesizers	*psaA*	Cubic (R^2 = 0.45)	Logit	Logit	Cubic (R^2 = 0.54)
	PSII-binding protein	Photosynthesizers	*psbA*	Cubic (R^2 = 0.39)	Logit	Logit	Cubic (R^2 = 0.65)

Table PII. 4 Best fitting model results for studied gene abundances. Results are based on the model displaying the lowest value of the residual standard error (RSE). For polynomial equations (i.e. linear, quadratic and cubic), values of R^2 are indicated in parentheses.

Discussion

Experimental design and treatment conditions

Our experimental approach had the advantage of combining rigorous control over anthropogenic stressors and sufficient statistical power to detect individual and combined effects. However, experimental designs relying on the use of artificial ecosystems might fail to reproduce the communities that develop in natural environments. To assess this likely limitation, we compared the community in our artificial streams with that of the natural reference stream from where the sediment was extracted (Llémena River, Girona, EU). Results indicate that in terms of bacterial OTUs, ~92 % of the OTUs retrieved from Llémena River were detected in our artificial streams at the end of the acclimation period, representing a Bray-Curtis similarity of ~72 %. Thus, despite the limitations associated to performing the study on an artificial environment, the similarity of bacterial community with that on the natural reference site places our results in context and provides reliability to our conclusions.

WWTP effluent strongly interacted with sediment type, which is known to be one of the major drivers of bacterial community composition in river beds (Zeglin, 2015). Also, the triple interaction between WWTP effluent, desiccation and sediment type (We * D * S) was significant, suggesting that the combined effect of WWTP effluents and desiccation is particularly important when sediment type is considered. Importantly, desiccation did not affect bacterial community composition at high taxonomic levels (i.e. phylum), and its interaction with WWTP effluent and sediment type was weak. This adds further empirical evidence to previous work aiming at finding the best taxonomic resolution for studying multiple-stressor effects (R. K. Salis *et al.*, 2017). In their work, Salis and collaborators argued that the order level might represent the best compromise between stressor sensitivity and coverage of bacterial community. Here we show that lower taxonomic levels better respond to the stress caused by a desiccation event. Associated to this, higher taxonomic levels (i.e. phylum level) are more likely to contain taxa with opposing sensitivities to desiccation, likely blurring the overall response.

The range of main nutrient (nitrate and phosphate) concentrations that we were able to achieve in our treatments span from the less polluted river basins in Europe, containing < 0.02 mg $P\text{-}PO_4^{3-}$ L-1 and < 0.8 mg $N\text{-}NO_3^-$ L-1 to the most polluted European basins, containing up to 0.40 mg $P\text{-}PO_4^{3-}$ L-1 and 11.3 mg $N\text{-}NO_3^-$ L-1 (Grizzetti et al., 2017; European Environment Agency (EEA), 2018). The nutrient values in the 100 % streams (i.e. the treatment containing pure WWTP effluent) were 10.1 ± 3.5 mg $N\text{-}NO_3^-$ L-1, 0.72 ± 0.16 mg $P\text{-}PO_4^{3-}$ L-1 and 14.3 ± 0.60 mg DOC L^{-1}. Levels of nutrients and DOC in WWTP effluents depend on treatment processes and influent conditions, and the levels tested in this study represent overall nutrient

concentrations observed in WWTP effluents across Europe (European Environment Agency (EEA), 2018).

Proposed community thresholds

Results presented here show a significant overall effect of WWTP effluents on bacterial community composition, as determined by Illumina sequencing and quantitative PCR. Threshold Indicator Taxa Analysis (TITAN) was used to identify community thresholds and indicator taxa. TITAN outcomes revealed that WWTP effluent proportion around 50 % of the total flow resulted in the greatest change in sediment bacterial community structure. The steep shift of taxa relative abundance near the 50 % of WWTP effluent identified by TITAN analysis suggests that any river with the physicochemical characteristics that we achieved in the 43-56% treatments might be considered at risk. As an example, a recent study in the US showed that over 6 % of the studied rivers (~15 000) had WWTP effluent contributions > 50 % of the total river flow, and that global change might worsen this situation by reducing natural dilution capacity (Rice and Westerhoff, 2017). This 50 % of WWTP effluent was associated with moderate to high nutrient levels (i.e. 4.6 - 5.2 mg N-NO_3^- L^{-1}, 0.21 - 0.32 mg P-PO_4^{3-} L^{-1} and 7.09 - 9.00 mg DOC L^{-1}). Bacterial communities in fine sediments experienced higher changes in their composition at lower WWTP effluent proportions, indicating their higher sensitivity to WWTP effluents. Since bacterial communities inhabiting fine sediments are known to be major contributors to the accumulation and degradation of organic matter, changes in their composition might affect carbon dynamics at the ecosystem level (Romaní *et al.*, 2004). Together with this, larger confidence intervals were retrieved from fine sediment samples, probably due to the fact that bacterial communities in fine sediments are richer and more diverse than bacterial communities in cobbles, therefore making their response to the gradient of WWTP effluent more complex. Taken together, our results indicate that rivers receiving WWTP discharges under reduced dilution capacity might potentially risk of dramatic bacterial community structure changes.

Potential indicator taxa

Members of Actinobacteria and Cyanobacteria might be seen as good positive indicator taxa, as they increased in their relative abundance while showed narrow confidence intervals. On the other hand, results point towards members of Gammaproteobacteria as good negative indicator species. Although Gammaproteobacteria contain several pathogenic bacteria that are known to be discharged from WWTPs, their abundance was recently reported to decrease 100 meters downstream of a WWTP discharge point (Marti and Balcázar, 2014). This decrease is reflected in the qPCR results as a decrease in the number 16S rRNA copies. In fine sediment samples, this decrease in the 16S rRNA copy numbers is not observed, probably due to a counteracting effect caused by increased abundances of Cyanobacteria and *Gemmata* (class

Planctomycetia) at > 50 % of WWTP effluent. We observed that a WWTP effluent proportion > 40-50 % of the total flow resulted in decreased relative abundance of keystone taxa, such as *Phyllobacteriaceae* and Bacilli. The former contains members with the unique ability to fix diatomic nitrogen into forms that are usable for most organisms (Mergaert and Swings, 2015), while the latter are capable to degrade organic matter (Ludwig *et al.*, 2015). Decreases in the relative abundance of these groups might imply changes that go beyond the structure of the bacterial community, as their ecological roles are decisive for the ecosystem integrity of small rivers receiving large WWTP discharges.

The increase in the relative abundance of Cyanobacteria is in line with increased copy numbers of the genes *psaA* and *psbA*, both of them involved in the photosynthesis and present in the Cyanobacteria. The abundance of 16S rRNA in non-desiccated coarse sediments was highest at 100 % of WWTP effluent, resulting in a cubic-type response pattern. This increase was related to the dominance of taxa associated to WWTP effluent-dominated sites, such as members of *Rhodobacteraceae* and Verrucomicrobia (Drury *et al.*, 2013). Drury *et al.*, 2013 showed increased relative abundance of members affiliated to these groups 50 meters downstream of a WWTP discharge point. *Rhodobacteraceae* are deeply involved in sulphur and carbon biogeochemical cycling (Pujalte *et al.*, 2014), and Verrucomicrobia have recently been proposed as major polysaccharide degraders in freshwaters (He *et al.*, 2017). Several microbial functions assessed by the METAGENassist functional predictor were potentially affected in coarse sediment samples exposed to WWTP effluent. Sporulating capacity was particularly affected; the decrease in the relative abundance of sporulating-associated bacterial sequences at > 43 % of WWTP effluent is in line with the observed decrease in relative abundance of spore-forming bacteria, such as members affiliated to the class Clostridia. Most members of Clostridia play an important role in degrading organic matter in the environment (Wiegel *et al.*, 2006), and their decrease could lead to the accumulation of organic carbon in river ecosystems affected by WWTP discharges. Although the functional annotation did not show any clear patterns for sulphate reduction, we observed a decay on the abundance of the sulphate-reducing bacteria gene marker (*dsrA*, quadratic model, R^2 = 0.34), and TITAN identified a sulphate-reducing Deltaproteobacteria (*Desulfomicrobium*) as a reliably decreasing taxon.

In desiccated sediments, reliably decreasing taxa included members of Alpha- and Gammaproteobacteria, as well as Bacteroidetes. It has been previously stated that desiccation of river sediments could drive the bacterial community towards those occurring in soils, with increased abundances of Alphaproteobacteria and Actinobacteria (Pohlon *et al.*, 2013, 2018). Here we found that, in desiccated sediments, members of Alphaproteobacteria usually isolated from soil (i.e. *Aminobacter*, *Kaistia* and *Sphingobium*) could be used as indicator taxa.

In the present study, the individual and interactive effects of a WWTP effluent dilution gradient and a 7-day desiccation period were assessed using artificial streams and sediment bacterial communities. The major conclusions of the work are:

- Among the 3 studied factors (i.e. WWTP effluent, desiccation and sediment type), the one showing highest individual effect was WWTP effluent.
- Lower taxonomic levels (i.e. order, family and genera) were more sensitive to the effects of desiccation.
- The strongest interactive effect was observed between WWTP effluent and sediment type.
- A WWTP effluent proportion between 21.5 - 72 % of the total stream flow and, particularly, > 50 % resulted in the greatest changes for bacterial community structure.
- Positive indicator taxa were mostly affiliated to Actinobacteria and Cyanobacteria, whereas negative indicator taxa were affiliated to Gammaproteobacteria.
- Desiccation increased the number of indicator taxa related to soil environments, such as members of Alphaproteobacteria.

PAPER II. Desiccation events change the microbial response to gradients of wastewater effluent pollution

Supplementary information

Ferran Romero[1]*, Sergi Sabater[1,2], Carme Font[1], José Luís Balcázar[1] and Vicenç Acuña[1].

1. ICRA, Catalan Institute for Water Research, Emili Grahit 101, University of Girona, 17003 Girona (Spain).
2. Institute of Aquatic Ecology, University of Girona, Campus de Montilivi, Girona 17071 (Spain).

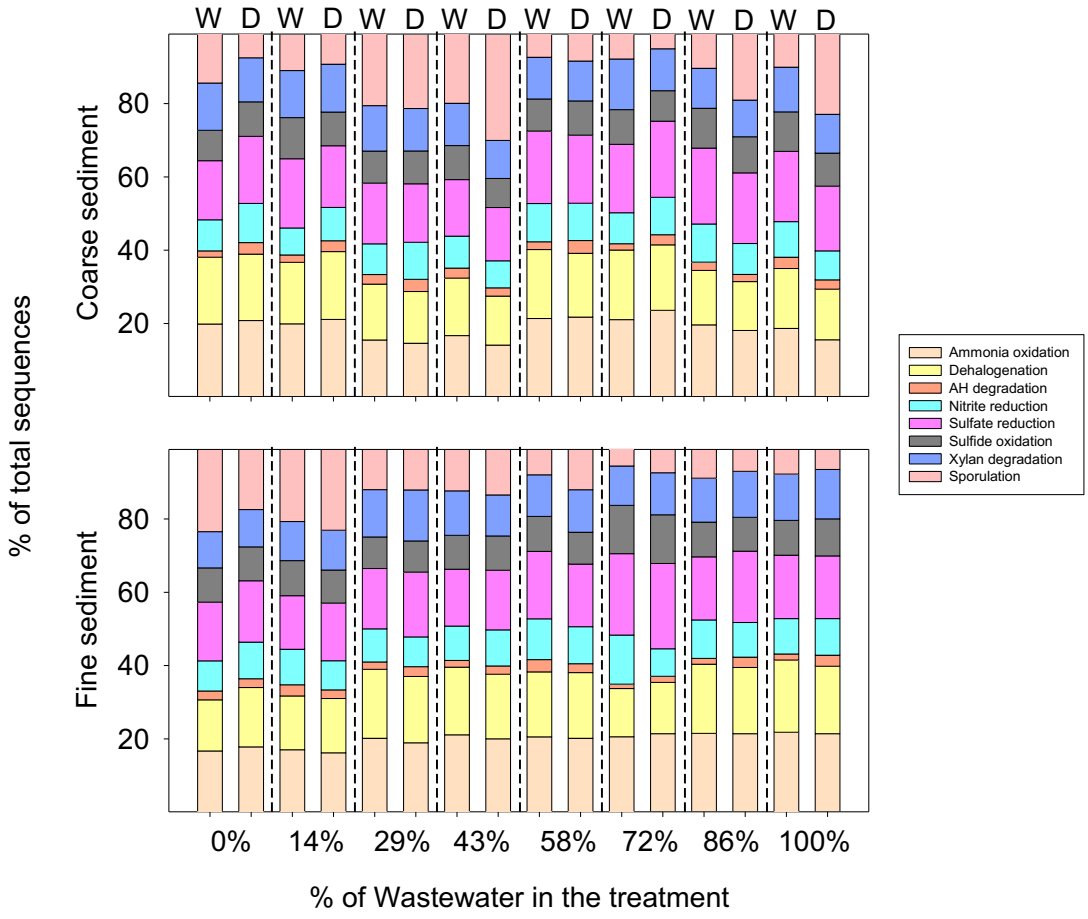


Figure PII.S1 Bacterial community functional prediction. Predicted functions of coarse and fine sediment bacterial communities across the gradient of WWTP effluent concentration. Results are shown for non-desiccated (wet, W) and desiccated (dry, D) samples. Please note that, given that one single OTU might be related to more than one phenotype, the sum of the relative abundances within a single treatment does not necessarily equals 100 %. A detailed version of these results is available at supplementary Table PII.S2.

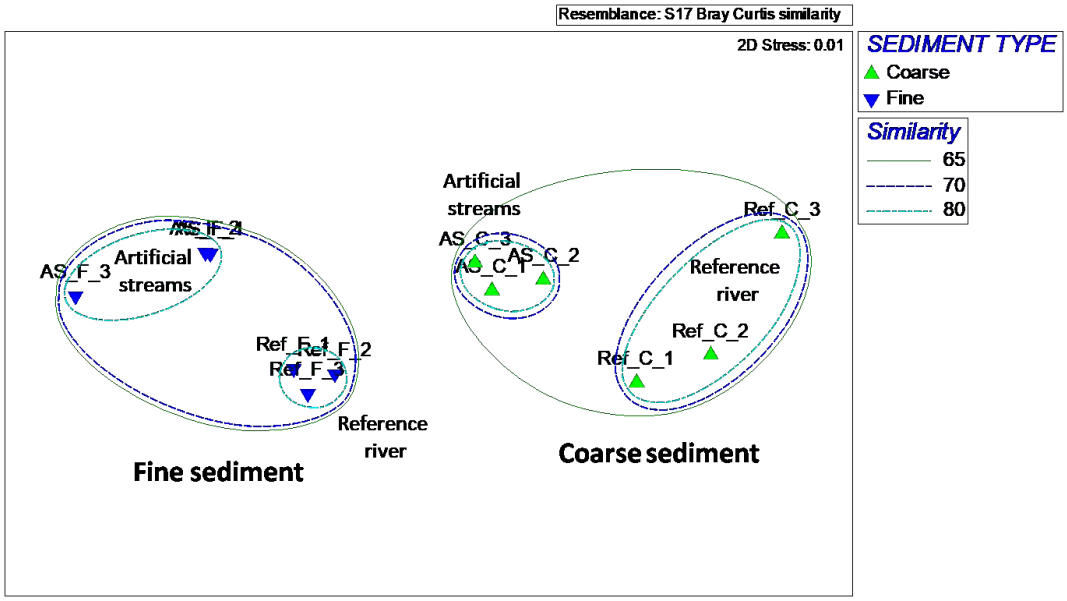


Figure PII.S2 Bacterial community structure in artificial streams and reference river. S17 Bray-Curtis similarity multidimensional scaling (MDS) plot showing sample similarity.

Target group	Target gene	Primer name	Primer sequence (5'-3')	Reference
Bacteria	16S rRNA	1048F	GTGSTGCAYGGYTGTCGTCA*	Maeda et al 2003
		1194R	ACGTCRTCCMCACCTTCCTC	
Photosynthesizers	psaA	psaA3F	TTAGAAGAAGTTTCTCGTAAAATTTT*	Romero et al 2018, and personal communication from Sandra K. Tiam (sandra.kimtiam@gmail.com)
		psaA3R	TGATGTGCAATATCACTTAACCATAA	
	psbA	psbA3F	GGTATCCGTGAGCCAGTAGCAGGTC*	
		psbA3R	GCTAAGAAGAAGTGTAAAGCACGAG	
Denitrifying bacteria	narG	narG1960F	TAYGTSGGSCARGARAA*	Philippot et al 2002
		narG2650R	TTYTCRTACCABGTBGC	
Sulphate-reducing bacteria	dsrA	DSR1F	ACBCAYTGGAARCACG*	Ben-dov et al 2007
		RH3-dsr-R	GGTGGAGCCGTGCATGTT	
Methanogenic archaea	mcrA	mlas-mod – F	GGYGGTGTMGGDTTCACMCARTA*	Angel et al 2012
		mcrA-rev - R	CGTTCATBGCGTAGTTVGGRTAGT	

Table PII.S1 Summary of quantitative PCR (qPCR) analyses and target groups addressed in this study. Genes indicated here were used to target some ecologically-relevant microbial groups. Abbreviations: 16S rDNA, ribosomal DNA 16S gene - psaA, photosystem I binding apoprotein A1 - psbA, photosystem II structural protein D1 gene - narG, nitrate reductase alpha chain gene - dsrA, dissimilatory sulphite reductase subunit A gene - mcrA, methyl coenzyme M reductase gene. Asterisks (*) indicate forward primer.

		0%	14%	29%	43%	58%	72%	86%	100%
CS	AO	19.79 ± 0.42	20.15 ± 1.90	15.49 ± 0.86	16.78 ± 1.41	21.35 ± 0.04	21.04 ± 0.70	19.61 ± 0.25	18.66 ± 0.32
	DH	18.12 ± 0.83	16.38 ± 2.85	15.24 ± 0.26	15.65 ± 0.56	18.83 ± 0.42	18.99 ± 0.12	14.80 ± 0.84	16.26 ± 0.79
	AH deg.	1.72 ± 0.08	1.97 ± 0.33	2.63 ± 0.15	2.69 ± 0.43	2.13 ± 0.06	1.73 ± 0.11	2.28 ± 0.15	3.06 ± 0.08
	NO_2- red.	8.49 ± 0.49	7.38 ± 0.08	8.36 ± 0.67	8.63 ± 0.91	10.44 ± 0.52	8.46 ± 0.40	10.38 ± 0.41	9.67 ± 0.39
	SO_4- red.	16.18 ± 0.58	19.27 ± 2.79	16.60 ± 0.42	15.40 ± 0.49	19.79 ± 0.24	18.66 ± 0.24	20.71 ± 0.15	19.18 ± 0.10
	SO_2- ox.	8.33 ± 0.39	11.55 ± 2.46	8.72 ± 0.22	9.29 ± 0.03	8.75 ± 0.16	9.47 ± 0.34	10.87 ± 0.23	10.83 ± 0.44
	XD	12.83 ± 0.59	12.55 ± 2.19	12.41 ± 0.31	11.47 ± 0.86	11.40 ± 0.56	13.82 ± 0.48	10.87 ± 0.60	12.10 ± 0.91
	Spor.	14.53 ± 1.43	10.74 ± 2.01	20.54 ± 2.24	20.09 ± 3.23	7.32 ± 0.57	7.83 ± 0.65	10.48 ± 1.55	10.23 ± 1.27
CS	AO	20.76 ± 0.90	21.10 ± 0.23	14.54 ± 0.90	14.05 ± 0.76	21.52 ± 1.39	23.68 ± 1.00	18.11 ± 0.15	15.53 ± 0.57
	DH	18.12 ± 0.40	18.52 ± 0.06	14.01 ± 0.82	13.38 ± 0.38	17.24 ± 0.85	17.91 ± 0.66	13.29 ± 0.89	13.75 ± 1.12
	AH deg.	3.16 ± 0.32	2.97 ± 0.05	3.25 ± 0.47	2.26 ± 0.27	3.41 ± 0.35	2.79 ± 0.13	1.96 ± 0.07	2.57 ± 0.29
	NO_2- red.	10.71 ± 1.42	9.11 ± 0.61	10.04 ± 0.75	7.33 ± 0.52	10.18 ± 0.52	10.21 ± 0.30	8.40 ± 0.29	7.86 ± 0.55
	SO_4- red.	18.32 ± 0.67	16.84 ± 0.41	15.83 ± 0.98	14.50 ± 0.26	18.56 ± 0.30	20.86 ± 0.86	19.26 ± 0.94	17.62 ± 0.78
	SO_2- ox.	9.44 ± 0.36	9.18 ± 0.19	8.93 ± 0.36	7.92 ± 0.26	9.44 ± 0.94	8.02 ± 3.08	9.89 ± 0.82	9.01 ± 0.22
	XD	12.02 ± 0.99	13.09 ± 0.68	11.44 ± 0.96	10.36 ± 0.77	10.83 ± 0.37	11.49 ± 0.43	9.94 ± 0.67	10.54 ± 1.04
	Spor.	7.48 ± 0.64	9.19 ± 0.95	21.96 ± 4.48	30.20 ± 2.77	8.82 ± 0.12	5.03 ± 0.92	19.14 ± 3.73	23.13 ± 3.73
FS	AO	16.70 ± 0.57	16.99 ± 1.13	20.22 ± 0.70	21.10 ± 0.38	20.47 ± 1.03	20.55 ± 1.12	21.46 ± 0.90	21.79 ± 0.59
	DH	13.99 ± 0.65	14.57 ± 0.85	18.77 ± 0.64	18.47 ± 0.49	17.73 ± 0.14	13.14 ± 0.54	18.88 ± 0.28	19.70 ± 0.05
	AH deg.	2.41 ± 0.35	3.03 ± 0.16	2.04 ± 0.55	1.87 ± 0.15	3.33 ± 0.15	1.21 ± 0.04	1.59 ± 0.08	1.67 ± 0.17
	NO_2- red.	8.23 ± 0.23	9.69 ± 1.31	9.09 ± 0.45	9.32 ± 0.70	11.17 ± 0.42	13.48 ± 0.84	10.47 ± 0.60	9.64 ± 0.29
	SO_4- red.	16.04 ± 0.26	14.51 ± 0.97	16.53 ± 0.74	15.51 ± 0.15	18.45 ± 0.66	22.16 ± 0.56	17.28 ± 0.82	17.32 ± 0.17
	SO_2- ox.	9.34 ± 0.11	9.59 ± 1.30	8.61 ± 0.39	9.25 ± 0.39	9.53 ± 0.18	13.12 ± 0.88	9.44 ± 0.36	9.48 ± 0.17
	XD	9.91 ± 0.65	10.51 ± 1.54	12.85 ± 0.96	12.15 ± 0.49	11.38 ± 0.23	10.74 ± 0.76	12.04 ± 0.80	12.65 ± 0.13
	Spor.	23.39 ± 2.51	21.11 ± 4.72	11.89 ± 1.11	12.33 ± 0.46	7.95 ± 0.12	5.60 ± 0.67	8.85 ± 0.62	7.75 ± 0.41
FS	AO	17.21 ± 2.38	15.79 ± 2.63	18.92 ± 0.37	19.95 ± 0.95	20.17 ± 0.47	21.43 ± 1.14	21.42 ± 0.40	21.43 ± 0.31
	DH	15.80 ± 1.55	14.52 ± 1.93	18.09 ± 0.48	17.52 ± 1.12	17.92 ± 0.28	13.95 ± 0.74	18.05 ± 0.12	18.38 ± 0.39
	AH deg.	2.39 ± 0.17	2.38 ± 0.19	2.69 ± 0.35	2.25 ± 0.16	2.41 ± 0.56	1.67 ± 0.25	2.83 ± 0.26	3.05 ± 0.30
	NO_2- red.	9.87 ± 0.67	7.82 ± 0.86	8.11 ± 0.56	9.82 ± 0.61	10.12 ± 0.26	7.50 ± 0.29	9.45 ± 0.54	9.99 ± 0.59
	SO_4- red.	16.51 ± 0.94	15.47 ± 1.95	17.69 ± 0.24	16.21 ± 0.97	17.11 ± 1.42	23.31 ± 0.41	19.45 ± 0.47	17.15 ± 1.42
	SO_2- ox.	9.21 ± 0.27	8.83 ± 1.20	8.49 ± 0.16	9.32 ± 0.59	8.68 ± 0.22	13.30 ± 0.83	9.23 ± 0.15	10.10 ± 0.47
	XD	10.22 ± 0.21	10.77 ± 0.66	13.92 ± 0.53	11.17 ± 0.69	11.61 ± 0.34	11.45 ± 0.71	12.58 ± 0.27	13.46 ± 0.49
	Spor.	18.79 ± 5.36	24.42 ± 8.93	12.09 ± 1.39	13.77 ± 4.59	12.00 ± 1.74	7.39 ± 0.86	7.00 ± 0.91	6.44 ± 1.17

Table PII.S2 Functional community composition. Average relative abundance of dominant functions calculated for each of the 3 replicates per treatment (± SE) as predicted by METAGENassist predicting tool. Abbreviations: CS, coarse sediment; FS, fine sediment.

gene	R^2 standard curve	efficiency	copy number g biofilm^{-1}
16S rRNA	0.993 ± 0.002	96.88 ± 2.13	$1.31 \times 10^9 \pm 1.07 \times 10^8$
psbA	0.983 ± 0.006	81.43 ± 3.92	$1.87 \times 10^7 \pm 3.22 \times 10^6$
psaA	0.997 ± 0.001	92.90 ± 0.44	$2.92 \times 10^7 \pm 6.43 \times 10^6$
dsrA	0.979 ± 0.015	120.3 ± 8.74	$4.66 \times 10^3 \pm 8.05 \times 10^2$
narG	0.970 ± 0.012	90.80 ± 0.05	$1.64 \times 10^6 \pm 2.18 \times 10^5$
mcrA	0.991 ± 0.002	91.68 ± 2.94	$2.55 \times 10^4 \pm 4.84 \times 10^3$

Table PII.S3 Information about quantitative PCR analyses. Mean values (n = 4) for calculated R^2, quantification efficiency and copy number are indicated (± SE).

Factor	Tax.level	d.f.	SS	MS	F-value	R2	p-value
We	Phylum	7	0.34	0.05	5.19	0.15	< 0.001
	Class	7	0.95	0.14	8.11	0.26	< 0.001
	Order	7	1.60	0.22	6.07	0.21	< 0.001
	Family	7	1.94	0.28	5.82	0.20	< 0.001
	Genus	7	2.19	0.31	5.29	0.18	< 0.001
	Function	7	0.16	0.02	4.54	0.15	< 0.001
D	Phylum	1	0.01	0.01	1.42	< 0.01	0.220
	Class	1	0.04	0.04	2.54	0.01	0.040
	Order	1	0.15	0.15	3.92	0.02	< 0.001
	Family	1	0.25	0.25	5.25	0.03	< 0.001
	Genus	1	0.32	0.32	5.44	0.03	< 0.001
	Function	1	< 0.01	< 0.01	0.85	< 0.01	0.370
S	Phylum	1	0.04	0.04	4.36	0.02	0.010
	Class	1	0.05	0.05	2.96	0.01	0.020
	Order	1	0.14	0.14	3.73	0.02	0.010
	Family	1	0.18	0.18	3.85	0.02	< 0.010
	Genus	1	0.22	0.22	3.79	0.02	0.010
	Function	1	0.02	0.02	3.52	0.02	0.050
We * D	Phylum	7	0.08	0.01	1.17	0.03	0.290
	Class	7	0.16	0.02	1.36	0.04	0.110
	Order	7	0.31	0.04	1.17	0.04	0.210
	Family	7	0.41	0.06	1.22	0.04	0.180
	Genus	7	0.45	0.07	1.09	0.04	0.310
	Function	7	0.04	0.01	1.19	0.04	0.290
We * S	Phylum	7	1.04	0.15	15.88	0.49	< 0.001
	Class	7	1.32	0.19	11.22	0.34	< 0.001
	Order	7	2.55	0.36	9.65	0.33	< 0.001
	Family	7	3.21	0.46	9.62	0.33	< 0.001
	Genus	7	4.07	0.58	9.81	0.33	< 0.001
	Function	7	0.49	0.07	14.05	0.45	< 0.001
D * S	Phylum	1	0.03	0.03	3.06	0.01	0.040
	Class	1	0.03	0.03	1.60	0.01	0.160
	Order	1	0.04	0.04	1.18	0.01	0.270
	Family	1	0.05	0.05	1.06	0.01	0.340
	Genus	1	0.06	0.06	1.06	0.01	0.330
	Function	1	0.02	0.02	3.07	0.01	0.070
We * D * S	Phylum	7	0.13	0.02	2.01	0.06	0.020
	Class	7	0.27	0.04	2.25	0.07	0.001
	Order	7	0.46	0.01	1.74	0.06	0.010
	Family	7	0.64	0.09	1.92	0.07	< 0.010
	Genus	7	0.87	0.12	2.10	0.07	< 0.010
	Function	7	0.04	0.01	1.10	0.04	0.360

Table PII.S4 Overall effects of factors on bacterial community. Overall comparisons of coarse and fine sediment bacterial community composition and functioning analyzed by PERMANOVA

using Bray-Curtis distances. Abbreviations: We, WWTP effluent; D, desiccation; S, sediment type; d.f., degrees of freedom; SS, sum of squares; MS, mean sum of squares.

Model	Equation	Parameters
Linear	$y = a + bx$	a, intercept (gene copy number at WWTP concentration = 0 %) b, slope
Exponential growth	$y = ae^{bx}$	a, intercept (gene copy number at WWTP concentration = 0 %) b, increase (decrease when b < 0) rate
Power	$y = y_0 + ax^b$	a, coefficient b, exponent (defines curvature of function) y_0, intercept (gene copy number at WWTP concentration = 0 %)
Logistic	$y = \frac{a}{1 + e^{-b(x-x_0)}}$	a, maximum gene copy number b, steepness of the curve x_0, x value of the sigmoid's midpoint
Monod	$y = a\frac{x}{b + x}$	a, maximum gene copy number b, concentration value when gene copy number is at its half maximum
Quadratic	$y = a + bx + cx^2$	a, intercept (gene copy number at WWTP concentration = 0 %) b, slope c, curvature
Logit (inverse of the logistic function)	$y = y_0 + \frac{1}{b}\log\left(\frac{x+1}{a-(x+1)}\right)$	a, maximum gene copy number b, steepness of the curve y_0, value of the sigmoid's midpoint
Cubic	$y = a + bx + cx^2 + dx^3$	a, intercept (gene copy number at WWTP concentration = 0 %) b, slope c,d, curvature

Table PII.S5 Fitted functions to measured WWTP effluent concentrations (x) and gene copy numbers standardized by gram of biofilm (y).

% We	SpCond (µS cm^{-1})	DO (mg L^{-1})	pH
0	240 ± 1.00	8.68 ± 0.24	8.69 ± 0.16
14	236 ± 4.50	9.03 ± 0.10	8.90 ± 0.16
29	242 ± 2.51	9.11 ± 0.12	8.94 ± 0.05
43	252 ± 1.16	8.94 ± 0.12	8.86 ± 0.14
58	255 ± 0.57	8.92 ± 0.10	8.89 ± 0.07
72	259 ± 0.57	8.88 ± 0.03	8.89 ± 0.03
86	253 ± 0.02	8.70 ± 0.03	8.82 ± 0.05
100	257 ± 0.57	8.62 ± 0.07	8.81 ± 0.05

Table PII.S6 Physical-chemical properties before the experimental manipulation. Mean ± standard deviation of specific electrical conductivity (SpCond), dissolved oxygen (DO) and pH in each treatment before the onset of the experimental manipulation. Mean values result from 3 independent experimental replicates. The proportion of WWTP effluent to the total flow that was applied during the experimental manipulation is indicated as "% We".

phylum	**class**	**order**	**family**	**genus**
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Rhodobacter
(49.47 ± 5.42)	(18.61 ± 5.42)	(5.71 ± 0.72)	(5.16 ± 0.42)	(4.06 ± 0.41)
		Sphingomonadales	Sphingomonadaceae	*Kaistobacter*
		(4.04 ± 0.17)	(3.00 ± 0.17)	(1.00 ± 0.15)
	Betaproteobacteria	Burkholderiales	Comamonadaceae	unclassified genus
	(7.78 ± 1.43)	(4.34 ± 0.17)	(3.49 ± 0.19)	(2.72 ± 0.12)
	Gammaproteobacteria	Thiotrichales	Thiotrichaceae	*Thiothrix*
	(13.53 ± 2.57)	(1.88 ± 0.27)	(1.66 ± 0.27)	(1.66 ± 0.27)
		Xanthomonadales	Xanthomonadaceae	*Luteimonas*
		(6.66 ± 0.27)	(5.45 ± 0.27)	(1.28 ± 0.15)
				unclassified genus
				(3.26 ± 0.19)
	unclassified class	unclassified order	unclassified family	unclassified genus
	(4.09 ± 0.54)	(4.09 ± 0.54)	(4.09 ± 0.54)	(4.09 ± 0.54)
Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	*Planctomyces*
(8.68 ± 0.34)	(8.03 ± 0.34)	(1.66 ± 0.10)	(1.66 ± 0.10)	(1.66 ± 0.27)
			Gemmataceae	*Gemmata*
			(2.91 ± 0.23)	(2.21 ± 0.21)
		Pirellulales	Pirellulaceae	unclassified genus
		(2.81 ± 0.15)	(2.81 ± 0.15)	(2.56 ± 0.15)
Verrucomicrobia	unclassified class	unclassified order	unclassified family	unclassified genus
(8.50 ± 0.38)	(3.01 ± 0.30)	(3.01 ± 0.30)	(3.01 ± 0.30)	(3.01 ± 0.30)
	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	*Luteolibacter*
	(2.81 ± 0.14)	(2.81 ± 0.14)	(2.81 ± 0.14)	(1.91 ± 0.13)
Cyanobacteria	Oscillatoriophycideae	Chroococcales	Cyanobacteriaceae	*Cyanobacterium*
(5.01 ± 0.60)	(1.92 ± 0.24)	(1.84 ± 0.23)	(1.73 ± 0.23)	(1.73 ± 0.23)
Bacteroidetes	Saprospirae	Saprospirales	Chitinophagaceae	unclassified genus
(9.77 ± 0.36)	(5.17 ± 0.17)	(5.17 ± 0.17)	(3.33 ± 0.12)	(2.04 ± 0.09)

Table PII.S7 Main bacterial genera derived from the investigated treatments. Only genera representing > 1.00 % of total reads is showed. Each genus (right column) is classified in its corresponding family, order, class and phylum. All taxa are accompanied by its total relative abundance ± standard error (under the name of the taxon, in parentheses).

Paper III

Effects of multiple stressors on river biofilms depend on time scale

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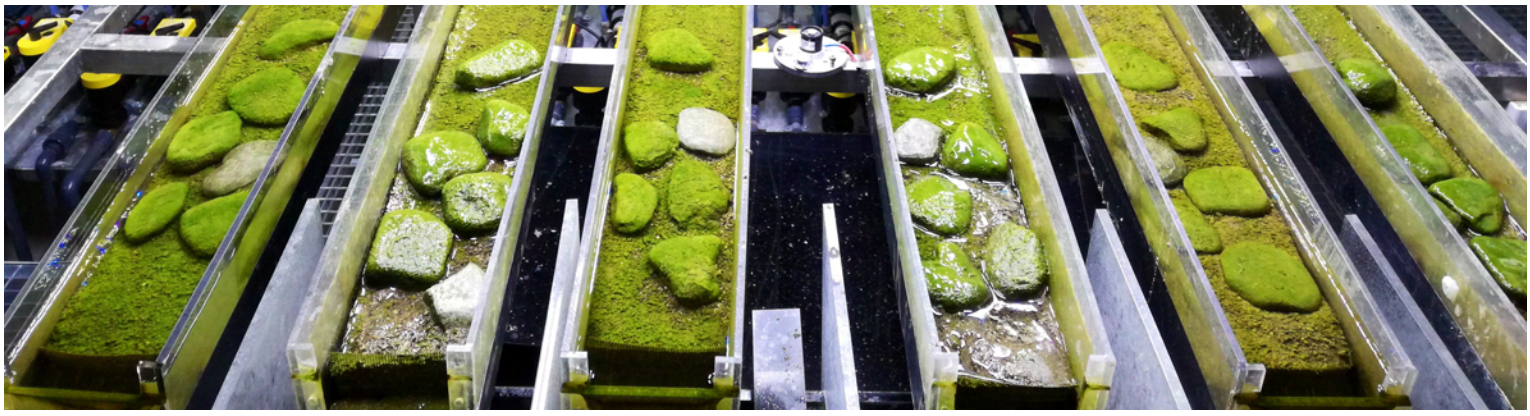


PAPER III. Effects of multiple stressors on river biofilms depend on time scale

Ferran Romero[1,2], Vicenç Acuña[1,2], Carme Font[1,2], Anna Freixa[1,2], Sergi Sabater[1,3]

1 - Catalan Institute for Water Research (ICRA), C. Emili Grahit 101, 17003 Girona (Spain).
2 – Universitat de Girona (UdG), Girona (Spain)
3 - Institute of Aquatic Ecology (IEA), University of Girona, Campus de Montilivi, 17003 Girona (Spain).

Abstract

Global change exposes ecosystems to a myriad of stressors differing in their magnitude, frequency and temporal/spatial scale. Among freshwater ecosystems, rivers and streams are subject to physical, chemical and biological stressors, which interact with each other and might produce diverging effects depending on time scale. We conducted a manipulative experiment using 24 artificial streams to examine the individual and combined effects of warming (1.6 °C increase in water temperature), hydrological stress (simulated low-flow situation) and chemical stress caused by pesticide exposure (15.1 – 156.7 ng L^{-1}) on river biofilms. We examined whether co-occurring stressors could lead to non-additive effects, and if these differed at different exposure times. Specifically, structural and functional biofilm responses were assessed after 48 hours (short-term effects) and after 30 days (long-term effects) of exposure. Hydrological stress caused strong negative impacts on river biofilms, whereas effects of warming and pesticide exposure were less intense, although increasing on the long term. Most stressor combinations (71 %) resulted in non-significant interactions, suggesting overall additive effects, but some non-additive interactions also occurred. Among non-additive interactions, 59 % were classified as antagonisms after short-term exposure to the different stressor combinations, rising to 86 % at long term. Our results indicate that a 30-day exposure period promotes antagonism by inducing changes in biofilm community functioning. Overall, the impacts of multiple-stressor occurrences appear to be hardly predictable from individual effects, highlighting the need to consider multiple stressor effects over time when searching reliable forecasts of global change on river and stream ecosystems.

Introduction

Freshwater ecosystems are currently threatened by global pressures on land use and climate, affecting ecosystem stability and biodiversity (Johnson and Penaluna, 2019). Among freshwater ecosystems, rivers and streams are particularly vulnerable to stressors derived from land-use and climate change, and multiple stress occurrences have been identified as responsible for river biodiversity loss (Jackson *et al.*, 2016). However, these effects are difficult to predict because of the complexity of the interactions between stressors (Crain *et al.*, 2008; Jackson *et al.*, 2016; Nõges *et al.*, 2016). Multiple stressors may interact in additive or in complex (non-additive) ways, where the responses of the combined effects of multiple stressors may be greater (synergistic) or smaller (antagonistic) than what would be predicted based on the individual stressor effects involved (Folt *et al.*, 1999). Recent analyses have emphasized that interactions in river ecosystems may account for 40 % to 69 % of all ecological responses (Crain *et al.*, 2008; Jackson *et al.*, 2016; Schinegger *et al.*, 2016b) and that non-additive interactions may be as frequent as additive responses (Nõges *et al.*, 2016), indicating that multiple stressor effects are hard to predict based on effects attributed to single stressors. A recent literature review suggested that the differences observed may depend on the type of ecosystem and the organization level studied, from individual species to populations and whole ecosystems (Côté *et al.*, 2016). Understanding these often overlooked multiple-stressor effects is still seen today as one of the most pressing challenges in ecology.

Global change and its associated stressors, such as warming, river flow reductions and chemical exposure due to land-use changes are particularly urgent issues in riverine areas. The Intergovernmental Panel on Climate Change (IPCC) indicates that greenhouse gases emissions will increase global temperatures by up to 4.5 °C in the next 50 years (IPCC, Summary for Policymakers - Special Report, 2018). Combined with the expected lower-than-average precipitation events, this could suppose a reduction in river flow by 16 to 35% compared to pre-industrial periods in areas already suffering from limited water resources such as the Mediterranean region (Marx *et al.*, 2018), with potential implications for habitat conditions and biodiversity (Döll and Zhang, 2010). Global environmental change also affects land uses associated to high urbanization (Grimm *et al.*, 2008) and increasing demand for food production (Godfray *et al.*, 2010), thus shifting natural land use from forest to agricultural fields (Donchyts *et al.*, 2016). Streams and rivers draining agricultural catchments are highly impacted by elevated levels of dissolved nutrients (Withers *et al.*, 2014), deposited fine sediments (Piqué *et al.*, 2017) and pesticides (Kuzmanovic *et al.*, 2015; Kuzmanović *et al.*, 2016). Thus, climate and land-use changes force multiple stress scenarios onto river ecosystems, which may produce uncertain outcomes.

River biota is directly impacted by multiple stressors. Amongst river and stream organisms, river biofilms play a key role in nutrient processing and river functioning (Battin, Besemer, Bengtsson, and Romani, 2016). Biofilms occupy different habitats on the riverbed, which favor the occurrence of compositional variability and complexity (Romaní and Sabater, 2001). Biofilms developing on hard river surfaces (cobbles and rocks) are known as *epilithic biofilms*. When dissolved nutrients are not limiting and light reaches the riverbed, epilithic biofilms are usually dominated by primary producers (algae, cyanobacteria), whereas under light limitation as it might occur in small streams with dense canopies, heterotrophs become more important (Romani *et al.*, 2017) Conversely, biofilms that develop on sub-superficial fine sediments (e.g. sand) are known as *epipsammic biofilms*, and are mostly composed by heterotrophic microorganisms, such as bacteria and fungi. Because of the higher porosity of fine sediments, epipsammic biofilms are less affected than epilithic biofilms by hydrological stress (Timoner *et al.*, 2012). The different composition and attributes of epilithic and epipsammic biofilms may be involved in their specific response to single stressors (Besemer *et al.*, 2012; Besemer, 2015), differing both on the velocity of response as well as in the degree of tolerance (Proia *et al.*, 2011; Tiam *et al.*, 2014; Ylla *et al.*, 2014b; Freixa *et al.*, 2017; Romero *et al.*, 2018).

Several studies have already assessed the short-term (i.e. hours) effects of multiple interacting stressors on river biofilm communities (Proia *et al.*, 2011; Romero *et al.*, 2018). Others have also shown that effects can appear in the long term (L. Proia *et al.*, 2013; Stampfli *et al.*, 2013; Pohlon *et al.*, 2018). The photosynthetic efficiency of algae and cyanobacteria and the enzymatic activities of heterotrophs become rapidly altered after river biofilm exposition to herbicides (Proia *et al.*, 2011), or to physical stressors such as warming or hydrological stress (Freixa *et al.*, 2017; Romero *et al.*, 2018). There are indications that responses might differ according to the exposure time; a sustained stress can promote changes in a community, selecting the most resistant species (Tlili, Corcoll, *et al.*, 2011) and therefore favouring community adaptation to the new conditions (Tlili, Montuelle, *et al.*, 2011). In long-term exposures (e.g. weeks), ecosystem function may experience pronounced shifts (Chará-Serna and Richardson, 2018), which usually come along with structural changes (Tiam *et al.*, 2014; Ylla *et al.*, 2014b). Previous work with river biofilms has demonstrated that extended non-flow periods promote changes in the production-respiration ratios in biofilm communities, leading it towards heterotrophy (Acuña *et al.*, 2015). Thus, there is a need to produce experimental designs focusing on multiple stressor effects at different time scales, and including several structural and functional descriptors.

This study aimed to evaluate the individual and interactive effects of three stressors (namely hydrological stress, warming and a pesticide mixture) on river biofilms at two different time scales (i.e. after 48 hours and 30 days of stressor exposure). To do so, a full-factorial design (2^3) was used and river biofilms were exposed to either individual or combined stressors. We

could therefore produce an experimental design focused on the effect of exposure time on the size and direction of the interactive stressor effects. We hypothesized that (i) stressors associated to climate change (warming and hydrological stress) would cause the most pervasive effects, as they encompass multiple level effects derived from hindered resource acquisition and overall physiological disruption and (ii) antagonism would be the main non-additive interaction type, particularly in the long term, because of the high potential for adaptation of the biofilm community to stressors.

Material and methods

Experimental design

The experiment was performed at the indoor Experimental Streams Facility of the Catalan Institute for Water Research (Girona, EU), between July 3rd and August 22nd, 2017. Each of the 24 artificial streams was assigned one of seven treatments (W, warming; H, hydrological stress; P, pesticides; W:H, W:P, H:P and W:H:P), and a control (C), following a full-factorial replicated (n = 3) design with 3 factors (i.e. W, H, P) and 2 levels per factor (i.e. presence vs. absence of the stressor). River sediment was transported from an unpolluted reference site (see next section, 2.2) and allowed to acclimate under control conditions for 16 days. After the acclimation period, treatments were applied for 35 days. All the response variables were assessed after 48 hours (short-term effects) and after 30 days (long-term effects) of treatment exposure.

Experimental conditions

Each artificial stream consisted of an independent methacrylate channel (l – w – d = 200 cm – 10 cm – 10 cm) and a 70 L water tank from which water could be recirculated (Figure PIII.S1). Each artificial stream was filled with 5 L of fine sediment (i.e. sand) extracted from an unpolluted segment of the Llémena River (Sant Esteve de Llémena, Girona, EU), which is a permanent river draining a calcareous mountainous range (d_{50} = 0.74 mm). The extracted sand was transported in less than one hour to the artificial streams, and then evenly distributed to create a plane bed covering the bottom of the streams. At complete water saturation, the porosity of the sand yielded a water content of 25% of the wet weight. The sand was used to colonize the epipsammic biofilm, whereas, in order to assess the response of the epilithic biofilm, small flat cobbles (l – w – d = 2 cm – 2 cm – 1 cm) were extracted from the Llémena River, transported to the laboratory and distributed on the streams. Each stream received a constant flow of 60 mL s^{-1} from the tank, and operated as a closed system for 72 h, as water from all the streams was renovated every three days. Mean water velocity was 2 cm s^{-1}, and water depth over the plane bed was 3 cm. Daily cycles of photosynthetic active radiation (PAR) were defined as 10 h daylight + 14 h darkness and were simulated by LED

lights (Lightech, Girona, EU). PAR was held constant at 173.99 ± 33 µE m^{-2} s^{-1} during the daytime, and was recorded every 10 min using 4 quantum sensors located across the whole array of streams (sensor LI-192SA, LiCOR Inc, Lincoln, USA). Air temperature was maintained at 15 ° C during the acclimation period and at 20 ° C during the exposure period, at a constant air humidity of 30%. Water temperature was recorded every 10 min using VEMCO Minilog (TR model, AMIRIX Systems Inc, Halifax, NS, Canada) temperature data loggers (-5 to 35 ° C, ± 0.2 ° C).

All treatments were applied simultaneously after the 16-day acclimation period. Accordingly, Cryo-Compact Circulators (Julabo CF-31, Seelbach, Germany) were used to achieve an average water temperature increase of 2 °C in all the treatments including warming as a stressor. Hydrological stress was applied by reducing the water flow from 60 to 5 mL s^{-1}, for which the fine sediments covering the bottom of the artificial streams remained slightly wet, while the cobbles became completely desiccated. Pesticide exposure consisted of a mixture composed by two herbicides (i.e. Diuron and Simazine), two fungicides (i.e. Imazalil and Prochloraz) and one insecticide (i.e. Chlorpyrifos). Nominal concentrations of each compound in the mixture are presented in Table PIII.S1. All the used compounds were provided by Sigma-Aldrich. The mixture of pesticides was freshly prepared in each water renewal (each 2–3 days) at a concentration of 100 mg L^{-1} in 50% methanol: water (v: v). The total concentration of methanol reaching the artificial streams was 400 ng L^{-1}, representing 0.0005% of the total water volume. The same concentration of methanol was added in the treatments without pesticides. The mixture of pesticides was added using peristaltic pumps (IPC Microprocessor pump, IDEX Health & Science GmbH_Ismatec, Switzerland). The compounds included in the mixture and their nominal concentrations were selected because of their common occurrence and frequency in rivers draining agricultural catchments (see Table PIII.S2 for references).

Water physical and chemical properties

General descriptors

Dissolved oxygen, pH and specific conductivity were measured in each artificial stream using WTW (Weilheim, Germany) hand-held probes. Nutrient and dissolved organic matter concentrations were measured from the water collected from the stream outlet. Both physical and chemical parameters were measured after short (i.e. 48 h) and long-term (30 d) exposure to the experimental treatments in 12 randomly selected streams (out of a total 24). Water was filtered immediately through 0.2 µm pore nylon filters (Whatman, Kent, UK) into pre-washed polyethylene containers for nutrient analyses and through 0.7 µm glass fiber filters for DOC analyses. Detection and quantification of nutrients and DOC were performed according to standard procedures previously used in the Experimental Streams Facility (Corcoll *et al.*, 2015a).

Pesticide quantification

All standards for the target compounds were obtained from Sigma-Aldrich. Stock solutions of the pesticides Diuron, Imazalil, Prochloraz, Simazine and Chlorpyrifos were prepared from powder in methanol at 1 mg mL^{-1}, which was stored frozen at -20 °C. Chemical information and nominal concentrations of the pesticides are available in Table PIII.S1. Working standard solutions as well as the calibration standard curve were prepared by appropriate dilution in methanol:water (10:90, v:v) of the stock solution. Water samples for pesticide analyses (1000 mL) were collected 48 hours after the beginning of the experimental manipulation phase (short-term exposure) and after 30 days (long-term exposure) from all artificial streams. The collected samples were filtered through 0.45 µm polyvinylidene fluoride membrane filters (PVDF, Millipore) and analyzed using ultra-performance liquid chromatography (UPLC, Waters Corp. Milford, MA, USA) coupled to an hybrid quadrupole-linear ion trap mass spectrometer (5500 QTRAP, Applied Biosystems, Foster City, USA) (LC-MS/ MS system).

Sampling and sample processing

Biofilm variables (Chlorophyll-*a* concentration, Photosynthetic efficiency, Photosynthetic capacity, Chlorophyll basal fluorescence, Leucine aminopeptidase activity, organic substrate utilization, 16S rRNA gene abundance and metabolic rates) were measured after short (48 h) as well as long-term (30 days) exposure to stress conditions. Metabolic rates (i.e. community respiration and gross primary production) were measured from wire net baskets containing epilithic and epipsammic biofilm (see section 2.4.6); all the other variables were measured separately from epilithic and epipsammic biofilms. Photosynthetic efficiency, photosynthetic capacity and chlorophyll-*a* basal fluorescence were measured *in-situ* using Pulse Amplitude Modulated (PAM) fluorescence (see section 2.4.2). Chlorophyll-*a* concentration, Leucine aminopeptidase activity, substrate utilization and 16S rRNA gene abundance were measured from re-suspended biofilm in filtered (0.2 µm) stream water (see sections 2.4.1, 2.4.3, 2.4.4 and 2.4.5).

Algal biomass determination

Chlorophyll-*a* concentration was used to evaluate biofilm structural changes after a 90 % acetone extraction. The chlorophyll-*a* extraction was done overnight in dark conditions at 4 ºC, and quantified spectrophotometrically using a Lambda UV/VIS spectrophotometer (U-2000 Spectrophotometer; Hitachi, Tokyo, Japan). Chlorophyll-*a* concentration is expressed in $\mu g \cdot cm^{-2}$.

In vivo fluorescence measurements

Biofilms were analyzed *in-vivo* to determine three chlorophyll fluorescence-derived parameters; namely photosynthetic efficiency (Y_{eff}), photosynthetic capacity (Y_{max}) and chlorophyll basal fluorescence (F_0) using a Diving PAM (Pulse Amplitude Modulated) underwater fluorometer (Heinz Wlaz, Effeltrich, Germany). Y_{eff} was determined under steady-state conditions, whereas F_0 and Y_{max} were measured after a 30-min adaptation to dark conditions. Y_{eff} and Y_{max} indicate the fraction of light that is converted into chemical energy during photosynthesis, and can therefore be used as a measure to evaluate functional changes in the algal component of the biofilm after exposure to environmental disturbances (Kim Tiam *et al.*, 2015).

Leucine aminopeptidase activity

The degradation capacity of peptides was assessed by measuring the activity of the extracellular enzyme leucine aminopeptidase (LAP). LAP was here used as a functional parameter to assess the capacity of the bacterial compartment to degrade peptidic compounds. It was measured using fluorescent-linked substrata (aminomethyl-coumarin, AMC). Biofilms were incubated for 1 h in the dark at 12.5 °C immediately after collection. Blanks and standards of AMC (0–100 µmol L^{-1}) were also incubated. At the end of the incubation, a glycine buffer (pH 10.4) was added (1/1 vol/vol), and fluorescence was measured at 364/445 nm excitation/emission for AMC. Values were expressed as nmol of released AMC cm^{-2} h^{-1}.

Organic substrate utilization (Biolog Ecoplates)

Biolog Ecoplates (Biolog Inc. Hayward, California, USA) were used to assess the differences in the substrate utilization capacity of different biofilm samples. Each Biolog Ecoplate contains three replicated wells of 31 different carbon sources and a blank with no substrate. Biofilms were extracted and diluted using a Ringer solution (1:20). Then, Ecoplates were inoculated with 130 µL of biofilm extract, under sterile conditions and incubated at 20 °C in the dark. Plates were read every 24 h until an asymptote was reached, which took between 6 and 7 days at 590 nm using a microplate reader (Epoch microplate reader, Biotek instruments, Winooski, USA). Data treatment followed the procedure described in Freixa *et al.*, 2016. Briefly, raw absorbance data for each well was corrected by taking away the mean absorbance of the control wells (without substrate) and negative values, as well as low absorbance values (< 0.05) were set to zero. Finally, Shannon diversity index and substrate richness (i.e. the

number of positive wells) were calculated using data from wells when the Average Well Colour Development was closest to 0.5 (Garland *et al.*, 2001).

Abundance of 16S rRNA gene copies

The 16S rRNA gene was used as a proxy for bacterial abundance (Marti *et al.*, 2013; Romero *et al.*, 2019). Extraction of DNA was performed on samples of 200 mg of freshly detached biofilm using the FastDNA® SPIN kit for soils (MP Biomedicals) following manufacturer instructions. DNA concentration in each sample was measured using Qubit 2.0 fluorometer (Life Technologies; Carlsbad, CA, USA); its purity was determined by measuring A260/A230 and A260/A280 absorbance ratios using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific; Wilmington, USA). Standard quantitative PCR (qPCR) procedure was used to quantify abundance of 16S rRNA gene on DNA extracted from epilithic and epipsammic biofilms. Quantitative PCR conditions are detailed in Romero *et al.*, 2019.

Metabolic biofilm rates

Metabolic biofilm rates were assessed through changes in oxygen concentration (oxygen balance method) under light and dark conditions. Trays containing 34 cm^2 of fine sediment and one cobble from each artificial stream were removed and incubated in cylindrical acrylic chambers (volume 0.96 L). Each chamber was provided with a submersible water circulation pump to avoid the formation of zones of low diffusion within the chamber. The incubations for each metabolic rate (net primary production and community respiration) lasted for 45 min, and were carried out inside an incubator chamber (Radiber AGP-700-ESP, Barcelona, Spain) at the same temperature and light conditions than those of the artificial streams. Net primary production was measured under light conditions, and community respiration was measured in the dark. Dissolved oxygen concentration inside the chambers was measured continuously with oxygen sensors and logged at 15 s intervals (PreSens OXY-10 mini, Regensburg, Germany). Gross primary production and community respiration were calculated according to Acuña *et al.*, 2008.

Statistical approach

We examined the response of the different community-level metrics on epilithic and epipsammic biofilms, as well as the overall metabolic biofilm response. For each metric, we ran a mixed-model nested ANOVA with the factors *warming* (W; fixed factor, 2 levels),

hydrological stress (H; fixed factor, 2 levels), *pesticides* (P; fixed factor, 2 levels), *time* (T; random factor, nested in W, H and P, 2 levels) and *substratum type* (S; random factor, nested in W, H and P, 2 levels). ANOVA was carried out on univariate data using the *aov* function of the package *stats* on R (R Core Team, 2017). Two different response patterns where derived from the ANOVA results: *main effects* evaluated the mean performance in the treatments where a given stressor is present, as opposed to the treatments without the stressor. *Interactive effects* were used to evaluate whether the response of a given biofilm metric to the presence of one stressor changed at different levels of additional stressors. Significant interactive effects (ANOVA interaction term P-value < 0.05) were classified into antagonism and synergism according to Crain *et al.*, 2008. Accordingly, antagonism was assumed for stressor combinations resulting in responses less pronounced than predicted from additive effects, whereas synergism was assumed when the opposite pattern was observed (i.e. combined effects amplifying individual effects).

Results

Physical-chemical parameters

Water temperature averaged 18.5 ± 0.4 °C (n = 24 artificial streams) during the acclimation period. After experimental manipulation, it increased to 20.2 ± 0.1 °C in the artificial streams containing *warming* (W) as a stressor (i.e. n = 12; W, W:H, W:P, W:H:P), representing a 1.6 °C increase in water temperature. In the treatments where warming was not a stressor, water temperature averaged 18.5 ± 0.2 °C (n = 12). Added pesticides concentrations ranged from 15.1 to 156.7 ng L^{-1} in the treatments containing *pesticides* (P) as a stressor (i.e. n = 12; P, H:P, W:P, W:H:P). Unexpectedly, chlorpyrifos concentrations were below the detection limit in both the short and long-term measurements (Table PIII.1). Pesticide contamination did not occur in the other artificial streams (n = 12, i.e. C, W, H and W:H, data not shown).

	Short term	**Long term**
Dissolved oxygen (mg L^{-1})	9.23 ± 0.08	9.02 ± 0.11
Conductivity (µS cm^{-1})	231 ± 1.58	281 ± 8.75
pH	8.90 ± 0.03	8.37 ± 0.11
NO_2^- (mg N- NO_2^- L^{-1})	0.003 ± 0.001	0.003 ± 0.001
NO_3^- (mg N- NO_3^- L^{-1})	1.49 ± 0.06	1.37 ± 0.06
PO_4^{3-} (mg P- PO_4^{3-} L^{-1})	0.003 ± 0.001	0.003 ± 0.001
NH_4^+ (mg N- NH_4^+ L^{-1})	< LOQ [1]	< LOQ [1]
DOC (mg L^{-1})	2.84 ± 0.09	2.30 ± 0.03
Diuron (ng L^{-1})	156.7 ± 51.4	140.7 ± 3.9
Chlorpyrifos (ng L^{-1})	< LOD [2]	< LOD [2]
Imazalil (ng L^{-1})	15.1 ± 2.1	85.4 ± 3.1
Prochloraz (ng L^{-1})	< LOQ [3]	34.2 ± 1.0
Simazine (ng L^{-1})	50.3 ± 2.4	68.6 ± 2.1

Table PIII. 1 Physical-chemical characteristics (mean ± S.E, n = 12) of water in 12 randomly sampled artificial streams after 48 hours (i.e. short-term effects) and 30 days of exposure (i.e. long-term effects). Pesticide concentrations correspond only to water samples from contaminated streams (i.e. n = 12, treatments P, W*P, H*P and W*H*P). [1] The limit of quantification (LOQ) for NH_4^+ was 0.001 mg N-NH_4^+ L^{-1}. [2] The limit of detection (LOD) for Chlorpyrifos was 2.24 ng L^{-1}. [3] The limit of quantification (LOQ) for Prochloraz was 0.07 ng L^{-1}.

Biofilm responses to warming (W), hydrological stress (H) and pesticides (P)

Temporal variation

Biofilms in control artificial streams (containing biofilm without stressor addition; n = 3) progressively increased their algal biomass (Figure PIII.1, Figure PIII.2, Table PIII.2), as suggested by the increasing chlorophyll basal fluorescence (Figure PIII.1D, Table PIII.2). This increase in chlorophyll fluorescence was only translated into increased total chlorophyll-*a* concentration in the epipsammic biofilm (Figure PIII.1A; significant interaction between *time* and *substratum type* (S:T)*;* $F_{1,16}$ = 32.3, P < 0.001). Conversely, the number of 16S rRNA gene copies (targeting total bacteria) decreased (factor *time*; $F_{1,16}$= 32.1, P < 0.001), especially in the epilithic biofilm (Figure PIII.1F; significant interaction between *time* and *substratum type;* $F_{1,16}$ = 7.6, P = 0.013). Leucine aminopeptidase activity decreased with time in the epilithic biofilm only (Figure PIII.1E; S:T, $F_{1,16}$ = 16.5 P < 0.001). Overall, the shift towards algal biomass translated into increased production-respiration ratios (Figure PIII.2) in the biofilms (significant effect of *time*; $F_{1,16}$ = 18.80, P < 0.001).

Single stressor responses (main effects)

Hydrological stress (H) applied as reduced flow produced the most severe effects in the river biofilms employed in this experiment (Figures PIII.1 and PIII.2), significantly altering 8 out of the 11 response variables assessed (Table PIII.2). Hydrological stress significantly reduced total chlorophyll-a concentration ($F_{1,16}$ = 57.6, P < 0.001, Figure PIII.1A), basal chlorophyll fluorescence ($F_{1,16}$ = 107.4, P < 0.001, Figure PIII.1D), photosynthetic efficiency ($F_{1,16}$ = 249.4, P < 0.001, Figure PIII.1B) and photosynthetic capacity ($F_{1,16}$ = 61.1, P < 0.001, Figure PIII.1C). These effects were particularly intense for epilithic biofilm, making the interaction between hydrological stress and substratum type significant for most of the response variables assessed (Table PIII.S4). This translated into altered gross primary production in biofilms submitted to hydrological stress (Figure PIII.S2; $F_{1,16}$ = 30.1, P < 0.001). Water warming significantly altered 4 out of the 11 response variables assessed (Table PIII.2). Warming slightly decreased photosynthetic capacity (Figure PIII.1C; $F_{1,16}$ = 8.6, P = 0.010) and 16S rRNA gene abundance (Figure PIII.1F; $F_{1,16}$ = 33.7, P < 0.001). Warming had an overall significant main effect on community respiration ($F_{1,16}$ = 41.3, P < 0.001), although the levels of oxygen consumption in W treatment were comparable to those found on control streams. These effects on metabolic rates translated into altered production-respiration ratios (Figure PIII.2), with special impact of hydrological stress ($F_{1,16}$ = 35.7, P < 0.001). Pesticides significantly altered 4 out of the 11 response variables (Table PIII.2), namely photosynthetic efficiency (Figure PIII.1B; $F_{1,16}$ = 50.7, P < 0.001) and metabolic rates (Figure PIII.2, Figure PIII.S2). The

negative effects of pesticides on photosynthetic efficiency were more pronounced in the epilithic biofilm, making the interaction between pesticides and substratum type significant (Table PIII.S4).

Multiple stressor responses (interactive effects)

We assessed the effects of 4 different stressor combinations (i.e. W:H, W:P, H:P, W:H:P) on 11 response variables at 2 different time scales (short term vs. long term) and on 2 different substratum types (cobbles; epilithic and sand; epipsammic). Out of the 152 possible combinations, 108 resulted in non-significant interaction terms, suggesting additive effects (71 %), whereas 44 resulted in significant interactions (29 %). Detailed information on stressor combinations and interactive effects is available in Table PIII.S6. These interactions were mostly antagonistic, meaning that the combined effect of the stressors was less pronounced than the sum of the individual effects.

Chlorophyll-*a* concentration and photosynthetic efficiency antagonistically responded to the combination of hydrological stress and pesticides (H:P; $F_{1,16}$ = 10.5 and 5.6, P = 0.005 and < 0.001); when combined, these stressors resulted in chlorophyll-*a* concentration and photosynthetic efficiency values that were higher than the values that would be obtained assuming additive effects (Figure PIII.1A, B). In the case of chlorophyll-*a* concentration, this antagonistic interaction was only observed for epilithic biofilm, making the triple interaction between hydrological stress, pesticides and substratum type significant (Table PIII.S4). Antagonism was also observed for gross primary production (H:P; $F_{1,16}$ = 13.8, P = 0.002), community respiration (H:P; $F_{1,16}$ = 7.9, P = 0.013) and production-respiration ratios (H:P; $F_{1,16}$ =7.5, P = 0.015). The combination between warming and pesticides (W:P) resulted in an antagonistic interaction for basal chlorophyll fluorescence ($F_{1,16}$ = 5.5, P = 0.033). The warming-pesticides combination also decreased production-respiration ratios, favoring heterotrophic conditions, although the interaction was only significant for gross primary production ($F_{1,16}$ = 8.2, P = 0.011). The combination between warming and hydrological stress (W:H) mostly interacted to alter heterotrophic metabolism, as indicated by significant interaction terms for organic substrate utilization richness ($F_{1,16}$ = 10.0, P = 0.006), and community respiration ($F_{1,16}$ = 6.0, P = 0.026). The combination between the three stressors (W:H:P) was significant for basal chlorophyll fluorescence ($F_{1,16}$ = 5.1, P = 0.038) and community respiration ($F_{1,16}$ = 8.1, P = 0.012).

Single and multiple stressor responses at short and long-term exposure times

Single stressor responses were highly dependent on exposure time (Table PIII.3). The negative effects of hydrological stress (H) on primary producers were further amplified at long term, especially for photosynthetic capacity (H:T; $F_{1,16}$ = 90.5, P < 0.001) and basal chlorophyll fluorescence (H:T; $F_{1,16}$ = 108.5, P < 0.001). This was particularly the case for epilithic biofilm, as indicated by a significant triple interaction between hydrological stress, exposure time and substratum type (Table PIII.S5). The number of 16S rRNA gene copies dropped after long-term exposure to warming conditions especially in the epilithic biofilm (Figure PIII.1F, Table PIII.S5). As observed for hydrological stress, pesticides produced their negative impact on production-respiration ratios only after long-term exposure (Figure PIII.2), making the interaction between pesticides and exposure time significant ($F_{1,16}$ = 15.9, P = 0.001).

Overall, exposure time drove interactive effects towards antagonism. At short term, the 59 % of significant interactions were classified as antagonisms, and the 41 % as synergisms. On the other hand, the 86 % of the significant interactions were antagonisms at long term, and only the 14 % were synergisms by then (Table PIII.S6). The strongest dependence of an interaction on exposure time was observed for warming and pesticides (Table PIII.3). The negative effects of warming and pesticides on photosynthetic efficiency and basal chlorophyll-*a* fluorescence were mitigated after long-term exposure to both stressors ($F_{1,16}$ = 35.8, P < 0.001; $F_{1,16}$ = 13.4, P = 0.002). Similarly, the response to warming and hydrological stress in terms of community respiration became antagonism at long term (W:H:T; $F_{1,16}$ = 8.7, P = 0.010). Also in line with this, the little effect of the interaction between hydrological stress and pesticides on production-respiration ratios became antagonistic after long-term exposure to the same combination of stressors (H:P:T; $F_{1,16}$ = 7.4, P = 0.015).

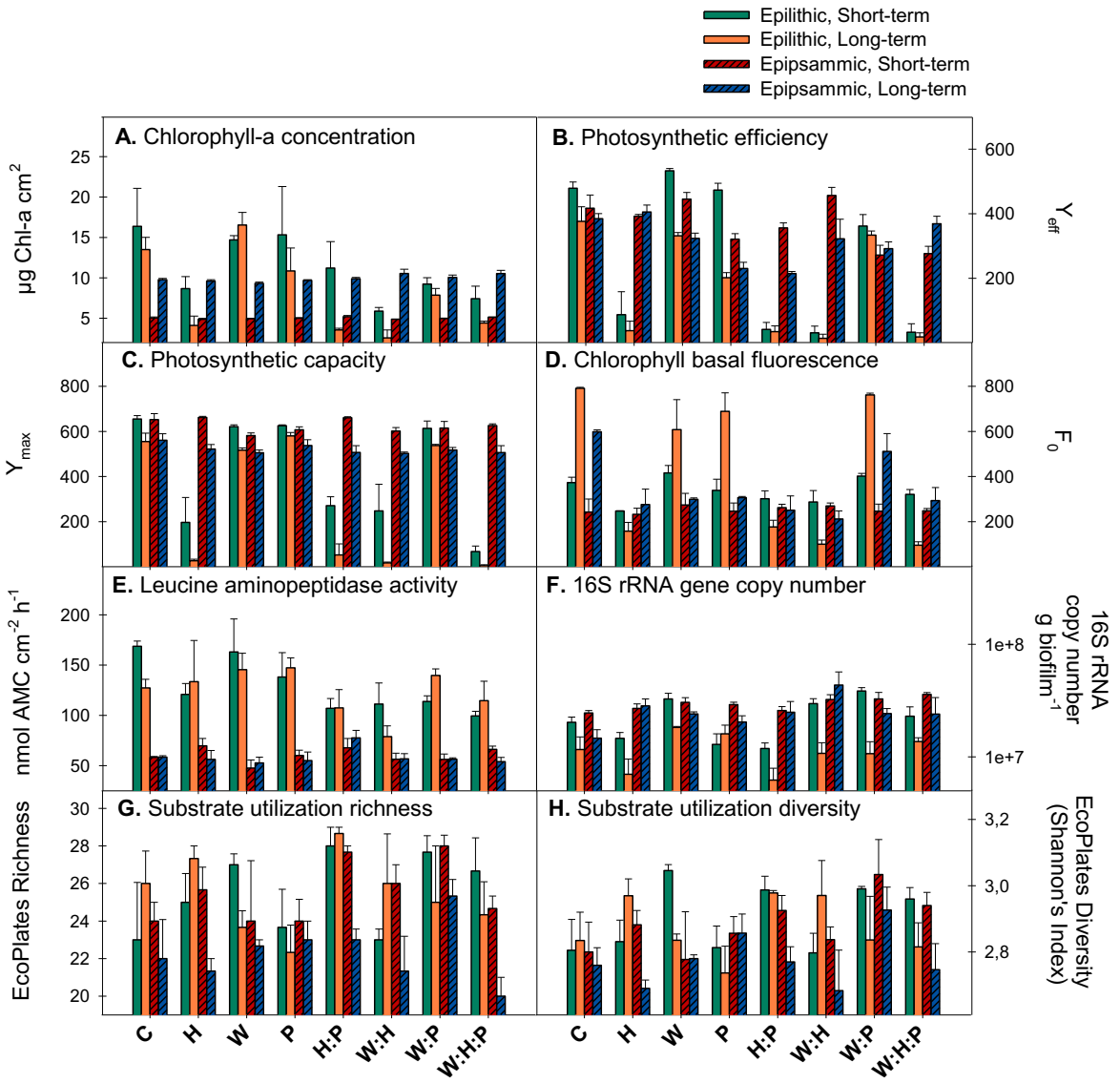


Figure PIII. 1 Changes in response variables for epilithic (smooth bars) and epipsammic (stripped bars) river biofilms after short and long-term exposure to the different treatments (hydrological stress; H, warming; W, pesticides; P, H:P, W:H, W:P, W:H:P) and in control biofilms (C). Plots represent averaged values of chlorophyll-a concentration (A), photosynthetic efficiency (B), photosynthetic capacity (C), chlorophyll basal fluorescence (D), leucine aminopeptidase activity (E), 16S rRNA gene copy number (F), and substrate utilization richness (G) and diversity (H). Error bars show standard errors between replicates (n = 3).

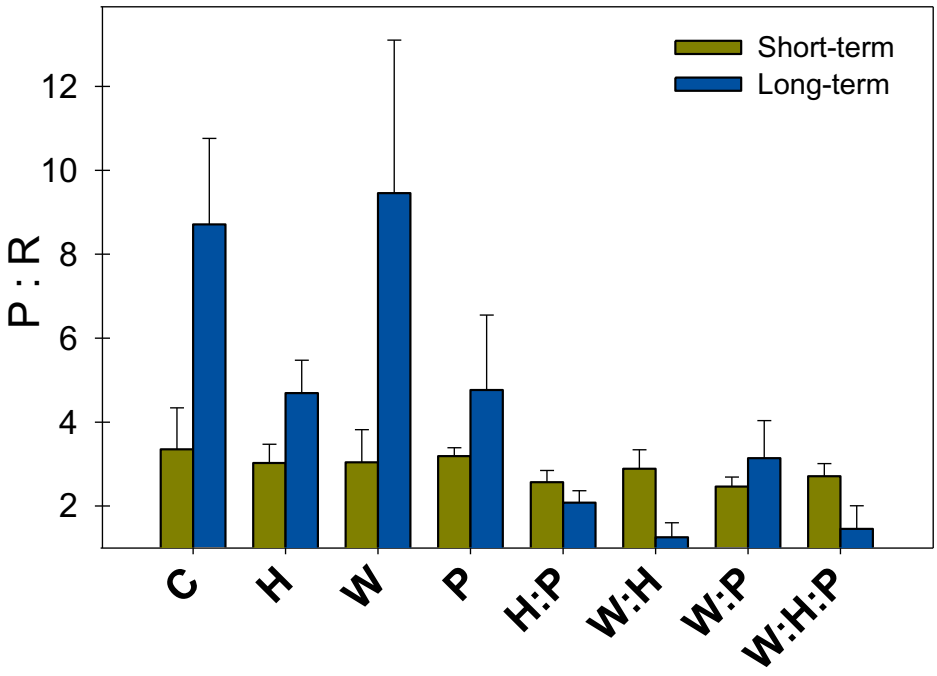


Figure PIII. 2 Changes in production-respiration ratios for river biofilms after short and long-term exposure to the different treatments (hydrological stress; H, warming; W, pesticides; P, H:P, W:H, W:P, W:H:P) and in control biofilms (C). Bars represent averaged values (n = 3), error bars represent standard errors (SE).

Response variable	Factor	df	SS	MS	F	P
Chlorophyll-*a* concentration	**Hydrological stress (H)**	**1**	**2.81E+02**	**2.81E+02**	**57.6**	**< 0.001**
	H:P	1	5.13E+01	5.13E+01	10.5	0.005
Photosynthetic efficiency	**Hydrological stress (H)**	**1**	**6.76E+05**	**6.76E+05**	**249.4**	**< 0.001**
	Pesticides (P)	1	1.37E+05	1.37E+05	50.7	< 0.001
	H:P	1	1.51E+04	1.51E+04	5.6	0.031
Photosynthetic capacity	Warming (W)	1	3.49E+04	3.49E+04	8.6	0.010
	Hydrological stress (H)	**1**	**2.48E+05**	**2.48E+05**	**61.1**	**< 0.001**
Basal chlorophyll fluorescence	**Hydrological stress (H)**	**1**	**1.07E+06**	**1.07E+06**	**107.4**	**< 0.001**
	W:P	1	5.42E+04	5.42E+04	5.5	0.033
	W:H:P	1	5.09E+04	5.09E+04	5.1	0.038
Leucine aminopeptidase activity	**Hydrological stress (H)**	**1**	**1.33E+04**	**1.33E+04**	**10.2**	**0.006**
16S rRNA gene abundance	**Warming (W)**	**1**	**1.56E+15**	**1.56E+15**	**33.7**	**< 0.001**
Substrate utilization richness	**W:H**	**1**	**8.44E+01**	**8.44E+01**	**10.0**	**0.006**
Gross primary production	Warming (W)	1	3.42E+03	3.42E+03	18.7	< 0.001
	Hydrological stress (H)	**1**	**5.52E+03**	**5.52E+03**	**30.1**	**< 0.001**
	Pesticides (P)	1	1.08E+03	1.08E+03	5.9	0.027
	W:P	1	1.50E+03	1.50E+03	8.2	0.011
	H:P	1	2.53E+03	2.53E+03	13.8	0.002
Community respiration	**Warming (W)**	**1**	**4.15E+03**	**4.15E+03**	**41.3**	**< 0.001**
	Hydrological stress (H)	1	1.65E+03	1.65E+03	16.5	< 0.001
	Pesticides (P)	1	1.63E+03	1.63E+03	16.2	< 0.001
	W:H	1	6.01E-01	6.01E-01	6.0	0.026
	H:P	1	7.93E-01	7.93E-01	7.9	0.013
	W:H:P	1	8.09E-01	8.09E-01	8.1	0.012
Production-respiration ratio	**Hydrological stress (H)**	**1**	**5.71E+01**	**5.71E+01**	**35.7**	**< 0.001**
	Pesticides (P)	1	3.69E+01	3.69E+01	23.1	< 0.001
	H:P	1	1.19E+01	1.19E+01	7.5	0.015

Table PIII. 2 Output for the mixed-model nested ANOVA (fixed factors). Significant results for single and multiple stressors are presented (P-value < 0.05). Acronyms: H = hydrological stress, P = pesticides, W = warming, df = degrees of freedom, SS = sum of squares, MS = mean of squares, F = F-value, P = P-value. The strongest effect (i.e. highest F-value) for each response variable is highlighted in bold. Substrate utilization diversity does not appear in the table as none of the factors included in the ANOVA were significant (P > 0.05).

Response variable	Factor	df	SS	MS	F	P
Photosynthetic efficiency	**Time (T)**	**1**	**1.11E+05**	**1.11E+05**	**54.4**	**< 0.001**
	H:T	1	3.04E+04	3.04E+04	14.9	0.001
	W:P:T	1	7.32E+04	7.32E+04	35.8	< 0.001
Photosynthetic capacity	Time (T)	1	6.92E+04	6.92E+04	24.6	< 0.001
	H:T	**1**	**2.54E+05**	**2.54E+05**	**90.5**	**< 0.001**
Basal chlorophyll fluorescence	Time (T)	1	1.88E+05	1.88E+05	31.5	< 0.001
	W:T	1	3.20E+04	3.20E+04	5.3	0.034
	H:T	**1**	**6.48E+05**	**6.48E+05**	**108.5**	**< 0.001**
	W:P:T	1	8.00E+04	8.00E+04	13.4	0.002
	W:H:P:T	1	3.53E+04	3.53E+04	5.9	0.027
Leucine aminopeptidase activity	**Time (T)**	**1**	**2.89E+04**	**2.89E+04**	**109.4**	**< 0.001**
	H:T	1	2.63E+03	2.63E+03	9.9	0.006
	P:T	1	1.44E+03	1.44E+03	5.4	0.033
16S rRNA gene abundance	**Time (T)**	**1**	**1.42E+15**	**1.42E+15**	**32.1**	**< 0.001**
	W:T	1	2.26E+14	2.26E+14	5.1	0.038
Substrate utilization richness	**Time (T)**	**1**	**6.34E+01**	**6.34E+01**	**17.4**	**< 0.001**
Substrate utilization diversity	**Time (T)**	**1**	**1.23E-01**	**1.23E-01**	**20.8**	**< 0.001**
	P:T	1	3.05E-02	3.05E-02	5.1	0.037
Gross primary production	**Time (T)**	**1**	**4.40E+01**	**4.40E+01**	**104.2**	**< 0.001**
	H:T	1	1.52E+01	1.52E+01	35.9	< 0.001
Community respiration	Time (T)	1	5.89E+03	5.89E+03	54	< 0.001
	W:T	**1**	**1.34E+03**	**1.34E+03**	**12.2**	**0.003**
	W:H:T	1	9.49E-01	9.49E-01	8.7	0.01
Production-respiration ratio	Time (T)	1	2.84E+01	2.84E+01	18.8	< 0.001
	H:T	**1**	**4.65E+01**	**4.65E+01**	**30.8**	**< 0.001**
	P:T	1	2.40E+01	2.40E+01	15.9	0.001
	H:P:T	1	1.12E+01	1.12E+01	7.4	0.015

Table PIII. 3 Output for the mixed-model nested ANOVA (random factor time). Significant interactions with time are presented (P-value < 0.05). Acronyms: H = hydrological stress, P = pesticides, W = warming, df = degrees of freedom, SS = sum of squares, MS = mean of squares, F = F-value, P = P-value. The strongest effect (i.e. highest F-value) for each response variable is highlighted in bold. Chlorophyll-a concentration does not appear in the table as none of the factors included in the ANOVA were significant (P > 0.05). For the complete ANOVA output, including non-significant effects and residuals, see supplementary information.

Discussion

Considerations on the experimental design and treatment conditions

Manipulative experiments in the laboratory can define causative relationships between stressors and the response to them (Sabater and Borrego, 2015). These experiments may also provide insight on the mechanisms involved and the effects over exposure time. Nevertheless, laboratory experiments are simplifications of the reality, as they replace the complexity of natural settings by only a few factors. In order to properly address the effects of Global change on ecosystems, manipulative experiments need to be able to reproduce natural communities under controlled conditions. The artificial streams used in this study were previously demonstrated to be able to reproduce up to 91.6 % of the bacterial operational taxonomic units (OTUs) present in the original site (i.e. Llémena River), sharing a 72.7 % Bray-Curtis similarity (Romero *et al.*, 2019). At the primary producers level, the artificial streams used in this study reproduce a typical river biofilm community, with dominance of diatoms (60 % of total abundance) and lower proportions of Cyanobacteria (16 %), Chlorophyta (13 %) and Rhodophyta (7 %) (Sabater-Liesa *et al.*, 2019). At the functional level, we measured photosynthetic efficiencies and organic matter degradation before any experimental manipulation (i.e. end of the acclimation phase), and we obtained results equivalent to those found for river biofilms in the Mediterranean region (Freixa and Romaní, 2014; Freixa *et al.*, 2016; Ponsatí *et al.*, 2016). A detailed comparison between the biofilms used in this study and those developing under realistic environmental conditions is presented in Table PIII.S3.

Our experimental conditions (i.e. light availability, water temperature, water velocity and/or available nutrients) favoured the prevalence of the phototrophic community. Basal chlorophyll fluorescence, chlorophyll-*a* concentration, and production-respiration ratios therefore significantly increased with time in control streams, whereas the abundance of total bacteria (predicted from 16S rRNA gene abundance) decreased. This trend was especially evident for the epilithic biofilm, where significant interactions between time and substratum type occurred. Whereas the average nutrient concentrations in our artificial streams were low (especially for nitrite, ammonia and phosphate), water temperature, light and flow conditions may have promoted algal growth in our artificial streams.

Stressor levels used in our experiment represented realistic current values as well as estimates from Climate Change projections (IPCC, Summary for Policymakers - Special Report, 2018; Marx *et al.*, 2018). Appropriately selecting stressor levels is critical to avoid one or few factors dominating over the others (Garnier *et al.*, 2017). So forth, the increase in water temperature we applied (see section 3.1) lies within the modelled projection of 0.3 – 4.8 °C increase in global mean surface temperature by the end of the 21st century, relative to 1986 – 2005 (IPCC, Summary for Policymakers - Special Report, 2018). Also, the low-flow situation

applied is aligned with future climate change projections (Marx *et al.*, 2018). We here applied a controlled low-flow situation on which surface flow is removed, but sub-surface flow remains. Finally, the mixture of pesticides represented a common combination in Mediterranean systems (Ricart *et al.*, 2010; Ccanccapa *et al.*, 2016; Casado *et al.*, 2019). A comparison between the pesticide concentrations achieved in our artificial streams and realistic concentrations in agricultural rivers and streams is presented in Table PIII.S2.

Single stressor effects

The largest main effect observed was caused by hydrological stress, which impedes resource acquisition through the limitation of organic matter and nutrient diffusion, combined with osmotic stress (Schimel *et al.*, 2007); it negatively altered both biofilm community structure and function, leading to 73 % of the response variables being significantly altered. This effect was especially relevant for epilithic biofilms, probably because of the low porosity of cobbles, which lead to severe desiccation and decreased productivity in biofilms subjected to hydrological stress. The negative effects of low-flow on the epilithic biofilm were observed at both short and long term, although the magnitude of the effect was higher after long-term exposures (i.e. 30 days). In this situation, the interaction between hydrological stress and time was significant for 54 % of response variables. These results partially confirm our first hypothesis predicting that climatic stressors would cause the most pervasive effects on the biofilm.

The large impact of hydrological stress on the autotrophic compartment (i.e. affecting photosynthetic parameters) also had an indirect effect on heterotrophic functioning. We observed a decrease in the decomposition rate of peptidic compounds (measured as the leucine aminopeptidase activity, LAPA), suggesting decreased availability of primary producers-derived organic compounds. The effects on LAPA were smaller in the long term, suggesting that heterotrophic microorganisms in epilithic biofilms could become adapted to hydrological stress, shifting towards the use of other substrates, as indicated by the increase in substrate utilization diversity after 30 days of exposure.

A 1.6 ° C increase in water temperature significantly altered a 36 % of response variables, suggesting that water warming produce smaller effects than hydrological stress on river biofilms. Water temperature caused an overall positive effect in the 16S rRNA gene abundance, while it reduced the photosynthetic capacity of the epipsammic biofilm. So forth, temperature increase may have favored the bacterial heterotrophic capacity within the biofilms, confirmed by the reduction in gross primary production after long-term exposure to

warming. Our observations match those of previous studies showing the positive effects of temperature on bacterial growth and organic carbon degradation (Ylla *et al.*, 2012, 2014b).

Finally, pesticides impaired mainly the autotrophic compartment, with a 36 % of response variables being significantly altered. The reduction in photosynthetic efficiency was immediate in the epipsammic biofilm (i.e. after 48 hours), and only at long term (i.e. 30 days) we could observe reduced photosynthetic efficiency in the epilithic biofilm. This lower tolerance of epipsammic biofilms to pesticide exposure might be associated with the particularly high sensitivity of microorganisms in this biofilm (Widenfalk *et al.*, 2008), as well as the increased porosity and retention capacity of fine sediments which facilitate the accumulation of toxicants, promoting biofilm exposure (Magnusson *et al.*, 2013).

Multiple stressor effects

Biofilm responses to multiple stressors depend on the ability of its organisms to respond to each stressor and on the possible occurrence of positive or negative co-tolerance mechanisms (Tlili, Corcoll, *et al.*, 2011; Stampfli *et al.*, 2013). An exposure to a stressor combined with a positive co-tolerance should reduce the impact of a second stressor, while a negative co-tolerance would have the adverse effect (D. Vinebrooke *et al.*, 2004). We here applied a null model comparison (as our null hypothesis predicted additive effects), and found that the majority of the stressor combinations (71 %) did not result in significant interaction terms in the mixed-model nested ANOVA, suggesting the existence of overall additive effects. This prevalence of additive effects is consistent with previously published research, including analyses with higher statistical power (Jeremy J Piggott, Niyogi, *et al.*, 2015a; Elbrecht, Beermann, Goessler, Neumann, Tollrian, R??diger Wagner, *et al.*, 2016; Schinegger *et al.*, 2016b). However, we also found non-additive significant interactions (29 % of all stressor combinations) regarding the three studied stressors (warming, hydrological stress and pesticides). We found that antagonisms prevailed among significant interactions and that exposure time lead the overall multiple-stressor response towards increased number of antagonistic interactions (from 59 % of all significant interactions at short term to 86 % at long term). The occurrence of these antagonistic effects agrees with previous research indicating that antagonisms are common at the community level in freshwaters (Côté *et al.*, 2016; Jackson *et al.*, 2016).

The antagonistic interaction between warming and pesticides (W:P) particularly occurred after long-term exposure; the negative individual effects of W and P on photosynthetic efficiency and basal chlorophyll fluorescence were partially mitigated. This antagonistic interaction was enhanced with exposure time, especially in the epilithic biofilm. Other studies have also

shown partial mitigation of individual effects on river biofilms when warming and pesticides co-occur (Larras *et al.*, 2013; Romero *et al.*, 2018). Warming and hydrological stress (W:H) resulted in antagonistic interactions concerning the heterotrophic activity. Organic substrate utilization richness and overall CR responded analogously. The organic substrate utilization richness in the epilithic biofilms for the W:H treatment reached values resembling more the controls than those under single-stressor treatments W and H. Strikingly, the W and H single-stressor treatments did not differed from the controls in terms of CR, but reached a 4.7-fold increase in oxygen consumption after 30 days of exposure when the two stressors co-occurred. In line with this, the lowest production-respiration ratios were recorded in the W:H streams, suggesting that this stressor combination promotes heterotrophy. The *ecological surprises* arising from the interaction between warming and hydrological stress have recently been highlighted on river biofilms (Romero *et al.*, 2018), and might be due to the metabolic activation of desiccation-tolerant taxa by temperature (Marcus, Jennifer K Wey, *et al.*, 2014).

Hydrological stress and pesticides (H:P) produced antagonistic interactions on both chlorophyll-*a* concentration and photosynthetic efficiency. This antagonism was especially relevant for epilithic biofilms after long-term exposure, which is probably related to the time lapse between the negative effects produced by hydrological stress (i.e. immediate) and those of pesticides (i.e. mostly after long-term exposure). Both hydrological stress and toxicant exposure have recently been reported to co-occur in 10 – 25 % of rivers and transitional coastal waters worldwide (Nõges *et al.*, 2016). The accumulation of extracellular polymeric substances (EPS) may lower the sensitivity of biofilms to organic chemicals (Flemming and Wingender, 2010; Polst *et al.*, 2018). As shown for monospecific biofilms (Chang *et al.*, 2007), the 30-day exposure to hydrological stress applied in this study could have favoured the accumulation of EPS, hindering the penetration of the pesticides through the biofilm matrix. This antagonistic response was in line with production-respiration ratios, which decreased in the H:P treatment with respect to control streams, but less than expected based on individual H and P results.

Finally, the co-occurrence of warming, hydrological stress and pesticides (W:H:P) lead to the lowest values of photosynthetic efficiency, photosynthetic capacity and basal chlorophyll-*a* fluorescence in the epilithic biofilm. This interaction was however antagonistic for basal chlorophyll-*a* fluorescence, which showed levels above the additive prediction at long term. Overall, interactive effects among the three stressors (i.e. 3-way interactions) had smaller effect sizes (i.e. smaller computed F-values) than single stressors (i.e. main effects) and 2-way interactions. Interactions between 2 stressors drove the overall responses in our multiple stressors experiment, in a similar manner as indicated by other studies with higher statistical power (Lange *et al.*, 2011; Jeremy J Piggott, Salis, *et al.*, 2015c; R. K. Salis *et al.*, 2017). The inclusion of climatic stressors (i.e. warming and hydrological stress) amongst the analyzed

stressors is probably driving this pattern, as the W:H:P and the W:H combinations resulted in similar production-respiration ratios at long term, indicating little effects of pesticides in the triple interaction.

Overall, our study reveals that river biofilms exposed to multiple global change stressors may partially adapt through changes in community structure and function, leading to antagonistic interactions, with combined effects that deviate from *a priori* predictions. Importantly, multiple stressor scenarios shifted the community metabolism towards heterotrophy, particularly when climatic stressors were at play. Ours study results may help mark the way forward for future studies assessing the nature of multiple stressor interactions across food webs in both artificial and natural settings.

PAPER III. Effects of multiple stressors on river biofilms depend on time scale

Supplementary information

Ferran Romero[1,2], Vicenç Acuña[1,2], Carme Font[1,2], Anna Freixa[1,2], Sergi Sabater[1,3]

1 - Catalan Institute for Water Research (ICRA), C. Emili Grahit 101, 17003 Girona (Spain).
2 – Universitat de Girona (UdG), Girona (Spain)
3 - Institute of Aquatic Ecology (IEA), University of Girona, Campus de Montilivi, 17003 Girona (Spain).

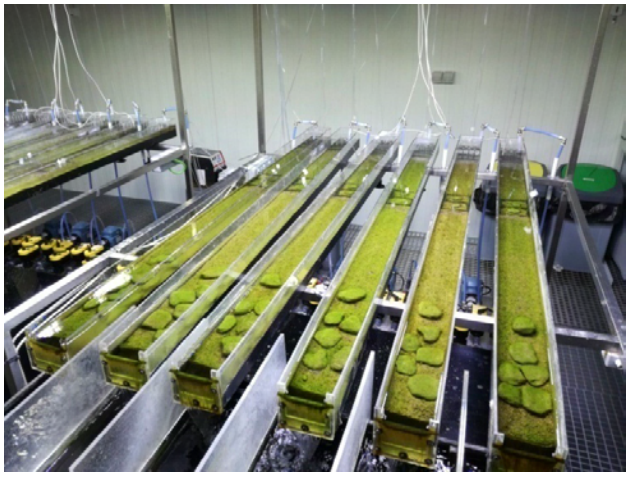


Figure PIII.S1 Picture of 6 (out of 24) artificial streams used in this study installed at the Experimental Streams Facility. Each artificial stream consists of an independent methacrylate channel (l – w – d = 200 cm – 10 cm – 10 cm) and a 70 L water tank from which water can be recirculated.

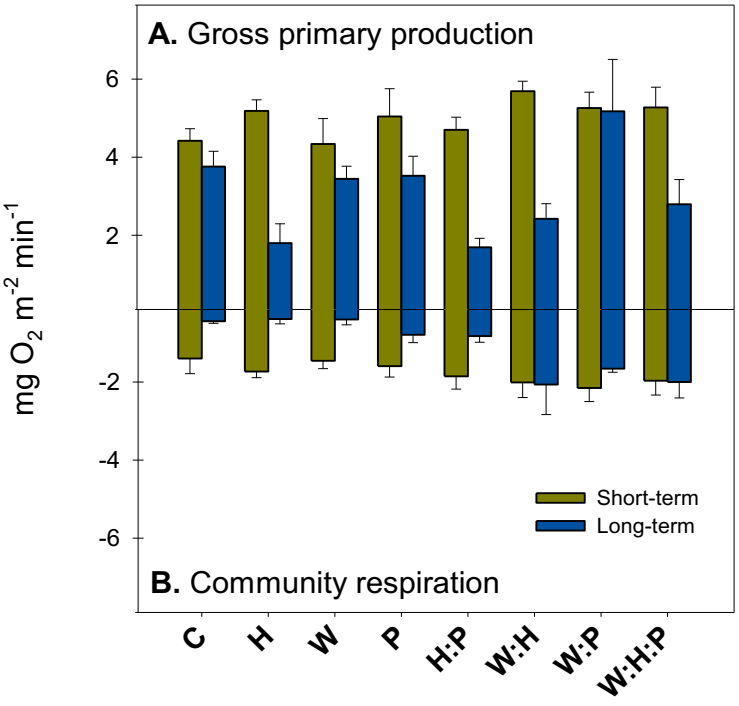


Figure PIII.S2 Changes in metabolic rates (A; gross primary production, B; community respiration) for river biofilms after short and long-term exposure to the different treatments (H, W, P, H:P, W:H, W:P, W:H:P) and in control biofilms (C). Bars represent averaged values (n = 3), error bars represent standard errors (SE).

Pesticide	Target	Nominal conc. (ng L^{-1})	CAS number	Molecular weight (g mol^{-1})	Solubility (mg L^{-1})
Diuron	Herbicide	150	330 – 54 – 1	233.09	42.0
Chlorpyrifos	Insecticide	20	2921 – 88 – 2	350.59	1.4
Imazalil	Fungicide	120	35554 – 44 – 0	297.18	180.0
Prochloraz	Fungicide	30	67747 – 09 – 5	376.67	9.1
Simazine	Herbicide	50	122 – 34 – 9	201.66	5.0

Table PIII.S1 Pesticides employed in this experiment and nominal concentrations applied. Solubility is indicated for the given pesticides in water at 25° C.

Parameter	This study - artificial streams (influent water)	Field conditions			
		site	Season	value	reference
Diuron (ng L^{-1})	140.7 - 156.7	Zadorra River	A	150.96	(Ccanccapa *et al.*, 2016)
Chlorpyrifos (ng L^{-1})	< 2.24	Ebro River	A	1.01 - 16.40	(Ccanccapa *et al.*, 2016)
Imazalil (ng L^{-1})	15.1 - 85.4	Segre River	A	120	(Ccanccapa *et al.*, 2016)
Prochloraz (ng L^{-1})	0.07 - 34.2	Segre River	A	34.2	(Ccanccapa *et al.*, 2016)
Simazine (ng L^{-1})	50.3 - 68.6	Llobregat River	SP	53.6	(Ricart *et al.*, 2010)

Table PIII.S2 Comparison between pesticide concentrations used in this study and field conditions. All rivers in column "site" are located in the Iberian Peninsula (south of Europe). Abbreviations: A; autumn, SP; spring.

Parameter	This study - biofilm before experimental manipulation (average ± SD , n = 24)		Field conditions				
	Epilithic	Epipsammic	site	Biofilm type	Season	value	reference
Chlorophyll-a concentration (µg cm^{-2})	11.05 ± 3.08	5.21 ± 0.18	Siurana River	Epilithic	A	8 - 10	(Ponsatí *et al.*, 2015, 2016)
Photosynthetic efficiency (Y_{eff})	474 ± 60	373 ± 63	Cinca River	Epilithic	S	300 - 400	(Ponsatí *et al.*, 2015, 2016)
Photosynthetic capacity (Y_{max})	630 ± 30	604 ± 42	Montsant River	Epilithic	A	500 - 600	(Ponsatí *et al.*, 2015, 2016)
Leucine aminopeptidase activity (nmol AMC cm^{-2} h^{-1})	181 ± 31	26 ± 17	Cinca River	Epilithic	S	100 - 200	(Ponsatí *et al.*, 2015, 2016)
EcoPlates - Substrate utilization diversity (Shannon's Index)	2.81 ± 0.10	2.87 ± 0.14	Llobregat River	Epipsammic	A	2.0 – 3.0	(Freixa and Romaní, 2014)

Table PIII.S3 Comparison between biofilm parameters before experimental manipulation (i.e. at the end of the acclimation period) and the same parameters in field conditions. Abbreviations: S; summer, W; winter, A; autumn, SP; spring.

Response variable	Factor	df	SS	MS	F	P
Chlorophyll-a concentration	Substratum type (S)	1	1.01E+02	1.01E+02	20.5	< 0.001
	W:S	1	2.49E+01	2.49E+01	5.0	0.039
	H:S	1	3.20E+02	3.20E+02	65.2	< 0.001
	H:P:S	1	4.96E+01	4.96E+01	10.1	0.006
Photosynthetic efficiency	Substratum type (S)	1	4.09E+05	4.09E+05	298.4	< 0.001
	H:S	1	7.89E+05	7.89E+05	575.1	< 0.001
	P:S	1	1.69E+04	1.69E+04	12.3	0.003
Photosynthetic capacity	Substratum type (S)	1	6.92E+04	6.92E+04	24.6	< 0.001
	H:S	1	2.54E+05	2.54E+05	90.5	< 0.001
Basal chlorophyll-a fluorescence	Substratum type (S)	1	1.57E+05	1.57E+05	27.7	< 0.001
	H:S	1	3.79E+05	3.79E+05	66.7	< 0.001
Leucine aminopeptidase activity	Substratum type (S)	1	2.89E+04	2.89E+04	109.4	< 0.001
	H:S	1	2.63E+03	2.63E+03	9.9	0.006
	P:S	1	1.44E+03	1.44E+03	5.4	0.032
16S rRNA gene abundance	Substratum type (S)	1	2.52E+15	2.52E+15	33.6	< 0.001
	H:S	1	6.84E+14	6.84E+14	9.1	0.008
Substrate utilization richness	Substratum type (S)	1	5.70E+01	5.70E+01	9.3	0.007
Substrate utilization diversity	Substratum type (S)	1	7.65E-02	7.65E-02	10.1	0.006
	H:S	1	5.18E-02	5.18E-02	6.8	0.019
	P:S	1	6.05E-02	6.05E-02	8.0	0.012

Table PIII.S4 Output for the mixed-model nested ANOVA (random factor substratum type). Significant results for single and multiple stressors are presented (P-value < 0.05). Acronyms: S = substratum type, H = hydrological stress, P = pesticides, W = warming, df = degrees of freedom, SS = sum of squares, MS = mean of squares, F = F-value, P = P-value.

Response variable	Factor	df	SS	MS	F	P
Chlorophyll-a concentration	S:T	1	3.93E+02	3.93E+02	32.3	< 0.001
Photosynthetic efficiency	H:S:T	1	1.99E+04	1.99E+04	7.4	0.015
	W:H:P:S:T	1	2.99E+04	2.99E+04	11.1	0.004
Photosynthetic capacity	S:T	1	6.12E+05	6.12E+05	133.1	< 0.001
	H:S:T	1	3.78E+05	3.78E+05	82.2	< 0.001
Basal chlorophyll-a fluorescence	H:S:T	1	1.49E+05	1.49E+05	50.4	< 0.001
	W:P:S:T	1	1.99E+04	1.99E+04	6.8	0.019
Leucine aminopeptidase activity	S:T	1	2.34E+04	2.34E+04	16.5	< 0.001
16S rRNA gene abundance	S:T	1	2.88E+14	2.88E+14	7.6	0.013
	W:S:T	1	2.76E+14	2.76E+14	7.3	0.015
	W:H:P:S:T	1	2.36E+14	2.36E+14	6.3	0.023
Substrate utilization richness	S:T	1	5.70E+01	5.70E+01	5.9	0.027
Substrate utilization diversity	H:S:T	1	1.16E-01	1.16E-01	5.9	0.027

Table PIII.S5 Output for the mixed-model nested ANOVA (random factors S:T). Significant results for single and multiple stressors are presented (P-value < 0.05). Acronyms: T = time, S = substratum type, H = hydrological stress, P = pesticides, W = warming, df = degrees of freedom, SS = sum of squares, MS = mean of squares, F = F-value, P = P-value.

			W:H (T:S)	W:P (T:S)	H:P (T:S)	W:H:P (T:S)
Chlorophyll-a concentration	Short-term	epilithic	n.s.	n.s.	A	n.s.
		epipsammic	n.s.	n.s.	A	n.s.
	Long-term	epilithic	n.s.	n.s.	A	n.s.
		epipsammic	n.s.	n.s.	A	n.s.
Photosynthetic efficiency	Short-term	epilithic	n.s.	S	S	S
		epipsammic	n.s.	S	A	S
	Long-term	epilithic	n.s.	A	A	A
		epipsammic	n.s.	A	S	A
Photosynthetic capacity	Short-term	epilithic	n.s.	n.s.	n.s.	n.s.
		epipsammic	n.s.	n.s.	n.s.	n.s.
	Long-term	epilithic	n.s.	n.s.	n.s.	n.s.
		epipsammic	n.s.	n.s.	n.s.	n.s.
Basal chlorophyll fluorescence	Short-term	epilithic	n.s.	A	n.s.	A
		epipsammic	n.s.	A	n.s.	S
	Long-term	epilithic	n.s.	A	n.s.	A
		epipsammic	n.s.	A	n.s.	A
Leucine aminopeptidase activity	Short-term	epilithic	n.s.	n.s.	n.s.	n.s.
		epipsammic	n.s.	n.s.	n.s.	n.s.
	Long-term	epilithic	n.s.	n.s.	n.s.	n.s.
		epipsammic	n.s.	n.s.	n.s.	n.s.
16S rRNA gene abundance	Short-term	epilithic	n.s.	n.s.	n.s.	A
		epipsammic	n.s.	n.s.	n.s.	A
	Long-term	epilithic	n.s.	n.s.	n.s.	S
		epipsammic	n.s.	n.s.	n.s.	A
EcoPlates - substrate utilization richness	Short-term	epilithic	A	n.s.	n.s.	n.s.
		epipsammic	S	n.s.	n.s.	n.s.
	Long-term	epilithic	A	n.s.	n.s.	n.s.
		epipsammic	S	n.s.	n.s.	n.s.
EcoPlates – substrate utilization diversity	Short-term	epilithic	n.s.	n.s.	n.s.	n.s.
		epipsammic	n.s.	n.s.	n.s.	n.s.
	Long-term	epilithic	n.s.	n.s.	n.s.	n.s.
		epipsammic	n.s.	n.s.	n.s.	n.s.
Gross primary production	Short-term		n.s.	A	A	n.s.
	Long-term		n.s.	A	A	n.s.
Community respiration	Short-term		S	n.s.	A	A
	Long-term		A	n.s.	A	A
Production-respiration ratio	Short-term		n.s.	n.s.	S	n.s.
	Long-term		n.s.	n.s.	A	n.s.

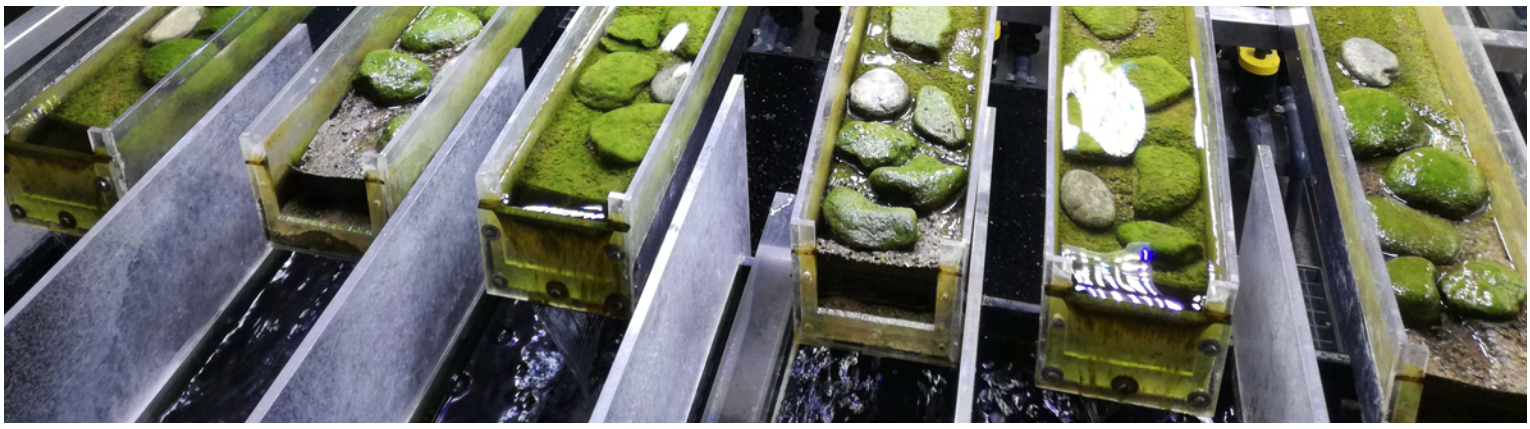
Table PIII.S6 Stressor combinations assessed in this study (n = 152). Factors are: W; warming, H, hydrological stress, P; pesticides, T; time and S; substratum type. Parentheses indicate random (i.e. nested) factors. Out of the 152 stressor combinations, 44 (29 %) were significant at $P < 0.05$ (A; antagonism, S; synergism). Considering only short-term significant interactions (n = 22), 13 were classified as antagonisms (59 %), and 9 as synergisms (41 %). At long-term exposure (n = 22), 19 interactions were antagonistic (86 %) and 3 synergistic (14 %).

Paper IV

Multiple stressors determine structure and estimated function of river biofilm bacterial communities

Romero, F., Acuña, V., and Sabater, S.

Manuscript



PAPER IV. Multiple stressors determine structure and estimated function of river biofilm bacterial communities

Ferran Romero[1,2], Vicenç Acuña[1,2], Sergi Sabater[1,3]

1. Catalan Institute for Water Research (ICRA), Emili Grahit 101, 17003 Girona (Spain)
2. Faculty of Sciences - University of Girona (UdG), Campus de Montilivi, 17003 Girona (Spain)
3. Institute of Aquatic Ecology (IEA), University of Girona, Campus de Montilivi, 17003 Girona (Spain)

Abstract

Freshwater ecosystems receive multiple stressors worldwide. The effects of multiple stressors (including their interactions) remain largely unexplored, particularly on benthic bacteria, which are responsible for key stream ecosystem processes. We here used 24 artificial streams to investigate the effects of warming, hydrological stress and pesticide exposure on epilithic and epipsammic stream biofilms using 16S rRNA gene metabarcoding. Bacterial community composition and estimated function were assessed after a 30-day exposure period to individual and combined stressors. Hydrological stress significantly altered 57 % of the most abundant bacterial taxa, followed by warming (21 %) and pesticide exposure (11 %). Out of all stressor combinations, the 16 % resulted in significant interaction effects on bacterial community composition, and the same was observed regarding estimated function. Antagonistic responses prevailed among interaction effects, although synergistic responses also occurred, particularly on the epilithic biofilm. Overall, multiple stressors shaped the composition of the bacterial communities and their estimated function. Antagonistic and synergistic responses were associated to specific bacterial taxa, suggesting that multiple-stressor scenarios could lead to unexpected shifts in the community composition and function of bacterial communities in river biofilms.

04

General discussion

General discussion

This thesis investigated the effects of single and multiple stressors on river biofilms using artificial systems, i.e. micro- and mesocosms. The single and multiple-stressor effects on river biofilms were assessed through usual response parameters (e.g. enzymatic activity, net metabolism, photosynthetic efficiency...) as well as molecular tools targeting gene expression (Paper I), gene abundance (Paper II, III) and bacterial community composition (Paper II, IV). Regarding single-stressor effects, the results presented in this thesis highlight the importance of hydrological stress as a decisive stressor for river and stream ecosystems compared to warming or pollutant exposure. Also, our results highlight the relative importance of significant interactions within a multiple-stressor context. In view of our results, significant interactions represented between 14.5 % (Paper I) and 29 % (Paper III) of all biofilm responses. Among significant interactions, antagonisms dominated in all cases, representing up to 89 % (Paper IV) of all biofilm responses.

In this general discussion, the main conclusions of the constituent papers are summarized and combined to present general agreements and discuss discrepancies.

The use of artificial systems to assess the effects of multiple stressors on river biofilms

The response of complex microbial communities such as biofilms to multiple stressors is not fully understandable from in situ monitoring of rivers and streams, as natural settings usually encompass a variety of uncontrolled factors derived from natural heterogeneity that might impact response variables, leading to confounding results and misinterpretations (Romaní *et al.*, 2004; Singer *et al.*, 2006). Artificial systems (e.g. micro- and mesocosms) aim at reducing experimental variability and suppose a more controlled environment with less variability compared to field conditions (Figure D.1), providing a good opportunity to establish cause-effect relationships (Sabater and Borrego, 2015). Generally, smaller artificial systems, such as microcosms (Paper I), allow for increased number of experimental units and replicates, as well as treatment conditions, thus increasing the statistical strength of the observations derived from manipulative experiments (Brennan and Collins, 2015). However, microcosms replace a myriad of environmental conditions and biotic interactions by a few elements, usually only a laboratory-grown culture and the water, thus jeopardizing the ecological realism of the approach and making direct extrapolations of the results complicated (Fraser and Keddy, 1997). On the other hand, larger systems such as experimental streams (i.e. mesocosms, Paper II, III and IV) are better suited to reproduce complex biological communities than

microcosms, usually at the expense of reducing replication and control over confounding variables (Battin *et al.*, 2003; Petersen and Englund, 2005).

Moving from single-species assessment to community analysis is crucial to understand the impact of occurring stressors on freshwater ecosystems, as it provides experimental approaches with high degrees of ecological realism (Besemer *et al.*, 2012). Moreover, single species have a limited range of tolerance to climatic stressors such as warming, which hinders our capacity to predict interaction effects between climatic stressors and pollutants. Community-level approaches, in turn, are better suited for investigating multiple-stressor interactions (Clements and Rohr, 2009; Ponsatí *et al.*, 2016). If experimental mesocosms are placed outdoors, fed with a source of colonists (e.g. by diverting river water into the system) and subjected to climatic variations, the complexity of the ecological processes taking place within them provides experimentation with high degrees of realism (Figure D.1), although the control over confounding variables decreases (Ledger *et al.*, 2011). In this thesis, I have addressed the lack of a source of colonists by employing non-sterilized natural river sediment (Paper II, III and IV) already containing the biofilm communities from the source site. Overall, the use of artificial systems in ecological research can be conceived as a multiple-scale approach directed to achieve the best trade-off between ecological realism and control over the confounding variables. Finally, observations from artificial systems, including micro- and mesocosms, need validation with analogous field studies, to assess the relative importance of confounding factors and put the results obtained through manipulative experiments into an environmentally-realistic context.

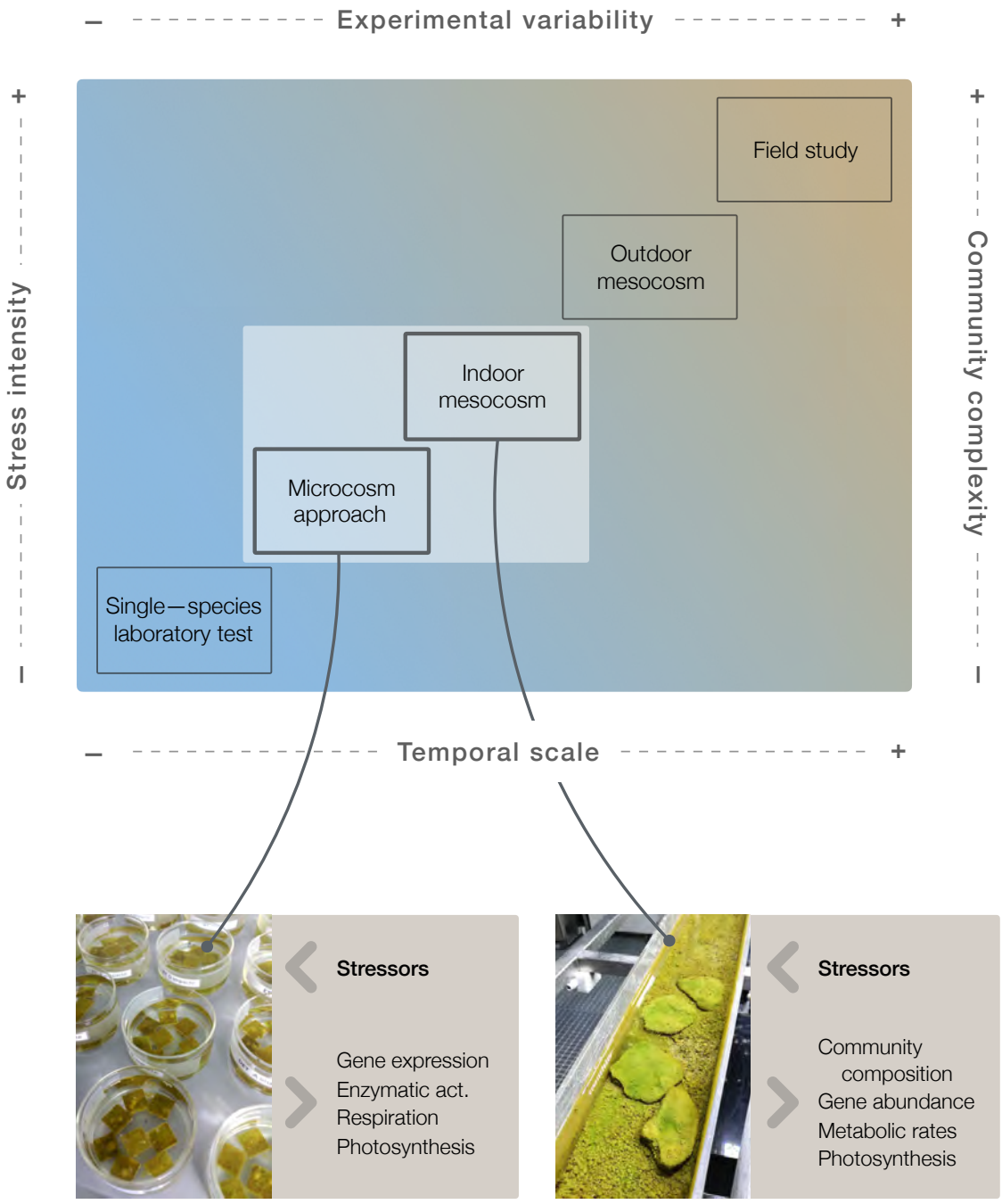


Figure D. 1 Experimental approaches to the study of stressors on freshwater ecosystems. Pictures (bottom) show a detail of the micro- (left) and mesocosms (right) used in this thesis, together with response variables employed.

Single-stressor responses: consistencies and discrepancies

In this section, I compare stressor effects across the four papers (i.e. three experiments) presented in this thesis (Table D.1). Regarding experiment 2, I also include 3 additional works which derive from the same experiment (Acuña *et al.*, 2019; Pereda *et al.*, 2019; Sabater-Liesa *et al.*, 2019) and which I use to disentangle how the results obtained at the community composition level (Paper II) translate into shifts in ecosystem processes (i.e. nutrient dynamics, metabolic rates, enzymatic activities, and photosynthesis).

As shown in Table D.1, single pollutants mainly had effects in line with their mode of action. Accordingly, the antibiotic erythromycin reduced the expression of the bacterial gene marker 16S rRNA (Paper I). This is in line with the described mode of action of erythromycin, which binds and blocks the bacterial ribosome, inhibiting protein synthesis (Prescott *et al.*, 2000). Conversely, erythromycin exposure increased the community respiration, suggesting that erythromycin-tolerant individuals might have promoted their respiratory activity after sensitive individuals have declined. On the other hand, the herbicide diuron slightly decreased the expression of the photosynthesis-related gene *psbA*. Conversely, Kim Tiam *et al.*, 2012 found that a suspension of the biofilm-forming diatom *Eolimna minima* up-regulated the expression of *psbA* upon exposure to cadmium. Although the pollutant and exposure times differed, this suggests that single-species approaches might not be representatives of the community-level response when dealing with toxicant effects on gene expression. In this thesis, I also show that a slight decrease in *psbA* gene expression upon exposure to diuron translated into a significant decrease in photosynthetic efficiency (Y_{eff}). This finding is in line with the mode of action of diuron, which blocks the chloroplast electron transport chain at the photosystem II (PSII) level, leading to the inhibition of photosynthesis (Moreland, 1980). In line with this, exposure to a pesticide mixture decreased photosynthetic efficiency (Paper III), but increased the relative abundance of Cyanobacteria (Paper IV). In view of the results presented here, I suggest that the capacity of some Cyanobacteria to degrade pesticides might suppose a competitive advantage upon their niche competitors (e.g. green algae) in polluted environments (Aislabie and Lloyd-Jones, 1995), helping to keep key ecosystem processes by maintaining functions such as photosynthetic efficiency and primary production.

Apart from individual pollutants (i.e. erythromycin, diuron [Paper I]) and simple artificial mixtures (pesticide mixture [Paper III, IV]), this thesis also investigated the effects of a complex chemical stressor, i.e. WWTP effluent (Paper II) on river biofilms. Wastewater effluents include a huge variety of organic and inorganic molecules, including pollutants (e.g. antibiotics, pesticides), organic matter in the form of dissolved organic carbon (DOC) and inorganic nutrients (NH_4^+, PO_4^-). In line with this, the response of river biofilms to WWTP effluents (Paper II) was by far more complex than in the other experiments presented in this

thesis (Table D.1). The rationale behind this complex response is probably related to the complex mixture of chemicals that were present in the WWTP effluent, including pharmaceuticals and heavy metals (Sabater-Liesa 2019, STOTEN). I here show that WWTP effluent proportions > 50 % of the total flow lead to significant changes in bacterial community composition of exposed sediments. In the same experiment, (Sabater-Liesa *et al.*, 2019) showed algal community changes in exposed sediments, with increased abundances of green algae (i.e. Chlorophyta) at WWTP effluent proportions between 30 - 70 % of the total flow. Concurrently, Acuña *et al.*, 2019 and Pereda *et al.*, 2019 observed that effluent proportions > 50 % seriously impaired NH_4^+ and PO_4^- uptake capacity. Despite the negative effects of pollutant exposure on photosynthetic activity observed in Paper I and Paper III, a positive effect of WWTP effluent on photosynthetic efficiency was observed (Sabater-Liesa *et al.*, 2019) as well as increased respiration and primary production rates (Pereda *et al.*, 2019). I here strengthen the need for linking molecular initiating events, such as the ones presented in Paper I, to community responses and ecosystem processes, as it is the necessary path to close the loop between pollutant exposure and ecosystem function shifts (Besemer *et al.*, 2012; Zeglin, 2015). In view of my results, WWTP effluent proportions > 50 % might compromise river ecosystem stability via changes in community composition that escalate until reaching key ecosystem processes such as those related to nutrient dynamics and metabolic rates.

Stressor theory predicts that less frequent stressors (e.g. climatic stressors) are the ones associated to higher stress intensity, and therefore cause the strongest and most pervasive effects on biological communities (Sabater, 2017) (Figure D.2). In line with what I expected, hydrological stress was the strongest and most pervasive stressor across all the experiments presented in this thesis. Hydrological stress significantly decreased the expression of the photosynthetic gene *psbA* (Paper I), which could be due to the fact that stressed microbial communities tend to reduce the expression of growth-related genes in favour of stress-response genes, such as the ones related to DNA repair (Schimel *et al.*, 2007). This translated into reduced photosynthetic efficiency (Paper I, Paper III) and primary production (Paper III). At the community level, hydrological stress favoured the *Alphaproteobacteria* (Paper II, and IV), while decreased other bacterial groups, such as the *Planctomycetes*. While members of *Alphaproteobacteria* are known to degrade a wide range of organic compounds (Rosenberg, 2013), members of *Planctomycetes* perform key ecological functions, i.e. NH_4^+ oxidation (Hu *et al.*, 2011). Warming conditions also decreased *psbA* expression, and this translated into decreased photosynthetic efficiencies (Table D.1), although the effect was low compared to hydrological stress. Conversely, warming stimulated bacterial enzymatic activity in river biofilms (Paper I) and the abundance of 16S rRNA copies (Paper III). At the community level, heterotrophic bacteria such as members of *Alphaproteobacteria* and *Firmicutes* were favoured under warming conditions (Paper IV). Taking it from gene expression to community

composition and ecosystem processes, this suggests that streams and rivers subjected to climatic stressors such as those derived from hydrological stress and warming might shift towards heterotrophic metabolism, as it has been shown under field conditions (Acuña *et al.*, 2015).

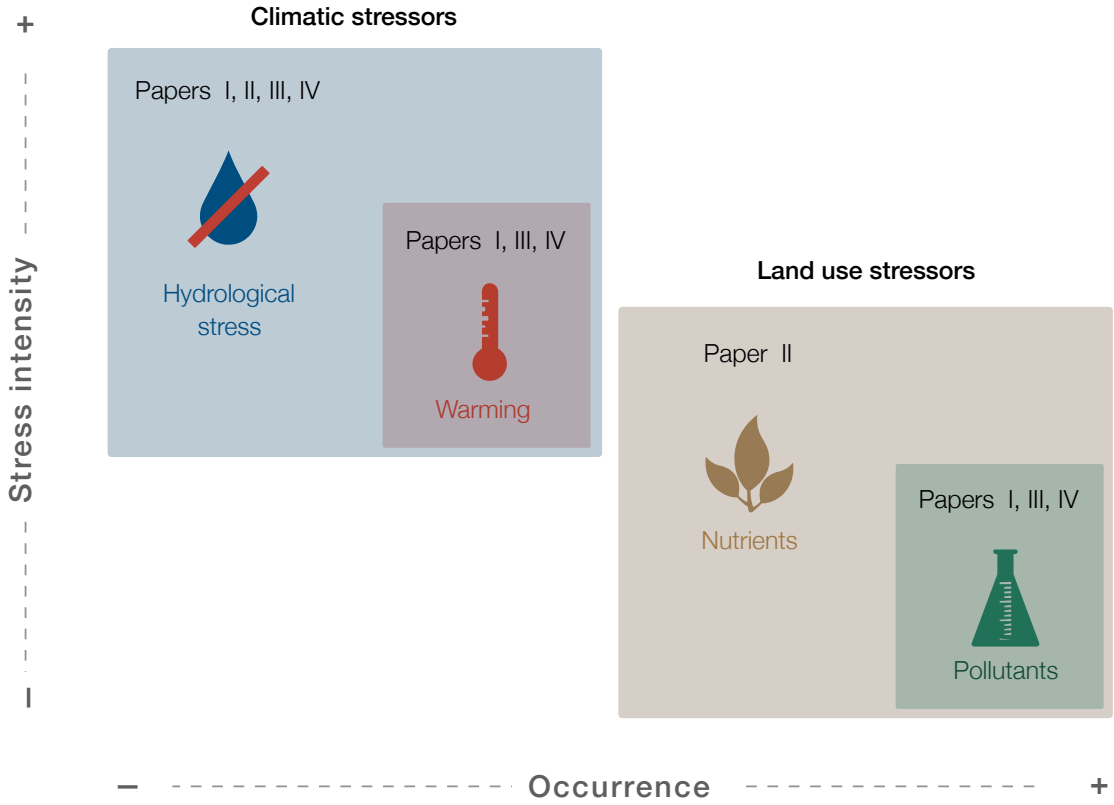


Figure D. 2 Stressor types used in this thesis, together with stress intensity and relative occurrence in freshwater environment. Adapted from Sabater, 2017.

	Experiment 1		Experiment 2			Experiment 3	
	Paper I (this thesis)	**Paper II (this thesis)**	**Acuña et al., 2019**	**Pereda et al., 2019**	**Sabater-Liesa et al., 2019**	**Paper III (this thesis)**	**Paper IV (this thesis)**
POLLUTANT	↑ Respiration ↓ Photosynthesis	Community shift at > 50 % WWTP effluent	↓ NH_4^+ balance ↓ PO_4^- balance	↑ Respiration ↑ Primary production	↓ Enzymatic activity ↑ Photosynthesis	↓ Photosynthesis	↑ Cyanobacteria
HYDROLOGICAL STRESS	↓ Enzymatic activity ↓ Photosynthesis	↑ Alpha--proteobacteria	Not assessed	Not assessed	Not assessed	↓ Enzymatic activity ↓ Photosynthesis ↓ Primary production	↑ Alpha--proteobacteria ↓ Planctomycetes ↓ Bacteroidetes
WARMING	↑ Enzymatic activity ↓ Photosynthesis	Not assessed	Not assessed	Not assessed	Not assessed	↓ Photosynthesis	↑ Alpha--proteobacteria ↑ Firmicutes

Table D. 1 Stressor effects summarized across experiments in this thesis. Experiment 2 is presented together with three additional papers (i.e. Acuña et al., 2019; Pereda et al., 2019 and Sabater-Liesa et al., 2019) in order to discuss results from Paper II within an integrative perspective.

Multiple-stressor responses: consistencies and discrepancies

Biofilm responses to multiple stressors depend on the ability of its organisms to respond to each stressor and on the potential occurrence of positive or negative co-tolerance mechanisms (D. Vinebrooke *et al.*, 2004). An exposure to a stressor combined with positive co-tolerance should reduce the impact of a second stressor, leading to an antagonistic response, while a negative co-tolerance would have the adverse effect (synergism).

In line with our predictions, additive effects (i.e. no interaction whatsoever) dominated biofilm responses under multiple-stressor scenarios, and this trend was consistent across the three papers specifically addressing this question in this thesis (Paper I, III and IV). In Paper I, additive effects represented 86 % of all responses; 71 % in Paper III and 84 % in Paper IV. Previous research assessing multiple-stressor effects on different biotic freshwater components (e.g. fish, invertebrates) also points to the prevalence of additive effects (Jeremy J Piggott, Niyogi, *et al.*, 2015b; Elbrecht, Beermann, Goessler, Neumann, Tollrian, Rüdiger Wagner, *et al.*, 2016; Schinegger *et al.*, 2016a). In line with the results presented in this thesis, Piggott *et al*., 2015 assessed single and multiple-stressor effects of warming, nutrients and sediment addition on organic matter decomposition rates, and found that 82 % of all responses were additive. The prevalence of additive effects when biofilm communities are exposed to multiple-stressor scenarios could be linked to stressors differing in their modes of action (Breitburg *et al.*, 1998) Accordingly, if the microbial taxa within a biofilm affected by two independent stressors (X and Y) do not overlap (i.e. stressor X affects species A, whereas stressor Y affects species B), we could assume that the combined effect of X and Y will be additive (species A and B affected). This assumption is challenged in the context of the stressors used in this thesis, as I have used complex stressors such as warming and hydrological stress, which do not have a well-defined mode of action, as they encompass a myriad of physiological constraints including shifts in substrate diffusion, osmotic stress and DNA damage (Schimel *et al.*, 2007). I therefore argue that more multiple-stressor studies under controlled conditions are required to fully disentangle the interactive nature of complex stressors with less well-defined modes of action, such as warming and hydrological stress.

I have also observed that, among non-additive responses, antagonisms dominated. In Paper I, 75 % of all non-additive interactions were antagonistic; in Paper III, antagonisms represented 59 % of all interactions after 48 hours of exposure, and 86 % after 30 days. The prevalence of antagonistic effects is in line with previously published research (Larras *et al.*, 2013; Lawes *et al.*, 2017; Birrer *et al.*, 2018; Nuy *et al.*, 2018),

and it has been related to physiological and structural processes. Physiological processes imply molecular mechanisms activated as a response to one stressor conferring tolerance to a second stressor (D. Vinebrooke *et al.*, 2004). In this thesis, the dominant stressor (i.e. hydrological stress) probably activated generalized stress-tolerance mechanisms such as protection against reactive oxygen species, membrane disruption or DNA damage (Schimel *et al.*, 2007). These tolerance mechanisms might have also been implied in protection against cellular damage caused by other stressors, such as warming and pollutant exposure. Concurrently, Stampfli *et al.*, 2013 discussed that the strong individual effect of hydrological stress might prevent the appearance of significant synergisms. I cannot fully discard that the dominance of hydrological stress over the other stressors used in this thesis is masking the appearance of synergistic interactions. On the other hand, structural processes leading to antagonism include compositional adaptation of the community, especially at long term (Feckler *et al.*, 2018). Based on this, the selection pressure of the dominant stressor (here, hydrological stress), could have driven the community composition towards increased abundance of tolerant taxa, which, in turn, might be also more tolerant to secondary stressors, thus leading to an antagonistic overall response.

Regarding the interaction effects of warming, the results presented in Paper I and Paper III indicate that increased temperature partially mitigated the toxic effect of pollutants, especially pesticides affecting phototrophic organisms via reduced photosynthetic efficiencies. Unexpectedly, this antagonistic interaction was not accompanied by a shift in the expression of the photosynthesis-related gene *psbA*, which might be indicating regulation at the protein level. In Paper III, the interaction between the pesticide mixture and warming conditions followed the same trend only at long term. I conclude that the lower concentration of the pesticides used in Paper III (i.e. ng L^{-1}) was insufficient to produce reliable interactions at short term, especially in the epilithic biofilm, where biofilm subjected to pesticides showed only a 1.3 % reduction in Y_{eff} with respect to controls. Paper IV, in turn, indicates that this antagonistic interaction between warming and pesticides might be the result of increased abundances of photosynthetic bacteria under multiple-stressor situations. Accordingly, the artificial streams containing both warming and pesticides (Paper III and IV) showed the highest relative abundances of Cyanobacteria, including the genus *Leptolyngbya*. In line with this, Larras *et al.*, 2013 found that eukaryotic algae were also more sensitive to herbicides at lower temperatures, suggesting that the response of eukaryotic and prokaryotic oxygenic photosynthesizers to the combined action of warming and pesticide exposure might be similar.

Apart from showing the strongest main effect, the results obtained in this thesis highlight the importance of hydrological stress in modulating the response of river biofilms to additional stressors. It is crucial to understand how hydrological stress and other stressors (e.g. pollutants) interact, as they have been reported to co-occur in 10-25 % of rivers and transitional coastal waters worldwide (Nõges *et al.*, 2016). In Paper I, a 40-h exposure to the combined action of diuron and hydrological stress synergistically reduced photosynthetic capacity (Y_{max}). In line with this, the photosynthetic efficiency (Y_{eff}) values obtained at short term (i.e. 48 h) in the artificial streams submitted to pesticides and hydrological stress (Paper III) were 91.3 % lower than controls (epilithic biofilm). However, the combination between pesticides and hydrological stress was antagonistic at long term (Paper III). According to the results of Paper IV, there was a long-term increase in the relative abundance of *Cyanobacteria* in artificial streams submitted to both hydrological stress and pesticides, particularly in epilithic biofilms (~12 % of total bacterial abundance). This might be indicating that the enrichment of *Cyanobacteria* in multiple-stressor treatments could help maintain basal levels of photosynthetic activity and primary production under multiple-stressor scenarios. In line with this, the interaction between hydrological stress and WWTP effluent (Paper II) was only significant when substratum type was also considered, and led to increased numbers of desiccation-tolerant *Alphaproteobacteria* being favoured by the wastewater effluent in the epilithic biofilm. From these results, I argue that epilithic biofilms are more likely than epipsammic biofilms to be involved in significant interactions when considering multiple-stressor effects; and that substratum type should be taken into account when dealing with the effects of multiple stressors on benthic microbial organisms.

Future research directions

1. In the future, the results obtained in this thesis could be used to build strong, knowledge-based hypotheses for field-based experimental designs, in order to investigate to which extent our results are equivalent to those found under field conditions.

2. As presented in this thesis, future studies should include stressor-response assessment at the community level. This will enlarge the amount of data that could be used to develop new null mathematical models that open the path toward a predictive understanding of multiple-stressor responses. This theoretical framework could be inspired in the compositional null model

(Thompson *et al.*, 2018), which predicts stressor response at the individual level, and then aggregates the responses at the community level.

3. We have observed that multiple stressors severely impact river biofilms, which are the base of virtually all food webs in river ecosystems (Weitere *et al.*, 2018). However, it remains to be explored the single and multiple-stressor effects on food-web dynamics through, for example, bottom-up cascade effects that might be studied using stable isotopes.

4. Highly controlled laboratory studies could be useful to elucidate the mechanisms by which the studied stressors interact, particularly as the mechanisms behind the effects of hydrological stress and warming on the different components of river biofilms are not well understood.

5. Advanced molecular approaches, including *omic* approaches, could help elucidate the mechanisms by which multiple stressors interact. These approaches could span from the study of the whole set of genes present under single and multiple-stressor scenarios (i.e. *metagenomics*), to the expression of these genes (i.e. *metatranscriptomics*) and the resulting metabolic profile (i.e. *metabolomics*).

05

Conclusions

Conclusions

1. Hydrological stress applied both as a reduction in water flow or as complete sediment desiccation was the stressor showing the largest impact on river biofilm community composition and functioning.

2. The impacts of hydrological stress were particularly strong on epilithic biofilms, which showed a sharp decrease in photosynthetic rates and enzymatic activity, together with deep bacterial community shifts.

3. Climatic stressors, and especially hydrological stress, drove the bacterial community composition towards increased relative abundances of *Alphaproteobacteria* and *Firmicutes*, whereas other bacterial groups declined.

4. Individual pollutants (erythromycin, diuron) and an artificial pesticide mixture showed effects following their mode of action.

5. River biofilms exposed to a WWTP effluent dilution showed a more complex response than when exposed to individual pollutants, with bacterial community composition shifts noticeable at > 50 % of wastewater effluent proportion to the total flow.

6. Multiple-stressor scenarios affected river biofilm community composition and function following additive effects in most of the cases (≥ 70 %), indicating a prevalence of additive effects over significant interactions.

7. Antagonisms represented up to the 89 % of all non-additive responses, dominating interaction effects, and being especially prevalent at long-term exposure times.

8. Epilithic and epipsammic biofilms showed contrasting responses to multiple-stressor scenarios, which was derived from the strong impact of hydrological stress on epilithic biofilms.

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