Effects of urban pollution on stream ecosystem functioning

Olatz Pereda Iriondo

PhD Thesis

Supervised by Arturo Elosegi and Sergi Sabater

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Gezurra dirudien arren, jadanik, lau urte igaro dira tesia egiteko diru-laguntza lortu nuenetik. Emozio askok betetako lau urte izan dira, gogo eta kemen handiz lan egitera behartu nautenak, haserrealdi eta amorrualdi asko ere eragin dizkidatenak, baina batez ere, guzti horren gainetik, erabat disfrutatu ditudan lau urte eder eta oparoak izan dira. Nekez ahaztuko ditut lan hau gauzatzen bizi izandako momentuak eta, tarte honetan nire ondoan izan ditudan pertsona guztiak. Askori zor baitizuet lan hau hasi, eta, batez ere, bukatu izana! Zuetako bakoitzari, bihotz-bihotzez, MILESKER!

Lehenik eta behin, zuri eman nahi dizkizut eskerrak Arturo! Asko gustatu zait zurekin lan egitea eta uste dut tesian zehar profesionalki hazteko aukera mugagabeak ez ezik, pertsonalki hazteko aukera ere eman izan didazula. Beraz, bihotzez milesker zurekin tesia egiteko aukera ekaini izanagatik eta hortik aurrera, momentu guztietan nitaz fidatu, nigan sinetsi eta nigatik borrokatu izanagatik! Y la verdad, que ni en sueños habría imaginado que alguien como Sergi Sabater acompañaría a Arturo para respaldar mi trabajo. Aunque muchas veces haya sido desde la distancia, me siento muy afortunada de haberte tenido como codirector. Pero sobre todo, muchas gracias por haberte preocupado tanto por mí y por haberme transmitido el lado más humano que puede tener la ciencia. He aprendido que la calidad es tan importante como la cantidad, y que este trabajo no hubiera sido posible sin ese 5% que complementa el trabajo en su totalidad. Bioi, bihotzez, mila esker!

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litzateke berdina izango, *oilategi* berezi hori osatu izan duzuen eta osatzen duzuen zuetako bakoitzarengatik izango ez balitz. Eskerrik asko ni sartu nintzenean, eredu izan zintuztedan guztiei: Lorea, Ibon, Aitor, Aingeru, Silvia, Javi, Jon. Aipamen berezia egin nahiko nizuke Maite, gure eredu izateagatik, Libe eta biok hazi eta hezteagatik, beti laguntzeko prest egoteagatik eta lehenengo momentutik, hori guztia zure irribarre goxo horrekin eskaintzeagatik. Aupa Gaztek! Eskerrik asko gaur egun ere laborategian agintzen geratzen zareten neska-talde sendo horietako bakoitzari: Mireni, Ioarri, Vicki eta Naiarari. Makina batzuk zarete eta ziur nago, arrakastaz beteriko tesiak izango dituzuela zuek ere. Azkenik, guztien artean zuri eskertu nahiko nizuke hau guztia, Libe. Askotan pentsatu izan dut zeinen diferentea izango litzatekeen etapa hau zuregatik izan ez balitz! Nahiz eta ez diguten utzi batera lan egiten nahiko genukeen bezain beste, zu izan zara momentu txarretan eduki dudan euskarririk sendoena, baina baita momentu onetan barrez leherrarazi, disfrutarazi eta gozarazi egin didan barne-indar garrantzitsuena. Eskerrik asko lau urte hauetan zehar emandako guztiagatik Libe; asko botako zaitut faltan hemendik aurrera.

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laguntzeko prest, zure animo, irribarre eta hitz goxoenekin. Ez dakit non egongo den gutako bakoitza etorkizun urrunago batean, baina ziur nago tesiarekin batera hasi genuen adiskidetasun honek luze iraungo duela! Eskerrik asko, bene-benetan, nirekin zaren modukoa izategatik.

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Izas zuk erakutsi didazu benetako adiskidetasuna, zintzoena, edozein distantziaren gainetik manten daitekeela. Biologiako ikasketak egiten hasi ginenetik denbora asko igaro da, baina momentu horretatik eduki dudan babes sendoenetariko bat izan zara niretzat. Nahiz eta karrera amaitu eta gure egunerokotasuna urrundu izan, eskerrik asko etxe azpiko kantoian edukitako solasaldi amaigabeengatik, nire urduritasunak eta zalantzak entzuteagatik eta haiekiko interesa edukitzeagatik, gauzak erlatibizatzen irakasteagatik eta beti nigan sinetsi izanagatik. Zuk ulertu izan bainauzu askotan beste inork baino hobeto.

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sinesteagatik, denarekin ahal dudala pentsatzeagatik eta zure indar eta animo guztiak ematearren behar izan dudan momentu bakoitzean. Baina bereziki, eskerrik asko bizitza lan egiteaz ez ezik, beste gauza asko ere badela gogoratzearren eta, noiz-behinka, kalera ateratzera ere derrigortzearren. Oksi, eskerrik asko bost urte zenituenetik uda bakoitzean gu guztiok pertsona hobeak izaten irakastearren. Urrun zaitugun arren, etxean sentitzen zaitudalako. Eskerrik asko familia, bihotz-bihotzez! Ezin nitzake ahaztu, ordea, prozesu honetan amaierara heltzen lagundu izan didaten Mikel, Itzi, Tia Izaskun, Tio Iñaki eta amamak. Eskerrik asko beti eman didazuen babesagatik.

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CONTENTS

Summary

During the last century pollution generated in urban areas has become a prevalent impact in most streams and rivers worldwide. Although many waste water treatment plants (WWTP) have been implemented to reduce pollution, they do not eliminate it completely and their effluents still contribute complex mixtures of pollutants. The ecological effects of these effluents depend on their chemical composition and the final concentration in the receiving water body, which varies with the water flow of the receiving river or stream. While the effects of urban effluents on stream water quality are relatively well studied, we still lack a clear picture about their effects on ecosystem processes. This dissertation analysed the effects of urban pollution on stream ecosystem functioning, by combining observational and manipulative experiments.

The effects of pollution can be exacerbated when stressors operate in concert. Among these, hydrological alterations are of special concern, either natural or human-induced, as low flows reduce the dilution capacity of the streams. Thus, I first assessed the joint effects of urban pollution and water stress on stream functioning. I studied several ecosystem processes in 13 Mediterranean streams of contrasting dilution capacity, and defined in each stream a control site and an impact site, upstream and downstream from the sewage inputs, respectively. Urban effluents caused complex effects on ecosystem functioning, but most ecosystem rates increased in proportion to pollutant subsidies of labile organic matter, nutrients and pharmaceutically active compounds. The main driver of the variability observed in ecosystem functioning, were variations in water chemistry, especially in the concentration of pharmaceutical drugs. These results show the effects of pollution to depend on the effluent nature and its dilution in the receiving stream, and that even highly concentrated urban effluents tend to subsidize biological activity.

The effects of WWTP effluents are difficult to measure in the field by comparing reaches upstream and downstream from the effluent input, as the upstream sites are usually affected by other sources of pollution. This situation calls for a manipulative experiment, which I performed following a Before-After/Control-Impact design. Part of the effluent of a large tertiary urban WWTP was diverted into a small nearly unpolluted stream and the effects were assessed during a whole year. Despite of being highly diluted, the effluent subsidized most of the measured processes, only a few remained unaffected, and biofilm nutrient uptake capacity was the only being reduced. These results show that even highly diluted effluents can exert significant effects on stream ecosystem functioning.

Theoretically, we expected WWTP effluents to cause a subsidy-stress response on ecosystem functioning depending on their final dilution, but the two studies so far discussed showed no such a pattern. Therefore, we performed a laboratory experiment in which a series of artificial streams were subjected to a gradient of WWTP effluent concentration, ranging from pure stream water to pure effluent. Biofilm biomass accrual was the only process following the subsidy-stress pattern, whereas WWTP effluent subsidized most processes, although they showed complex responses to their

concentration. Biofilm nutrient uptake capacity was again the only process stressed by the effluent. Most of the processes showed clear legacy effects, although they followed contrasting patterns. These results can help to improve the understanding of the effects of urban pollution on ecosystem functioning, but highlight the complexity of the response.

Overall, this dissertation showed that urban effluents exert significant but complex effects on stream ecosystem functioning, which can be especially detrimental for the processes mediated by microbial communities. Therefore, these results highlight that it is crucial to maintain environmental water flows and adjust to the thresholds proposed by current pollutant legislations, not to reduce the dilution capacity of streams and rivers. Further investigation is needed to determine the overall ecosystem effects of these mixtures of pollutants, in order to optimize treatment processes and reduce their ecological impacts.

CHAPTER 1

General introduction

1. Urban pollution: a global environmental problem

1.1. Something new under the sun

Humans have been altering their physical environment throughout their 4 million years of history, although as McNeill (2000) reported, "*there has never been anything like the 20th century*". During this last century, the unprecedented growth in the human population has been accompanied by increases in resource extraction and economic development (Fig. 1; Krausmann *et al.*, 2009). Furthermore, environmental change has kept rising during the first two decades of the 21st century (Ripple *et al.*, 2017).

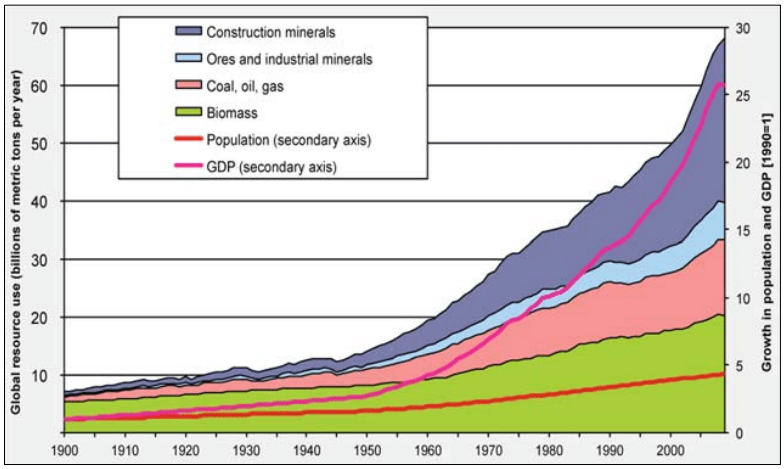

Figure 1. Exponential increase of human population, use of resources and economical development during the 20th century. From Krausmann *et al.*, 2009.

These changes, however, are not evenly distributed across the globe and usually, they are intensified associated to urban areas. Although cities only cover about 3% of the global terrestrial surface, they are responsible for more than 75% of the world consumption of resources (Madlener & Sunak, 2011). Urbanization is one of the main forces shaping this last century, prompting a dramatic shift from rural areas to cities. By the end of the last century, more than 50% of the global population were already urban dwellers and this percentage is still increasing (Jones & O´Neill, 2016). Human activity is leaving a pervasive and persistent signature on Earth, to the point that some of the pre-defined boundaries for the resilience and sustainability of our planet have already been trespassed (Steffen *et al.*, 2015). The vast extents of these transformations have encouraged scientists to propose that a new geological epoch has begun, coined by the name of Anthropocene (Waters *et al.*, 2016), in which humans are the main geophysical force shaping the planet (Foley *et al.*, 2005). The Anthropocene is characterized by changes in land use and cover, altered biogeochemical cycles, altered climate and air quality and by reduced biodiversity (Dudgeon *et al.*, 2006). Therefore, it is irrefutable that human activities are drastically changing the environment and affecting every ecosystem on Earth (UNE, 2019).

1.2. Streams and rivers: walking on thin ice

Streams and rivers are among the most threatened ecosystems on Earth, (Vörösmarty *et al.*, 2010; Dudgeon, 2013). Running waters are dynamic ecosystems, which host a large biodiversity not only limited to the wetted channel. They occupy the lowest-lying areas of the landscape and thus, they can be influenced by the upstream drainage network, surrounding land, riparian and hyporheic zones and downstream reaches (Fig. 2) (Sabater *et al.*, 2009).

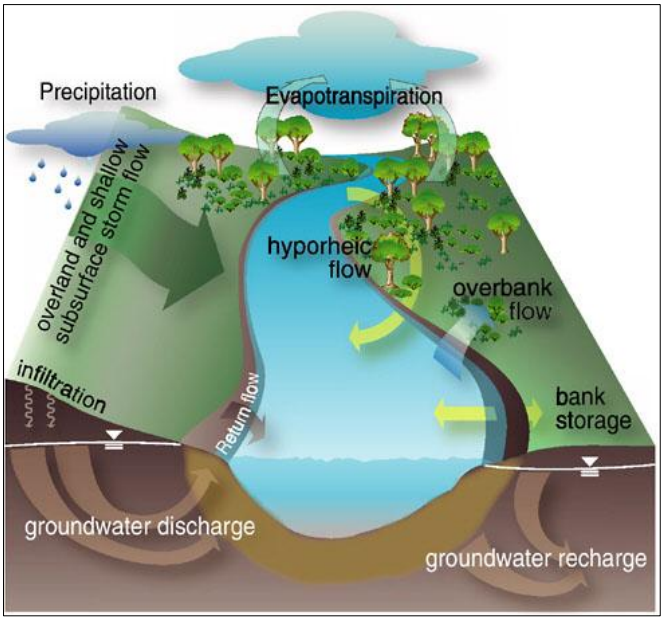

Figure 2. A three-dimensional structure of a riverbed, which takes into account the longitudinal, lateral and vertical dimensions. The figure is of public domain.

Historically, cities have grown along river watercourses, floodplains and deltas due to their water availability (Grimm *et al.*, 2008). Water is one of the most essential natural resources for humans, and necessary in many domestic, industrial or sanitation processes. But humans have radically adapted freshwater ecosystems to their needs and have threatened most streams and rivers worldwide, impairing ecosystem sustainability, energy reserves, water security and ecosystem health (Grant *et al.*, 2012), to the point that few of these systems are unaltered right now. Vörösmarty and collaborators (2010) showed that nearly 65% of the freshwater ecosystems are already from moderately to highly threatened, and that water security is at stake for more than 80% of the worldwide population. The main threats for streams and rivers encompass among others over-exploitation of water resources (Fig. 3a), water pollution and destruction or degradation of the drainage area (Fig. 3b), flow modification (Fig. 3c), stream channel simplification or channelization (Fig. 3d), invasion of non-native species (Fig. 3e) and climate change (Fig. 3f).

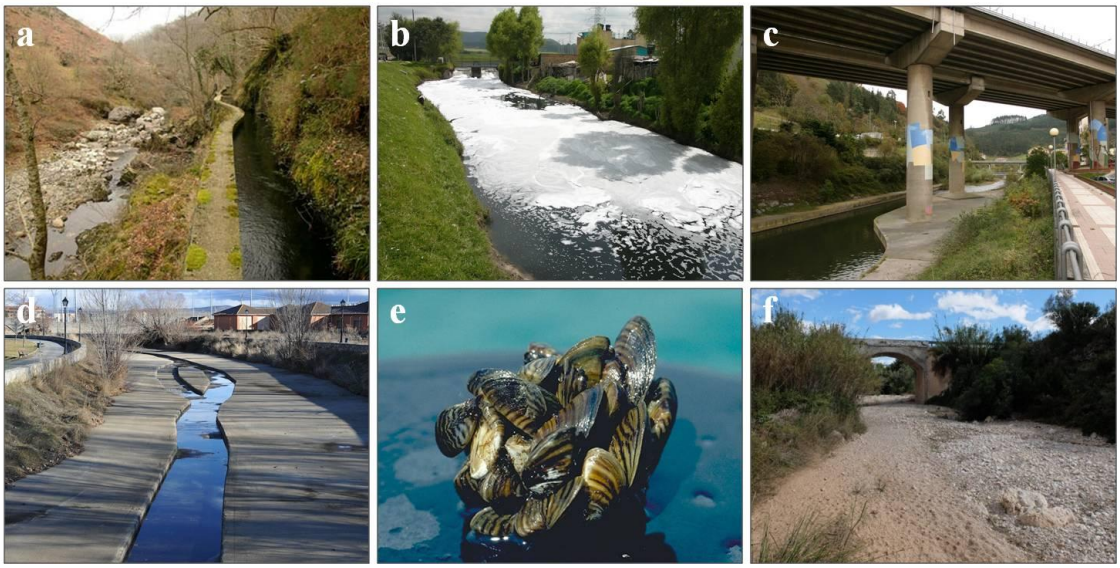

Figure 3. Photographs of different urban pressures threatening freshwater ecosystems: (a) irrigation canals for water exploitation, (b) point-source pollution and degradation of the riparian area, (c) diversion schemes for water abstraction, (d) simplification of the stream channel, (e) invasion of alien species and (f) water scarcity. All photographs are provided by Arturo Elosegi.

1.3. Forces acting at the same time: the role of multiple stressors

Stressors do not act as single factors in river ecosystems but co-occur and trigger complex ecological responses (Sabater *et al.*, 2018). Multiple stressors do affect riverine biological communities (Dudgeon, 2010; Sabater *et al.*, 2016) and river ecosystem functioning (Johnston *et al.*, 2015), which can ultimately affect goods and services the society derives from them (MEA, 2005). Sometimes, however, stressor interactions can produce ecological surprises, which can be driven by direct and indirect stressor interactions (Romero *et al.*, 2018), the high variability among biological responses (Berthelsen *et al.*, 2018) and by the different resistance and resilience of ecosystems (Jackson *et al.*, 2016). Overall, the number of stressors affecting river ecosystems and their geographical extent has increased dramatically during recent decades. For instance, the European Environment Agency (EEA, 2012) showed that the main pressures affecting European water bodies were pollution (both point and diffuse sources) and hydromorphological alterations. More recent reports also recognized nutrient pollution to be one of the most prevalent drivers shaping European streams and rivers (EC, 2015). Nevertheless, these stressors are usually examined in isolation (O´Brien *et al.*, 2019), which yields a simplistic view of the real situation (Dafforn *et al.*, 2016). In order to face the constraints imposed by these single-stressor approaches, several projects and studies have been developed. For instance, Birk and collaborators (2018), in an exhaustive review of multistressor-impact across Europe, showed the interaction between nutrient stress and hydrological alteration to be the most common, affecting nearly 60% of the European rivers (Fig. 4).

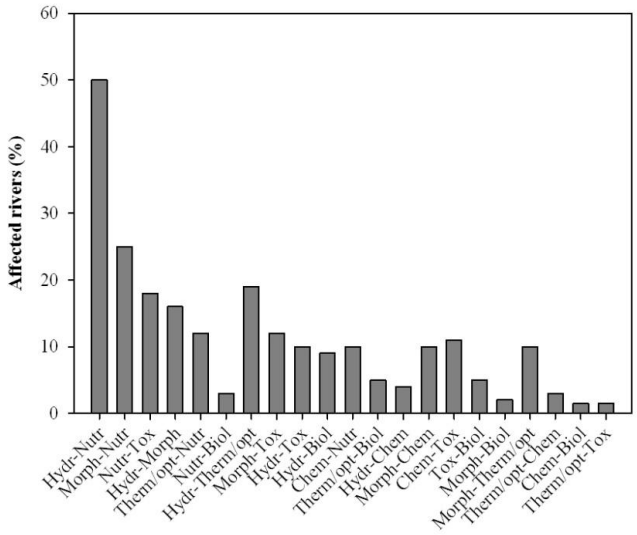

Figure 4. Frequency of multiple stressors occurring in European streams and rivers (Y axis). In the X axis combinations among two different types of stressors are shown: "*Hydr*" = hydrological alterations, "*Nutr*" = pollution by excessive nutrient concentrations, "*Morph*" = morphological alterations of the stream channel, "*Tox*" = pollution by toxic substances, "*Therm/opt*" = stress from warm water temperature, "*Biol*"= invasion of alien species, and "*Chem*" = chemical pollution. Modified from Birk *et al.*, (2018).

1.4. Increasing worldwide pollution

During the last century huge amounts of biologically active compounds are being released into freshwater ecosystems (Petrovic *et al.*, 2013), undermining their biodiversity and functioning (Kaushal *et al.*, 2012; Rosi-Marshall *et al.*, 2015). Chemical pollution is a multiple stressor on its own, due to the ever-changing mixture of compounds reaching aquatic environments (*e.g.*, Jackson *et al.*, 2016), which include organic matter (Carey & Migliaccio, 2009), excessive nutrient concentrations, such as nitrogen and phosphorus (Meyer *et al.*, 2005), fine sediments (Miserendino *et al.*, 2008) and organic micropollutants, including pesticides, metals, pharmaceutically active compounds, personal care products or illicit drugs (Rosi-Marshall *et al.*, 2015; Sabater *et al.*, 2016; Aymerich *et al.*, 2016). Streams and rivers are net receivers of these substances and affect most streams and rivers across Europe. Many of these suffer from moderate to high concentrations of both nitrogen (Fig. 5a) and phosphorus (Fig. 5b) (Grizzetti *et al.*, 2017). The composition and concentration of these substances, however, depend on different factors. On the one hand, their chemical composition varies according to the prevailing land use, such as agricultural practices, industrial activities or human conurbations (Posthuma *et al.*, 2008), which are making up the mixture (Altenburger *et al.*, 2015). On the other hand, their final concentration varies according to the water flow of the receiving river or stream, as it will correspond to the dilution capacity of the receiving water body (Rice & Westerhoff, 2017). This situation is predicted to exacerbate in the near future corresponding to stream flow reductions due to climate change and increased water abstraction and increasing concentration of pollutants (Rice & Westerhoff, 2017), which will impair even more the ecological status of streams and rivers.

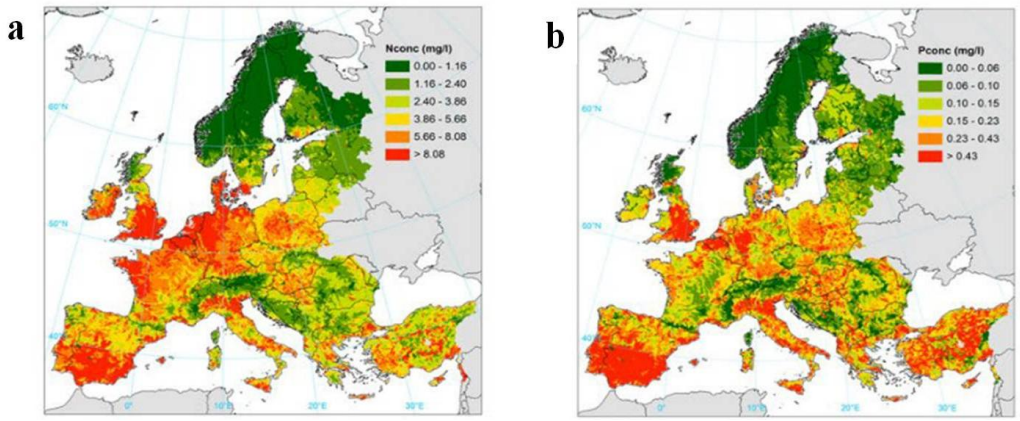

Figure 5. Concentrations of nitrogen (left) and phosphorus (right) across European streams and rivers. From Grizzetti *et al.*, (2017).

1.5. Facing the problems

During the last decades, the rising concern about the deterioration of freshwater ecosystems has resulted in new legislative frameworks, such as the Water Framework Directive (WFD) on the European Union (EU), the Clean Water Act (CWA) developed by the United States Environmental Protection Agency or the Canadian Protection Act (CPA). In the EU the WFD defined in the year 2000 is an ambitious policy to reduce anthropogenic pressures and achieve good ecological status for the EU member water bodies. Accordingly, all EU member states must assess the ecological status of freshwater ecosystems in their territory and establish programs of measures to reduce impacts and improve ecological status. As a consequence, management and legislative objectives shifted from the control of pollution sources to ecosystem integrity (Borja *et al.*, 2011; Birk *et al.*, 2012). Furthermore, additional legislations have also been developed to reduce the amount of pollutants entering freshwater systems, with the aim of protecting the environment from any adverse effect caused by sewage inputs. For instance, the European Commission Directive 91/271/EEC established in the year 1991 the Urban Waste Water Treatment Directive (EC, 1991), which encouraged the collection, treatment and discharge of urban and industrial wastewater. Since its establishment, this legislation has been continuously updating and broadening the number of substances and compounds required fulfilling ecological rates. Examples include the legislation outlined by the European Commission in the year 2008 (EC, 2008) by which ecological control rates were defined for a wide range of organic and inorganic pollutants, or the one outlined in the year 2012 (EC, 2012), which described pharmaceuticals as emerging contaminants, and thus as priority hazardous substances. As a result, with the aim of achieving the standards established by these laws, there has been a boom in the infrastructures to collect, store, treat and transport wastewater of anthropogenic origin, especially in wastewater treatment plants (WWTP). During the last decades, for example, more than 2,500 and 39,000 WWTPs have been put in operation in Spain (Serrano, 2007) and Europe (EUROSTAT, 2017), respectively.

Wastewater treatment plants (WWTP) are the most important technological advances to treat the sewage generated in urban areas. During the 20th century, they were introduced on a large scale to avoid public health risks derived from the high concentration of pollutants in residual water, so that countries that could afford their costs greatly benefited human health and water security (Vörösmarty *et al.*, 2010). In year 2015, 7 out of 10 people in the world had access to safe drinking water, completely free of contamination, and 4 out of 10 people had safe sanitation services for an appropriate wastewater treatment (WHO & UNICEF, 2017). However, there are huge differences among countries in respect to drinking water and sanitation. In Europe, for example, the proportion of population connected to at least secondary WWTPs exceeds the 80%, and those connected to tertiary treatment ranges from 20 to 80%, depending on the EU member (EUROSTAT, 2017). The situation is much worse in developing countries, where around 850 million people still lack access to safe drinking water (Fig. 6a), 2.3 billions lack the access to basic sanitation services (Fig. 6b) and many millions die each year due to waterborne diseases (WHO & UNICEF, 2017). Existing pollution legislation in developing countries is generally weak and not adequately implemented due to its high economic cost (Beyene *et al.*, 2009). Consequently, more than 160 million people collect drinking water directly from surface water sources in Sub-Saharan Africa and Oceania, and more than 900 million still practice open defecation in Sub-Saharan Africa, Oceania and Central-Southern Asia (WHO & UNICEF, 2017).

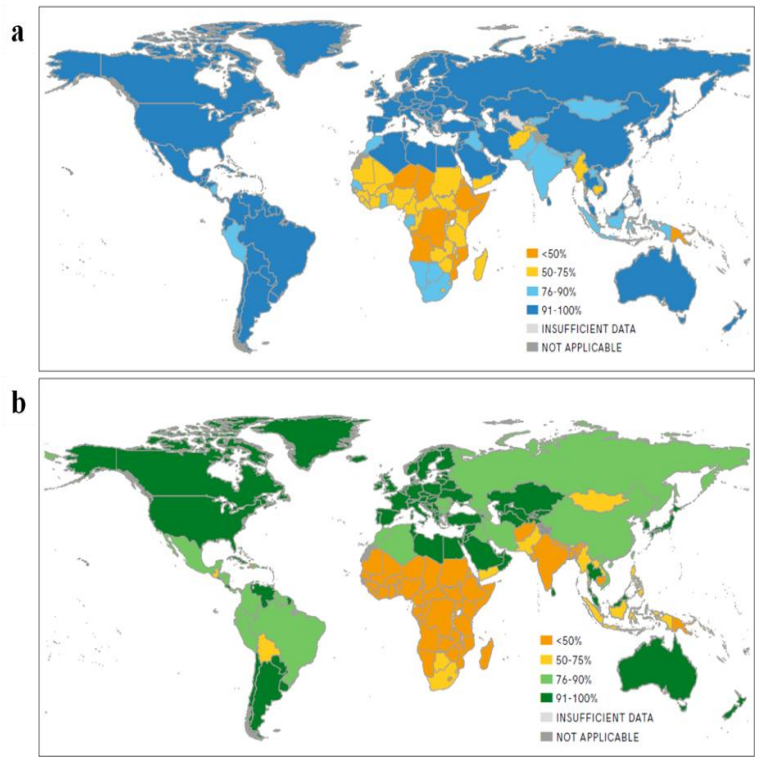

Figure 6. Proportion of the global population using at least basic drinking water services (top) and basic sanitation services in 2015 (bottom). Modified from WHO & UNICEF (2017).

Over the course of time, a significant progress has been made in improving wastewater treatment processes, and current sewage treatment systems can include primary, secondary and

tertiary stages (Fig. 7). Primary treatment consists of a set of physical processes to remove solid materials and floating substances such as oils. After separation of the heaviest and lightest solids, they are removed and the remaining liquid is subjected to secondary treatment. During the second stage, dissolved or suspended nutrients and organic compounds are digested through oxidation processes, reducing biochemical oxygen demand and toxicity of the wastewater. The slurry so produced is separated to remove microorganisms, organic matter and toxic substances from the treated water. Finally, the slurry is subjected to tertiary treatment, which involves a series of additional steps after secondary treatment to a further reduction of recalcitrant organic compounds, nutrients, turbidity, metals and pathogens (Gerba & Pepper, 2015).In general, tertiary treatment is applied by micro-filtration or synthetic membranes which involve physicochemical treatments, including coagulation, filtration, activated carbon adsorption of organic compounds, reverse osmosis, aerobic granulation, ozonation or photocatalytic degradation, among others (Gerba & Pepper, 2015; Ameta, 2018). Thanks to this final stage effluent water quality is greatly improved before being released into the environment.

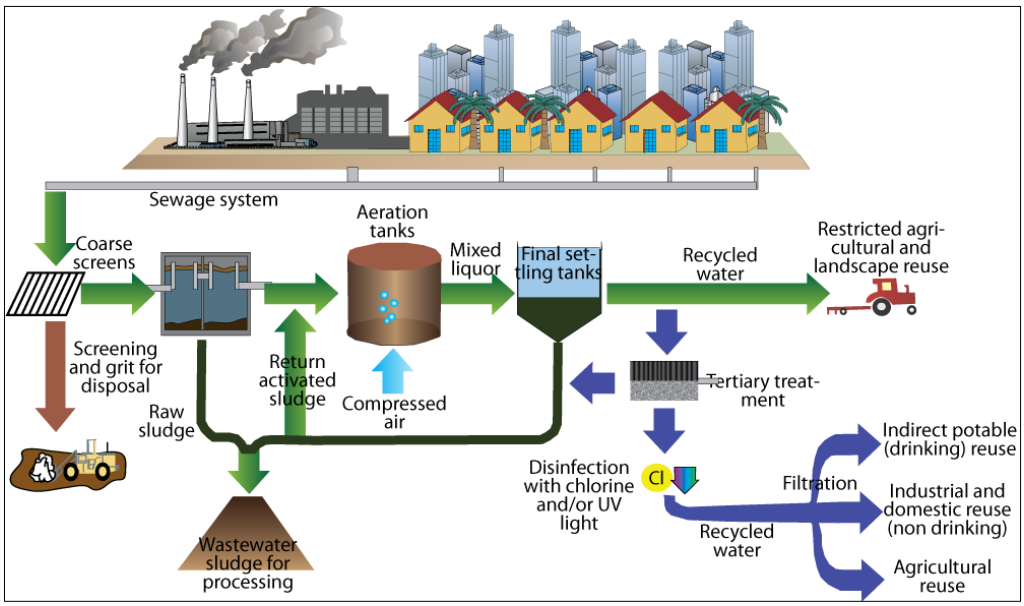

Figure 7. Schematic picture of a complex WWTP operation system which includes primary, secondary and tertiary treatments. The figure shown is of public domain.

2. Urban pollution and river ecosystems

2.1. Not all that glitters is gold

WWTPs are a necessary step to improve wastewater quality before it is discharged to surface or groundwater and re-enter water supplies (Carey & Migliaccio, 2009), and to reduce the symptoms associated to high pollution levels, such as anoxia or eutrophication (Brack *et al.*, 2007). For instance, Arroita *et al.* (2018) showed that stream water concentration of ammonium significantly

reduced from 1.02 ± 0.6 to 0.15 ± 0.1 mg N L^{-1} after putting in operation a WWTP in the Oria River, Spain (Fig. 8).

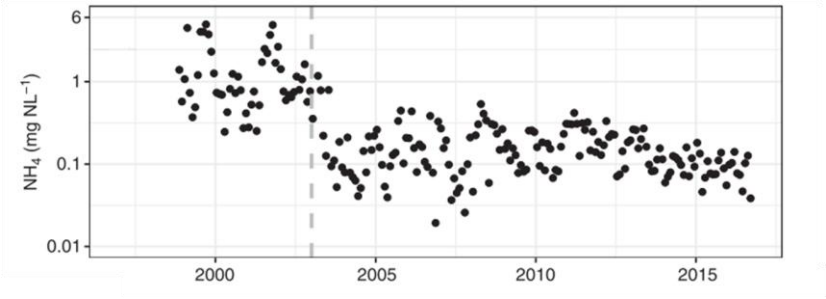

Figure 8. Decline in monthly means of ammonium concentration (NH_4) after putting in operation a wastewater treatment plant (dashed grey line) in the Oria River, Spain. From Arroita *et al.* (2018).

Nevertheless, WWTPs are unable to effectively remove all contaminants from sewage and their effluents still contribute complex mixtures of substances to freshwater ecosystems (Rodriguez-Mozaz *et al.*, 2015). Substances that pass through biological wastewater treatments without being fully degraded include nutrients (Waiser *et al.*, 2011), organic matter (Spänhoff *et al.*, 2007), fine sediments (Wakelin *et al.*, 2008), pathogens (Subirats *et al.*, 2017), as well as other emerging contaminants, such as metals (Holeton *et al.*, 2011), pesticides (Kuster *et al.*, 2008), pharmaceuticals (Petrovic *et al.*, 2005), personal care products (de Solla *et al.*, 2016) or illicit drugs (Rosi-Marshall *et al.*, 2015). As an example, Carey & Migliaccio (2009) reported that even a WWTP completing a secondary treatment can produce effluents with high nitrogen and phosphorus concentrations, ranging from 15 to 25 mg N L^{-1} and from 4 to 10 mg P L^{-1}, respectively. As a consequence, WWTP effluents cannot match the characteristics of the water in the receiving systems and therefore, induce detrimental consequences on ecosystem health (Hubbard *et al.*, 2016). On the one hand, effluents can alter water chemical properties, impairing physico-chemistry, by decreasing oxygen (Walsh *et al.*, 2005) and increasing nutrient concentrations (Waiser *et al.*, 2011). On the other hand, they can also modify hydrology of the receiving streams by disturbing their natural flow regime (Merseburger *et al.*, 2011).

2.2. Why should we care about ecosystem level responses?

During the last decades, concern about ecosystem deterioration due to WWTP effluent inputs has greatly increased and with that, the research addressing this issue. According to a search in Science Direct for research articles published during the last decade (2009-2019) with the terms "*wastewater treatment plant*" or "*wastewater treatment plant effluent*", a total of 67,200 articles were computed, among which 4,600 have been published only during the first months of the present year 2019. When including the term "*effects of*" to the previous search, the initial number was reduced to 14,600 research articles. However, when analyzing the latter search, among the most relevant 100 articles ranked by Science Direct, 40 articles focused on technological advances or methodological optimisations of the WWTPs. The next most abundant articles (35) analyzed the impacts of WWTP

effluents on water quality, focusing on both surface and drinking waters. Another 10 studies focused on restoration methods and tools for a safe reuse of these sewage waters. And finally, only 15 out of the 100 studies assessed ecological effects and impacts of WWTP effluents on ecosystem-level responses. While most of them focused on ecosystem structure (14), just a single study analyzed the effects on ecosystem functioning (1). This literature search highlights that, despite that many efforts have been channelled to mitigate the impacts of these anthropogenic inputs, there has been little evaluation of their success; even if rivers provide fundamental services that depend on their structure and functioning (Perrings *et al.*, 2010).

3. The crux of the matter: impacts on ecosystem structure and function

Urban pollution can affect both ecosystem structure and functioning. Ecosystem structure refers to the physico-chemical features of the ecosystems, such as channel morphology, water quality or the composition of biological communities (from microbes to animals). Ecosystem functioning, on the other hand, refers to the set of processes that regulate the fluxes of energy and matter as a consequence of the joint activity of physical features and biological communities (von Schiller *et al.*, 2017). These include organic matter decomposition and retention, biofilm exo-enzymatic activities, nutrient retention and ecosystem metabolism, among others (Sandin & Solimini, 2009). Thus, both approaches range from purely physical processes to more biologically mediated ones (Palmer & Febria, 2012) and can cover different spatial and temporal scales, from patch/habitat to river segment (Frissell *et al.*, 1986) and from hours to years.

In general, all these processes respond to a wide variety of environmental stressors, such as chemical pollution (Aristi *et al.*, 2015; Ferreira *et al.*, 2015), flow regulation or water abstraction (Arroita *et al.*, 2016; von Schiller *et al.*, 2015) and water flow interruption (Acuña *et al.*, 2005; Zoppini *et al.*, 2016). Ecosystem structure and functioning influence each other and thus, they could be viewed as the two sides of the same coin (von Schiller *et al.*, 2017), but the relation among them is not always straightforward and often one cannot automatically inferred from the other (Cardinale *et al.*, 2012). Traditionally, most efforts have been conducted to the development of methods for characterization of ecosystem structure, neglecting ecosystem functioning (Palmer & Febria, 2012). It has only been during the last decades that assessment of ecosystem functioning has gained popularity among scientists and managers (Jax, 2010), mainly because it is a key point of ecosystems services (MEA, 2005) and an integral component of ecological status (EC, 2000).

3.1. Impacts on ecosystem structure

As above-mentioned, WWTP effluents can alter hydrological and the chemical properties of the receiving streams. Concerning hydrological alterations, effluents can disturb the natural flow regime of streams, by increasing stream water flow, water velocity, mean channel depth and width (Merseburger *et al.*, 2011). These inputs are especially relevant during periods of lower discharge

(Wakelin *et al.*, 2008) or in arid regions (Brooks *et al.*, 2006), where they can make up most of the stream water flow (Rice & Westerhoff, 2017) transforming intermittent streams into perennial systems. This situation represents a paradox in which the flow needed to support the development of biological communities is provided by urban effluents (Boyle & Fraleigh, 2003), which simultaneously affects water quality and development of the biological communities (Canobbio *et al.*, 2009). In addition, WWTP effluents can also affect water quality, increasing temperature, electrical conductivity (Merbt *et al.*, 2015) as well as the concentration of nutrients (Carey & Migliaccio, 2009) and pollutants (Rodriguez-Mozaz *et al.*, 2015), while decreasing water pH (Englert *et al.*, 2013) and oxygen concentration (Walsh *et al.*, 2005). As mentioned above, all these conditions will undermine the structure of biological communities by affecting their growth, survival, dispersal and reproduction, from microbes (Drury *et al.*, 2013) and algae (Corcoll *et al.*, 2015), to macrophytes (Gücker *et al.*, 2006), benthic invertebrates (Ortiz & Puig, 2007) and fish (Northington & Hershey, 2006). These hydrological and chemical alterations induced by WWTP effluents condition the composition and relative abundance of riverine biological communities, as they establish environmental filters and additional selective pressures. As a consequence, some species abandon the reach and look for a refuge in other nearby reaches (James *et al.*, 2008), while other are replaced by more tolerant and opportunistic species (Miserendino *et al.*, 2008).

3.2. Impacts on ecosystem functioning

Even if all these structural changes are likely to affect stream ecosystem functioning (Elosegi & Sabater, 2013), we still lack a clear picture about the effects of urban pollution on ecosystem functioning. Ecosystem processes constitute a major pathway of energy transfer and nutrient cycling in rivers (Tank *et al.*, 2010), which is mainly driven by the joint activity of the above-mentioned biological communities, from microbes to fish (Tilman *et al.*, 2014). Consequently, as they depend on physical features and biological communities, they are likely to be also affected by WWTP effluents.

Biofilm activity and nutrient uptake are among the most sensitive processes to anthropogenic pollution, especially to wastewater discharges (Martí *et al.*, 2009). Stream biofilms are complex biological communities formed by autotrophic and heterotrophic organisms, including algae, cyanobacteria, bacteria, fungi and microfauna, growing on solid substrata embedded in a matrix of polysaccharides and other polymers (Lock, 1993; Romaní, 2010). These organisms produce extracellular enzymes for the degradation of organic matter into smaller molecules, which then become available for bacterial growth and microbial nutrient uptake (Romaní *et al.*, 2012). Biofilms are major sites for uptake, storage and transformation of dissolved organic matter and nutrients, and thus, play a key role in stream biogeochemistry, contributing to nutrient dynamics, primary production, CO_2 emission and organic matter decomposition (Battin *et al.*, 2016). Indeed, biofilms can contribute to transform and retain up to 50-75% of the nitrogen and 30% of the phosphorus entering streams (Mulholland, 2004), which can alleviate the effects of anthropogenic

nutrient inputs (Ribot *et al.*, 2013). Therefore, biofilms can be highly responsive to environmental stressors (Sabater *et al.*, 2007) and thus, respond to pollution derived from WWTP effluents (Proia *et al.*, 2013).

Likewise, biofilms occupy a key position at the basis of food webs, although their resource availability is usually determined by other ecosystem-level processes, such as carbon and nutrient retention (Rowe & Richardson, 2001). In particular, nutrient retention reflects the process by which dissolved nutrients are removed from the water column and immobilized in particulate form or transformed into gaseous form, leaving permanently the system (Newbold, 1996). It is strongly related to the self-purification capacity of running waters, one of the most important ecosystem services provided by rivers, as uptake can reduce the load of nutrients transported downstream (Schlesinger & Bernhardt, 2013). Whole-ecosystem nutrient uptake can be controlled by physical mechanisms such as turbulence or hyporheic flow, chemical mechanisms such as sorption processes, as well as by biological uptake (Mulholland & Webster, 2010). Nutrient uptake can also be assessed for specific compartments of the ecosystem, including biofilm growing on rocks, leaf litter, macrophytes or sediments among others (Mulholland & Webster, 2010), which allows disentangling their specific role or contribution relative to the whole-ecosystem process (Hoellein *et al.*, 2009). Both measurements for nutrient uptake are controlled by the biomass and activity of autotrophic and heterotrophic organisms, as well as by physico-chemical features, such as water discharge and nutrient concentrations. As a consequence, both can be strongly affected by multiple environmental stressors, such as water diversion or flow regulation (Arroita *et al.*, 2016; von Schiller *et al.*, 2016), altered sediment dynamic and siltation (Ryan *et al.*, 2007), salinization (Arce *et al.*, 2014) or WWTP derived effluents (Gücker *et al.*, 2006; Proia *et al.*, 2017). Stream nutrient uptake capacity is generally higher under relatively pristine conditions, but this capacity is easily overwhelmed while ambient nutrient concentrations increase (Ensign & Doyle, 2006).

Ecosystem metabolism is another process potentially sensitive to urban inputs. It is an ecosystem process that integrates the joint metabolic activity of organisms and reflects the energy transfer and organic carbon fluxes along fluvial network (Battin *et al.*, 2008). Ecosystem metabolism typically includes gross primary production (GPP), which is the synthesis of new organic matter from solar energy and inorganic carbon, and ecosystem respiration (ER), which is the oxidation of the organic matter to obtain energy (Odum, 1956). GPP is controlled by light availability (Hill *et al.*, 2001), temperature (Gillooly *et al.*, 2001) and nutrient concentration (Mulholland *et al.*, 2001; Roberts *et al.*, 2007), whereas ER is mainly controlled by temperature (Yvon-Durocher *et al.*, 2012) and organic matter availability (Acuña *et al.*, 2004). Similar to nutrient uptake, ecosystem metabolism can also be assessed for specific compartments, such as the biofilm growing on rocks or the macrophytes (Tank *et al.*, 2010), which allows disentangling their specific contribution to the whole ecosystem (Acuña *et al.*, 2011). Both whole ecosystem and compartment specific approximations are good indicators of the ecological status of streams and rivers, as both have key implications for the energy flow and the cycling of materials in ecosystems and determine the life-

supporting capacity of riverine biological communities (Young *et al.*, 2008; Bunn *et al.*, 2010). In most running waters, photosynthesis and aerobic respiration are predominant ways to synthesize and oxidize organic matter. Thus, as both processes affect oxygen concentration, most techniques to measure stream metabolism are based on oxygen changes (Izagirre *et al*., 2008; Demars *et al.*, 2015). Due to the intrinsic characteristics above-mentioned, stream metabolism can be strongly affected by multiple environmental stressors, such as flow regulation (Uehlinger, 2000; Hall *et al.*, 2015), drought (Timoner *et al.*, 2012), channelization (Hope *et al.*, 2014), altered sediment dynamics (Young *et al.*, 2008) or WWTP derived effluents (Aristi *et al.*, 2015; Corcoll *et al.*, 2015). Ecosystem metabolism varies across seasons, years or rivers due to natural variations in environmental factors (Tank *et al*., 2010; Bernhardt *et al*., 2018). However, direct and indirect effects of human activities are likely to alter the potential or the efficiency of rivers to perform the key ecosystem services of nutrient assimilation and retention (Fellows *et al.*, 2006; Mulholland *et al.*, 2008), which could consequently exhacerbate the sensitivity of metabolism to human impacts by altering the magnitude and timing of both GPP and ER (Arroita *et al.*, 2018; Bernhardt *et al.*, 2018). Increased nutrient and organic matter loads, as well as exposure to contaminants, are among the most common impacts driving ecosystem metabolism (Arroita *et al.*, 2018).

In the same way, organic matter decomposition is another process sensitive to anthropogenic inputs. Decomposition of coarse particulate organic matter constitutes one of the major pathways of energy transfer and nutrient recycling in rivers (Tank *et al.*, 2010). Organic matter entering streams suffers complex changes until it is finally decomposed to inorganic matter, this detritus pathway being essential for the recycling of nutrients and other materials (Gessner *et al.*, 2010). The process involves different biotic and abiotic processes, which include leaching of soluble compounds, microbial conditioning by fungi and bacteria, and fragmentation by invertebrates or physical abrasion (Hieber & Gessner, 2002), all of them overlapping in time (Wantzen *et al.*, 2008). Organic matter decomposition generally involves placing pre-weighted substrates in the stream and estimating the mass lost over time (Benfield, 2006). Traditionally, the "leaf litter method" has been the most used approach, but during the last years artificial substrates, such as wooden sticks or standard cotton strips, have gained popularity among scientists, as a simpler, more standardised method that responds in the same way as leaf litter (Arroita *et al.*, 2012; Tiegs *et al*., 2013). Organic matter decomposition is an integrative process that is a good indicator of impaired river ecosystem functioning (Young *et al.*, 2008). It can be affected by a wide range of environmental factors (Chauvet *et al.*, 2016), such as flow regulation (Arroita *et al.*, 2015), channelization (Elosegi & Sabater, 2013), drought (Datry *et al.*, 2011), salinization (Gómez *et al.*, 2016) or effluents derived from WWTPs (Peters *et al.*, 2013; Ferreira *et al.*, 2015). Elevated nutrient concentrations have been shown to increase organic matter decomposition rates, which can be driven by an improvement in the palatability of leaves (Ferreira *et al.*, 2015) and by an increase in the density of invertebrates (Pascoal *et al.*, 2003). However, under other contaminants, such as heavy metals, decreased organic

matter breakdown rates have been reported (Niyogi *et al.*, 2001), driven by deterioration in food resources and consumer communities.

4. "*Beautiful hypotheses and ugly facts*"

In 1996, Dennis Chitty (Chitty, 1996), a world-class population biologist, summarized his whole career in a book titled "*Do lemmings commit suicide? Beautiful hypotheses and ugly facts*". In this book, Professor Chitty told of the countless experiments he had performed over the years seeking to test hypotheses on the reasons behind large fluctuations in the populations of small rodents, such as lemmings and voles. Every time a "*beautiful hypothesis*" seemed too logical not to be true, the experimental facts went against that hypothesis. I guess most ecologists have experienced this feeling at times. I certainly have, what led me to think on the reasons behind facts contradicting seemingly impeccable hypotheses, and what could also explain contradictory results obtained under similar experimental settings.

4.1. Reasons behind contrasting results

Despite large efforts to understand the effects of urban pollution on ecosystem functioning, we still lack a clear, coherent picture (Peters *et al.*, 2013; Kaushal *et al.*, 2014, 2018; Ferreira *et al.*, 2018). Many studies have been carried out on streams and rivers receiving urban inputs by measuring ecosystem processes such as nutrient uptake, ecosystem metabolism or organic matter decomposition. However, they often yield contrasting results. For instance, while most of the works report decreased uptake rates for both nitrogen and phosphorus (*e.g.*, Martí *et al.*, 2004, 2009; Merseburger *et al.*, 2005, 2011; Kunz *et al.*, 2017), some report enhanced nutrient uptake rates, especially for ammonium, below WWTP effluents (*e.g.*, Ruggiero *et al.*, 2006; Stutter *et al.*, 2010), or no overall effects (*e.g.*, Haggard *et al.*, 2001, 2005; Sánchez-Perez *et al.*, 2009). Similarly, most works show increased GPP and ER below effluent inputs (*e.g.*, Izagirre *et al.*, 2008; Aristi *et al.*, 2015; Arroita *et al.*, 2018), although some works showed a reduction (*e.g.*, Rodríguez-Castillo *et al.*, 2017) or non-significant changes (*e.g.*, Sánchez-Perez *et al.*, 2009). Finally, contrasting responses have also been shown for organic matter decomposition, which can either increase (*e.g.*, Pascoal *et al.*, 2003; Ferreira *et al.*, 2015; Solagaistua *et al.*, 2018), decrease (*e.g.*, Lecerf *et al.*, 2006; Piscart *et al.*, 2009, 2011; Englert *et al.*, 2013) or remain unaffected (*e.g.*, Chadwick & Huryn, 2003; Abelho & Graça, 2006; Baldy *et al.*, 2007).

These contrasting results suggest that the response of ecosystem functioning to urban pollution may also depend on other factors. On the one hand, most of the streams and rivers are simultaneously subjected to multiple stressors (Sabater *et al.*, 2018), such as hydrological variability in freshwater ecosystems (Ponsatí *et al.*, 2016), warmer temperatures (Acuña *et al.*, 2008) or diffuse pollution (Brett *et al.*, 2005), which can mask the effects of chemical pollution (Stevenson & Sabater 2010). On the other hand, the effects of WWTP effluents can also vary depending on their final

concentration in the receiving water body, which is mainly determined by the size of the WWTP (*i.e.*, the amount of people it services), and the dilution capacity of each river (Rice & Westerhoff, 2017). The dilution capacity changes temporally with the hydrological regime of each river, which varies between climatic regions (Martí *et al.*, 2009) and seasons (Petrovic *et al.*, 2011). Therefore, the final concentration of WWTP effluents can span a large range. For instance, in the province of Gipuzkoa (northern Spain) it averages 35% (Province Government of Gipuzkoa, 2015), whereas it can reach 100% in Mediterranean regions during periods in which streams naturally cease to flow (*e.g.*, Merseburger *et al.*, 2011), leading to obviously different effects. Additionally, the effects can also vary depending on the composition of the biological community in the receiving freshwater system (Segner *et al.*, 2014). Nevertheless, the effects of chemical pollution do not only depend on the intensity of the stress, but also on the timing of the exposure (Camargo & Alonso, 2006) as well as on the legacy effects that effluents can exert even when they are reduced or no longer received (Jarvie *et al.*, 2013). For instance, Arroita *et al.* (2018) showed that adequate treatment of wastewater after the implementation of a WWTP greatly improved stream water quality and ecological status, although the recovery of the system can take a long time. Finally, most of the studies assessing the effects of urban pollution on ecosystem functioning, are limited to the comparison between reaches located up- and downstream from a WWTP effluent (*e.g.*, Drury *et al.*, 2013), neglecting the influence that can exert on the response all these potentially confounding factors affecting urban streams.

4.2. The Subsidy-Stress Hypothesis

Among all the possible reasons listed above, one of the most important factors behind contrasting results in ecosystem functioning is likely to be the final concentration of WWTP effluents in the receiving water body. According to the classic "*Subsidy-Stress hypothesis*" (Odum *et al*., 1979), pollutants and other environmental factors can be grouped in two main groups according to their effects on stream biological communities (Fig. 9). Some substances, such as nutrients and organic matter, can promote biological activities up to a threshold, where they are no longer assimilable for the river biota, become toxic and start reducing biological activities below "normal" levels (*i.e.*, Stress). Some other substances, such as heavy metals or pesticides, are always detrimental for the river biota and they tend to reduce biological activities roughly in proportion to their concentration.

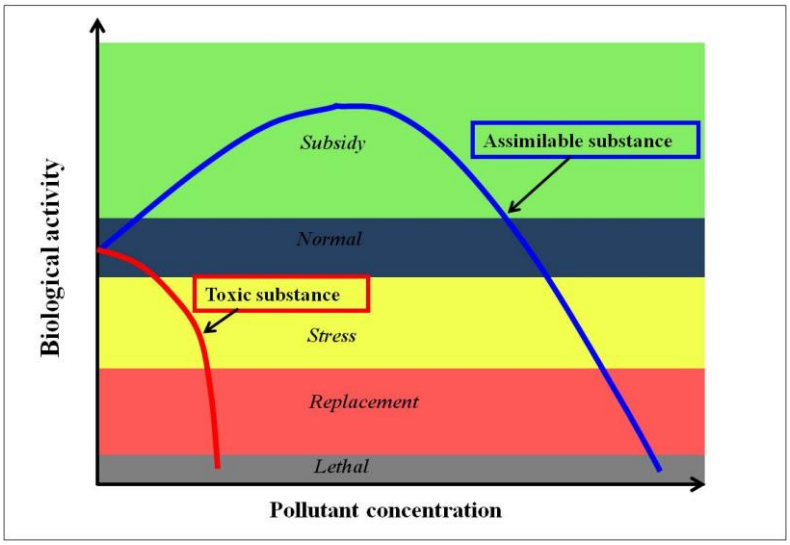

Figure 9. The "*Subsidy-Stress Hypothesis*". Modified from Odum *et al.* (1979).

Both assimilable and toxic compounds affect stream ecosystem functioning (*e.g.*, Aristi *et al.*, 2015, 2016), although depending on their mixed composition and their resulting concentration in the receiving water body, the final effect of WWTP effluents is difficult to predict, either a subsidy or a stress for biological activity. Besides, it is also likely that pollution will have different effects depending on the biological activity and thus, different biological activities could show different response curves to the concentration of WWTP effluents (Fig. 10). For some biological activities (*e.g.*, diversity of the biological community), pollution will be a stress and thus, it will decrease the process even at low concentrations of pollution. Otherwise, for some other biological activities pollution will play as a subsidy and promote them, although they could also differ in the response pattern. For instance, some of the processes (*e.g.*, nutrient uptake, breakdown rates, gross primary production or Chlorophyll-*a* concentration) will follow the Subsidy-Stress scheme, being subsidized up to a maximum and afterwards, being reduced and decreased below "*normal* " levels. Nevertheless, they could also differ according to the maximum concentration at which they reach peak activities, some substances reaching this peak at lower concentrations of pollution (*e.g.*, nutrient uptake), while others reaching it at higher concentrations (*e.g.*, Chlorophyll-*a* concentration). Finally, some other biological activities (*e.g.*, ecosystem respiration or biofilm biomass), will be subsidized by the effluent without following the Subsidy-Stress pattern, but being permanently promoted. For this reason, in this dissertation I have analyzed a large variety of ecosystem processes, instead of analysing a single one, in order to obtain a more clear picture about the effects of urban pollution on ecosystem functions.

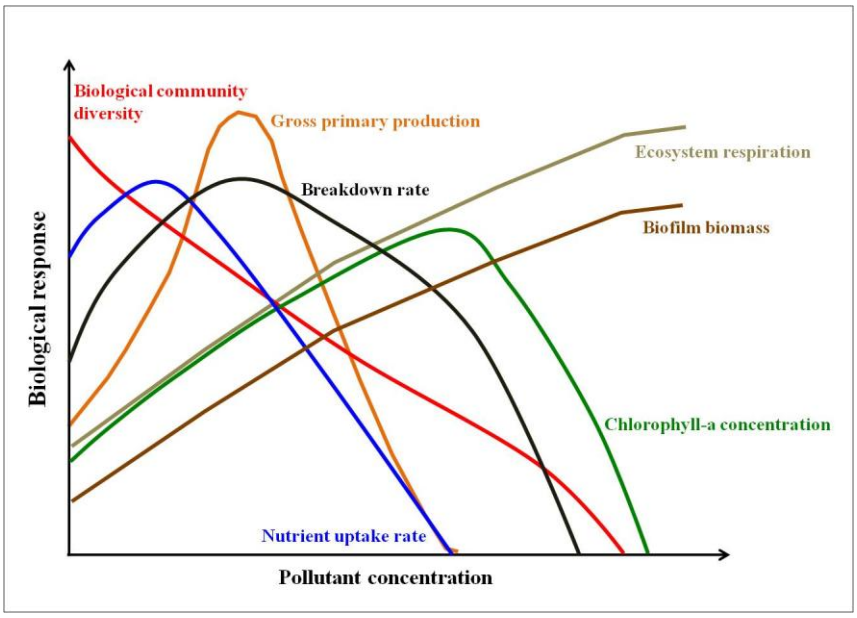

Figure 10. Schematic picture of the different response patterns that could be expected for different biological activities in relation to the concentration of WWTP effluent in the receiving water body. Therefore, I would expect urban effluents to stress some of the biological responses measured (*e.g.*, diversity of biological communities), to subsidize some other responses (*e.g.*, ecosystem respiration or biofilm biomass) or instead, cause subsidy-stress response patterns in some other biological responses (*e.g.*, nutrient uptake, gross primary production or descomposition rates).

5. Objectives

This PhD dissertation studies the effects of urban pollution on stream ecosystem functioning by combining field and laboratory experiments and by measuring the response at different levels, from the biofilm to the whole ecosystem. More specifically, we try to answer the following questions:

1. What are the combined effects of urban pollution and water scarcity on the functioning of stream ecosystems?
2. What are the effects of a well-treated and highly diluted WWTP effluent input on stream ecosystem structure and functioning?
3. How do stream ecosystem processes respond to a gradient of WWTP effluent contribution? What are the pollution legacy effects and how can they modulate the recovery capacity of the system?

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CHAPTER 2

Combined effects of urban pollution and hydrological alterations on the functioning of Mediterranean streams

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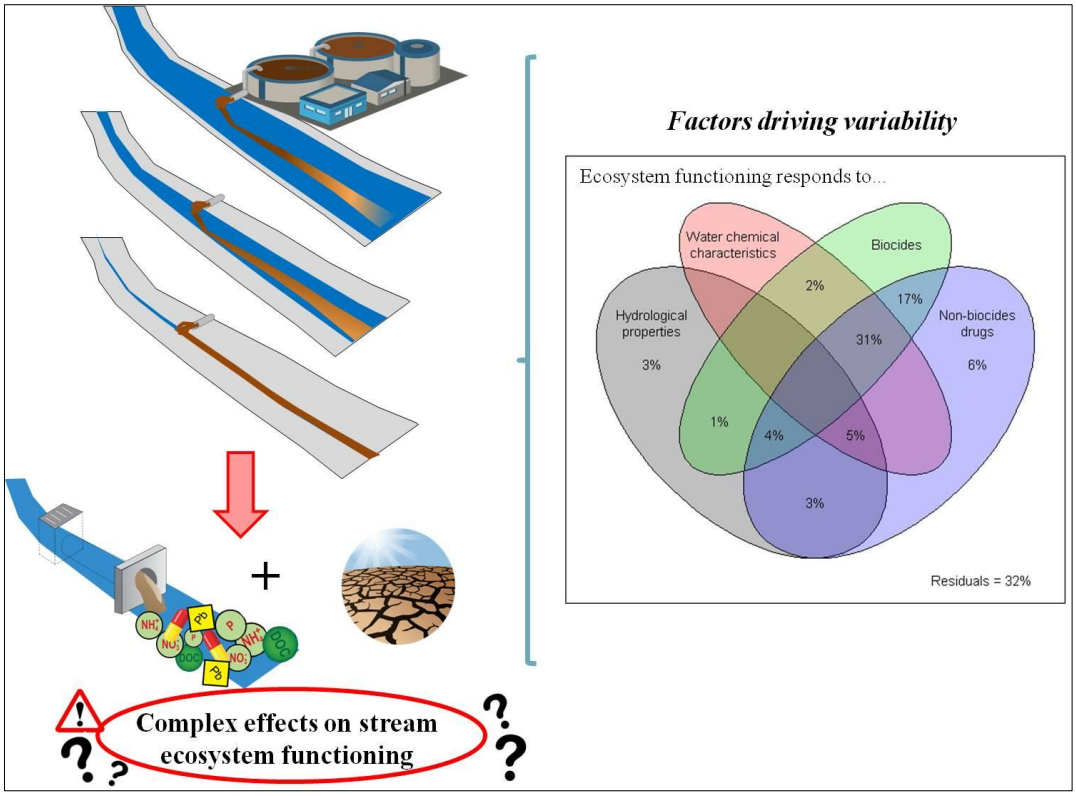
Abstract

Urban streams are affected by pollution and hydrological alterations, which determine their dilution capacity and thus the final concentration of pollutants. We assessed the combined effects of urban pollution and water scarcity in 13 Mediterranean streams covering a wide range in their dilution capacity. In each stream, we defined a control reach upstream and an impact reach downstream from an input of urban sewage. In each reach, we measured biofilm biomass accrual and phosphorus uptake capacity, organic matter decomposition, and whole-reach nutrient uptake and metabolism. Sewage caused complex effects on ecosystem functioning. It subsidized most functional processes, except organic matter decomposition and biofilm phosphorus uptake capacity, which decreased with increasing pollutant concentrations. Most processes showed a linear response to pollution, and none of them followed the subsidy-stress pattern we expected. The variability observed in ecosystem functioning was driven by chemical characteristics, especially the concentration of drugs, although the response depended on the process. Overall, the response of stream ecosystem functioning to urban pollution depended mainly on the nature of the effluent and on the dilution capacity of the receiving water body.

Keywords: Wastewater; nutrients; pharmaceutically active compounds (PhACs); hydrology; subsidy-stress; ecosystem functioning.

Graphical Abstract



Introduction

Increasing urbanization and human population growth affect water quality and the ecosystem functioning of urban streams (Kaushal *et al.*, 2015). Urban areas continuously release complex mixtures of biologically active compounds into aquatic environments (Rice & Westerhoff, 2017). While sewage is commonly treated in wastewater treatment plants (WWTPs) in more affluent countries, in less favored countries two thirds of urban wastewaters are still released to streams and rivers without any treatment (EEA, 2018). WWTPs greatly reduce nutrients, organic matter and pathogens reaching the environment but do not completely eliminate them, and their effluents still contain a wide range of pollutants, including nutrients and organic matter (Carey & Migliaccio, 2009), heavy metals (Deycard *et al.*, 2014), and organic micro-pollutants such as pesticides, personal care products or pharmaceutically active compounds (PhACs) (Kuzmanovic *et al.*, 2015; Mandaric *et al.*, 2018). Some of these substances, such as heavy metals, are toxic and particularly stress biological activity, whereas others, such as nutrients, can subsidize (*i.e.*, promote) biological activity up to a threshold, but stress it at higher concentrations, resulting in the so-called Subsidy-Stress pattern (*sensu* Odum *et al.*, 1979).

The effects of pollution can be exacerbated in streams subjected to co-occurring stressors, such as increased temperature or changes in hydrology (Sabater *et al.*, 2018). Among these, hydrological stress is of special concern, especially where it converts perennial watercourses into temporary ones as a consequence of climate change and water abstraction (Acuña *et al.*, 2014). Arid and semi-arid regions such as the Mediterranean are amongst the most threatened by hydrological stress, and further deterioration could lead to critical environmental problems. Under natural conditions, Mediterranean streams are characterized by large seasonal variations in discharge, with large floods during autumn and spring and strong flow reductions in summer (Sabater & Tockner, 2010), when their dilution capacity is severely reduced. Under global change scenarios, WWTP effluents are expected to make up a larger proportion of water flow in many receiving ecosystems (Döll & Schmied, 2012; Rice & Westerhoff, 2017). Water use and management in these ecosystems makes them particularly sensitive to the combined effect of chemical and hydrological stressors (Skoulikidis *et al.*, 2017).

While the effects of water pollution and hydrological alteration on stream biota have been relatively well studied (*e.g.*, Sabater *et al.*, 2016; Rodríguez-Castillo *et al.*, 2017; Romero *et al.*, 2018; Tornés *et al.*, 2018), implications for ecosystem functioning are still unclear. Ecosystem functioning reflects the fluxes of energy and matter in ecosystems, but its relevance is seldom considered in monitoring schemes aiming to assess the ecological river status (von Schiller *et al.*, 2017). Previous analyses of effects of chemical stress on ecosystem functioning have yield inconsistent results. For instance, among the works studying the effect on ecosystem metabolism, most of them show increased metabolic rates below sewage inputs (*e.g.*, Aristi *et al.*, 2015), although

there are some other works that show a reduction (*e.g.*, Rodríguez-Castillo *et al.*, 2017) or a non-significant effect (*e.g.*, Sánchez-Perez *et al.*, 2009). Similarly, most of the works studying the effects of urban pollution on nutrient dynamics report decreased uptake rates below sewage inputs (*e.g.*, Martí *et al.*, 2004), although there are some others that report increased (Stutter *et al.*, 2010) or unaffected (*e.g.*, Haggard *et al.*, 2005) uptake rates. In the same way, contrasting results have also been reported for organic matter decomposition, with increased (*e.g.*, Pascoal *et al.*, 2003), decreased (*e.g.*, Englert *et al.*, 2013) or even unaffected decomposition rates (*e.g.*, Baldy *et al.*, 2007).Therefore, these inconsistencies likely derive from the specific characteristics of the effluents, the dilution capacity of each site, or the particular response of each ecosystem function, some being subsidized and others stressed (Pereda *et al.*, 2019).

Here, we examined the combined effects of urban pollution and hydrological alteration on stream ecosystem functioning. With this purpose, we selected 13 Mediterranean streams that received either treated or untreated sewage inputs and differed in their dilution capacity. We assumed that the type of sewage as well as the final concentration in the receiving ecosystem would define the impact of urban pollution. We predicted that (i) sewage inputs would deteriorate physico-chemical conditions and increase the concentration of pollutants, (ii) these changes in water chemical characteristics would alter ecosystem functioning below sewage inputs, by increasing metabolic rates, decomposition or biofilm biomass, while decreasing nutrient uptake capacity, and (iii) the interaction between urban pollution and hydrology would explain the variability in ecosystem functioning.

Material and Methods

Study sites and experimental design

We conducted this study in small tributaries of the lower Ebro River (NE, Iberian Peninsula) (Fig. 1). We selected 13 streams ranging in order from 2 to 4, which drained calcareous basins mainly dominated by forested and dry land agriculture. All streams experienced strong seasonal flow variability as a consequence of the Mediterranean climate, compounded by water abstraction and groundwater exploitation. In each stream we selected two reaches, one upstream (Control) and another downstream (Impact) from urban effluents. The sites were studied from autumn 2015 to spring 2016.

The experiment followed a natural repeated measures complete block design (RCB, Casella, 2008), on which the 13 streams acted as blocks, reaches represented the experimental manipulation, and the two sampling campaigns represented repeated measurements. While water chemical characteristics, including basic physico-chemical variables, nutrients and pharmaceutically active compounds (PhACs), were measured during both sampling campaigns (autumn 2015 and spring 2016), hydrology and stream functioning were only measured during the second sampling campaign.

Control and Impact reaches were set as close as possible from each other, the distance among them ranging from a few hundred meters in the smaller streams to a few kilometres in the larger ones. Special care was taken not to include any dam or tributary along the watercourse, which could obscure the effect of the urban input. Impact reaches received either treated or untreated sewage from small towns ranging from 500 to 7,000 population equivalents (Fig. 1).

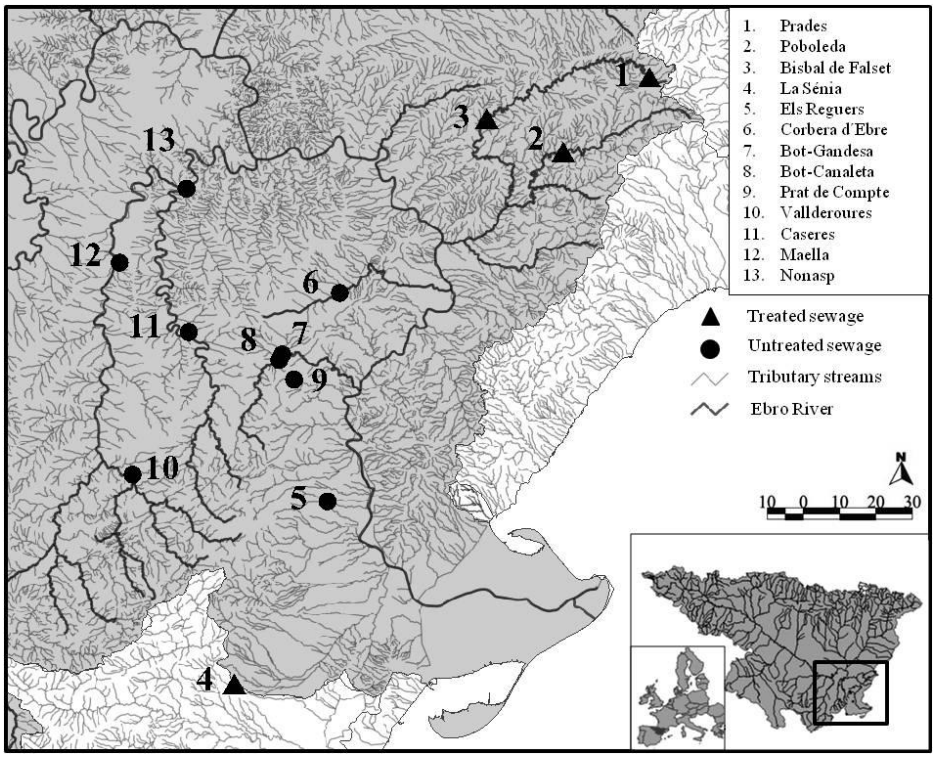


Figure 1. Sampling sites in the lower Ebro River Basin. Black triangles represent streams receiving effluents from WWTPs, while dots represent untreated urban sewage inputs. Thicker line represents the main Ebro River and thinner lines tributary streams. The bottom right inset shows the location of the sampling sites within the Ebro River Basin, and its location in Europe.

Water chemical characteristics

Temperature (T), pH, electrical conductivity (EC) and dissolved oxygen (DO) concentration were measured using hand-held probes (WTW multiparameter handheld meters Multi 350i and WTW 340i SET, WTW Wissenschaftlich, Weilheim, Germany; YSI ProODO handled; YSI Incorporated, Yellow Springs, OH, USA). Collected water samples were immediately filtered (0.7-µm pore size pre-combusted glass-fiber filters, Whatman GF/F, Whatman International Ldt., Kent, UK) and frozen until analysis. We determined the concentration of soluble reactive phosphorus (SRP) [molybdate method (Murphy & Riley, 1962)], on a manual double-beam UV-1800 UV-Vis spectrophotometer (Shimadzu, Shimadzu Corporation, Kyoto, Japan). Concentrations of anions [nitrate (NO_3^-) and nitrite (NO_2^-)] and cations [ammonium (NH_4^+)] were determined by ion chromatography on a Dionex ICS-5000 (Dionex Corporation, Sunnyvale, USA). Dissolved organic

carbon (DOC) concentration was determined by catalytic oxidation using a Shimadzu TOC-V CSH coupled to a TNM-1 module (Shimadzu Corporation, Kyoto, Japan).

We quantified the concentration of pharmaceutically active compounds (PhACs), as they are one of the most abundant groups of micro-contaminants in urban waters and are considered pseudo-resistant contaminants causing adverse effects on living organisms and aquatic environment (Ellis, 2006). We analysed the concentration of PhACs following the method described by Gros *et al.* (2012). Briefly, we carried out the analyses with an off-line solid phase extraction followed by ultra-high-performance liquid chromatography coupled to ion trap tandem mass spectrometry (UHPLC-QqLIT-MS2). Chromatographic separation was carried out with a Waters Acquity Ultra-Performance™ liquid chromatography system, coupled to a 5500 QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) with a turbo Ion Spray source. Quantification was carried out by isotope dilution. Finally, we acquired and processed all data using Analyst 1.5.1 software. Detailed information about method performance is described in Mandaric *et al.* (2018).

Hydrology

We estimated water velocity and discharge from the time *vs.* conductivity curves obtained with pulse additions of NaCl conducted on the same day in the Control and Impact reaches of the same stream. We calculated mean water velocity (m s^{-1}) dividing reach length by the time elapsed between the pulse addition and the conductivity peak. We calculated water discharge based on the mass-balance approach, using electrical conductivity as a surrogate of the chloride concentration (Martí & Sabater, 2009). Additionally, we characterized the physical habitat of the reaches measuring width and depth of the wet channel at ten equidistant transects. We also placed a water level datalogger per reach (Solinst Levelogger Edge 3001; Solinst Canada Ltd., Georgetown, USA), to measure absolute pressure and temperature during the experiment. These data were then corrected for the atmospheric pressure measured by additional loggers (Barologger, Solinst Levelogger Edge 3001) to obtain continuous water level data. Using these data we calculated a proxy of the hydrological stress per reach, which was computed as the total number of days during the study period in which the average water level remained below 5 cm (Acuña *et al.*, 2005).

Response processes

We assessed the functional response of streams by measuring several ecosystem processes at both habitat and ecosystem scales. Habitat-scale functioning was analyzed by the activity of the biofilm, measuring its biomass accrual and its capacity to retain SRP, and of the microbial heterotrophic community, by measuring its capacity to decompose organic matter. Ecosystem scale measurements, on the other hand, included reach-scale nutrient uptake and metabolism.

Biofilm functioning

We measured the biofilm biomass accrual rate and SRP uptake capacity in biofilm carriers. These are artificial substrata with high surface-to-volume ratio used in aquaria to promote biofilm attachment and which have been used as standard substrata in stream studies (Baldwin *et al.*, 2003; Elosegi *et al.*, 2018). We used plastic cubic carriers (2.5 cm in side, SERA GmbH D52518, Heinsberg, Germany). Five biofilm carriers per reach were deployed randomly during the first sampling campaign (October 2015), tied with nylon line to roots or to metal bars, and recovered after 6 months of incubation (April 2016). After recovering, they were stored in stream water inside dark plastic containers and carried to the laboratory, where they were immediately subjected to a bioassay to measure SRP uptake capacity. Once this was performed, they were individually frozen in plastic bags for posterior biomass determination.

To measure SRP uptake capacity (SRPUC, µg P h^{-1}), biofilm carriers were first acclimated individually for 30 min in an orbital shaker (Agitator-Incubator INF-66123 Multitron Standard, BIOGEN Científica S.L., Madrid, Spain) at 100 rpm shaking speed, 20 ºC and 180 µmol m^{-2} s^{-1} light. The carriers were immersed in an acclimation solution of 1:5 v/v mixture of Perrier carbonated mineral water (Nestlé, Vergèze, France) in deionized water designed to ensure a sufficient supply of micronutrients. After acclimation, biofilm carriers were individually incubated in 60-mL of the same solution but spiked with phosphate (K_2HPO_4, 10 mM = 310 mg P L^{-1}) to achieve a final concentration of 5 µM P (155 µg P L^{-1}), and incubated under the same conditions for 1 h. This concentration was chosen to ensure saturating conditions for the biofilm while allowing the nutrient decline during the incubation and the subsequent determination of uptake. After the incubation, 10 mL from each vial were filtered (0.7-µm pore size, Whatman GF/F) and frozen until analysis. Control treatments using non-colonized biofilm carriers were also used. SRP uptake was calculated as the difference between the mean SRP concentration of the control and the colonized substrates, in the incubation volume (L) and time (h).

To determine biomass accrual rates (BAT, g m^{-2} d^{-1}) during the incubation period, biofilm from the carriers was detached in 100 mL of deionized water using a sonifier ultrasonic cell disruptor (Branson Ultrasonic TM, Branson Ultrasonic Corporation, Emerson Electric, USA) combining 3 min of pulses (70% of amplitude) and 2 min of continuous mode at the same amplitude. We filtered one subsample of the biofilm solution on pre-weighed filters (0.7-µm pore size, Whatman GF/F), which were oven-dried (70 ºC, 72 h), weighed, combusted (500 ºC, 5 h) and reweighed to obtain the ash-free dry mass (AFDM). AFDM values were then divided by the area of the artificial substrata and by the number of days incubated within the stream, in order to express the results per surface unit and day of accrual rate.

Organic matter decomposition

We measured organic matter decomposition (OMD) using tongue depressors (sticks, 15 x 1.8 x 0.2 cm) made of untreated Canadian poplar wood (*Populus nigra x canadensis*, Moench) (Arroita *et al.*,

2012). Wooden sticks were individually numbered, punched, oven-dried (70 ºC, 72 h) and weighed. Each depressor was individually tied with fishing line and deployed in the streams at five sites randomly distributed per reach, tied with nylon line to roots or to metal bars. Sticks were retrieved after 6 months of incubation. Upon removal, sticks were stored individually in laboratory filter paper and carried to the laboratory. Once there, depressors were washed and brushed to remove mineral particles, oven-dried (70 ºC, 72 h), weighed, combusted (500 ºC, 5 h) and re-weighed to estimate the ash-free dry mass (AFDM). Initial dry mass of sticks was corrected for leaching following Arroita *et al.* (2012). During the experiment, some of the sticks were broken, so we extrapolated the total mass of these sticks from the bits recovered using their remaining area as a reference. Organic matter decomposition rates were calculated following the negative exponential model (Petersen & Cummins, 1974). However, as streams differed significantly in temperature, we used the coefficients based on thermal sums ($k_{degree\text{-}day}$) to correct for its effect (Cummins *et al.*, 1989). For degree-day calculations, we calculated the thermal sum in degree days above 0 ºC on the collection day (*i.e.*, the sum of daily mean water temperature over the incubation period measured by water level dataloggers).

Whole-reach nutrient uptake

We measured whole-reach nutrient uptake for ammonium (NH_4^+, as NH_4Cl) and soluble reactive phosphorus (SRP, as KH_2PO_4) using the pulse addition technique (Martí & Sabater, 2009). We dissolved both nutrients in 30 L of stream water together with NaCl as a hydrological conservative tracer (Bencala *et al.*, 1987). Three reaches were dry or only presented some big and disconnected pools with stagnant water, so we could not measure the nutrient uptake. On each addition, we poured the solution to the stream in a single pulse at the head of the selected sub-reach, where appropriate mixing was ensured. At the downstream end, we automatically recorded EC every 10 s using a portable conductivity meter (WTW 340i SET) up to the return to basal conditions. During the breakthrough curve, we collected water samples for each nutrient, which were filtered (0.7-µm pore size, Whatman GF/F) and frozen until analysis. Finally, we used the mass-balance approach to estimate areal uptake (U, µg min^{-1} m^{-2}) for SRP (SRPAU) and NH_4^+ (NH4AU), which reflects the nutrient uptake capacity at reach scale (Martí & Sabater, 2009).

Metabolism

We measured ecosystem metabolism from diel DO changes by the open-channel method (Odum, 1956). We first set hydraulically homogeneous reaches in each of our study sites, and recorded DO and T at 5-min intervals during 24 h by means of optical oxygen probes (YSI 6150 connected to YSI 600 OMS; YSI Inc., Yellow Springs, OH, USA) deployed at the beginning and end of each reach. Depending on the hydraulic conditions of each reach we analyzed the results with either the single-station or the double-station method. We used the single-station method in five reaches that were longer than three times the ratio between water velocity (v) and re-aeration coefficient (k) ($3v\ k^{-1}$)

and the two-station method in the rest of reaches (Reichert *et al.,* 2009). Before the deployment and after field measurements, all probes were immersed together in the same bucket with aerated water to inter-calibrate them. We estimated reaeration coefficients with the night-time regression method (Hornberger & Kelly, 1975). We used the whole night data for the regression, but if it did not result significant we used different time intervals after sunset (*e.g.* 5 or 3 h) to improve the regression values. Nominal water travel time (*T*, min) was calculated by the time elapsed between the peaks of the breakthrough curves at the upstream and downstream sampling stations following several NaCl additions (Hubbard *et al.*, 1982). Finally, we calculated ecosystem respiration (*ER*), net ecosystem metabolism (*NEM*) and gross primary production (*GPP*) from DO variations (all expressed in g O_2 m^{-2} d^{-1}). ER was calculated as the sum of net metabolism rate during night and respiration rate during day, which was estimated by the linear interpolation between values of net metabolism rate of sunrise and sunset of the nights before and after the day of interest. NEM was calculated as the sum of the net metabolism over the 24 h of interest. GPP was calculated as the difference between ER and NEM.

Data analysis

Using the information above, we generated six data matrices. The first included the eight water chemical characteristics (*i.e.*, pH, EC, DO and concentrations of NH_4^+, NO_2, NO_3, SRP and DOC). We classified the *ca.* 80 PhACs in water samples according to their chemical structure and mechanism of action, and derived new variables by summing the relevant concentrations (Table S1 in Appendix S1). Thus, the second data matrix specified the eight groups of PhACs classified as biocides (*i.e.*, AbMacrolide, Metronidazole, Ofloxacin, Ronidazole, Sulfamethoxazole, Tiabendazole, Trimethropin and Levamisole), whereas the third defined the twenty one groups classified as non-biocide drugs (*i.e.*, Acetaminophen, ARBs, Benzodiazepine, BetaBlocker, Carbazepamine, CCB, Clopidogrel, Fibrate, Glibenclamide, H_2Antagonist, Iopromide, LoopDiuretic, Loratadine, NSAID, Opioid, Salbutamol, SSRI, Statin, Tamsulosin, Trazodone and Venlafaxine). The fourth data matrix described the six hydrological properties (*i.e.*, Temperature, Water flow, Width, Mean velocity, Depth and Hydrological stress). The fifth data matrix included the seven functional processes: biofilm biomass accrual (BAT), SRP uptake capacity (SRPUC), organic matter decomposition (OMD), whole-reach nutrient uptake of SRP (SRPAU) and NH_4^+ (NH4AU), and metabolism (GPP and ER). And finally, the sixth data matrix included the geographical location of the sampling units (*i.e.*, latitude and longitude).

Once the data shape and co-linearity were checked (Tukey, 1977), we applied univariate and multivariate techniques to test for the effects of factor Treatment (Control *vs.* Impact). Besides, in those variables linked to water chemical characteristics (*i.e.*, physico-chemical variables and concentration of nutrients, biocides and non-biocide drugs), which were measured during both sampling campaigns, we also tested for the effect of factor Time (autumn *vs.* spring) to see whether

chemical differences depended on the season. The effect of Time was not tested on hydrology and stream functioning, as they were measured only during the second campaign.

Non-metric multidimensional scaling (NMDS) (Legendre & Legendre, 2012), a technique for unconstrained ordination, was applied to disclose the main gradients in water chemical characteristics, hydrology and stream functioning, using the Euclidean distance. Multivariate analysis of variance (Anderson, 2001; McArdle & Anderson, 2001), also Euclidean-distance based, was applied to test for the main effects of Treatment and Time, as well as the interaction effect. This was achieved via functions metaMDS() and adonis2() of package vegan (Oksanen *et al.*, 2018); p-values were obtained using 9999 permutations. In the univariate case, to test for the same effects as in the multivariate case, we used mixed effects models (Pinheiro &Bates, 2000), obtaining the corresponding effect size. Effect size values were computed from parameter estimation obtained after linear mixed modelling applied to the individual environmental and functional variables. These were fitted with function lme of package nlme (Pinheiro *et al.*, 2018). The packages MuMIn (Barton, 2018) and sjPlot (Lüdecke, 2018) were used to obtain R^2 statistics and graphs, respectively.

Multivariate and univariate techniques were also applied to test for the relationship between stream functioning and water chemical characteristics together with hydrology. Redundancy analysis (RDA) (Legendre & Legendre, 2012), combined with stepwise variable selection and variation partitioning (Borcard *et al.*, 1992), was used in the multivariate case. Since stream functioning variables were measured at differing scales, they were standardized by subtracting the mean and then dividing by the standard deviation prior to analysis. Likewise, since these variables were found to be spatially autocorrelated, they were spatially detrended prior to analysis (spatial autocorrelation unduly affects model selection procedures). Since we detrended the response matrix, we also detrended all potentially explanatory variables. Stepwise variable selection was applied in two steps. In the first step, this procedure was used to find the best (four) partial models, *i.e.* models explaining multivariate variation in stream ecosystem functioning in terms of only water physico-chemical variables, only biocides, only non-biocides drugs, and only hydrology. We call these explanatory models partial (independent) RDA models. By combining the variables selected in each of the above procedures we obtained a non-parsimonious model explaining ecosystem functioning in terms of thirteen explanatory variables of all four kinds. Since the procedure above described took into account co-linearity between explanatory variables in each group (biocides, hydrology…), but not between explanatory variables classified in different groups, variance inflation factors and subsequent partitioning of variation showed that multiple sources of co-linearity were present in this non-parsimonious RDA model. For this reason, we applied a second stepwise selection procedure on the set of the previously selected 13 explanatory variables. As a result, we obtained a parsimonious RDA model. In the univariate case, generalised linear modelling was applied (Madsen &Thyregod, 2010).

R software was used for data analysis (R Core Team 2017). R coding (Appendix S2) and data (Appendix S3) sufficient to reproduce completely our methods are provided.

Results

Water chemical characteristics

Sewage inputs affected significantly water chemical characteristics of the Impact reaches in the thirteen streams (Table 2A.1, Table S3 and Fig. S1). The pH and DO concentration significantly decreased below sewage inputs, while EC did not vary significantly between reaches (Table 1A.1, Tables S4-S5). Effluents increased significantly the concentrations of most nutrients, including NO_2^-, NH_4^+, SRP and DOC, with the only exception of NO_3^-, which did not vary significantly between control and impact reaches (Table 1A.1, Table S4-S5). Besides, some of these chemical characteristics differed significantly between seasons, although the interaction between treatment and time was not statistically significant, showing that differences among treatments were not season dependant (Table 2A.1, Tables S4-S5). Nonetheless, except in the case of DO (R^2 = 37%), pH (R^2 = 24%) and NO_2^- (R^2 = 94%), the amount of variability in water chemical characteristics that was explained by sewage inputs or repeated sampling was minor.

Table 1. Effect size of sewage inputs (Treatment) and sampling campaigns (Time) for environmental and functional variables, expressed as the percentage of change observed in Impact reaches with respect to Control reaches and in spring with respect to autumn, respectively. Note that the size of the effect for Time (*i.e.* autumn vs. spring) is computed only for *Water chemical characteristics*, because the rest of the variables were measured only once (spring). If either Treatment or Time were not significant, effect sizes are not shown (this is indicated by the hyphen mark). If either Treatment or Time was significant, effect sizes are specified by their corresponding values, with green upward looking arrows indicating a positive change and red down looking arrows indicating a negative change.

Response	**EffectSize$_{Treatment}$ (%)**	**EffectSize$_{Time}$ (%)**
A) *Water chemical characteristics*		
1) *Physico-chemical variables and nutrients*		
pH	↓ 3	↑ 2
EC (µS cm^{-1})	-	↓ 11
DO (mg L^{-1})	↓ 40	↑ 25
NO_3^- (mg N L^{-1})	-	-
NO_2^- (mg N L^{-1})	↑ 1000	-
NH_4^+ (mg N L^{-1})	↑ 107	↓ 0.3
SRP (mg P L^{-1})	↑ 48	↓ 3
DOC (mg C L^{-1})	↑ 59	-
2) *Biocide concentration*		
AbMacrolide (ng L^{-1})	↑ 259	-
Levamisole (ng L^{-1})	↑ 3071	-
Metronidazole (ng L^{-1})	-	↓ 31500
Ofloxacin (ng L^{-1})	↑ 730	↓ 48

Ronidazole (ng L^{-1})	-	-
Sulfamethoxazole (ng L^{-1})	-	↓ 87
Tiabendazole (ng L^{-1})	-	-
Trimethropin (ng L^{-1})	-	-
3) *Non-biocide drugs concentration*		
Acetaminophen (ng L^{-1})	↑ 23629	↑ 93
ARBs (ng L^{-1})	↑ 2369	-
Benzodiazepine (ng L^{-1})	↑ 471	-
BetaBlocker (ng L^{-1})	↑ 19840	-
Carbazepamine (ng L^{-1})	↑ 1242	-
CCB (ng L^{-1})	↑ 370	-
Clopidogrel (ng L^{-1})	↑ 2000	↓ 100
Fibrate (ng L^{-1})	↑ 3836	-
Glibenclamide (ng L^{-1})	-	-
H_2Antagonist (ng L^{-1})	-	-
Iopromide (ng L^{-1})	↑ 800	↑ 767
LoopDiuretic (ng L^{-1})	↑ 1545	-
Loratadine (ng L^{-1})	-	-
NSAID (ng L^{-1})	↑ 2210	-
Opioid (ng L^{-1})	↑ 4833	-
Salbutamol (ng L^{-1})	-	↑ 600
SSRI (ng L^{-1})	-	-
Statin (ng L^{-1})	↑ 912	-
Tamsulosin (ng L^{-1})	-	-
Trazodone (ng L^{-1})	↑ 2000	-
Venlafaxine (ng L^{-1})	↑ 1350	-
B) *Hydrology*		
Temperature (ºC)	-	-
Water Flow (m^3 s^{-1})	-	-
Width (m)	-	-
Mean Velocity (m s^{-1})	-	-
Depth (m)	-	-
Hydrological Stress (days)	-	-
C) *Ecosystem functioning*		
Biofilm biomass accrual (g m^{-2} d^{-1})	↑ 83	-
Biofilm SRP uptake capacity (µg P h^{-1})	↓ 111	-
Whole-reach SRP areal uptake (µg P m^{-2} min^{-1})	-	-
Whole-reach NH_4 areal uptake (µg N m^{-2} min^{-1})	↑ 11650	-
Gross primary production (mg O_2 m^{-2} d^{-1})	-	-
Ecosystem respiration (mg O_2 m^{-2} d^{-1})	↑ 138	-
Organic matter decomposition ($dd^{-1}.10^{-5}$)	-	-

Sewage inputs (Treatment), season (Time), and Treatment:Time were found to affect water biocide concentration (Table 2A.2, Table S7 and Fig. S3). Some biocides, such as Metronidazole, Ronidazole, Sulfamethoxazole, Tiabendazole and Trimethoprim, showed no significant differences

between reaches, while others, including AbMacrolides, Levamisole and Ofloxacin, increased significantly in the Impact reaches (Table 1A.2, Tables S8-S9). Biocide concentration differed significantly between sampling campaigns, and the interaction between treatment and time was also statistically significant (Table 2A.2, Tables S8-S9), showing that the concentration of biocides depends on season. For Levamisole, Metronidazole and Ofloxacin, both sewage inputs and season explained more than 90% of observed variation.

Table 2. Permutational multivariate analyses of variance applied to environmental and functional variables. In each case, multivariate variation was represented by an Euclidean distance matrix, which was partitioned among the factors Treatment (*i.e.* Control *vs.* Impact), Time (*i.e.* autumn *vs.* spring, only when possible) and the interaction between them. Two of the sampled streams (La Sènia and Els Reguers) were not included in the analyses due to missing data points (this technique cannot handle unbalanced data); thus, data for only eleven streams were considered. Degrees of freedom may differ according to the number of available sampling campaigns (one *vs.* two).

Source	**n, *d.f.***	**MS**	***F*-value**	***p*-value**	**R^2 (%)**
A) *Water chemical characteristics*					
1) Physico-chemical variables and nutrients					
Treatment	1, 43	44.67	8.92	**0.014**	13
Time	1, 43	11.21	4.10	**0.004**	3
Treatment x Time	1, 43	1.83	0.67	0.637	-
2) Biocide concentration					
Treatment	1, 43	17.87	3.05	0.110	-
Time	1, 43	14.54	3.03	**0.001**	6
Treatment x Time	1, 43	9.35	1.99	**0.021**	4
3) Non-biocide drugs concentration					
Treatment	1, 43	115.17	4.60	**0.058**	13
Time	1, 43	22.19	2.28	**0.041**	3
Treatment x Time	1, 43	21.67	2.23	**0.046**	2
B) *Hydrology*					
Treatment	1, 21	178.60	1.06	0.331	-
Time	-	-	-	-	-
C) *Ecosystem functioning*					
Treatment	1, 21	19.31	4.76	**0.002**	16
Time	-	-	-	-	-

The concentration of non-biocide drugs was higher in Impact than in Control reaches (Table 2A.3, Table S11 and Fig. S5). Although most PhACs increased in the impact reaches, this was especially remarkable for Acetaminophen, ARBs, β-Blockers, Fibrates, Loop Diuretics and NSAIDs (Table 1A.3, Tables S12-S13). However, some PhACs, such as Glibenclamides, H_2Antagonists, Loratidine, Salbutamol, SSRIs and Tamsulosin, did not significantly increase (Table 1A.3, Tables S12-S13). Season (Time), as well as the interaction between Treatment and Time, were found to be significant (Table 2A.3, Tables S12-S13), suggesting that season affected differences between reaches.

Hydrology

All hydrological properties remained unaltered, including mean water velocity, flow, temperature, wet-channel width, depth and hydrological stress in the Impact reaches (Table 2B, Tables S16-S17). Therefore, sewage effluents had no significant overall effects on stream hydrology (Table 2B, Table S15 and Fig. S7).

Ecosystem functioning

Stream functioning was significantly altered by sewage inputs, although the effects were especially noticeable when streams received untreated sewage (Table 2C, Fig. 2 and Table S19). However, the direction of change differed among processes. While biofilm biomass accrual, whole-reach NH_4^+ areal uptake and ecosystem respiration were subsidized, biofilm SRP uptake capacity decreased significantly. The rest of measured processes, including whole-reach SRP areal uptake, gross primary production and organic matter decomposition, remained unaltered (Table 1C, Tables S20-S21).

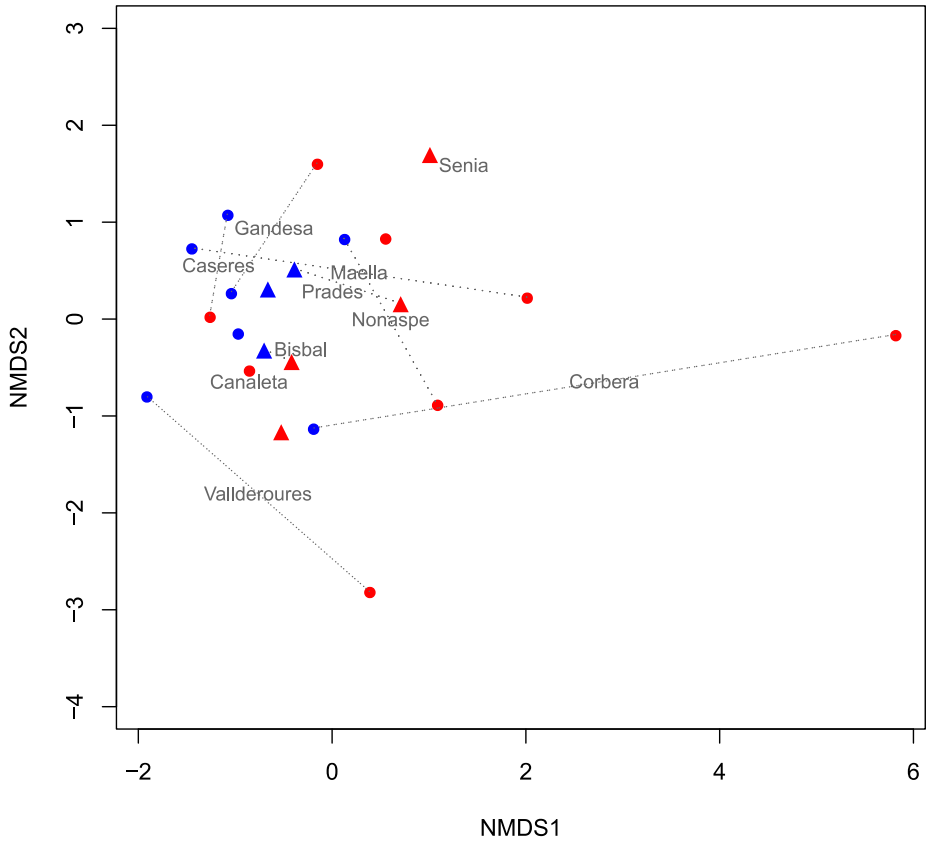


Figure 2. Main gradients in stream ecosystem functioning, as described with non-metric multidimensional scaling (NMDS). Impact reaches are indicated in red and Control reaches in blue. Note that distinction between streams receiving treated (triangles) and untreated (dots) sewage is also made. Stress = 0.12; non-metric goodness-of-fit of the ordination: R^2 = 0.987; linear goodness-of-fit of the ordination: R^2 = 0.961. Poboleda and Bisbal (not represented in the diagram to avoid crowding) are close to PratCompte and Prades, respectively. See also Table 2C.

Results-oriented to predict and explain stream functioning: non-parsimonious RDA models

We built four partial (independent) RDA models to explain multivariate variation in stream ecosystem functioning in terms of heterogeneity in only: (A) water chemical characteristics (Table 3A; adj.-R^2 = 14%, using two explanatory variables: SRP and DOC), (B) biocides (Table 3B; adj.-R^2 = 52%, using four explanatory variables: AbMacrolides, Tiabendazole, Ofloxacin and Sulfamethoxazole), (C) non-biocide drugs (Table 3C; adj.-R^2 = 64%, using five explanatory variables: Opioid, Iopromide, Salbutamol, NSAID and SSRI) and (D) hydrology (Table 3D; adj.-R^2 = 34%, using two explanatory variables: Water flow and Width). These partial models, which used only a subset of the available variables, differed greatly in their capacity to explain variability in ecosystem functioning and in the number of variables selected (Table 3).

The partitioning of variation applied to a non-parsimonious RDA model obtained by the addition of the four partial models (Fig. 3) explained ca. 70% of the variation observed in stream ecosystem functioning using a total of 13 explanatory variables. The largest contribution corresponded to the fraction shared by physico-chemical variables and nutrients, and the concentrations of biocide and non-biocide drugs (adj.-R^2 = 31%). The fraction shared by biocides and non-biocide drugs was second in importance (adj.-R^2 = 17%).

Table 3. Partial (independent) redundancy analysis (RDA) models explaining multivariate variation in stream ecosystem functioning according to heterogeneity in only water chemical characteristics (A), only biocides (B), only non-biocides drugs (C) and only hydrology (D). The response matrix is in every case constituted by the same spatially detrended and standardized functional variables. In each case (A-D), the explanatory variables were selected by means of a stepwise procedure. *p*-values were estimated using permutation tests (9999 permutations).

Source	***d.f.***	**Variance**	***F*-value**	***p*-value**	**Variance inflation**
***A**. Model for water physico-chemical variables and nutrients*					
SRP	1	1.847	10.4	< **0.0001**	1.8
DOC	1	0.408	2.3	**0.060**	1.8
Residual	19	3.360			
Total	21	5.616			
Adjusted R^2 = 34%					
***B**. Model for biocide concentrations*					
AbMacrolides	1	1.888	14.7	< **0.0001**	1.2
Tiabendazole	1	0.657	5.1	**0.009**	1.2
Ofloxacin	1	0.471	3.7	**0.048**	1.1
Sulfamethoxazole	1	0.418	3.3	**0.030**	1.3
Residual	17	2.181			
Total	21	5.616			
Adjusted R^2 = 52%					
C. Non-biocides drugs model					
Opioid	1	2.305	23.6	< **0.0001**	3.5
Iopromide	1	0.323	3.3	**0.042**	1.5
Salbutamol	1	0.460	4.7	**0.016**	4.8
NSAIDs	1	0.472	4.8	**0.018**	4.6

SSRI	1	0.493	5.0	**0.003**	5.6
Residual	16	1.563			
Total	21	5.616			
Adjusted R^2 = 64%					
D. Hydrology model					
Water flow	1	0.611	2.7	**0.050**	1.3
Width	1	0.646	2.8	**0.036**	1.3
Residual	19	4.359			
Total	21	5.616			
Adjusted R^2 = 14%					

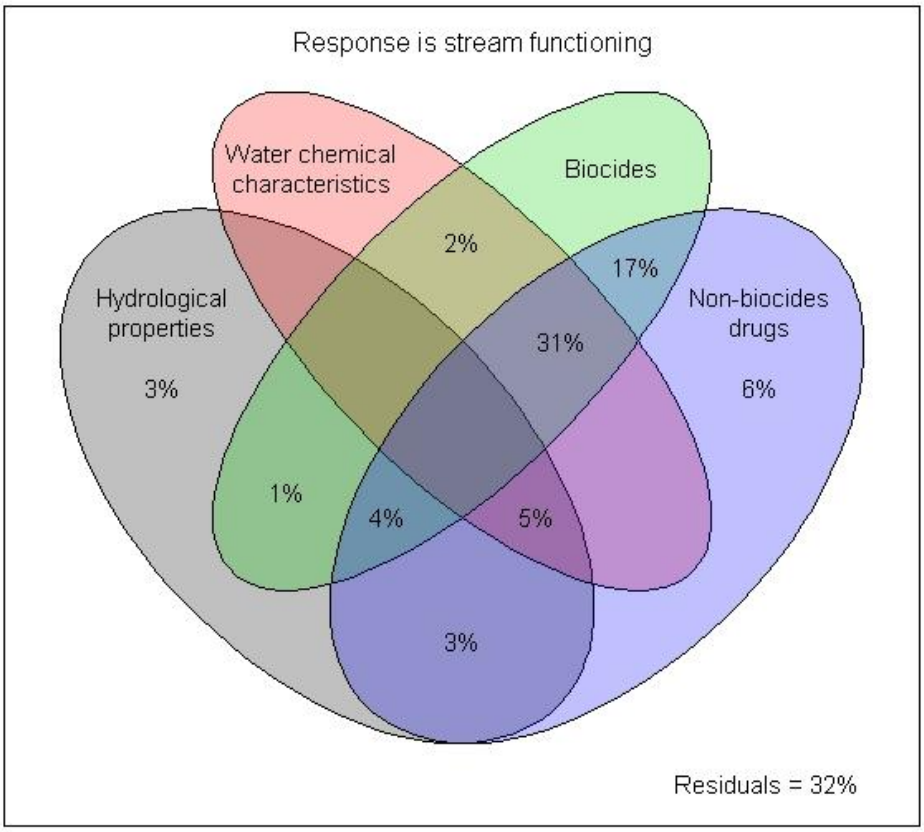


Figure 3. Venn diagram showing the partitioning of multivariate variation in stream ecosystem functioning among hydrological, chemical, biocides (incl. antibiotics, antifungals, and parasiticides) and non-biocide drugs (*i.e.* pharmaceutical drugs other than biocides) components. The values shown correspond to adjusted R^2 (%). This non-parsimonious model, which explains ca. 70% of multivariate variation in stream ecosystem functioning, was obtained by the addition of four partial models (detailed in Table 3). The hydrological component includes Water flow and Width. The chemical component includes SRP and DOC. The biocides component includes AbMacrolides antibiotics (Clarithromycin and Erythromycin), the antibiotics Ofloxacin and Sulfamethoxazole, and the antifungal and parasiticide Tiabendazole. The non-biocide drugs component includes Opioids (Oxycodone and Codeine), Iopromide (a contrast medium), Salbutamol (a β_2 adrenergic receptor agonist), NSAIDs (Diclofenac, Ibuprofen, Indomethacine, Ketoprofen, Naproxen, Piroxicam, Salicylic acid, and Phenazone), and SSRI (Seproxetine, Fluoxetine, and Citalopram). The variables AbMacrolides, Opioids, NSAIDs and SSRI were obtained by adding up the concentrations of the biocides and drugs named in parentheses.

Predicting stream ecosystem functioning: parsimonious RDA model

The model above (Fig. 3) predicted and, to some extent, explained the observed multivariate variability in stream ecosystem functioning using a subset of 13 environmental variables (out of the originally considered ca. 100 potentially explanatory variables). This model, though, was non-parsimonious and presented multiple co-linearities between the selected explanatory variables in the different groups (*e.g.* between biocide and non-biocide variables). Therefore, in order to just predict multivariate variation in stream ecosystem functioning in a parsimonious manner, as observed in the Baix Ebre area, we subsequently built a second (parsimonious) model.

The subsequent application of a second variable selection procedure led to a parsimonious RDA model (Table 4, Figs. 4-5), which explained 65% of the observed variability in stream ecosystem functioning considering only four environmental variables: two biocides (AbMacrolide, a class of antibiotics that inhibit protein synthesis in microorganisms, and the antifungal Tiabendazole) and two non-biocide drugs (Iopromide, a contrast medium used in computerized tomography scan, and NSAIDs, which includes common nonsteroideal anti-inflammatory drugs, such as Diclofenac and Ibuprofen). The strongest associations occurred between AbMacrolide concentration and ecosystem respiration (positive), biomass accrual (positive), and biofilm SRP uptake capacity (negative); between NSAIDs and ecosystem respiration (positive) and biofilm SRP uptake capacity (negative); and between Tiabendazole concentration and SRP areal uptake (positive). Once these four variables were selected, all other variables presented in the four partial models were found to be statistically non-significant. Overall, excluding the shared fraction (adj.-R^2 = 6%), in this parsimonious model, biocides contributed more than non-biocide drugs to the explanation of stream functioning variability (adj.-R^2 = 34% *vs.* adj.-R^2 = 25% respectively; Fig. 5).

Table 4. Parsimonious redundancy analysis (RDA) model explaining multivariate variation in stream ecosystem functioning according to heterogeneity in four stepwise-selected environmental explanatory variables: Tiabendazole and AbMacrolides (biocides), and NSAIDs and Iopromide (non-biocide drugs). The response matrix is constituted by the spatially detrended and standardized functioning variables. The environmental explanatory variables were selected by means of a second stepwise procedure that started from the set of the thriteen variables (SRP, DOC, AbMacrolides, Tiabendazole, Ofloxacin, Sulfamethoxazole, Opiod, Iopromide, Salbutamol, NSAIDs, SSRIs, Water flow and Width) previously selected variables in the RDA partial models (Table 3).

Source	***d.f.***	**Variance**	***F*-value**	***p*-value**	**Variance inflation**
NSAIDs	1	1.701	18.0	< **0.0001**	1.4
Tiabendazole	1	0.896	9.5	< **0.0001**	1.8
AbMacrolides	1	0.917	9.7	< **0.0001**	1.4
Iopromide	1	0.492	5.2	**0.014**	1.6
Residual	17	1.610			
Total	21	5.616			
Adjusted R^2 = 65%					

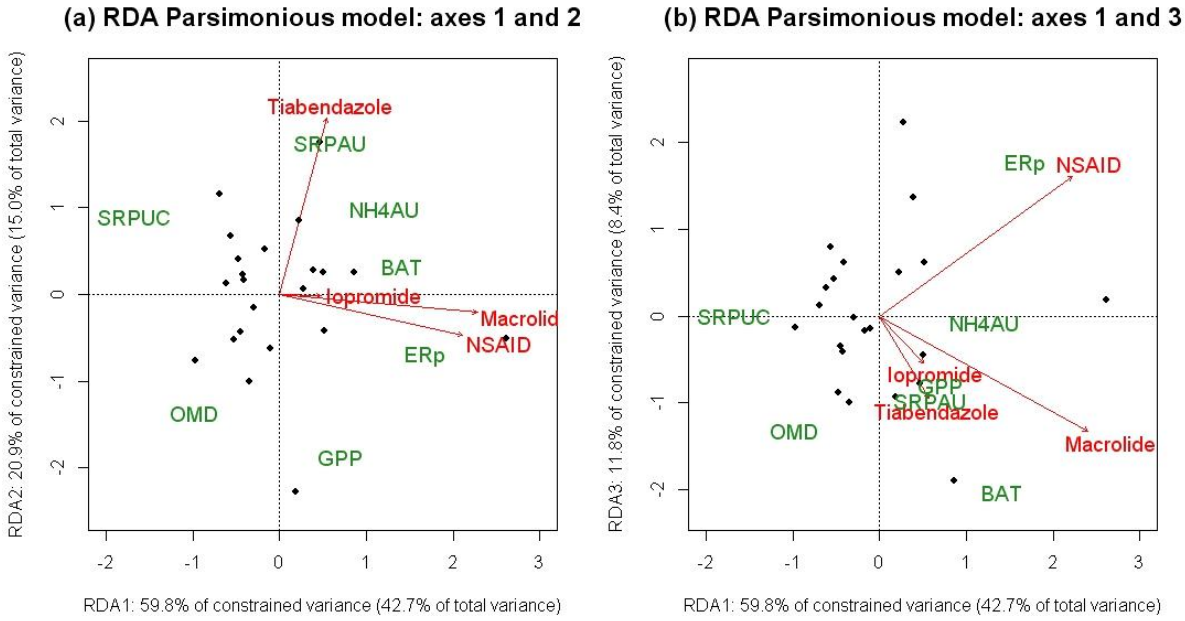


Figure 4. Biplots corresponding to the parsimonious redundancy analysis (RDA) model summarized in Table 4: (a) axis 1 vs. axis 2; (b) axis 1 vs. axis 3. The strongest associations occur between AbMacrolides and ERp (positive), BAT (positive), and SRPUC (negative); between NSAID and ERp (positive) and SRPUC (negative); and between Tiabendazole and SRPAU (positive). Note that acronyms for ecosystem functioning mean the following: BAT = biofilm biomass accrual, SRPUC = biofilm SRP uptake capacity, SRPAU = SRP areal uptake, NH4AU = NH_4^+ areal uptake, GPP = gross primary production, ER = ecosystem respiration and OMD = organic matter decomposition. See variance partitioning in Figure 5.

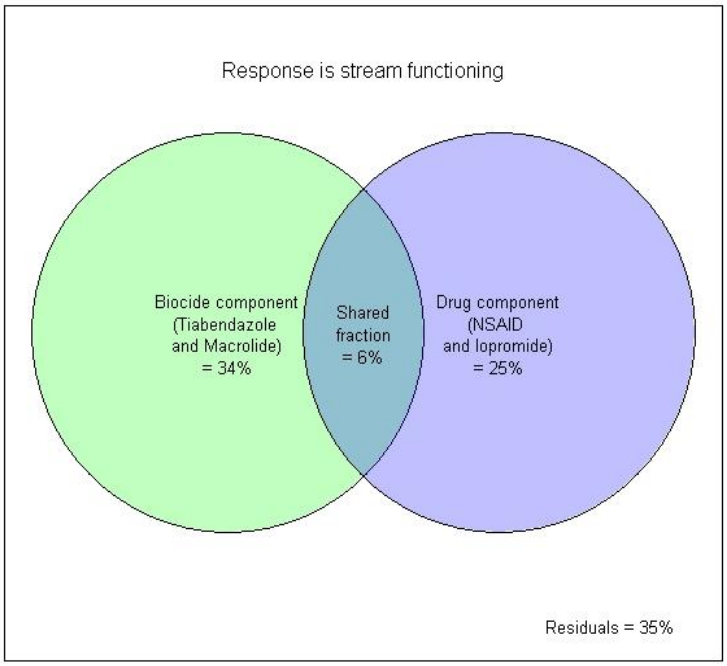


Figure 5. Venn diagram showing the partitioning of multivariate variation in stream ecosystem functioning between a biocide component and a non-biocide drug component. The values correspond to adjusted R^2 (%). This

parsimonious model, which explains ca. 65% of multivariate variation in stream ecosystem functioning, was obtained by further variable selection applied on the non-parsimonious model. The biocide component includes just AbMacrolide antibiotics and the antifungal and parasiticide Tiabendazole. The non-biocide drugs component includes only Iopromide and NSAIDs (Diclofenac, Ibuprofen, Indomethacine, Ketoprofen, Naproxen, Piroxicam, Salicylic acid, and Phenazone). The variables AbMacrolides and NSAIDs were computed by adding up the concentrations of the biocides and drugs named in parentheses.

Predicted individual ecosystem processes vs. individual environmental predictors

To analyse the form and strength of the relationships between the seven functional variables and the four environmental explanatory variables selected in the parsimonious RDA model, we applied GLMs (Table 5). Most significant relationships were linear (L), with the only exceptions of the relationships found between biofilm SRP uptake capacity and ecosystem respiration with AbMacrolide concentration. These relationships followed a curvilinear pattern (CL), but not the Subsidy-Stress pattern we expected. In addition, most relationships were positive, indicating that most processes were subsidized with increasing pollutant concentrations. However, there were also some negative relationships (*i.e.*, between organic matter decomposition and NSAIDs, biofilm SRP uptake capacity and AbMacrolides, and between biofilm SRP uptake capacity and NSAIDs). The explained variation showed a large variability, ranging from R^2_{GLM} = 17%, in the case of gross primary production and AbMacrolide concentration, to R^2_{GLM} = 83%, in the case of whole-reach SRP areal uptake and Tiabendazole concentration.

Table 5. Summary of GLMs used to test for the relationship between ecosystem functioning and environmental explanatory variables selected in the parsimonious RDA model. Note that the form of the response is also specified, either if it is linear (L) or curvilinear (CL), positive (+) or negative (-). Only significant relationships are shown.

Functional var.	**Explanatory var.**	**Null deviance (*d.f.*)**	**Residual deviance (*d.f.*)**	**R^2_{GLM} (%)**	***p*-value**	**Form**
NH_4^+ areal uptake	AbMacrolides	11.3 (21)	7.4 (20)	34	**0.001**	L+
NH_4^+ areal uptake	NSAIDs	11.3 (21)	8.3 (20)	27	**0.007**	L+
NH_4^+ areal uptake	Tiabendazole	11.3 (21)	6.9 (20)	39	< **0.0001**	L+
NH_4^+ areal uptake	Iopromide	11.3 (21)	8.8 (20)	22	**0.018**	L+
Biofilm SRP uptake capacity	AbMacrolides	19.2 (21)	6.3 (19)	67	< **0.0001**	CL-
Biofilm SRP uptake capacity	NSAIDs	19.2 (21)	9.8 (20)	49	< **0.0001**	L-
Ecosystem respiration	AbMacrolides	19.4 (21)	9.5 (19)	51	< **0.0001**	CL+
Ecosystem respiration	NSAIDs	19.4 (21)	3.5 (20)	82	< **0.0001**	L+
Biofilm accrual rates	AbMacrolides	20.9 (21)	6.8 (20)	67	< **0.0001**	L+
Gross primary production	AbMacrolides	18.2 (21)	15.0 (20)	17	**0.040**	L+
Organic matter decomposition	NSAIDs	18.9 (21)	14.3 (20)	24	**0.011**	L-
SRP areal uptake	Tiabendazole	10.0 (21)	1.7 (20)	83	< **0.0001**	L+
SRP areal uptake	Iopromide	10.0 (21)	7.6 (20)	24	**0.012**	L+

Discussion

Water chemistry: main factor driving variability

Pollution and hydrological alteration are some of the major pressures affecting European streams (EC, 2015). Among them, sewage inputs stand out as one of the most common stress factors, although it is often difficult to predict their effects because most streams are subjected to multiple stressors (Sabater *et al.*, 2018). Our results showed that sewage inputs significantly altered stream ecosystem functioning, mainly via changes in water chemical characteristic. Two physico-chemical variables (SRP and DOC) and, specially, the concentration of nine biocides (including AbMacrolide antibiotics and the antifungal Tiabenzazole) and non-biocide drugs (including Iopromide and NSAIDs), accounted for most (ca. 70%) of the multivariate variation observed in stream ecosystem functioning, and were found to be associated with most of the ecological processes measured. In this non-parsimonious RDA model, the contribution of hydrological properties to the explanation of stream ecosystem functioning was minor.

Additionally, we also used the data from this observational experiment to build a stringently parsimonious and purely predictive RDA model. This second RDA model, which is almost free of co-linearity issues, succeeded at accounting for 65% of multivariate variation in stream ecosystem functioning in terms of just four environmental variables (AbMacrolide antibiotics, Tiabendazole, Iopromide and NSAIDs), out of the nearly 100 originally measured. Since prediction is not the exactly the same as explanation, this parsimonious RDA model is not to be taken as an indication that stream ecosystem functioning can be explained in terms of just these four variables, *i.e.* this model does not indicate that these four variables caused 65% of the variation in ecosystem function. Rather, this model, which maximizes the amount of multivariate variation accounted for and minimizes the number of predictive variables, indicates that these four variables, taken together, summarize and optimize the predictive power of the nearly 100 environmental variables originally measured. It is optimized prediction, without explicit causal explanation.

Sewage inputs increased the concentration of nutrients and contaminants, as it is common in polluted streams subjected to the "*Urban Stream Syndrome*" (Walsh *et al.*, 2005; Brack *et al.*, 2007). Effluents decreased pH as well as DO up to anoxic conditions in some streams (< 1 mg L^{-1}), but increased the concentration of DOC, and nutrients, especially of SRP and NH_4^+, with maximum values reaching 51, 2 and 22 mg L^{-1}, respectively. These results agree with previous studies assessing the effects of urban effluents, in which pH (Englert *et al.*, 2013) and DO (Walsh *et al.*, 2005) decreased, while concentrations of OM and nutrients increased (Carey & Migliaccio, 2009), especially those of phosphorus and nitrogen (Waiser *et al.*, 2011). Concentrations in some of the sites are among the highest measured across European streams (Grizzetti *et al.*, 2017). Besides, sewage inputs also increased the concentration of PhACs, especially those of non-biocide drugs. Biocides, on the contrary, did not show significant overall differences between reaches, appearing

also at high concentrations in some of the studied Control reaches (Mandaric *et al.*, 2018). The most ubiquitous biocide was Ofloxacin, and the most ubiquitous non-biocide was NSAIDs. Their concentrations varied from a few ng L^{-1} to almost 9 µg L^{-1}, within the ranges reported in previous studies analysing the occurrence of PhACs in urban wastewaters (Gros *et al.*, 2012; Verlicchi *et al.*, 2012). These complex mixtures of contaminants can deteriorate physico-chemical conditions and induce other effects (Proia *et al.*, 2013). They can increase biological oxygen demand and thus, accentuate oxygen depletion (Calapez *et al.*, 2017). Furthermore, they can promote reductive conditions and proliferations of sulphate reductive and methanogenic microbial communities (Atashgahi *et al.*, 2015). In any case, these indirect effects can overwhelm some of the direct effects and thus, exacerbate the effects of pollution in the receiving streams.

As a consequence of large inter-site variability, sewage inputs did not have an overall significant effect on the hydrology of the study sites, in contrast to results elsewhere (*e.g.*, Merseburger *et al.*, 2011). At some streams, such as Els Reguers, Prat de Compte or La Sènia, sewage inputs converted perennial streams into temporary, whereas in larger streams, such Caseres, Nonaspe, Maella and Vallderoures), urban effluents had no detectable effects on hydrology.

The dilution capacity of the receiving water body and the quality of the sewage input determined the effect on ecosystem functioning. Thus, changes in ecosystem processes between Control and Impact reaches were smaller when urban effluents were treated and released into larger streams. However, our results showed that chemical characteristics prevailed over hydrology explaining most of the variability observed in stream ecosystem functioning. These results agree with other studies that highlight the role of emerging contaminants on streams (Kuzmanovic *et al.*, 2015), and recognize pollution to be the main factor influencing the structure of biological communities (Karaouzas *et al.*, 2018), the functioning of streambed biofilm (Proia *et al.*, 2013) or whole-reach processes, such as nutrient uptake (Marcé *et al.*, 2018).

Predicting variability in stream ecosystem functioning

Sewage inputs caused complex and process-specific responses in terms of ecosystem functioning. However, none of the processes followed the subsidy-stress pattern we expected and instead, most of them linearly increased with increasing levels of pollution. For instance, whole-reach NH_4^+ areal uptake significantly increased below sewage inputs and was positively correlated to the increasing concentration of drugs. Sewage inputs are an important source of emerging contaminants, OM and nutrients, especially of nitrogen and phosphorus (Waiser *et al.*, 2011). In our experiment, sewage inputs increased ambient NH_4^+ concentrations up to 3.5 mg N L^{-1}, which is in the lower range of concentrations described below effluents (0.1-10 mg N L^{-1}) and still far from saturation (Bernal *et al.*, 2017). Thus, these inorganic N loads could have stimulated stream nitrification, promoting ammonium areal uptake (Merbt *et al.*, 2015; Bernal *et al.*, 2017). In contrast, sewage inputs did not affect whole-reach SRP areal uptake in Impact reaches. However, individual relationships between

ecosystem processes and environmental explanatory variables, predicted that it could increase with increasing concentrations of certain drugs, such as biocides. According to the "*Efficiency loss hypothesis*" (O´Brien *et al.*, 2007; Mulholland *et al.*, 2008), most works report reduced SRP uptake in streams receiving sewage inputs, as P-demand decreases with increasing ambient concentration (*e.g.*, Haggard *et al.*, 2001, 2005; Martí *et al.*, 2004; Merseburger *et al.*, 2005; Gibson & Meyer, 2007). Besides, this possible increase in SRP areal uptake also contrasts with the observed results at the biofilm scale, where the SRP uptake capacity was seriously reduced. Consequently, our results suggest that SRP uptake at whole-reach scale was compensated, and thus increased, by other ecosystem compartments and biological communities (Withers & Jarvie, 2008). Specifically, fine sediments (Withers & Jarvie, 2008) and macrophytes (Riis *et al.*, 2012), either growing in the main channel or near stream banks, could have accounted for these differences.

Sewage inputs also promoted ecosystem metabolism. Ecosystem respiration significantly increased below urban effluents and besides, the individual relationships between ecosystem processes and environmental explanatory variables predicted that it could be positively correlated to increasing concentration of drugs. Conversely, gross primary production remained unaltered below sewage inputs and individual relationships only predicted a slight increase with increasing concentration of certain drugs, such as biocides. It is well known that nutrients and organic matter promote ecosystem respiration (Bernhardt *et al.*, 2018), but we still lack a clear picture about how these substances interact with toxic compounds, such as heavy metals or pharmaceuticals. As observed in some previous studies (Izagirre *et al.*, 2008; Aristi *et al.*, 2015), ecosystem respiration increased with increasing pollutant concentrations. It has been suggested that ecosystem respiration can be promoted by pharmaceutical exposure (Corcoll *et al.*, 2015), as well as by a mixture of assimilable and toxic compounds (Aristi *et al.*, 2016). Gross primary production, on the other hand, was not significantly affected by urban effluents and only had a weak positive response to the concentration of certain drugs. While some authors describe increased primary production below urban effluents (*e.g.*, Gücker *et al.*, 2006), some others do not show clear effects, and attribute this lack of response to the fact that primary production can be mediated by other environmental factors, such as light (Aristi *et al.*, 2015), riparian vegetation (Bernot *et al.*, 2010) or turbidity (Izagirre *et al.*, 2008). Among these factors, turbidity could have limited production rates in our streams (Hall *et al.*, 2015), especially in those receiving raw effluents, which can be very turbid. In addition, other authors (Hill *et al.*, 2001; Acuña *et al.*, 2004) showed increased ecosystem metabolic rates coupled with high biofilm biomass accrual rates below sewage inputs. In the present experiment, however, biofilm biomass was unexpectedly correlated to certain drugs, such as AbMacrolides, which are antibiotics that inhibit the protein synthesis in bacteria. Although we can only speculate about this correlation, our results suggest that sewage inputs affected the biofilm community, favouring more resistant and tolerant species, which maintained functional rates (Rosi *et al.*, 2018); also, autotrophic biomass could have overwhelmed bacterial heterotrophic communities (Proia *et al.*, 2013). Similar results were found in some parallel works to this experiment, in which pollution-tolerant diatom

(Tornés *et al.*, 2018) and invertebrate (Mor *et al.*, 2019) taxa prevailed in the studied reaches below sewage inputs. Another possible explanation is that biofilms developed antibiotic resistance genes, as shown in another parallel work to this experiment (Subirats *et al.*, 2017).

Contrary to ecosystem-level processes, which showed less evident and contrasting response patterns, biofilm-scale processes showed more consistent response. Biofilm biomass accrual rates were subsidized significantly below sewage inputs, whereas biofilm SRP uptake capacity and organic matter decomposition became stressed. Biofilm SRP uptake capacity significantly decreased below urban effluents and it was negatively correlated to increasing concentrations of drugs. Conversely, organic matter decomposition, even if it remained unaltered below sewage inputs, slightly decreased with increasing concentration of certain drugs, such as non-biocides. In the present experiment, SRP loads derived from sewage inputs could have made biofilm less effective in removing nutrients from the water column, even reducing uptake rates to zero due to biofilm saturation (Earl *et al.*, 2006), as it has been shown in a laboratory experiment (Pereda *et al.*, 2019). On the other hand, some of the toxic drugs derived from sewage inputs could have also reduced the biofilm capacity to retain dissolved nutrients, which has also been described in other studies (Proia *et al.*, 2017). Additionally, our results are in line with other recent studies (e.g. Smeti *et al.*, 2019), indicating that urban effluents can have toxic effects on stream microbial communities and trigger detrimental effects on their functioning, not only deteriorating their capacity to retain nutrients, but also their ability to consume organic matter.

Behind the Subsidy-Stress hypothesis

Individual relationships between ecosystem functioning and environmental variables showed linear responses to the gradient of pollution, none of them following the Subsidy-Stress pattern we expected. Specifically, we predicted urban effluents to subsidize functional processes at high dilution rates, but to stress them at high concentrations, resulting in the hump-shape response observed in other field and laboratory experiments (Carmargo & Alonso, 2006; Niyogi *et al.*, 2007; Wagenhoff *et al.*, 2011, 2012, 2013; Woodward *et al.*, 2012). However, functional processes only resulted in either positive or negative linear responses. Subsidy-stress patterns have been widely described when analyzing the effects of single stressors, such as fine sediment (Wagenhoff *et al.*, 2012) or nutrient concentration (Woodward e*t al.*, 2012), but these patterns usually disappear when assessing the combined effects of multiple stressors (Wagenhoff *et al.*, 2013). In that sense, urban wastewater pollution is recognized as a multiple stressor on its own, due to the complex and ever-changing mixture of contaminants (Jackson *et al.*, 2016).

Conclusions

In summary, our experiment shows that sewage inputs can seriously impair stream chemical characteristics and lead to complex effects on ecosystem functioning below sewage inputs. Urban effluents significantly affected most of the functional processes, although they did not follow the Subsidy-Stress pattern we expected, and instead showed either positive or negative responses to the gradient of pollution. Finally, biofilm-level processes showed more clear and consistent responses to sewage inputs than ecosystem-level ones.

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CHAPTER 3

Impact of wastewater effluent pollution on stream functioning: a whole-ecosystem manipulation experiment

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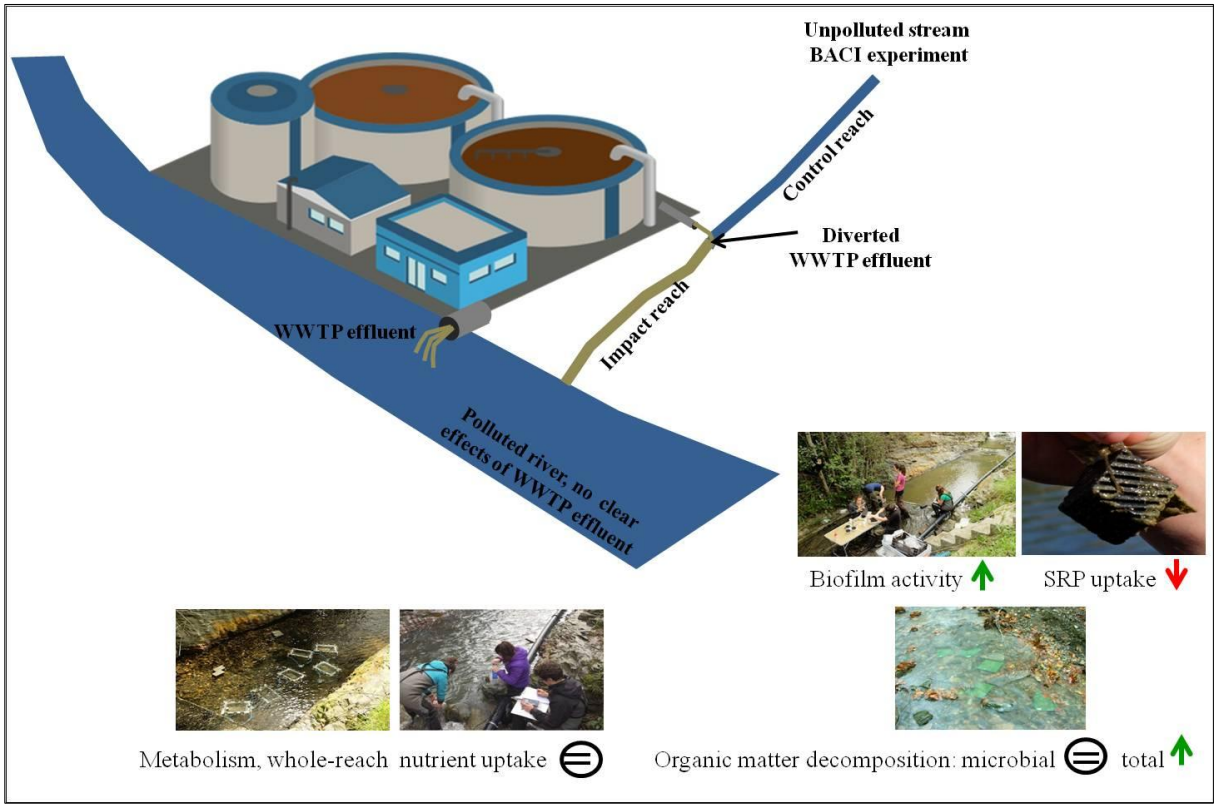
Environmental Pollution, *submitted*

Abstract

There is growing concern about the ecological effects of waste water treatment plant (WWTP) effluents on stream ecosystems. However, it is difficult to detect and isolate these effects because most streams receiving WWTP effluents are also affected by other stressors. Here, we show the results of a whole-ecosystem manipulation experiment following a BACI design (Before-After/Control-Impact), in which we diverted part of the effluent of a large tertiary urban WWTP into a small nearly unpolluted stream, and studied the structural and functional responses of the ecosystem. Despite of being highly diluted, the effluent caused an increase in biofilm chlorophyll-*a* and biomass, exo-enzymatic activities and invertebrate-mediated organic matter decomposition, whereas phosphorus uptake capacity of the biofilm was reduced. Biofilm metabolism, reach-scale nutrient uptake and microbial organic matter decomposition were not affected by the effluent. Our results indicate that even well treated and highly diluted WWTP effluents can have significant and complex effects on stream ecosystem structure and functioning.

Keywords: Urban pollution; BACI; ecosystem-level manipulation; effluent dilution; ecosystem response.

Graphical Abstract



Introduction

Recent economic development and demographic changes have prompted an unprecedented growth of urban population (Jones & O´Neill, 2016). As a side effect of this expansion, complex mixtures of biologically active substances are continuously released into aquatic environments, threatening freshwater ecosystems worldwide. Developed countries have invested large sums in sanitation and implementation of wastewater treatment plants (WWTP). For example, in Europe over 80% of the population is already connected to WWTP facilities (EEA, 2018). Nevertheless, WWTP effluents still consist of complex mixtures of pollutants, including organic matter and nutrients, as well as metals, pesticides and organic micro-pollutants (Aristi *et al.*, 2015; Kuzmanović *et al.*, 2015). Some of these substances are toxic and reduce biological activity, whereas others, such as nutrients, promote biological activity at low concentrations but reduce it above a threshold, resulting in the so-called Subsidy-Stress response (Odum *et al.*, 1979).

Effluents of WWTPs can affect all types of stream organisms, including microbes (Drury *et al.*, 2013), algae (Corcoll *et al.*, 2015), macrophytes (Gücker *et al.*, 2006), invertebrates (Ortiz & Puig, 2007) and fish (Northington & Hershey, 2006). Moreover, WWTP effluents impact stream ecosystem functioning by altering key processes, although the response can vary depending on the biological activity measured (Berthelsen *et al.*, 2018). For instance, WWTP effluents can enhance biofilm accrual (Ribot *et al.*, 2015) or exo-enzymatic activities (Carreiro *et al.*, 2000), which can consequently subsidize ecosystem metabolism (Aristi *et al.*, 2015) and litter decomposition (Ferreira *et al.*, 2015), while they can decrease whole-reach nutrient uptake (Martí *et al.*, 2009). However, some studies assessing the effects of WWTP effluents on stream ecosystem processes report contrasting results. For example, while some studies describe increased organic matter decomposition rates (*e.g.*, Solagaistua *et al.*, 2018), others show a reduction (*e.g.*, Englert *et al.*, 2013) or no effect (*e.g.*, Baldy *et al.*, 2007). These results suggest that the effects of WWTP effluents on streams ultimately depend on the concentration and chemical composition of the water in both the effluent and the receiving stream (Posthuma *et al.*, 2008; Altenburger *et al.*, 2015; Atashgahi *et al.*, 2015), as well as the dilution capacity of the latter (Rice & Westerhoff, 2017). Other factors can also influence the effects of WWTP effluents, such as the composition of the biological communities in the receiving stream (Segner *et al.*, 2014), the timing of the exposure (Camargo & Alonso, 2006), the legacy effects that effluents can exert even when they are reduced or no longer received (Jarvie *et al.*, 2013) and the different resistance and resilience capacity of the ecosystem (Jackson *et al.*, 2016). Finally, the fact that most streams can be simultaneously subjected to other co-occurring stressors will also modulate the effects of urban effluents (Sabater *et al.*, 2018), as these effects can be driven by direct and indirect interactions between stressors (Romero *et al.*, 2018).

The ecological effects of poorly treated and highly concentrated WWTP effluents can be obvious, but detecting the effects of well treated and highly diluted effluents in the real world is more difficult, especially because receiving streams are almost always also affected by other co-occurring stressors. This is especially true when studies describe the temporal changes of a stream after the implementation of a WWTP (Arroita *et al.*, 2018), or when we compare reaches located upstream and downstream from the

effluents (Drury *et al.*, 2013), as such designs cannot exclude potentially confounding factors generally affecting urban streams (Underwood, 1991). This situation calls for a powerful experimental design, which includes long-term responses and covers a variety of structural and functional variables (Downes *et al.*, 2002).

Here, we report the results of an unprecedented whole-ecosystem manipulation experiment, in which we assessed the effects of a well-treated and highly diluted effluent of a large tertiary urban WWTP on stream ecosystem structure and functioning following a BACI (Before-After/Control-Impact) design. Since the effluent was highly diluted in the receiving stream, thus precluding toxicity for the aquatic biota, we predicted that the effluent would subsidize ecosystem structure and functioning. Specifically, we predicted that the effluent addition would promote biofilm biomass, increasing equally both autotrophic and heterotrophic components. We also predicted that these structural changes would affect ecosystem functioning, subsidizing microbial activities, which would be metabolically more active, but at the same time reducing their capacity to retain dissolved nutrients. Finally, we predicted subsidized organic matter decomposition rates, as a consequence of enhanced microbial activity.

Material and Methods

Study site and experimental design

The WWTP of Apraitz (N Iberian Peninsula, 43°13'41" N, 2º23'54" W) consists of a sequential biological reactor (https://www.acciona-agua.com/areas-of-activity/projects/dc-water-treatment-plants/wwtp/apraitz/) that treats the sewage water of >90,000 population equivalents derived from urban and industrial sources. On average, 9,400 m^3 of wastewater per day is treated in batch reactors (Gipuzkoa Water Consortium, *unpublished data*), where the sewage water is first mixed with activated sludge, and alternatively subjected to aerobic and anaerobic conditions to reduce organic matter content. Then, it is subjected to a tertiary treatment to reduce nitrogen (denitrification tank) and phosphorus (precipitation with ferric sulphate) concentrations. The resulting effluent is released into the Deba River (mean discharge during our study = 10.9 ± 0.7 m^3 s^{-1}, http://gipuzkoa.eus/) through regularly pulsed releases of 20-40 min every 2 h. Ecotoxicological studies have shown this effluent to have little toxicity for microbes or invertebrates (Solagaistua *et al.*, 2018) and to cause no clear ecological effects below the release point of Apraitz WWTP, as it does not significantly affect water chemical characteristics nor alter the composition of the biological communities (URA, 2017).

Ten meters downstream from the WWTP effluent release point, the Deba River receives the waters of the Apraitz Stream, a small unpolluted stream draining a 7-km^2 catchment over sandstone and shale, with a mean discharge of 0.12 m^3 s^{-1}. In its lowermost 300 m, the Apraitz Stream runs by the side of the WWTP in a riffle-pool reach dominated by bedrock and cobbles under a young closed riparian forest dominated by black alder (*Alnus glutinosa*), hazel (*Corylus avellana*) and ash (*Fraxinus excelsior*).

Average annual rainfall and air temperature in the area are 1613 mm y^{-1} and 12.7 ºC, respectively (http://gipuzkoa.eus/).

To assess the effect of the WWTP effluent on stream ecosystem structure and functioning , we conducted a BACI experiment in the Apraitz Stream. In the lowermost part of this stream, we defined two 100-m long reaches: a Control (upstream) and an Impact (downstream). We studied both reaches bimonthly for 2 years (04/04/2016 to 30/06/2018), one year Before and another year After diverting part of the WWTP effluent to the Impact reach. The diversion was constructed by digging a 12 m-long and 4 m-deep trench to reach the WWTP outlet gutter, and inserting a 125-mm diameter pipe into the gutter to allow a discharge of 10 L s^{-1} into the Apraitz Stream during the periods of WWTP effluent release. The resulting dilution rate in the Apraitz Stream was similar to the one observed in the Deba River (0.2-4% and 0.1-9%, respectively, both calculated from mean daily values during the After period, Fig. 1).

Hydrology

We estimated hydraulic parameters from the time *vs*. electrical conductivity (EC) curves obtained with pulse additions of NaCl during periods of no effluent release. We calculated mean water velocity (m s^{-1}) dividing reach length by the time elapsed between the start of the pulse addition and the conductivity peak at the end of the reach. Water discharge was calculated based on a mass-balance approach, using EC as a surrogate of the chloride concentration (Martí & Sabater, 2009). On every sampling occasion, we obtained data for both reaches in less than 2 h. Additionally, we placed a water level datalogger (Solinst Levelogger Edge 3001; Solinst Canada Ltd., Georgetown, USA) per reach. These data were corrected for the atmospheric pressure measured with an additional datalogger (Barologger, Solinst Levelogger Edge 3001). Corrected water level was then regressed against discharge calculated from the time-EC curves, to obtain continuous discharge data. On each sampling occasion, we also measured wet channel width in equidistant transects every 10 m.

Water characteristics

We collected water samples and measured physico-chemical characteristics at the downstream end of both reaches and directly at the effluent during periods of effluent release (~10 min after the start of effluent release) and no release (~1 h after the end of the effluent release). We measured pH, temperature (T), EC and dissolved oxygen (DO) saturation using hand-held probes (WTW Multi 350i and WTW 340i SET, WTW Wissenschaftlich, Weilheim, Germany; YSI ProODO handled; YSI Incorporated, Yellow Springs, OH, USA). Water samples were immediately filtered (0.7-µm pore size pre-combusted glass-fiber filters, Whatman GF/F, Whatman International Ldt., Kent, UK) and frozen until analysis. We determined the concentration of soluble reactive phosphorus (SRP) [molybdate method (Murphy & Riley, 1962)] and ammonium (NH_4^+) [salicylate method (Reardon *et al.*, 1966)], on a spectrophotometer (Shimadzu UV-1800 UV-Vis, Shimadzu Corporation, Kyoto, Japan). The concentration of anions [nitrate (NO_3^-), nitrite (NO_2^-), sulphate (SO_4^{2-}) and chloride (Cl^-)] was determined with capillary ion electrophoresis (Agilent G1600AX

3D, Agilent Technologies, Wilmington, DE, USA) (Environmental Protection Agency, 2007). The concentration of dissolved inorganic nitrogen (DIN) was calculated as the sum of NO_3^-, NO_2^- and NH_4^+. Dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) were measured by catalytic oxidation using a Shimadzu TOC-L analyzer coupled to a TNM-L unit (Shimadzu Corporation, Kyoto, Japan). Additionally, we measured the concentration of emergent pollutants (herbicides, hormones, life style products, industrial chemicals and pharmaceuticals) during the first month after diversion (see Solagaistua *et al.* 2018 for details).

Biofilm

Three months before the beginning of the experiment, we deployed artificial substrata (granite paving stones of 20 x 10 x 8 cm) along both reaches to allow for biofilm colonisation. On each sampling occasion, we sampled the biofilm from five artificial substrata randomly collected in the wet channel of each reach, three of which were previously used to measure metabolism (see below). To sample biofilm, we scraped the whole surface of the substrata and collected the slurry in filtered stream water (0.7-µm pore size, Whatman GF/F). We obtained one slurry per substratum and stored individually in pre-washed polyethylene containers. Once in the laboratory, we measured the initial volume of the slurry and homogenized it with a grinder (IKA Ultra-Turrax T25 Basic, Werke GmbH & Co. KG, Staufen, Germany). Each sample was split into three subsamples: two subsamples for biomass and chlorophyll-*a* (Chl-*a*) were frozen until analysis, whereas the one for exo-enzymatic activities was processed immediately after each sampling.

To determine biofilm biomass, we filtered the corresponding subsamples trough pre-weighed filters (0.7-µm pore size, Whatman GF/F), which were then oven-dried (70 ºC, 72 h), weighed, combusted (500 ºC, 5 h) and reweighed to obtain the ash-free dry mass (AFDM). We corrected the obtained AFDM values by the volume of the filtered subsample and divided by the area of the artificial substrates, in order to express the results per surface unit (g AFDM m^{-2}). To determine Chl-*a* concentration, we filtered the subsamples (0.7-µm pore size) and extracted the filters in 90% acetone (4 ºC, 12 h in the dark) (Steinman *et al.*, 2006). To ensure the complete extraction of Chl-*a*, we sonicated (Selecta sonication bath, operating at 360 W power, 50/60 Hz frequency, JP Selecta S.A., Barcelona, Spain) and centrifuged (2000 rpm, G-value = 657.4, P-Selecta Mixtasel, JP Selecta S.A.) the samples. Then, we determined Chl-*a* concentration spectrophotometrically (Shimadzu UV-1800 UV-Vis, Shimadzu Corporation, Kyoto, Japan) by measuring the absorbance at 665 and 750 nm, following Jeffrey & Humphrey (1975). We also corrected the obtained Chl-*a* values by the volume of the filtered subsample and expressed per surface unit (mg Chl-*a* m^{-2}). We also calculated the biofilm autotrophic index (AI), computed as the ratio between AFDM and Chl-*a* concentration (Steinman *et al.*, 2006).

Exo-enzymatic activities related to the acquisition of organic phosphorus (alkaline phosphatase, AP) and the degradation of cellulose and hemicelluloses (β-glucosidase, BG) were determined following Saiya-Cork *et al.* (2002). We used the artificial fluorescent-linked substrata 4-methylumbelliferyl (MUF)-

phosphatase and (MUF)-β-D-glucopiranoside. Assays were carried out using 96-well micro plates (BRANDplates® microplates, BRAND GMBH + CO KG, Wertheim, Germany), in which biofilm samples were blended with acetate buffer (50 mM, pH = 5) and incubated for 30 min in the dark. Blanks without biofilm and a standard curve of MUF (0-100 µmol L^{-1}) were also incubated to correct for autofluorescence and quenching. NaOH buffer (1.0 M) was added (1/1, vol/vol) to stop the reaction. Fluorescence was measured at 365/455 nm excitation/emission (37 ºC) in a fluorometer microplate reader (Tecan GENios, Cavro Scientific Instruments Inc., Sunnyvale, California), and results were also corrected by the volume of the filtered subsample and expressed per surface unit (µmol h^{-1} m^{-2}).

To determine biofilm SRP uptake capacity, we used biofilm carriers, small artificial substrata (2.5 cm in side, SERA GmbH D52518, Heinsberg, Germany) commonly used in aquaria to encourage biofilm attachment due to their high surface-to-volume ratio. Before the onset of the experiment, we deployed the biofilm carriers in five sites randomly distributed per reach, tied with nylon line to roots or metal bars. On each sampling occasion, we recovered 1 biofilm carrier per site (totalling 5 per reach) and we deployed more to be colonised for the following sampling occasion. After collection, we stored them in stream water inside dark plastic containers. Once in the laboratory, biofilm carriers were acclimated in 200 mL of an acclimation solution [1:5 Perrier carbonated mineral water (Nestlé, Vergèze, France): deionized water], designed to ensure a sufficient supply of micronutrients (30 min, 100 rpm shaking speed, 20 ºC and ~180 µmol m^{-2} s^{-1} light) (Agitator-Incubator INF-66123 Multitron Standard, BIOGEN Científica S.L., Madrid, Spain). After acclimation, biofilm carriers were individually submersed in 45 mL of the same solution but spiked with phosphate (K_2HPO_4, 10 mM = 310 mg P L^{-1}), to achieve a final concentration of 5 µM (155 µg P L^{-1}), and incubated under the same conditions for 1 h. This concentration was chosen to ensure saturating conditions for the biofilm while allowing the nutrient decline during the incubation and the subsequent uptake estimation. After the incubation, 10 mL from each vial were filtered (0.7-µm pore size, Whatman GF/F) and frozen until analysis. Control treatments using non-colonised biofilm carriers were also used. SRP uptake capacity was calculated as the difference between the mean SRP concentration of the control and the colonised substrates, in the incubation volume and time (µg P h^{-1}) (Elosegi *et al*., 2018).

Biofilm metabolism was determined with closed chambers, since the high turbulence and the short length of our reaches prevented the use of open-channel methods (Bott *et al.*, 1978). On each sampling occasion, we randomly selected three artificial substrata per reach and enclosed them individually in methacrylate chambers (20 x 30 x 15 cm). Incubations were always carried out during no effluent release periods in a single pool located at the downstream end of the Control reach and all chambers with the same Control reach water. Thus, we ensured that the effluent release would not affect the measurements and consequently, exactly the same conditions of light, temperature and water chemistry in all the chambers. Although we are aware that photosynthesis from light incubations can stimulate heterotrophic activity during dark conditions (Espeland *et al.*, 2001), the number of chambers we had available prevented us from running simultaneously light and dark incubations while maintaining the same conditions in all the chambers. Inside the chambers, water was constantly recirculated by means of aquarium pumps (Syncra

Silent, Sicce, SICCE S.R.L., Pozzoleone, Italy). After 2 h of light incubation, we measured DO inside each chamber (YSI ProODO handled). After that, we covered all chambers with black opaque plastic and incubated them for 2 additional h and DO was measured again at the end. We used changes in DO concentration in dark conditions to calculate community respiration (CR) and those in light to calculate net community metabolism (NCM). Gross primary production (GPP) was calculated as the sum of NCM and CR (Fellows *et al.*, 2001). All parameters were computed from the difference in DO concentration between two measurements, in a specific time interval, accounting for the water volume in the chamber and the surface of the artificial substrata (mg O_2 h^{-1} m^{-2}). Finally, we also scaled metabolic rates per unit of biofilm biomass (*i.e.*, GPP per Chl-*a* concentration and CR per total biomass) to explore potential changes in biofilm metabolic efficiencies.

Whole-reach nutrient uptake

We measured whole-reach nutrient uptake for NH_4^+ (as NH_4Cl) and SRP (as $NaH_2PO_4.2H_2O$) using the pulse addition technique (Martí & Sabater, 2009). We dissolved both nutrients in 20 L of stream water together with NaCl as a hydrological conservative tracer (Bencala *et al.*, 1987). On each sampling occasion, we added the solution to the stream in a single pulse at the head of each reach, where appropriate mixing was ensured, during no effluent release. At the downstream end of both reaches, we automatically recorded EC every 10 s using a portable conductivity meter (WTW 340i SET) from the beginning of the addition until return to basal conditions. During the breakthrough curve, we collected ~25 water samples for each nutrient, which were filtered (0.7-µm pore size, Whatman GF/F) and frozen until analysis. We used the mass-balance approach to calculate three nutrient uptake metrics (Martí & Sabater, 2009): (i) uptake length (*Sw*, m), which reflects the distance travelled by a nutrient molecule before being removed from the water column, (ii) uptake velocity (*Vf*, m s^{-1}), which reflects the velocity at which a nutrient molecule is removed from the water column, and (iii) areal uptake (*U*, µg min^{-1} m^{-2}), which reflects the mass of nutrients taken up from the water column per surface area unit and time.

Organic matter decomposition

Freshly fallen black alder leaves (*Alnus glutinosa*) were collected in the field, air-dried to constant mass, and stored in dark plastic bags. For each sampling occasion, we enclosed leaves in fine (100 µm, 3 ± 0.05 g per bag) and coarse (5 mm, 4 ± 0.05 g per bag) mesh bags, to compare the contribution of different size consumers to organic matter decomposition. Fine bags were enclosed in coarse ones to ensure they were subject to the same conditions, and that invertebrates could only access the leaves in the coarse but not the fine-mesh bags, where decomposition was mainly microbial. In the field, bags were tied with nylon line in the same five sites randomly distributed per reach together with biofilm carriers and incubated for 3-4 weeks. After incubation, we collected the bags, stored them individually and carried them to the laboratory in a cool box. Once there, the remaining leaf material was rinsed to remove invertebrates and mineral particles, oven-dried (70 ºC, 72 h), weighed, combusted (500 ºC, 5 h) and re-weighed to obtain AFDM. An additional set of five fine bags were also incubated for two weeks in the Control reach, to determine

leaching rates and correct the initial dry mass of leaves used in the experiment. Decomposition rates were calculated by the ratio between final and initial AFDM according to the negative exponential model (Petersen & Cummins, 1974), which was expressed as AFDM consumed per day (d^{-1}).

Data analysis

For each variable, we calculated the effect size of the effluent addition following equation 1. Values <1 indicate that the effluent reduced the process, while values >1 indicate that the effluent increased it.

$$Eq.(1)\ \ Effect\ size = \frac{After\ \ (I/C)}{Before\ \ (I/C)}$$

We fitted our data to Gaussian models (Madsen & Thyregod, 2010; Zuur & Ieno, 2010), to test for the effect of the effluent addition on stream ecosystem response: biofilm structure (AFDM, Chl-*a*, AI) and functioning [AP, BG, SRP uptake capacity and metabolism (GPP, CR, GPP/Chl-*a*, CR/AFDM)], and organic matter decomposition (microbial, total). For parameter estimation, we used generalized least squares (Pinheiro & Bates, 2000), via the "gls" function of the nlme package (Pinheiro & Bates, 2016). The fixed structure of the model included period (Before/After) and reach (Control/Impact), fitted as discrete explanatory variables, and the interaction between them. We also added sampling occasion as random factor. The effect of the effluent addition was given by the interaction between period and reach (BA:CI). In some of the models, Akaike Information Criterion (AIC) comparisons revealed the need to add a variance structure to deal with heteroscedasticity and allow having different variances per sampling occasion. For water characteristics and whole-reach nutrient uptake (*Sw*, *Vf* and *U* for both SRP and NH_4^+), we analyzed the data using linear models and estimated the parameters using restricted maximum likelihood, via the "lme" function of the nlme package (Pinheiro & Bates, 2017), because we had a unique replicate per sampling occasion. The dataset was complete for all measured variables, except for whole-reach nutrient uptake and metabolism, which we were unable to measure correctly on some dates due to technical problems (*e.g.* sensor malfunctioning, sample loss). When necessary, data were log-transformed to fulfil the requirements of parametric analyses. In all cases, we accepted significance when $p < 0.05$. All statistical analyses were conducted using R software, ver. 3.4.0. (R Development Core Team, Vienna, Austria).

Results

Hydrology

During the experiment, the Apraitz Stream showed a flashy hydrograph with a mean water discharge of 118 L s^{-1}, a peak discharge of 1,095 L s^{-1} during periods of heaviest rainfall, and base flow values around 60 L s^{-1}. The mean daily contribution of the effluent to the Impact reach flow was 3%, ranging from 0.2 to 4% (Fig. 1).

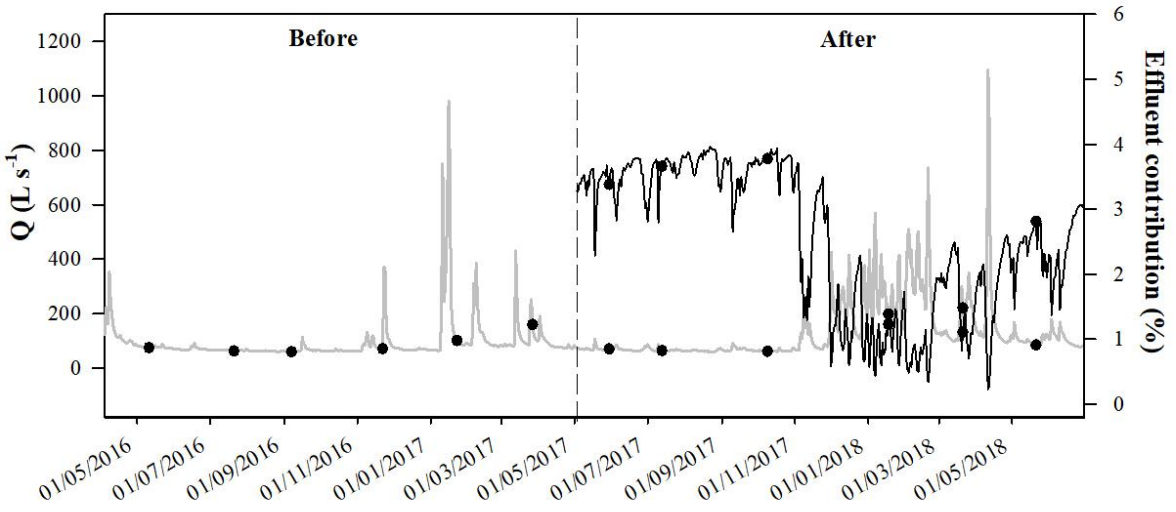


Figure 1. Daily mean stream discharge (Q) in the Control reach (L s^{-1}) (grey series, left axis) and effluent contribution to the Impact reach during the After period (%) (black series, right axis). Black dots represent each sampling campaign day, while the dashed line marks the beginning of the effluent addition.

Water characteristics

The WWTP effluent was circumneutral, on average 3 °C warmer than stream water, and with 3 times higher EC, 2 times lower DO saturation and 4 to 90 times higher concentrations of nutrients and DOC, being substantially higher for both DIN and TDN as well as for SRP (Table 1). Additionally, the effluent also showed high concentrations of emergent pollutants, especially those of ARBs (Angiotensin II Receptor Blockers –ARBs- that modulate the renin-angiotensin system and are used to treat hypertension, such as Valsartan, Irbesartan, Eprosartan and Telmisartan), caffeine (the most consumed stimulant of the central nervous system) and calorie-free sugar substitutes (such as Acesulfame and Sucralose). Most water physico-chemical variables in the Impact reach changed significantly during effluent release periods (Table 1, Table S1). The pH and DO saturation decreased to 0.9 times the initial values (BA:CI, $p = 0.007$ and $p = 0.04$, respectively), EC was 1.5 times higher (BA:CI, $p < 0.001$), whereas T was not significantly affected (BA:CI, $p = 0.20$). Nutrient concentrations in the Impact reach significantly increased during effluent discharge events, especially NH_4^+ (5.2 times: BA:CI, $p = 0.06$) and SRP (2.4 times: BA:CI, $p = 0.004$). These differences, however, almost disappeared in the phases between effluent release periods (Table 1, Table S1), with the only exception of NH_4^+, which was still 2.3 times higher (BA:CI, $p = 0.04$).

Table 1. Water characteristics of Control and Impact reaches during Before and After periods, together with WWTP effluent characteristics. In the After period, distinction between effluent discharge and no discharge periods is also made. Values shown are mean ± standard error (SE), calculated from 6 surveys (n = 6) during each experimental period.

	Stream water					**WWTP effluent**
Period	Before		After			
Reach	Control	Impact	Control	Impact- *Effluent release*	Impact - *No effluent release*	
pH	8.0 ± 0.1	7.9 ± 0.04	7.7 ± 0.3	7.1 ± 0.3	7.8 ± 0.1	7.1 ± 0.3
T (ºC)	14.1 ± 0.1	14.5 ± 0.04	13.3 ± 0.01	15.1 ± 0.2	13.3 ± 0.02	17.8 ± 0.02
EC (µS cm^{-1})	279 ± 18	277 ± 19	289 ± 18	427 ± 58	320 ± 44	791 ± 68
DO (%)	103 ± 2	101 ± 2	100 ± 1	92 ± 1	97 ± 1	57 ± 2
Cl^- (mg Cl L^{-1})	12 ± 1	13 ± 3	15 ± 2	27 ± 6	17 ± 2	107 ± 17
SO_4^{2-} (mg SO_4^{2-} L^{-1})	47 ± 4	43 ± 8	43 ± 4	33 ± 2	46 ± 7	46 ± 8
NO_2 (mg N L^{-1})	0.02 ± 0.00	0.03 ± 0.004	0.02 ± 0.00	0.5 ± 0.3	0.1 ± 0.1	1.5 ± 0.9
NO_3 (mg N L^{-1})	0.6 ± 0.1	0.7 ± 0.2	0.6 ± 0.1	1.3 ± 0.3	0.7 ± 0.1	6.2 ± 1.9
NH_4^+ (mg N L^{-1})	0.01 ± 0.001	0.01 ± 0.001	0.01 ± 0.002	0.2 ± 0.1	0.04 ± 0.002	0.5 ± 0.3
DIN (mg N L^{-1})	0.6 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	1.9 ± 0.3	0.8 ± 0.1	8.2 ± 1.3
DOC (mg C L^{-1})	3.5 ± 0.2	3.9 ± 0.5	3.4 ± 0.4	4.2 ± 0.9	3.0 ± 0.4	13.8 ± 1.9
TDN (mg N L^{-1})	1.1 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	2.3 ± 0.4	1.1 ± 0.1	10.2 ± 1.2
SRP (mg P L^{-1})	0.01 ± 0.003	0.02 ± 0.004	0.02 ± 0.01	0.2 ± 0.1	0.01 ± 0.002	1.1 ± 0.4

Biofilm

The WWTP effluent affected significantly biofilm structure, increasing both biofilm biomass and Chl-*a* (Tables S1). During the Before period, biofilm biomass was similar in both Control and Impact reaches (mean ± SE = 1.03 ± 0.25 and 0.84 ± 0.22 g AFDM m^{-2}, respectively), but the effluent addition increased it 2.1 times in the Impact reach (BA:CI, $p < 0.001$, Fig. 2a). Chl-*a* concentration showed a similar pattern, with similar values in both reaches during the Before period (Control: 5.62 ± 1.87 and Impact: 4.09 ± 1.38 mg m^{-2}), and an increase of 2.3 times in the Impact reach after the effluent addition (BA:CI, $p = 0.005$, Fig. 2b). The effluent addition did not change significantly the AI, *i.e.* the balance between the heterotrophic and autotrophic component of the biofilm (BA:CI, p = 0.23, Table S1).

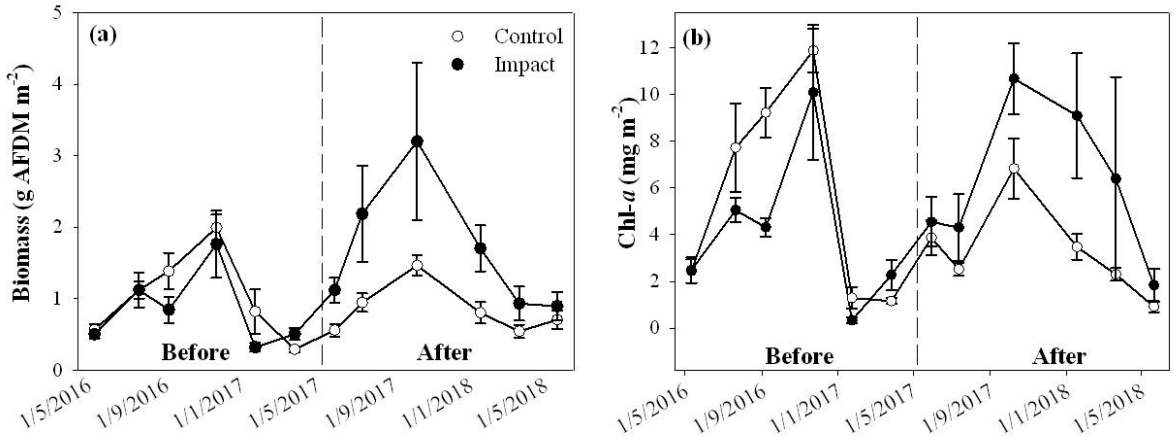


Figure 2. Biofilm (a) biomass and (b) chlorophyll-a (Chl-*a*) concentration in Control and Impact reaches Before and After the effluent addition. The dashed line marks the beginning of the effluent addition. Mean ± SE values are shown.

The effluent addition increased significantly biofilm exo-enzymatic activities (Table S1). During the Before period, AP values were higher in the Control than in the Impact reach (12.60 ± 5.10 and 7.78 ± 3.83 µmol h^{-1} m^{-2}, respectively). This pattern changed after the effluent discharge, as AP activity was 2.2 times higher in the Impact reach, specially just after the effluent addition (BA:CI, p = 0.015, Fig. 3a). BG activity showed a similar response, with higher values in the Control reach before the effluent addition (Control: 8.46 ± 3.98 and Impact: 5.34 ± 3.29 µmol h^{-1} m^{-2}) and an even more significant increase of 4.1 times in the Impact reach after the effluent addition (BA:CI, p = 0.002, Fig. 3b).

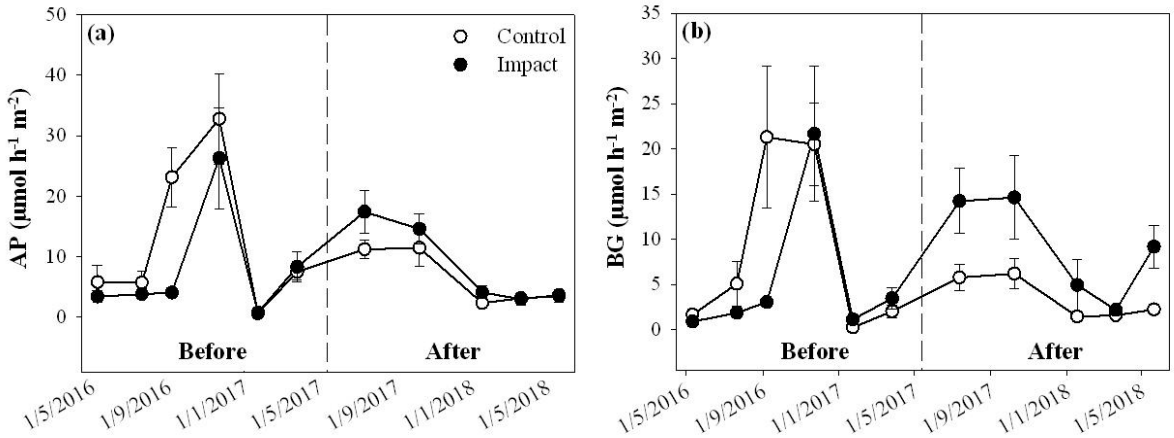


Figure 3. Biofilm (a) alkaline phosphatase (AP) and (b) β-glucosidase (BG) activities in Control and Impact reaches Before and After the effluent addition. The dashed line marks the beginning of the effluent addition. Mean ± SE values are shown.

Biofilm SRP uptake capacity was significantly impaired by the effluent addition. During the Before period, biofilm SRP uptake capacity showed no differences between Control and Impact reaches (3.17 ± 0.43 and 3.27 ± 0.39 µg P h^{-1}, respectively). However, this capacity was reduced to 0.5 times the initial values in the Impact reach after the effluent addition (BA:CI, p < 0.001, Fig. 4, Table S1).

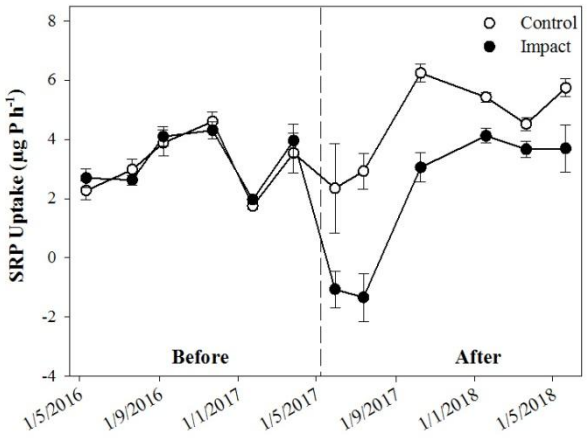


Figure 4. Biofilm SRP uptake capacity in Control and Impact reaches Before and After the effluent addition. The dashed line marks the beginning of the effluent addition. Mean ± SE values are shown.

Biofilm metabolism was not significantly affected by the WWTP effluent (Table S1). During the Before period, Control and Impact reaches showed similar rates of GPP (45 ± 18 and 29 ± 8 mg O_2 h^{-1} m^{-2}) and CR (-19 ± 6 and -15 ± 4 mg O_2 h^{-1} m^{-2}) , and the biofilm community was predominantly autotrophic (GPP:CR > 1). The effluent did not change this pattern, as it did not affect significantly neither GPP (BA:CI, p = 0.46, Fig. 5a), CR (BA:CI, p = 0. 96, Fig. 5b) nor the ratio between them (BA:CI, p = 0.28). The effluent significantly increased the ratio GPP/Chl-*a*, a proxy of the metabolic efficiency of biofilm autotrophs (BA:CI, p = 0.05). Conversely, the ratio CR/AFDM, a proxy of the metabolic efficiency of biofilm heterotrophs, was not significantly affected by the effluent addition (BA:CI, p = 0.65).

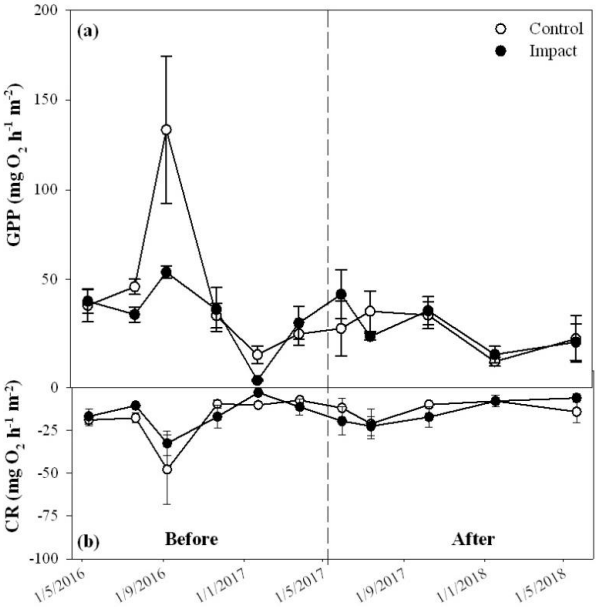


Figure 5. Biofilm (a) gross primary production (GPP) and (b) community respiration (CR) in Control and Impact reaches Before and After the effluent addition. The dashed line marks the beginning of the effluent addition. Mean ± SE values are shown.

Whole-reach nutrient uptake

The effluent addition did not affect significantly nutrient uptake metrics (Table S1). During the Before period, Control and Impact reaches showed similar *Sw* values for both SRP (499 ± 69 and 547 ± 107 m) and NH_4^+ (284 ± 50 and 297 ± 51 m), and the effluent addition did not affect them significantly (BA:CI, SRP $p = 0.54$ and NH_4^+ $p = 0.16$, Fig. 6a, b). Similarly, Control and Impact reaches also showed similar *Vf* values before the effluent addition for SRP (0.003 ± 0.002 and 0.004 ± 0.002 mm h^{-1}) and NH_4^+ (0.005 ± 0.002 and 0.005 ± 0.002 mm h^{-1}). However, while the effluent addition did not affect NH_4^+ *Vf* (BA:CI, $p = 0.19$, Fig. 6d), it caused a marginally significant reduction in SRP *Vf* below the input (BA:CI, $p = 0.06$, Fig. 6c). Finally, *U* for both nutrients was not significantly affected by the effluent (BA:CI, SRP $p = 0.13$ and NH_4^+ $p = 0.92$, Fig. 6e, f).

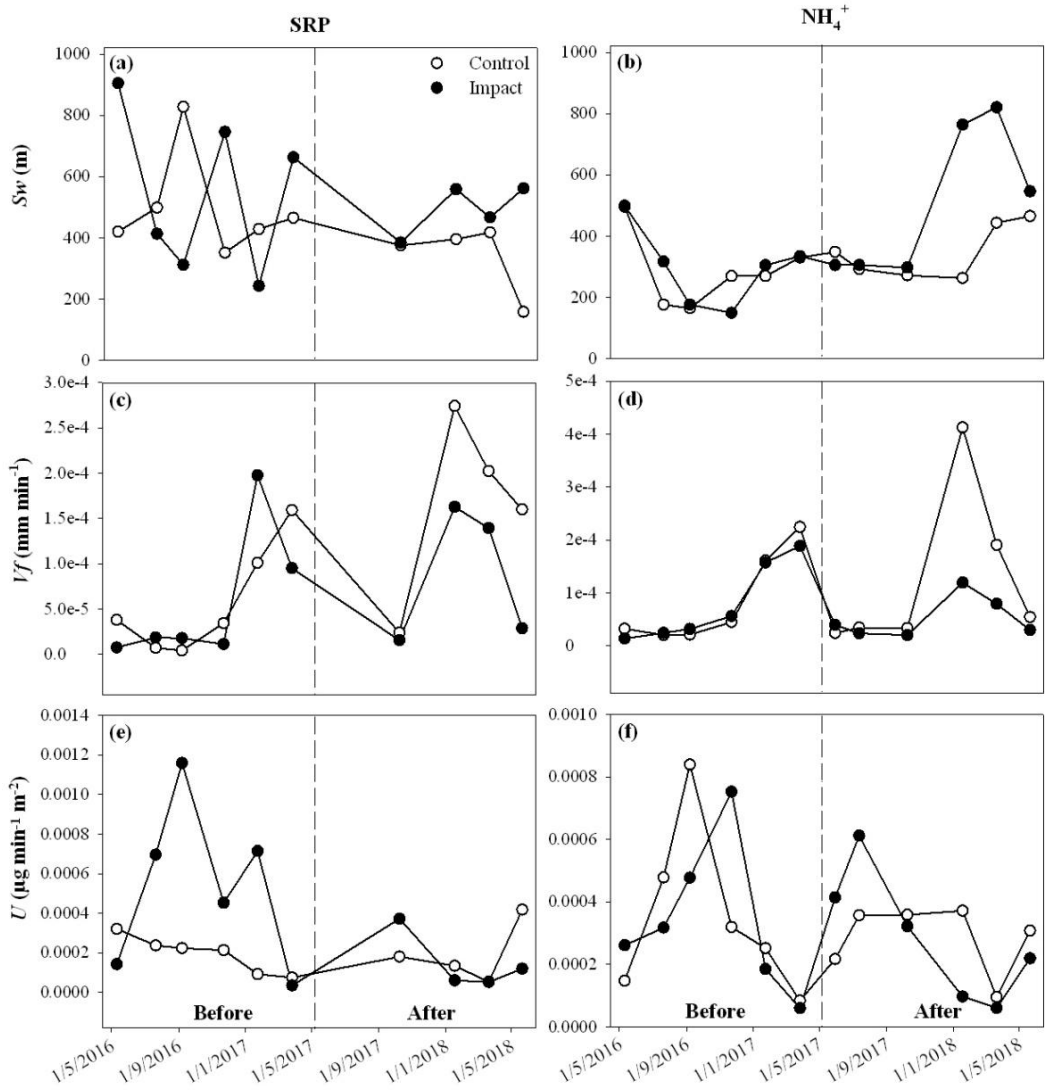


Figure 6. Whole reach nutrient uptake metrics (uptake length, *Sw*; uptake velocity, *Vf* ; areal uptake, *U*) for SRP (a, c, e) and NH_4^+ (b, d, f) in Control and Impact reaches Before and After the effluent addition. The dashed line marks the beginning of the effluent addition.

Organic matter decomposition

The effluent had different effects on microbial and total organic matter decomposition (Table S1). During the Before period, Control and Impact reaches showed similar rates of microbial (0.02 ± 0.004 and 0.03 ± 0.005 d^{-1}) and total decomposition (0.06 ± 0.02 and 0.05 ± 0.02 d^{-1}), even if the latter showed a huge reduction from the second sampling campaign on. Microbial decomposition was not affected by the effluent addition (BA:CI, p = 0.76, Fig. 7a), whereas total decomposition rates increased 1.4 times in the Impact reach (BA:CI, p = 0.003, Fig. 7b).

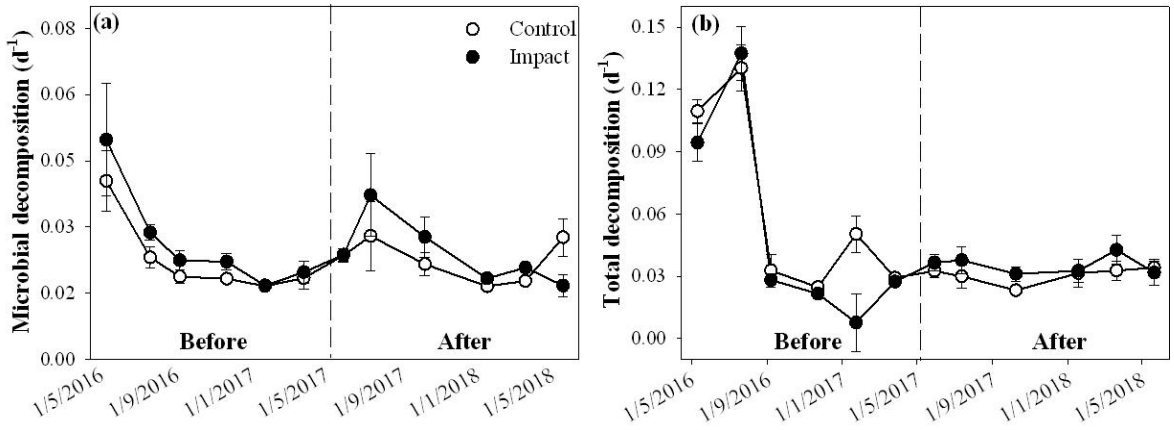


Figure 7. Leaf litter (a) microbial and (b) total decomposition in Control and Impact reaches Before and After the effluent addition. The dashed line marks the beginning of the effluent addition. Mean ± SE values are shown.

Discussion

The effluents of WWTPs represent relevant pollution sources to freshwater ecosystems (Rice & Westerhoff, 2017). However, the ecological effects of WWTP effluents are difficult to detect because most streams are subject to other co-occurring stressors (Sabater *et al.*, 2018). Our whole-ecosystem manipulation experiment following a BACI design isolated the effects of a WWTP effluent, thus allowing the detection of their impact on a wide range of variables at the different environmental conditions and over a relatively long time period. Our results show that, even if WWTP effluents are well treated and highly diluted, they can cause significant and complex effects on ecosystem structure and functioning, which could have remained unnoticed under less stringent experimental approaches. Overall, the effluent increased the measured biological activities, with only some of the measured activities showing no effect or a reduction (*i.e.* biofilm uptake capacity). These results suggest that the effluent investigated tends to subsidize biological activity, and has limited toxic or stress effects (*sensu* Odum *et al.*, 1979).

Our experiment was designed to mimic the high dilution rate of the Apraitz WWTP effluent in the Deba River, and thus, had a mean contribution of 3% to the Apraitz Stream flow. However, dilution rates of WWTP effluents in streams are highly variable and in cases of natural or human-induced water-scarcity the final concentration can be much higher. For example, mean effluent contribution can be as high as 35%

in other streams in the region, as a consequence of intensive human water exploitation (Province Government of Gipuzkoa, 2015). In the most extreme cases, effluents can make up most of the flow in the receiving streams (Rice & Westerhoff, 2017). It is likely that the environmental effects of WWTP effluents will depend on their final dilution rate, and thus, that the ecological impacts can be stronger than those detected in our experiment (Brooks *et al.*, 2006).

Despite the high dilution during our experiment, the intermittent effluent diverted to Apraitz Stream significantly affected water characteristics during release periods, reducing pH and DO while increasing EC and the concentrations of nutrients (NH_4^+ and SRP) and emerging pollutants. Similar effects of WWTP inputs on stream water quality have been reported in other systems (Martí *et al*., 2009), usually linked to streams subjected to the "*Urban Stream Syndrome*" (*sensu* Walsh *et al.*, 2005). However, excessive nutrient concentrations can trigger toxic effects in many aquatic organisms. In our experiment, the concentration of nutrients measured during effluent release periods (2.3 and 0.2 mg L^{-1} for TDN and SRP, respectively), corresponded to the low-medium pollution levels observed in European streams, which contained less than 2.4 mg N L^{-1} and 0.2 mg P L^{-1} (Grizzetti *et al.*, 2017). In another parallel work to this experiment, Solagaistua *et al.* (2018) reported that the concentrations of emerging contaminants detected in the effluent were also high, even if they were in the range as those detected in large monitoring surveys across Europe (Loos *et al.*, 2013). However, in our Impact reach, toxicity values established by Camargo & Alonso (2006) for salmonids, which are reported among the most sensitive organisms, were surpassed for NO_2^- (*i.e.*, 0.5 ± 0.3 mg L^{-1}) and NH_4^+ (*i.e.*, 0.2 ± 0.1 mg L^{-1}), although no strong toxic effects on stream biota were observed, as none of the five fish species occurring in the reach (*Salmo trutta*, *Phoxinus bigerri*, *Anguilla anguilla*, *Barbatula quignardi* and *Parachondrostoma miegii*) disappeared in the After period (de Guzmán, *unpublished data*). Similarly, laboratory experiments at full effluent concentration and field incubations in our Impact reach showed that the WWTP effluent had little or no toxic signs for the amphipod *Echinogammarus berilloni* (Solagaistua *et al*., 2018). Altogether, these results suggest low toxicity of the Apraitz WWTP effluent under the dilution rates of our experiment, which could also be aided by the short effluent pulses and the fast water renewal rate of this fast-flowing stream.

Biofilms are highly sensitive to anthropogenic alterations (Battin *et al.*, 2016) and thus, nutrient enrichment from WWTP effluents could have important consequences on their structure and functioning. In our experiment, the effluent increased significantly biofilm biomass as well as Chl-*a* concentration even if it did not affect significantly the autotrophic index, which could suggest a similar subsidy effect on both heterotrophs and algae, as has been reported elsewhere (*e.g.*, Ribot *et al.*, 2015). However, the effluent significantly increased the metabolic efficiency of biofilm autotrophs, which suggests that the effluent could have induced a change in the composition of the autotrophic community, favouring more resistant and physiologically more active taxa (Drury *et al.*, 2013; Proia *et al.*, 2013; Rosi *et al.*, 2018). These changes in biofilm structure could also affect their functioning and increase microbial processes, such as exo-enzymatic activities, since their expression is usually regulated by nutrient imbalances in water or within the biofilm (Romaní *et al.*, 2012). In general, AP activity decreases with increases in the ambient

concentrations of phosphorus due to a lower demand for it (Allison & Vitousek, 2005; Romaní *et al.*, 2012). However, we observed increased AP activity in response to the effluent addition, which could be explained by increased biological demand for phosphorus as a result of increased biofilm biomass (Romaní & Sabater, 2000). In addition, the thickness and complexity of the boundary layer likely increased with biofilm biomass, thereby reducing the hydraulic exchange and diffusion of nutrients (Battin *et al.*, 2003) and promoting internal cycling of the biofilm (Earl *et al.*, 2006), which can also contribute to increased AP activity. On the other hand, β-glucosidase activity is an exo-enzyme related to the degradation of carbon and it is involved in the last step of cellulose decomposition (Romaní *et al.*, 2012). Its activity is usually correlated with the degradation of organic compounds, either of algal origin or those dissolved in water (Jones & Lock, 1993). In our experiment, the effluent increased BG activity, which could also be explained by increased biofilm biomass, as cell lysis occurring during biofilm formation (Romaní *et al.*, 2008) and polysaccharides in algal exudates (Rier *et al.*, 2014), both can promote the activity of this enzyme.

Although the chemical composition of the effluent did not reach toxicity and increased most measured biofilm processes, it decreased the biofilm SRP uptake capacity. As ambient nutrient concentrations increase, biofilms tend to become less effective in nutrient removal, even reducing nutrient uptake to zero in cases of biofilm saturation (Earl *et al.*, 2006). In our experiment, the effluent addition substantially increased SRP concentrations in water (up to 2.4 times during periods of effluent release), which strongly decreased biofilm SRP uptake rates, even to negative values in which biofilm carriers released SRP, indicating biofilm saturation. Similar results of SRP release by biofilm carriers have been shown in a laboratory experiment with WWTP effluents (Pereda *et al.*, 2019). Although SRP release reflects the specific conditions of our bioassay, not necessarily what occurs in the field, it suggests that the ability of the biofilm to take up phosphorus was seriously impaired by the effluent. These negative values, however were only observed after long base flow periods, which is also when boundary layer thickness and complexity increase, reducing the hydraulic exchange and diffusion of nutrients (Battin *et al.*, 2003) and promoting internal cycling and nutrient release (Earl *et al.*, 2006).

The effluent had no significant effect on biofilm metabolism or whole-reach nutrient uptake, although both processes have been shown to respond to wastewater inputs (*e.g.* Gücker *et al.*, 2006). In our experiment, we only observed some weak tendencies towards higher metabolic rates, with increased GPP and CR, and longer nutrient uptake lengths, together with lower uptake velocities and areal uptake rates for both NH_4^+ and SRP. These tendencies agree with some previous works, in which nutrient enrichment promoted ecosystem metabolism, especially respiration (Aristi *et al.*, 2015), but limited nutrient uptake efficiency (Martí *et al.*, 2009). However, the lack of significant responses in our work could be due to the low replication of metabolism, or the lack of replication and the role of unmeasured compartments (*e.g.*, hyporheic sediments or the extensive roots of the riparian trees) in the case of whole-reach nutrient uptake.

The effluent addition did not affect microbial decomposition but increased total decomposition, although both have been shown to respond not only to toxic compounds, but also to

nutrient enrichment (Ferreira *et al.*, 2015). The lack of response of microbial decomposition in our study probably reflects the good nutritional quality of alder leaves (Petersen & Cummins, 1974), which could have been less affected than other leaf species by nutrient inputs. Nutritional characteristics of leaf species have been shown to play a key role controlling the magnitude of response to environmental stressors (Ferreira *et al.*, 2015). Invertebrate consumption, on the other hand, substantially decreased from the second sampling campaign on, a fact that could be attributed to the disappearance of the amphipod *Echinogammarus berilloni* in the studied reaches (Solagaistua, *unpublished data*). As expected, the effluent promoted invertebrate consumption, likely due to increased leaf palatability (Ferreira *et al.*, 2015) or increased densities of more resistant shredders communities below urban effluents (Pascoal *et al.*, 2003). These results agree with some other previous studies (*e.g.*, Woodward *et al.*, 2012), and suggest that leaf litter decomposition by invertebrates could be more responsive than microbial decomposition to urban pollution (Hieber & Gessner, 2002).

Conclusions

In conclusion, our experiment shows that even well treated and highly diluted WWTP effluents can cause subtle but complex responses in the structure of stream biological communities and ecosystem functioning. We believe that these effects could be exacerbated under more concentrated effluents, and even shift from subsidy to stress responses. Nevertheless, the fact that most receiving streams and rivers are already affected by other stressors may hamper detection of these effects under real field conditions. Whole-ecosystem manipulation experiments are a powerful approach to unveil these effects and to point to the variables most likely to be affected under multiple-stress situations.

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CHAPTER 4

Immediate and legacy effects of urban pollution on river ecosystem functioning: a mesocosm experiment

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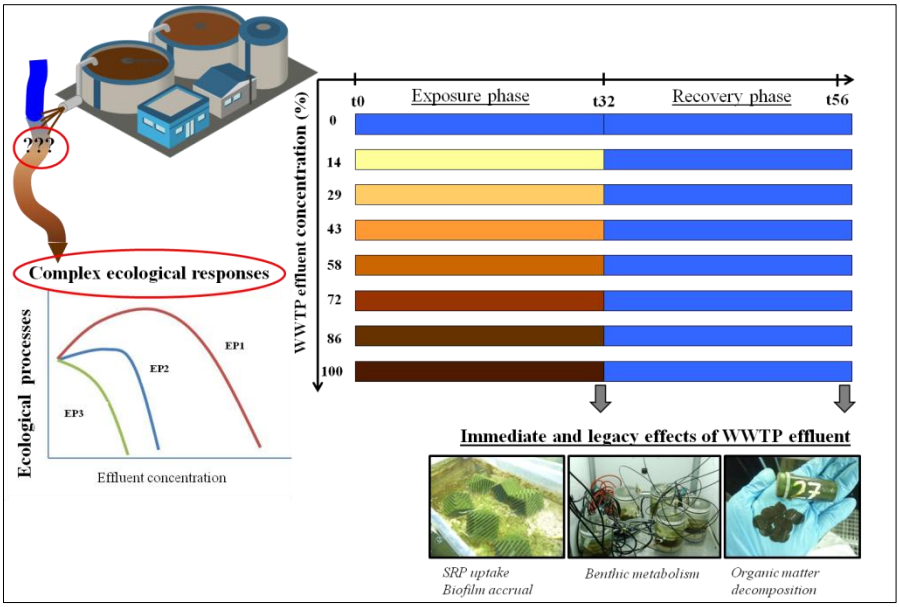
Abstract

Effluents from urban wastewater treatment plants (WWTP) consist of complex mixtures of substances that can affect ecosystem processes. Some of their components (toxic contaminants) stress biological activity at all concentrations, while others (*e.g.*, nutrients) subsidize it at low concentrations and stress it at high concentrations, causing subsidy-stress responses. Thus, the overall effects of WWTP effluents depend mostly on their composition and the dilution capacity of the receiving water bodies. We assessed the immediate and legacy effects of WWTP effluents in artificial streams, where we measured biofilm soluble reactive phosphorus (SRP) uptake, biomass accrual, benthic metabolism and organic matter decomposition (OMD). In a first phase (32 d), the channels were subjected to a gradient of effluent contribution, from pure stream water to pure effluent. WWTP effluent affected the ecosystem processes we measured, although we found no clear subsidy-stress patterns except for biofilm biomass accrual. Instead, most of the processes were subsidized, although they showed complex and process-specific patterns. Benthic metabolism and OMD were subsidized without saturation, as they peaked at medium and high levels of pollution, respectively, but they did not decrease below control levels. SRP uptake was the only process that decreased with increasing effluent concentration. In a second phase of the experiment (23 d), all channels were kept on pure stream water to analyse the legacy effects of the effluent. For most of the processes, there were clear legacy effects, which followed either subsidy, stress or subsidy-stress patterns. SRP uptake capacity was stressed with increasing pollution legacy, whereas algal accrual and benthic metabolism continued subsidized. Conversely, biofilm biomass accrual and OMD showed no legacy effects. Overall, the WWTP effluent caused complex and process-specific responses in our mesoscosm experiment, mainly driven by the mixed contribution of subsidizers and stressors. These results could help to improve our understanding of the effects of urban pollution on stream ecosystem functioning.

Keywords: Pollution; stream; decomposition; metabolism; subsidy-stress; biofilm.

Graphical Abstract



Introduction

Cities have been expanding exponentially as a consequence of population growth and migration from rural to urban areas (Jones & O´Neill, 2016). Associated to urban growth, inputs of sewage water, either raw or treated in waste water treatment plants (WWTPs), have become a relevant pollution point-source in river ecosystems (Vörösmarty *et al.*, 2010). WWTPs reduce urban pollution (Tchobanoglous & Burton, 1991; Serrano, 2007), but their effluents still consist of complex mixtures of substances including organic matter, nutrients (Merseburger *et al.*, 2005; Martí *et al.*, 2009), metals, pesticides and emergent pollutants such as pharmaceuticals, personal care products, or even illicit drugs (Santos *et al.*, 2013; Rosi-Marshall *et al.*, 2015; Aymerich *et al.*, 2017), which entail that the ecological effects of WWTP effluents are still far from clear (Aristi *et al.*, 2015). Given their content of assimilable and toxic compounds, they could either subsidise or stress ecosystem processes (*sensu* Odum *et al.*, 1979) depending on their exact composition, on their final concentration (Cardinale *et al.*, 2012; Rice & Westerhoof, 2017), as well as on the composition of the biological communities in receiving streams (Segner *et al.*, 2014). For instance, inorganic nutrients promote (subsidize) biological activity up to a threshold where they become toxic and start reducing it below "normal" levels (stress), whereas most heavy metals, pesticides or even antibiotics tend to suppress biological activity roughly in proportion to their concentration (Rodríguez-Mozaz & Weinberg, 2010; Peters *et al.*, 2013). Both assimilable and toxic compounds impair water quality (Beyene *et al.*, 2009; Ribot *et al.*, 2012), alter the structure of biological communities (Bundschuh *et al.*, 2011; Rosi-Marshall *et al.*, 2015), and affect the rates of different ecosystem processes (Aristi *et al.*, 2015; Corcoll *et al.*, 2015). On the other hand, the final concentration of these effluents depends on the dilution capacity of the receiving water body, which can be affected by human activities such as water abstraction (Arroita *et al.*, 2016) or climate change (Hisdal *et al.*, 2001; Englert *et al.*, 2013).

Ecosystem functioning reflects the fluxes of energy and matter in ecosystems (Tilman *et al.*, 2014; von Schiller *et al.*, 2017). Although explicitly included in the EU Water Framework Directive (WFD), which defines ecological status as "an expression of the structure and functioning of aquatic ecosystems associated with surface waters", ecosystem functioning is seldom considered in current monitoring schemes (Birk *et al.*, 2012). In addition to being an essential component of ecosystem health, it is at the basis of the services provided by river ecosystems (Millennium Ecosystem Assessment, 2005). However, we still largely ignore how ecosystem functioning responds to WWTP effluents and their potential legacy effects. Contradictory responses have been reported depending on the processes measured; for instance, organic matter decomposition (OMD) has been shown to increase in stream reaches receiving effluent inputs (*e.g.* Pascoal *et al.*, 2003), whereas nutrient retention can be unaffected (Haggard *et al.*, 2001; 2005) or reduced (Martí *et al.*, 2004; Merseburger *et al.*, 2005; 2011). Besides, effects on autotrophic and heterotrophic processes may differ when estimated at the site or at the whole-ecosystem scales (*e.g.* Aristi *et al.*, 2015), and the experimental

duration may also condition and modulate the response pattern (Aristi *et al.*, 2016). In addition, these effects may persist even when effluents are reduced or no longer received, this legacy delaying the recovery capacity of the ecosystem. Although the importance of assessing the legacy effects of stressors has been widely emphasized (Holeton *et al.*, 2011; Sharpley *et al.*, 2013), very few works have experimentally addressed this issue (Alvarez *et al.*, 2014).

The effects of WWTP effluents on ecosystem functioning depend on their final concentration in the receiving water, and thus, are difficult to assess in the real world. This situation prompts for laboratory experiments under controlled conditions, which provide powerful experimental techniques to isolate and analyse particular mechanisms that would go undetected *in situ* due the high complexity of natural communities (Benton *et al.*, 2007). In the case of river ecosystems, artificial indoor channels have been used to unveil complex ecological phenomena such as the interaction between nutrients and emerging contaminants (Aristi *et al.*, 2016), the importance of the duration of no-flow periods in intermittent waterways (Acuña *et al.*, 2015), the effect of warming and altered diel temperature patterns associated to Global Environmental Change (Freixa *et al.*, 2017) or the effects of the exposure to pharmaceuticals and illicit drugs on stream biological communities (Hoppe *et al.*, 2012; Lee *et al.*, 2016).

We investigated the impact of WWTP effluents on ecosystem processes by conducting a laboratory experiment using artificial streams, in which potentially confounding environmental factors were strongly simplified. We aimed at testing the following predictions: (1) biological processes would respond to the gradient of effluent dilution capacity following a subsidy-stress scheme, *i.e.*, showing an increase at low to moderate proportions of effluent, but a decrease below control (pure stream water) levels at high proportions; (2) the response pattern would diverge among processes; and (3) the effluent would produce legacy effects, *i.e.*, affect the recovery capacity of the process, which would be roughly proportional to the effluent concentration.

Material and methods

Experimental design

We conducted an experiment using a series of artificial streams located in the indoor Experimental Streams Facility of the Catalan Institute for Water Research (Girona, Spain). Each artificial stream was assigned to one of eight treatments, from non-polluted water (control treatment) to pure WWTP effluent (0, 14, 29, 43, 58, 72, 86 and 100% of WWTP effluent water). We used three replicates per treatment (8 treatments x 3 replicates = 24 artificial streams) distributed in four separate arrays of six artificial streams, with each treatment represented only once per array. The dilution values were designed to represent a regression design (Navarro *et al.*, 2000) from an unpolluted stream scenario with no WWTP contribution, to a temporary stream scenario during the dry phase, when 100% of

the water flow comes from the WWTP effluent. The design was also aimed at detecting tip-points and thresholds between subsidy and stress for each of the measured processes.

The experiment was developed between January 19th and March 31st, 2017. After an acclimation phase of 15 d, artificial streams were subjected to the different treatments during a first exposure phase (32 d), followed by a recovery phase (23 d), in which the flow of clean water was restored in all the artificial streams. These two phases allowed assessing both the immediate and the legacy effects of the WWTP effluent.

Experimental conditions

Each artificial stream consisted of an independent methacrylate channel (length-width-depth: 200 cm - 10 cm -10 cm), and a 70-L water tank from which water was recirculated. Each stream received a constant flow of 50 mL s^{-1} and operated as a closed system for 72 h, so all the water in each channel was renewed every three days. Water mean velocity was 0.71 cm s^{-1} and water depth over the plane bed ranged from 3 to 3.5 cm. Each artificial stream was filled with 5 L of sand and 14 cobbles collected from an unpolluted segment of the nearby Llémena River. The Llémena is a permanent Mediterranean oligotrophic calcareous stream, which has been previously used as a reference site for ecotoxicological laboratory experiments on biofilms due to its relatively low concentration of contaminants (Bonnineau *et al.*, 2010; Serra *et al.*, 2010; Corcoll *et al.*, 2015). The sediment and cobbles were transported in less than 1 h to the artificial streams and evenly distributed to create a plane bed to facilitate biofilm growth. During the acclimation phase, the biofilm was allowed to grow on the artificial streams from the inoculum present in these sediments and cobbles. After the exposure phase, additional cobbles from the Llémena River were included in the channels to mimic colonization from upstream reaches.

The artificial streams were fed with rainwater filtered through activated carbon filters, and WWTP effluent water added to every set of them following the above dilution scheme. Treated effluents were collected at the WWTP of Quart (Girona, Spain), transported in 200-L tanks, and transferred to the artificial streams in less than 2 h. Daily cycles of photosynthetic active radiation (PAR) in the channels were defined as 10 h daylight (09:00-19:00) + 14 h darkness (19:00-09:00) using LED lights (120 W; Lightech, Girona, Spain). PAR was held constant at 174 ± 33 µE m^{-2} s^{-1} during the daytime and 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 10 ºC during the acclimation phase and at 15 ºC during the exposure and recovery phases, with an air humidity of 30%, to allow a gradual acclimation of the biofilm from the stream conditions to the artificial channel conditions. Additionally, water temperature was held constant at 20 ºC over the whole experiment and it was recorded every 10 min using VEMCO Minilog temperature data loggers (-5 to 35 ± 0.2 ºC) (TR model, AMIRIX Systems Inc, Halifax, NS, Canada). 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

Background physico-chemical conditions [pH, temperature (T), conductivity and dissolved oxygen (DO) concentration and saturation] were measured at noon every 3-4 d from water collected on the channel outlet of each artificial stream using hand-held probes (WTW multiline 3310, Weilheim, Germany; YSI ProODO handled, YSI Inc., Yellow Springs, OH, USA). 24 h after the renewal of the artificial streams concentrations of nutrients, major anions and cations, and dissolved organic carbon (DOC) were measured from water collected from the channel outlet. Water for nutrient analyses was immediately filtered through 0.2-µm pore size nylon filters (Whatman, Kent, UK) into pre-washed polyethylene containers. The concentration of soluble reactive phosphorus (SRP) was determined colorimetrically using a fully automated discrete analyzer Alliance Instruments Smartchem 140 (AMS, Frépillon, France). The concentration of anions [nitrate ($N\text{-}NO_3^-$), nitrite ($N\text{-}NO_2^-$), sulphate (SO_4^{2-}), chloride (Cl^-) and bromide (Br^-)] and cations [ammonium ($N\text{-}NH_4^+$), calcium (Ca^{2+}), magnesium (Mg^{2+}), sodium (Na^+) and potassium (K^+)] were determined on a Dionex ICS-5000 ion chromatograph (Dionex Corporation, Sunnyvale, USA). Water for DOC analysis was immediately filtered through ashed 0.7-µm pore size glass fibre filters (Whatman GF/F, Kent, UK). The concentration of DOC was determined using a Shimadzu TOC-V CSH (Shimadzu Corporation, Kyoto, Japan). Heavy metal concentrations were analyzed on water samples of the most polluted treatment (100%) filtered through 0.45-µm pore size nylon filters (Whatman, Kent, UK) collected the last day of the first experimental phase (32 d) and determined by ICP-MS (7500c Agilent Technologies, Inc. Willington, DE).

Response processes

The stream ecosystem functional response was assessed by measuring the SRP uptake capacity (U_{SRP}) of the biofilm, its biomass accrual [Chlorophyll-*a* (Chl-*a*) and ash-free dry mass (AFDM)] and the metabolism of the benthic community [gross primary production (GPP) and community respiration (CR)]. The activity of the microbial heterotrophic community was also assessed by the capacity to consume available organic matter (organic matter decomposition, OMD).

Biofilm SRP uptake and biomass accrual

SRP uptake and biomass accrual of the biofilm were measured on biofilm carriers. These are artificial plastic substrata with a high surface-to-volume ratio, which are used in WWTPs and aquaria to encourage biofilm attachment. We used cubic polyethylene carriers 2.5 cm in side (SERA GmbH D52518, Heinsberg, Germany), deployed at the beginning of the exposure phase and wired to each of the streams in batches of 8 cubes. At the end of each phase (32 d and 55 d of incubation), we

recovered 1 biofilm carrier per stream (3 replicates per treatment), which were immediately subjected to a bioassay to measure SRP uptake capacity (see below, in the same section) and then frozen in individual labelled plastic bags. After thawing, biofilm was detached from the biofilm carrier using a Branson sonifier ultrasonic cell disruptor (Branson Ultrasonic TM, Branson Ultrasonic Corporation, Emerson Electric, USA) combining 3 min of pulse mode at 70% of amplitude and 2 min of continuous mode at the same amplitude in 100 mL of deionized water. The biofilm solution was filtered onto ashed 0.7-µm pore size glass-fibre filters (Whatman GF/F, Kent, UK) for the determination of Chl-*a* and AFDM.

The SRP uptake capacity (U_{SRP}) of the biofilm growing on the biofilm carriers was measured by a novel method developed by von Schiller *et al.* (*in prep*). Briefly, once the biofilm carriers were collected from each channel, they were individually incubated in 100-mL clean plastic vials with an acclimation solution (1:5 dilution of Perrier carbonated mineral water (Nestlé, France) in deionized water), designed to ensure a sufficient supply of micronutrients, for 30 min at 100 rpm shaking speed, 20 ºC and ~180 µmol m^{-2} s^{-1} light. After the acclimation phase, the biofilm carriers were placed individually in pre-washed 60-mL plastic vials with the same acclimation solution but spiked with a PO_4 solution (K_2HPO_4, 10.000 µM = $3.1 \cdot 10^5$ µg P L^{-1}) to achieve a final concentration of 5 µM P (155 µg P L^{-1}), and incubated under the same conditions for 1 h. This concentration was chosen as a compromise to ensure saturating conditions for the biofilm while allowing the nutrient decline during the incubation and the subsequent estimation of uptake. After the incubation, 20 mL of water were collected from each vial, and 10 mL were filtered through glass-fibre filters (0.7-µm pore size, Whatman GF/F, Kent, UK) into 15-mL plastic tubes and frozen until analysis. Together with the colonized substrates, control treatments using non-colonized biofilm carriers (n = 3 on each incubation) were also used. The SRP uptake capacity was calculated as the difference between the mean SRP concentration of the control treatments (non-colonized substrates) and the colonized substrates, in the incubation solution volume (L) per incubation time (h). Thus, uptake capacity results were expressed in µg P retained per h^{-1}. SRP uptake capacity was also standardized by the biofilm biomass (AFDM) to obtain a clear picture about the efficiency of the biofilm to take up phosphorus. SRP concentration was determined manually on a double-beam UV-1800 UV-Vis Spectrophotometer (Shimadzu, Shimadzu Corporation, Kyoto, Japan) following the method described by Murphy & Riley (1962). The high N/P ratio in our channels suggested phosphorus to be the limiting nutrient.

Chl-*a* was measured for each filter after extraction in 90% acetone for 12 h in the dark at 4 ºC (Steinman *et al.*, 2006). To ensure the complete extraction of Chl-*a*, samples were sonicated for 30 s, twice (30 s, 360 W power, 50/60 Hz frequency, JP Selecta S.A., Barcelona, Spain). After that, Chl-*a* concentration was determined spectrophotometrically (U-2000 Spectrophotometer; Hitachi, Tokyo, Japan) by measuring the absorbance at 665 and 750 nm wavelengths, following the method described in Jeffrey & Humphrey (1975). Results were expressed as µg of Chl-*a* cm^{-2} of biofilm

carrier surface area. Besides, AFDM was used as an estimate of biofilm biomass. For its determination, another subsample of the biofilm extract was filtered on pre-weighed filters, dried at 70 ºC for 72 h to constant weight, weighed, combusted at 500 ºC for 5 h using a muffle furnace (AAF 1100, Carbolite, UK) and reweighed. Results were expressed as mg of AFDM cm^{-2} of biofilm carrier surface area.

Benthic metabolism

We measured biofilm net community metabolism (NCM) and community respiration (CR) analysing the changes in DO concentration inside cylindrical (0.96 L) recirculating chambers, as described by Acuña *et al.* (2008). One tray (64 cm^2 in surface area) made of stainless-steel wire mesh (1 mm mesh size) was located on each channel at the beginning of the acclimation phase to allow for biofilm colonization. All the trays were filled with coarse sand (d_{50} = 0.74 mm median diameter grain size (48 cm^2 surface area)) and included a pebble *c.a.* 4.5-cm in diameter (16 cm^2 surface area), to collect the performance of epipsammic and epilythic biofilm. At the end of each phase, trays were extracted from the channels, placed in the recirculating chambers, filled with water from the corresponding treatment and incubated for 1 h in light plus 1 h in darkness. All the chambers were deployed inside an incubator chamber (Radiber AGP-700-ESP, Barcelona, Spain) to maintain the water temperature analogous to that of the artificial streams (20 ºC). NCM was measured under light conditions (constant PAR of 168 ± 2 µE m^{-2} s^{-1}, which was similar to the irradiance at the artificial streams), while CR was measured in darkness. Once metabolism measurements were performed, trays were returned to their corresponding artificial stream. DO concentration inside the chambers was measured every 15 s with oxygen sensors (PreSens OXY-10mini, Regensburg, Germany). Metabolism rates were calculated following Acuña *et al.* (2008), in which NCM and CR were computed from the difference in oxygen concentration between two consecutive measurements (mg O_2 L^{-1}), in a specific time interval (h), accounting for the water volume used in the chamber (L) and the active surface of the substrate used for the incubation (m^2). Gross primary production (GPP) was estimated as the sum of NCM and CR. In all cases, results were expressed in mg O_2 produced or consumed per surface area (m^{-2}) and time (h^{-1}).

Organic matter decomposition

We measured OMD using 12-mm diameter discs from freshly fallen leaves of black alder (*Alnus glutinosa* (L.) Gaertner). Discs were cut using a cork borer, arranged in sets of 15, weighed and enclosed all together in black PVC tubes to prevent algal growth. PVC tubes (2 cm diameter and 5 cm long) were individually numbered and covered with a fine mesh (400 µm mesh size) to preclude losing the disks while allowing microbial colonisation and water flow. PVC tubes were placed flat and longitudinally on the bed of the artificial streams at the beginning of the exposure phase in groups of 9, which allowed having 3 replicates per channel. At the end of the exposure phase, leaf discs inside PVC tubes were collected, oven-dried (72 h, 70 ºC), weighed, combusted (5 h, 500 ºC)

and weighed again to estimate AFDM. In the recovery phase, the same process was repeated with another set of discs previously prepared under the same conditions. To correct the initial mass of the leaves used during the experiment, the leaching rate was determined in the laboratory from an additional set of 20 tubes (10 per each experimental phase) incubated under the same experimental conditions. Decomposition rates were calculated according to the negative exponential model (Petersen & Cummins, 1974), and were expressed as the OM consumed per day (d^{-1}) on each experimental phase.

Data analysis

We aimed at identifying the response type of the functional processes we measured by comparing the fit of 8 different models (linear, exponential, power, logistic, logit, Monod, Haldane and quadratic, Table 1, Fig. S1) to the data. These models were selected to encompass the most common relationships between ecosystem processes and environmental factors. The linear and quadratic models were adjusted using linear models with the "lm" R function (Chambers, 1992), whereas the other models were adjusted using non-linear models with generalized least squares with the "gnls" R function (Pinheiro *et al.*, 2018). The number of models fitted was not the same for all the variables, as the specific conditions necessary to run some of the models were not always fulfilled. Thus, results for the models [*i.e.*, Akaike Information Criterion (AIC) and Relative Standard Errors (RSE)] were only computed when the data of each variable fulfilled these conditions. However, the simplest response (the linear model) was always computed. Among all the computed models, we selected the most appropriate in each case following a standard selection criterion: 1) lowest AIC value, 2) lowest RSE value and 3) the model computed must have ecological sense. This means that models without ecological sense, such as inverse Haldane or Quadratic models, although computed, were disregarded. Equally, when none of the models fit the data, the linear model was selected by default. Normality of the residuals was checked for the adjusted models in each case, to ensure a correct utilization of the AIC values. Pearson moment correlation analyses were also used with the averaged values of water characteristics (*i.e.*, physico-chemical variables and concentrations of nutrients, anions and cations and DOC) to identify the direction and strength of the response to the gradient of WWTP effluent. All statistical analyses were performed using R software, ver. 3.4.0. (R Development Core Team, Vienna, Austria).

Table 1. Conception of the different models tested, with their corresponding mathematical equations, the meaning of the parameters on each equation, their ecological interpretation in our experiment and some examples from the literature describing each type of response. The y variable corresponds to the measured functional process, while the x variable represents effluent concentration.

Model	Equation	Parameters	Interpretation	References
Linear	$y = a + bx$	**a**, intercept - rate at concentration 0 **b**, slope	Functional response changes at a constant rate with effluent concentration, showing either direct subsidy (positive slope) or stress (negative slope).	Wagenhoff *et al.*, 2011 Aristi *et al.*, 2016
Exponential	$y = ae^{bx}$	**a**, rate at concentration 0 **b**, increase rate	Subsidy or stress effects increase by equal ratio with each increase in effluent concentration	Vandermeer, 2010 Wagenhoff *et al.*, 2011, 2012
Power	$y = ax^b$	**a**, coefficient **b**, exponent (defines curvature of function)	Subsidy or stress effects increase potentially with effluent concentration	Peters, 1983 Marquet *et al.*, 2005
Logistic	$y = \frac{a}{1 + e^{-b(x-x_0)}}$	**a**, maximum rate **b**, slope of the curve $\mathbf{x_0}$, **x** value of the sigmoid's midpoint	Subsidy effect initially increases exponentially but then levels because of a limiting factor	Ricklefs, 1967 Vandermeer, 2010 Wagenhoff *et al.*, 2011
Logit	$y = y_0 + \frac{1}{b} + \log \frac{x+1}{a-(x+1)}$	**a**, maximum rate **b**, slope of the curve $\mathbf{y_0}$, **y** value of the inverse sigmoid´s midpoint	Inverse to the logistic, it shows stress to increase exponentially with concentration until it levels	McFadden, 1974 Hanley *et al.*, 1998 Carl & Kühn, 2007
Monod	$y = a\frac{x}{b + x}$	**a**, maximum rate **b**, concentration value when the rate is at its half maximum	Similar to Michaelis-Menten equation, describes a subsidy effect that saturates as effluent concentration increases	Monod, 1949 Mogens *et al.*, 2000
Haldane	$y = a\frac{x}{b + x + \frac{x^2}{c}}$	**a**, maximum rate **b**, concentration value when the rate is at its half maximum **c**, concentration value when the inhibition is at its half maximum	Subsidy-stress dynamics: functional response peaks at intermediate effluent concentrations but falls below normal at high concentrations. Rising and falling limbs can be asymmetric	Haldane, 1930 Camargo & Alonso, 2006 Woodward *et al.*, 2012 Wagenhoff *et al.*, 2011, 2012, 2013
Quadratic	$y = ax^2 + bx + c$	**a**, concentration values when the inhibition is maximum **b**, slope **c**, intercept, the rate at concentration zero	Similar to Haldane, but the rising and falling limbs are symmetric	Otto & Day, 2007 Weir & Pettit, 2000 Austin, 2002

Results

Water chemistry

The effluent used throughout the experiment had 7.9 ± 0.1 pH, 1348 ± 61 µS cm^{-1} conductivity and 6.9 ± 0.4 mg L^{-1} DO. Nutrient and DOC concentrations were high: 5.8 ± 2.0 mg N L^{-1} of $N\text{-}NO_2^-$, 16.1 ± 3.2 mg N L^{-1} of $N\text{-}NH_4^+$, 16.1 ± 4.2 mg N L^{-1} of $N\text{-}NO_3^-$, 0.70 ± 0.13 mg P L^{-1} of SRP and 14.3 ± 0.5 mg C L^{-1} of DOC (see Table 2 and Table S1 for more details). Copper (81.9 ± 1.6 µg L^{-1}), zinc (71.8 ± 4.8 µg L^{-1}), iron (51.9 ± 3.9 µg L^{-1}) and arsenic (7.3 ± 0.2 µg L^{-1}) were the most abundant heavy metals in the effluent (Table S1). Increased effluent contribution linearly reduced DO and pH ($R^2 = 0.87$, $p < 0.001$ and $R^2 = 0.93$, $p < 0.001$, respectively), and increased conductivity ($R^2 = 0.99$, $p < 0.001$), nutrients ($R^2 = 0.94$, $p < 0.001$ for $N\text{-}NO_2^-$; $R^2 = 0.99$, $p < 0.001$ for $N\text{-}NH_4^+$; $R^2 = 0.98$, $p < 0.001$ for $N\text{-}NO_3^-$; $R^2 = 0.95$, $p < 0.001$ for SRP) and DOC concentration ($R^2 = 0.99$, $p < 0.001$). However, T did not vary between treatments ($R^2 = 0.01$, $p = 0.84$; Table 3). The solutes increased linearly but in different proportion: $N\text{-}NO_2^-$ increased up to 100-fold, $N\text{-}NH_4^+$ up to 400-fold, whereas $N\text{-}NO_3^-$, SRP and DOC increased up to 5- to 20-fold. The entrance of unpolluted water in the recovery phase eliminated the differences among treatments (Table 2).

Table 2. Water characteristics in each treatment during the exposure and the recovery phases (all treatments pooled). Values shown are mean ± standard error (SE) calculated for pH, T, conductivity and DO concentration from 3 replicates per treatment, during 10 surveys in the exposure phase (n = 30) and 6 surveys in the recovery phase (n = 18)). Values for $N\text{-}NO_2^-$, $N\text{-}NH_4^+$, $N\text{-}NO_3^-$, SRP and DOC were calculated from 3 replicates per treatment during 12 surveys in the exposure phase (n = 36, except DOC where n = 30), and during 8 surveys in the recovery phase (n = 24, except DOC where n = 12).

Treatment	0%	14%	29%	43%	58%	72%	86%	100%	Recovery
pH	8.4 ± 0.1	8.4 ± 0.1	8.3 ± 0.1	8.2 ± 0.1	8.1 ± 0.1	8.0 ± 0.1	8.0 ± 0.1	7.9 ± 0.1	8.6 ± 0.0
T (ºC)	21.1 ± 0.3	21.0 ± 0.2	19.4 ± 0.2	19.1 ± 0.2	19.7 ± 0.2	19.9 ± 0.2	21.0 ± 0.2	21.2 ± 0.2	20.2 ± 0.3
Conductivity (µS cm^{-1})	220 ± 4	398 ± 9	584 ± 20	766 ± 28	934 ± 37	1112 ± 45	1205 ± 53	1348 ± 61	302 ± 4.3
DO (mg L^{-1})	8.9 ± 0.1	9.0 ± 0.1	9.2 ± 0.1	8.7 ± 0.2	8.3 ± 0.3	7.8 ± 0.4	7.3 ± 0.4	6.9 ± 0.4	9.2 ± 0.1
$N\text{-}NO_2^-$ (mg N L^{-1})	0.05 ± 0.0	0.4 ± 0.1	1.8 ± 0.4	2.9 ± 0.8	4.7 ± 1.2	5.5 ± 1.6	6.5 ± 2.0	5.8 ± 2.0	0.003 ± 0.0
$N\text{-}NH_4^+$ (mg N L^{-1})	0.04 ± 0.02	2.0 ± 0.5	5.4 ± 1.1	7.7 ± 1.6	9.4 ± 2.1	11.8 ± 2.5	13.4 ± 2.8	16.1 ± 3.2	0.002 ± 0.0
$N\text{-}NO_3^-$ (mg N L^{-1})	1.2 ± 0.0	3.7 ± 0.5	4.4 ± 0.9	6.9 ± 1.7	8.6 ± 2.3	11.9 ± 3.2	12.6 ± 3.6	16.1 ± 4.2	1.0 ± 0.1
SRP (mg P L^{-1})	0.04 ± 0.03	0.03 ± 0.01	0.06 ± 0.01	0.21 ± 0.06	0.31 ± 0.09	0.44 ± 0.11	0.53 ± 0.12	0.70 ± 0.13	0.03 ± 0.01
DOC (mg C L^{-1})	1.2 ± 0.1	3.0 ± 0.1	5.0 ± 0.1	7.1 ± 0.2	9.1 ± 0.3	11.3 ± 0.4	12.2 ± 0.5	14.3 ± 0.5	2.0 ± 0.0

Biofilm SRP uptake and biomass accrual

The WWTP effluent caused an immediate stress effect on SRP uptake capacity, which peaked in the control treatment (5.6 µg P h^{-1}, 0%) and decreased with increasing pollution proportions (Fig. 2a) to the point that above 70% of effluent contribution, it became negative, *i.e.* biofilm carriers released SRP (Fig. 2a, 2b). This decrease in the uptake capacity followed a logit model (Table 3, Fig. S2), which showed the most abrupt decrease at the lower levels of pollution and a more stable but negative response from medium levels of effluent contribution. The effluent also caused important legacy effects on SRP uptake capacity, which followed the same decreasing pattern from the control treatment (5.7 µg P h^{-1}, 0%) to the most polluted one (1.4 µg P h^{-1}, 100%), fitting again the logit model (Table 3, Fig. S2).

The immediate effects of the WWTP effluent on biofilm SRP uptake efficiency (*i.e.*, uptake capacity standardized by biofilm biomass) consisted on a decreased uptake efficiency (Fig. 2b), which was again the highest in the control treatment (1.0 µg P mg $AFDM^{-1}$ h^{-1}, 0%), and decreased down to -0.2 µg P mg $AFDM^{-1}$ h^{-1} in the most polluted treatment. Once more, data fitted best the logit model (Table 3, Fig. S3), suggesting a pure stress effect. Legacy effects also peaked at low levels of effluent contribution (highest uptake: 0.8 µg P mg $AFDM^{-1}$ h^{-1}, 14%; lowest uptake: 0.2 µg P mg $AFDM^{-1}$ h^{-1}, 100%). This relationship, however, followed the Haldane model, which suggested a possible subsidy-stress legacy effect (Table 3, Fig. S3).

Maximum Chl-*a* values during the exposure phase occurred at medium levels of effluent contribution (17.5 µg cm^{-2}, 29%, Fig. 2c), while the lowest values (3.9 µg cm^{-2}) were observed in the control treatment. Thus, the immediate effects of pollution consisted on subsidizing Chl-*a* at all proportions of pollution tested, but the data fitted best the Haldane model (Table 3, Fig. S4), thus suggesting a saturating subsidy effect which became inhibited at higher levels of effluent contribution. The WWTP effluent had a noticeable legacy effect on Chl-*a*, as values were lower in the control treatment (14.9 µg cm^{-2}) and peaked at intermediate levels of pollution (45.1 µg cm^{-2}, 58%). However, although the pattern was best described by the quadratic model (Table 3, Fig. S4), the falling limb of the hump did not fall below the control, which showed again a saturating subsidy legacy effect with inhibition at higher levels of pollution legacy.

Immediate effects of pollution on biofilm biomass accrual (AFDM) suggested a subsidy-stress response (Fig. 2d): AFDM peaked at low-medium levels of effluent (1.8 mg cm^{-2}, 14%) and the lowest values occurred in the most polluted treatment (0.9 mg cm^{-2}), the data fitting best the Haldane model (Table 3, Fig. S5). Thus, AFDM showed a saturating subsidy effect which became stressed in the highest levels of effluent contribution. Pollution also showed important legacy effects for AFDM, as biofilm biomass accrual peaked in treatments previously exposed to medium levels of effluent contribution (2.9 mg cm^{-2}, 58%), being the lowest again in the most polluted treatment (1.2

mg cm^{-2}). The data best fitted the quadratic model (Table 3, Fig. S5), thus showing similar legacy effects of subsidy-stress.

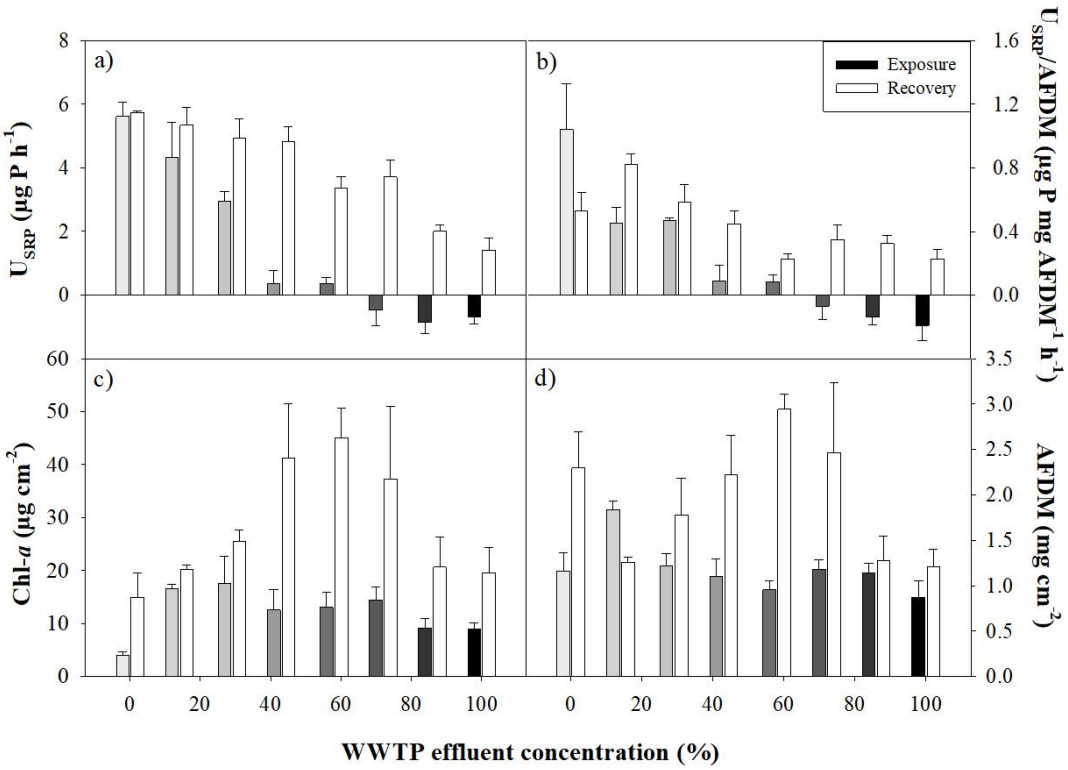


Figure 2. Immediate (exposure phase, shaded columns) and legacy effects (recovery phase, white columns) of WWTP effluent pollution on processes measured on biofilm carriers. (a) Soluble reactive phosphorus (SRP) uptake capacity (b) SRP uptake efficiency (SRP uptake standardized by biofilm biomass), (c) chlorophyll-*a* (Chl-*a*) and (d) biofilm biomass accrual (AFDM). Values shown are mean ± standard error (SE).

Benthic metabolism

Immediate effects of the WWTP effluent promoted GPP from 225 mg O_2 m^{-2} h^{-1} in the control treatment to 785 mg O_2 m^{-2} h^{-1} at medium levels of effluent contribution (58%), following the quadratic model (Table 3, Fig. S6). However, as values of the most polluted treatments did not fall below the control ones, it showed a saturating subsidy effect, which was most accentuated at medium concentrations and became inhibited at high levels of effluent contribution. The legacy effects followed a similar pattern (lowest production rate: 298.2 mg O_2 m^{-2} h^{-1}, 0%; highest production rate: 499.3 mg O_2 m^{-2} h^{-1}, 58%). Data fitted best the quadratic model (Table 3, Fig. S6), and treatments with the highest legacy did not provide values below the control ones, suggesting the same subsidy effect accentuated at medium concentrations.

Immediate effects of the WWTP effluent promoted CR from -83.8 mg O_2 m^{-2} h^{-1} in the control treatment to -518.4 mg O_2 m^{-2} h^{-1} in the most polluted, fitting a logit model which suggested a pure subsidy effect (Table 3, Fig. S7). A pollution legacy effect could also be seen, by the similar

pattern followed by the treatments at the end of the recovery phase (lowest oxygen consumption rate: -76.8 mg O_2 m^{-2} h^{-1}, 0%; highest oxygen consumption rate: -183.2 mg O_2 m^{-2} h^{-1}, 100%). Data fitted best again the logit model (Table 3, Fig. S7), indicating the same pure subsidy legacy effect.

All treatments were predominantly autotrophic, since the average production-to-respiration ratio (GPP:CR) calculated for exposure and recovery phases (mean ± standard error) were above 1, *i.e.* of 2.17 ± 0.21 and 3.52 ± 0.30, respectively, and peaked at intermediate levels of pollution and pollution legacy.

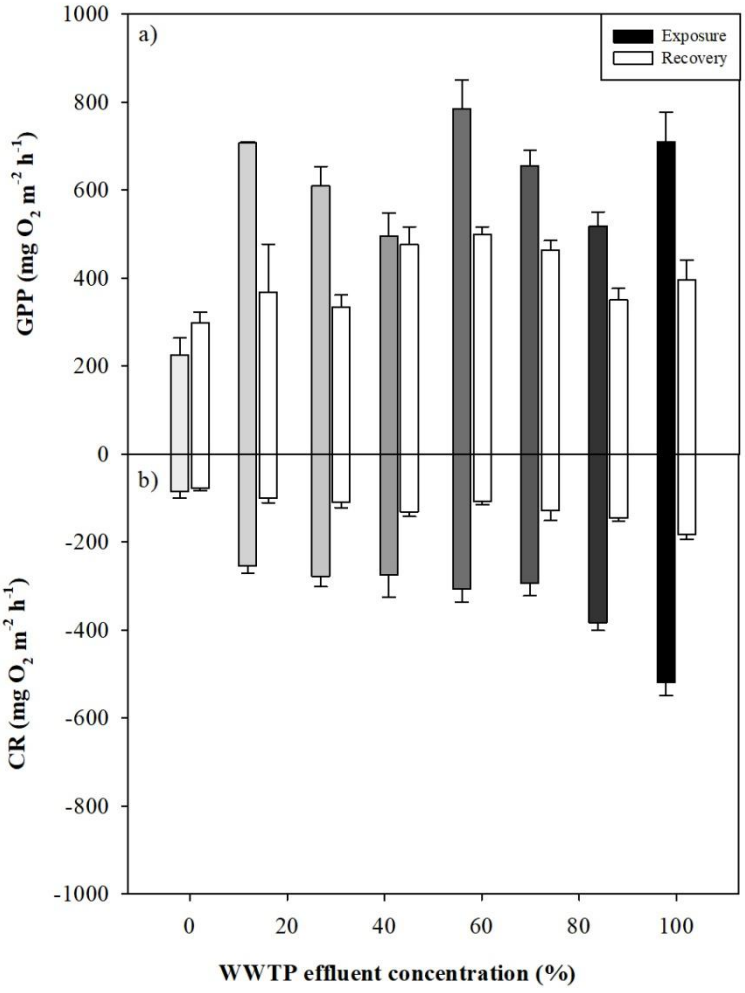


Figure 3. Immediate (exposure phase, shaded columns) and legacy effects (recovery phase, white columns) of WWTP effluent pollution on benthic metabolism. (a) Gross primary production (GPP) and (b) Community respiration (CR) measured on epipsammic and epilithic biofilm. Values shown are mean ± standard error (SE).

Organic matter decomposition

Immediate effects of the WWTP effluent promoted OMD to values up to 0.005 d^{-1} in the most polluted treatment (Fig. 4), whereas it was lowest at medium levels of effluent contribution (0.003 d^{-1}, 43%). The results best fitted a linear model (Table 3, Fig. S8), indicating a non-saturating subsidy

effect on OMD. Legacy effects were weak: the slowest OMD rate (0.011 d^{-1}) occurred at medium levels of effluent contribution (in the treatment 43%) and the fastest (0.013 d^{-1}) in the control treatment. However, process fitted best a weak linear pattern (Table 3, Fig. S8), showing almost no legacy effect.

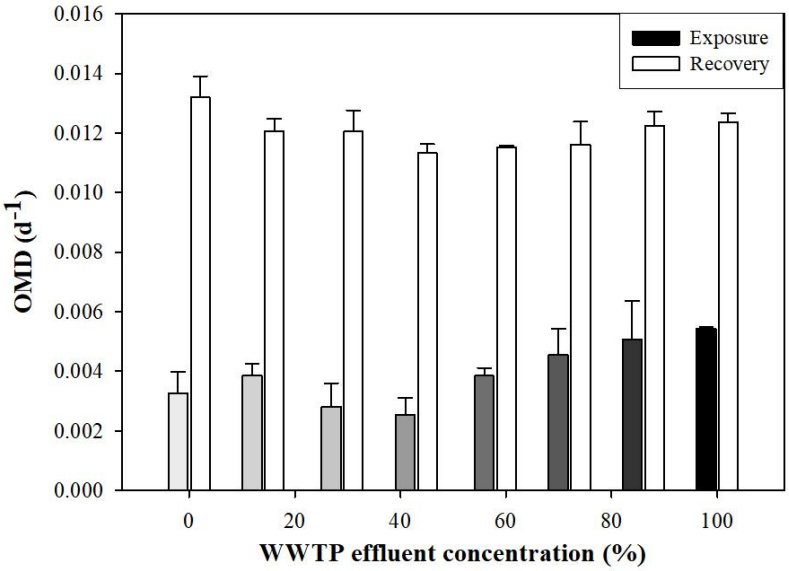


Figure 4. Immediate (exposure phase, shaded columns) and legacy effects (recovery phase, white columns) of WWTP effluent pollution on organic matter decomposition (OMD). Values shown are mean ± standard error (SE).

Table 3. Linear and best-fitting model results for soluble reactive phosphorus (SRP) uptake capacity (U_{SRP}), SRP uptake efficiency (U_{SRP}/AFDM), chlorophyll-*a* (Chl-*a*), total biomass (AFDM), gross primary production (GPP), community respiration (CR) and organic matter decomposition (OMD), for immediate (measured at the end of the exposure phase) and legacy effects (measured at the end of the recovery phase). Akaike Information Criterion (AIC) and Relative Standard Errors (RSE) results are shown for the best fitted model on each case (Best fit model, Best AIC, Best RSE), but also for the simplest response, given by default in all the cases (Lin. AIC, Lin. RSE). These values verify whether the best fitted model improved the linear response results or not. Finally, pollution and legacy effects refer to whether the WWTP effluent increases (Subsidy), reduces (Stress) or produces both outcomes (Subsidy-Stress) beyond the control values (pure stream water). For more detailed information about the fitting models see Figures 2-8, available as Supplementary Material to this paper (Fig. S2- S8).

Immediate effects							
Process	U_{SRP}	U_{SRP}/AFDM	Chl-*a*	AFDM	GPP	CR	OMD
Lin. AIC	78.4	3.2	158.1	16.7	317.5	274.5	-248.2
Lin. RSE	1.14	0.24	6.01	0.32	166.36	67.95	0.001
Best fit model	Logit	Logit	Haldane	Haldane	Quadratic	Logit	Linear
Best AIC	77.2	-3.3	145.3	11.5	315.1	259.8	-248.2
Best RSE	1.09	0.20	4.51	0.28	155.35	49.11	0.001
Pollution effects	**Stress**	**Stress**	**Subsidy**	**Subsidy-Stress**	**Subsidy**	**Subsidy**	**Subsidy**
Legacy effects							
Lin. AIC	59.3	-13.3	202.6	64.3	291.4	221.4	-258.9
Lin. RSE	0.77	0.17	15.18	0.85	96.48	22.47	0.001
Best fit model	Logit	Haldane	Quadratic	Quadratic	Quadratic	Logit	Linear
Best AIC	56.9	-22.8	192.3	62.5	286.6	214.5	-258.9
Best RSE	0.72	0.14	12.02	0.81	85.73	19.12	0.001
Legacy effects	**Stress**	**Subsidy-Stress**	**Subsidy**	**Subsidy-Stress**	**Subsidy**	**Subsidy**	**No legacy**

Discussion

Ecosystem response to rising concentrations of WWTP effluents

The gradient of effluent contribution in our experiment mimicked the situation occurring in the field, where in extreme cases wastewater discharges can contribute most of the in-stream flow, transforming these streams into effluent dominated systems (Rice & Westerhoff, 2017). Despite our extreme gradient, the expected subsidy-stress scheme was only followed by biofilm biomass accrual. Otherwise, the effluent acted as a subsidy for most processes, except SRP uptake, which decreased dramatically with increasing effluent concentration. However, the patterns as well as the concentrations at which activities peaked followed process-specific responses.

Some studies (*e.g.* Clapcott *et al.*, 2011; Woodward *et al.*, 2012) have reported stream variables to show hump-shaped responses to stressors such as nutrient inputs, and this pattern has been often attributed to the subsidy-stress scheme described by Odum *et al.* (1979). Nevertheless, Odum defined stress as values of biological activity below "normal", which means that not all hump-shaped curves reflect subsidy-stress. In our case, most measured processes did not follow the subsidy-stress scheme, but were rather subsidized by the WWTP effluent, even at 100% effluent contribution. Only a few functional processes were impaired by the effluent, such as, the biofilm SRP uptake capacity and the biofilm biomass accrual, although they showed different thresholds of stress. While the biofilm SRP uptake capacity was purely stressed from the lowest levels of effluent contribution, biofilm biomass accrual followed the subsidy-stress pattern, shifting from being subsidized to be stressed at medium levels of effluent contribution. These results suggest that the WWTP effluent used in the present experiment had low toxicity for microbially-mediated processes. According to the EU-funded ENERWATER research project (ENERWATER European Project 2018, URL: http://www.enerwater.eu/), the effluents from WWTPs worldwide span a very large range of nutrient concentration (0.1-95 mg N L-1 and 0.1-9 mg P L-1 for total nitrogen and total phosphorus, respectively) (ENERWATER Benchmarking Database, 2018). Our effluent fits in the low range of both nutrients, meaning that more stress responses could have been detected with effluents fitting higher ranges of nutrient concentrations.

The response of some of the functional processes measured (quadratic for GPP, logit for CR and linear for OMD) were best fitted by models indicating that the effluent produced a non-saturating subsidy effect. In those cases, the effluent did not become toxic, even at the highest concentrations, and also none of the experimental abiotic factors became limiting. Probably, the high nutrient supply (up to 400-fold times higher than in the control), absence of light limitation and warm water temperature in our experiment promoted these microbially-mediated processes, as has been shown in open streams placed below WWTP effluents (Stelzer *et al.*, 2003; Albek, 2003; Martí *et al.*, 2004). These results are in line with previous studies where high nutrient concentrations and warm water temperatures were reported as the main drivers of faster decomposition rates in effluent-dominated streams (Spänhoff *et al.*, 2007). Still, we expected that the high concentrations of both assimilable and toxic compounds in the treatments with lower dilution

capacity (*i.e.*, higher levels of effluent contribution), would have stress effects and limit processing rates, but this was not the case. Our results for benthic metabolism also support that the higher nutrient concentrations derived from wastewater effluents would promote overall ecosystem metabolism (Gücker *et al*., 2006; Izagirre *et al.*, 2008). At medium pollution levels, however, primary production became saturated while respiration increased dramatically. After 15 d of exposure to the WWTP effluent, some of the most polluted treatments started to show hypoxic conditions during the night (DO concentrations down to 4 mg L^{-1}), and reached anoxic conditions (1 mg L^{-1}) after 25 d of exposure. Overall, these are signals of eutrophication in the most polluted treatments (Smith, 2003; Brack *et al.*, 2007).

Some other response models (Haldane for Chl-*a* and AFDM) indicate that the effluent had a saturating subsidy effect on these processes, with inhibition at higher levels of effluent contribution. Medium pollution concentrations were saturating for both processes and the highest pollution levels induced inhibition, and even toxicity in the case of biofilm biomass. This saturating subsidy effect has been widely described in the literature (Paul & Meyer, 2001), together with the toxic effects that could be produced by higher nutrient concentrations (Ribot *et al.*, 2015). Interestingly, this saturation threshold was achieved at lower levels of effluent contribution for both Chl-*a* and AFDM than for GPP, suggesting that structural variables could be more sensitive than those related to ecosystem functioning. However, the decreasing tendencies caused by inhibition at higher levels of effluent contribution, do not fit the non-saturating tendencies observed for both OMD and CR, which suggests that effects might depend on the type of organism. It is well known that nutrients may induce changes in species composition (Bernhardt & Likens, 2004; Domingues *et al.*, 2011), and this could affect both the primary producers as well as the heterotrophs existing in our artificial streams, which could have favoured the presence of a more resistant community.

Finally, the logit model showed that the effluent had a pure stress effect on SRP uptake capacity and efficiency, as both activities decreased since the lowest levels of pollution. The demand for nutrients dissolved in the water column is affected by the balance between the biofilm and the water column, as internal recycling gains importance when the external supply is comparatively low (Mulholland, 1996; Hall *et al.*, 2002). Thus, under high external nutrient supply, biofilms become less efficient in nutrient removal from the water column (Proia *et al.*, 2017), to the most extreme cases where no nutrient removal occurs due to a saturation of the system (Earl *et al.*, 2006). Moreover, in our experiment, SRP concentrations in the most polluted treatments were higher than in the bioassay standard solution, which could have led to abiotic desorption of SRP by the biofilms. Therefore, our results point to a combination of biotic saturation of SRP removal and shifts in abiotic sorption-desorption mechanisms as the main causes of the observed decline in SRP uptake capacity and efficiency along the gradient of increasing pollution contribution.

Legacy effects

Most processes showed clear legacy effects of pollution, despite a higher biological activity at the end of the recovery phase than at the end of the exposure phase. However, these legacy effects followed either subsidy, stress, or subsidy-stress dynamics.

For most of the processes, the response model for the legacy effect was the same as for the immediate effect despite the inclusion of colonized cobbles from the unpolluted Llémena River at the onset of the recovery phase. This fact suggests some degree of resistance to change of the old biofilm community to the new inoculum. During the whole experiment, the water flow was very slow, which has been shown to enhance biofilm biomass, thickness and complexity (Battin *et al.*, 2003). These thick biofilm structures reduce the hydraulic exchange between the biofilm and the water column, promoting internal cycling processes (Earl *et al.*, 2006; Johnson *et al.*, 2015). Thus, thick biofilm can store large concentrations of nutrients, as we observed in the experiment even weeks after the effluent flow ceased. The storage of nutrients and other pollutants within the biofilm could be the main cause for the persistence of the similar subsidy, stress or subsidy-stress patterns for immediate and legacy responses. Results found by Acuña *et al.* (*In prep.*) in another add-on work to this experiment support this idea, as they found phosphate desorption from the sediment and biofilm during the recovery phase.

Most studies assessing the effects of urban pollution on stream ecosystem functioning are usually based on the comparison between reaches located upstream and downstream from existing point-source inputs (*e.g.*, Sánchez-Pérez *et al.*, 2009). Although field studies offer greater realism than laboratory experiments, the latter offer the opportunity to ask questions impossible to answer with just observational studies, such as the effect of the dilution rate of WWTP effluents.

Conclusions

Urban pollution from WWTPs affected the functioning of our mesocosm ecosystems, although mostly not following the subsidy-stress scheme we expected. Instead, subsidy responses were the most common among the variables measured, even though complex and process-specific patterns were observed. Legacy effects occurred for most of the studied processes and followed a similar pattern to that of immediate effects, although responses became more complex, mainly driven by the internal cycling occurring within biofilms. Overall, the effluent used in the present experiment produced complex effects, and we could, thus suspect even more complex responses in real-world freshwater ecosystems.

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CHAPTER 5

General discussion

1. Stream and effluent characteristics: crucial role determining the effects

The present dissertation studied contrasting stream types that received different types of urban effluents, under a relatively large geographical area and followed several ecosystem processes seeking to describe a consistent functional response. However, functional processes showed contrasting response patterns. While urban effluents subsidized most of the processes measured, the only being consistently stressed was the biofilm nutrient uptake capacity (Chapters 2, 3 and 4), and biofilm biomass accrual was the only process showing a clear subsidy-stress pattern during the laboratory experiment (Chapter 4). This suggests that some other factors could have influenced our results and therefore, that should be considered to assess the global magnitude of urban pollution.

1.1. How could multiple stressors modulate the effects of urban pollution?

Freshwater ecosystems are usually subjected to the joint pressure of multiple stressors (Sabater *et al.*, 2018b), and thus during the last decades the concern about their ecological effects on biological communities (Sabater *et al.*, 2016) and ecosystem functioning (Johnston *et al.*, 2015) has significantly increased. Interactions among stressors, however, are not always straightforward and sometimes yield unexpected results, due to the indirect relationships among stressors (Romero *et al.*, 2018), the variability on the biological response (Berthelsen *et al.*, 2018) and the resistance capacity of each ecosystem (Jackson *et al.*, 2016). Indeed, our field observational experiment which assessed the joint effects of urban pollution and hydrology (Chapter 2) showed that even if effluents subsidized most functions, the response pattern was complex and process-specific. Nevertheless, this complexity was maintained when the number of stressors was reduced to the minimum (Chapters 3 and 4), emphasizing that the balance between pollutants can also affect differently ecosystem processes. In both studies urban effluents stressed microbially-mediated functional processes, although it was not translated into ecosystem-level responses.

Many stressors can exacerbate the effects of urban pollution on stream ecosystem functioning. For instance, diffuse nutrient inputs derived from agricultural practices can increase stream background concentrations of nutrients and pesticides, which could increase toxicity levels. As a consequence, these pollutants can saturate stream biotic uptake, decreasing the efficiency of nutrient removal (Merseburger *et al.*, 2011) and increasing the load of nutrients downstream (Earl *et al.*, 2006). On the other hand, most freshwater ecosystems are also intensively managed by human practices such as weirs, dams, channelization, groundwater exploitation or water abstraction (Elosegi & Sabater, 2013; Sabater *et al.*, 2018a). All these impacts decrease stream flows, alter sediment transport and channel morphology, increase water temperature and impair water chemistry (Arroita *et al.*, 2016), all of which are likely to interact with pollution. In the field observational experiment (Chapter 2), lower stream flows reduced the dilution capacity of streams, increasing the concentrations of pollutants (Rice & Westerhoff, 2017), which affected key ecosystem processes, such as the biofilm capacity to retain nutrients. However, at the same time, lower stream flows enhance hydrological stability and hydraulic retention, which could favour the transformation, degradation and assimilation of pollutants (Arenas-Sánchez *et al.*, 2016). Some authors

described a significant growth of biological communities during periods of hydrological stability (Aristi *et al.*, 2014), which can promote ecosystem processes, such as nutrient retention or metabolism (Sabater *et al.*, 2018a). Finally, human practices, such as agriculture, intensive forestry or mineral extraction, promote sediment erosion and increase the proportion of fine sediments on streams and rivers (Wood & Armitage, 1997). Fine sediments can interact with urban effluents, also recognized to be an important source of them (Miserendino *et al.*, 2008), and alter the structure of biofilm (Wagenhoff *et al.*, 2013) and invertebrate communities (Piggot *et al.*, 2015), which can ultimately affect key ecosystem processes (Niyogi *et al.*, 2003; Young *et al.*, 2008). In that sense, field experiments showed that urban effluents stressed biofilm capacity to retain nutrients, but these responses were not translated into whole-ecosystem processes, suggesting that other compartments could have played a key role mitigating the negative effects observed at the biofilm scale.

Figure 1. Some of the stressors that can interact with urban pollution: pesticides, channelization, siltation. Photographs provided by Arturo Elosegi.

In streams and rivers a wide variety of compartments can be biogeochemically active and play a determinant role on ecosystem processes, including nutrient uptake and metabolism (Fig. 2). In our field manipulative experiment (Chapter 3), the effluent contribution was low and the stream was relatively little affected by additional human activities. The streambed sediment was dominated by bedrock, hyporheos seemed to be of little importance, but the riparian trees there had extensive roots protruding into the channel, which could have played a role on nutrient retention. In our field observational experiment, on the contrary, reed-type macrophytes were likely major players for ecosystem functioning at some streams. Additionally, urban effluents (Miserendino *et al.*, 2008) and agricultural practices (Wood & Armitage, 1997) caused thick deposits of fine sediments on some reaches. Fine sediments can clog top sediment layers and reduce the connectivity between ecosystem compartments, including the hyporheos (Descloux *et al.*, 2013). Because of that, in those streams other compartments such as fine sediments (Withers & Jarvie, 2008) or macrophyte communities (Riis *et al.*, 2012), could have played a key role explaining the differences observed between biofilm and whole-reach scales. Nevertheless, as I only measured the ecosystem functional response at two levels, without considering other compartments that could also be involved, I can only speculate about it. Therefore, I think that it would be interesting to measure the role of other ecosystem compartments in the Apraitz Stream and in the lower Ebro River tributaries, in order to disentangle their specific contribution to the whole-ecosystem processes and thus, detect the main

compartment modulating the ecosystem response under multiple stress scenarios (Hoellein *et al.*, 2009; Acuña *et al.*, 2011).

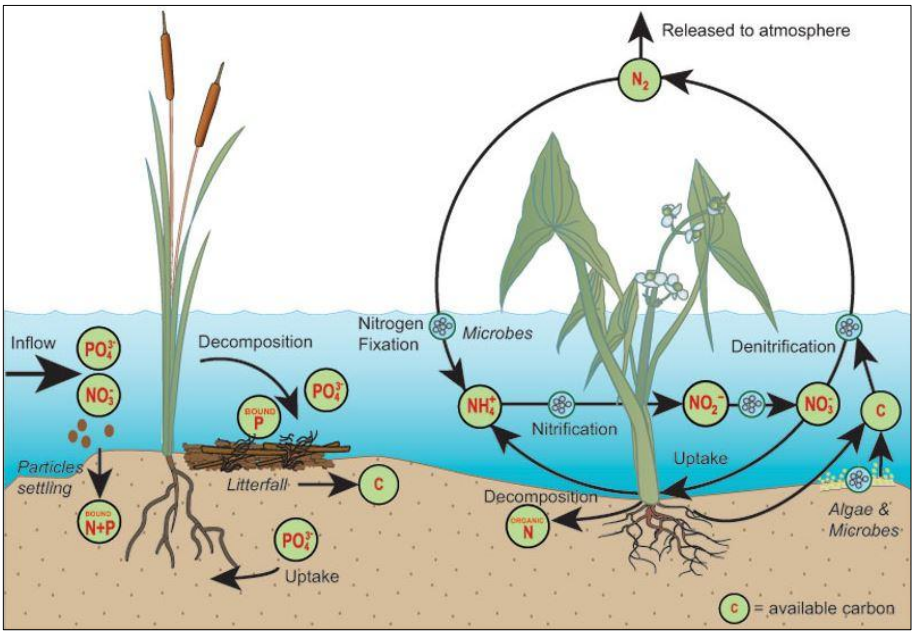

Figure 2. Ecosystem compartments that can play a key role in whole-reach nutrient uptake and metabolism. The figure is of public domain.

1.2. How can the nature of the effluent modulate the response?

During the last years, there have been many technological advances and optimisation of WWTPs. Consequently, human health and water security have improved in those countries able to afford their economic costs (Vörösmarty *et al.*, 2010). However, huge amounts of wastewater are still released into the environment without any treatment, especially in developing countries (WHO & UNICEF, 2017). Even if WWTPs do not remove all contaminants, they are necessary to reduce the loads of pollutants and pathogens to acceptable levels before releasing the effluents into the environment (Alfonsín *et al.*, 2014; Aymerich *et al.*, 2016). Some authors, comparing raw and treated effluents, showed how WWTPs reduce the concentrations of pollutants, such as pharmaceuticals (Mandaric *et al.*, 2018), personal care products (Kasprzyk-Horden *et al.*, 2009), heavy metals (Cantinho *et al.*, 2016) and pathogens (Vaz-Moreira *et al.*, 2014; Subirats *et al.*, 2017). Due to this ever-changing mixture of pollutants, chemical pollution is widely considered a multiple stressor on its own (Jackson *et al.*, 2016). But many authors highlight the need for assessing the exact composition of these mixtures (Altenburger *et al.*, 2015), as the mode of action of each compound (Cleuvers, 2003) could determine the balance between pollutants and thus, affect ecosystem functioning in different ways. In that sense, prevailing land-use in the surrounding area is one of the main factors influencing the chemical composition of sewage inputs (Posthuma *et al.*, 2008), as agricultural practices can increase the loads of nutrients and pesticides (Kuster *et al.*, 2008), industrial activities heavy metal concentrations (Holeton *et al.*, 2011), while urban areas the loads of other micro-contaminants, such as pharmaceuticals, personal care products or illicit drugs (Kuzmanovic *et al.*, 2015; Rosi-Marshall *et al.*,

2015; de Solla *et al.*, 2016). Finally, the effects of WWTP effluents depend on their final concentration in the receiving water body, which depends on the dilution capacity of the receiving stream, the wastewater treatment efficiency and the volume of effluents released. Also, temporal variability in effluent release can have important effects. For instance, Nyman *et al.* (2013) showed in a laboratory experiment that the exposure to a constant insecticide concentration reduced the activity and physiology of the amphipod *Gammarus pulex*, but that the same concentration added in pulses did not exert any significant effect.

In the present dissertation I have not directly assessed these above-mentioned factors. Therefore, I can only speculate about it. Nevertheless, I think that the precise nature of the effluent must be considered when assessing the effects of urban pollution on stream ecosystem functioning (Jackson *et al.*, 2016). In fact, if we assume that functional processes or biological activities respond to a gradient of pollution following the Subsidy-Stress pattern (Odum *et al.*, 1979), inconsistent results from different studies could simply derive from the specific effluent contribution or from the balance between assimilable and toxic compounds. Some studies can describe promoted biological activities below urban effluents, as a consequence of lower effluent concentration or because assimilable substances prevail over toxic ones (Green box, Fig. 3). On the contrary, other studies can describe stressed biological activities, due to higher effluent concentration or because toxic substances prevail over assimilables (Red box, Fig. 3). Whereas some other studies, can report unaffected ecosystem processes as a consequence of intermediate levels of effluent contribution or of a balance between assimilable and toxic substances (Blue box, Fig. 3).

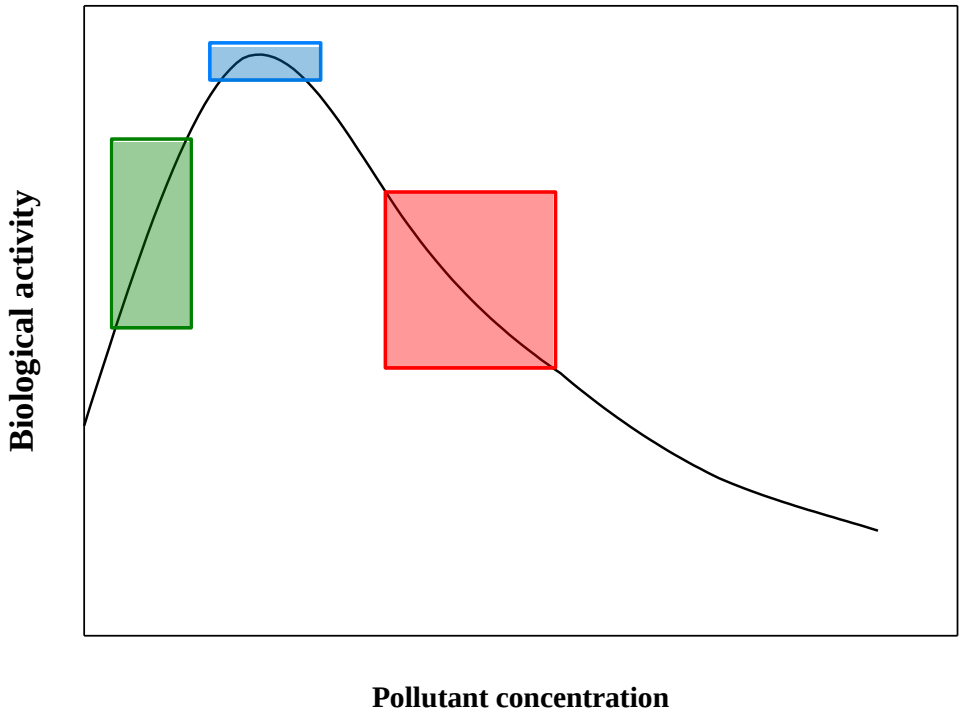

Figure 3. Hypothetical Subsidy-Stress type response of biological activity to the concentration of urban pollutants, and potential sources of contrasting results among studies focusing on the effects of pollutant concentration on ecosystem functioning. An experiment in the range of the green box would conclude that urban pollution subsidizes biological activity,

an experiment in the range of the red box would conclude there is an stress effect, whereas an study with two concentrations, one in the green range and one in the red range, would conclude that urban pollution has no effect on biological activity.

1.3. How does stream dilution capacity modulate the effluent effects?

The proportion of effluent in the total river flow defines the dilution capacity of a river (Rice & Westerhoff, 2017) and it does modulate the effects on ecosystem functioning. The study sites used in the present dissertation covered a large gradient of effluent contribution, from 3% to more than 90% in natural streams, in Apraitz Stream and tributaries of the lower Ebro River (*e.g.*, Els Reguers or La Sènia), respectively, and from 0 to 100% in laboratory artificial streams. Although impacts should increase with increasing effluent contribution, sometimes results yield ecological surprises (Jackson *et al.*, 2016) showing non-linear responses (Pereda *et al.*, 2019). Therefore, here we look at the effect of effluent contribution on stream ecosystem functional processes measured through the three chapters of the dissertation (Figs. 4-5), by computing the ratio between Impact and Control reaches (I:C) against the effluent contribution.

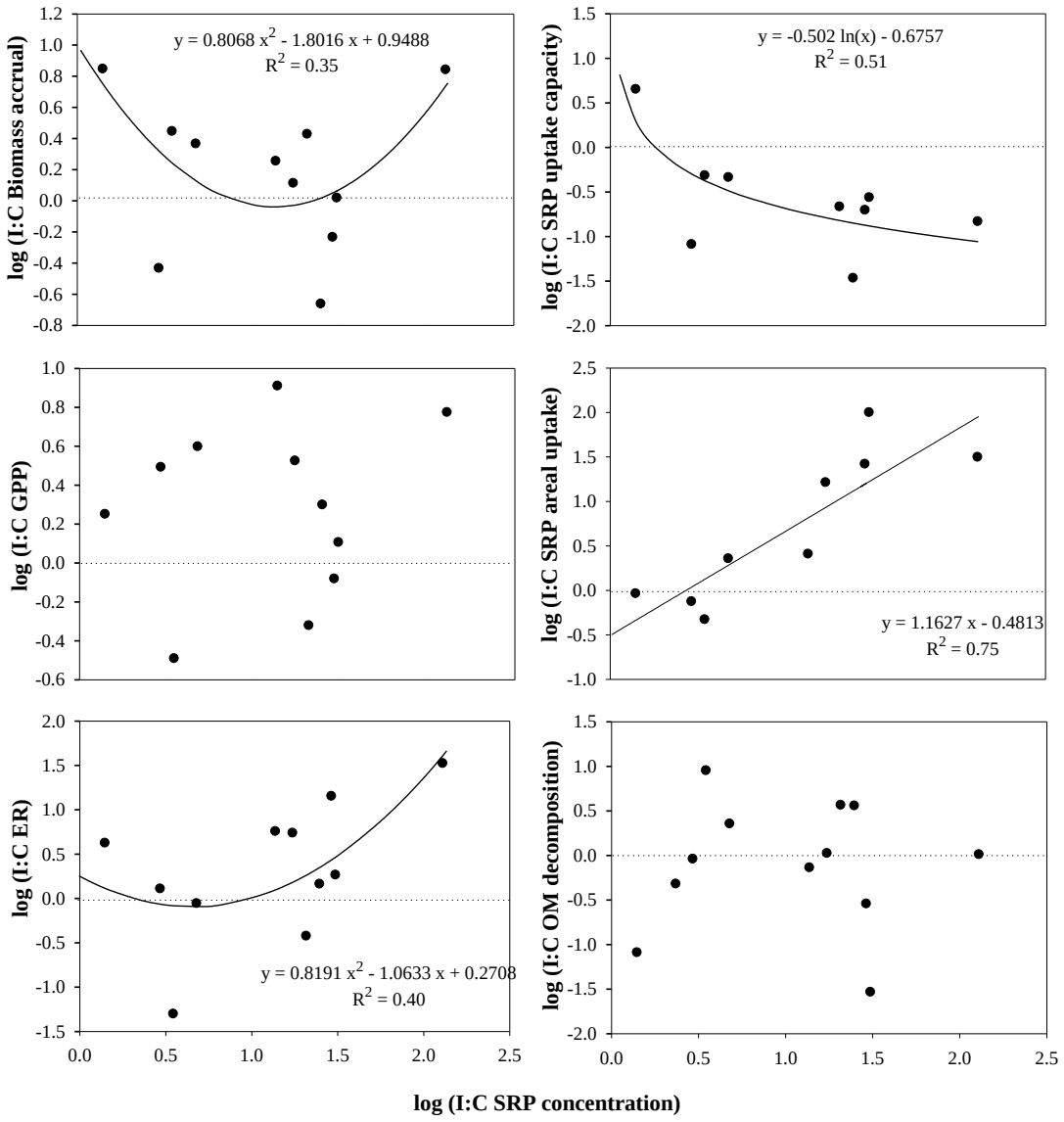

Figure 4. I:C ratios of some of the functional processes measured in the lower Ebro River *vs.* the I:C ratio of SRP concentration (Chapter 2). In this experiment we could not calculate the effluent contribution to the receiving stream. Thus, we show the ration of SRP concentration on Impact versus Control reaches as a proxy of the effluent contribution. Results are shown in logarithmic scale.

Results from the field observational experiment (Chapter 2, Fig. 4) showed that effluent contribution caused different effects on stream ecosystem processes. On the one hand, biofilm biomass accrual, ecosystem respiration and phosphorus areal uptake increased with increasing levels of effluent contribution, although they followed different response patterns. While biomass accrual and ecosystem respiration showed weak non-linear responses which were more accentuated at higher levels of effluent contribution (from I:C ratios between 5 and 10), phosphorus areal uptake showed a strong linear positive relationship to increasing effluent contribution. On the other hand, biofilm capacity to retain phosphorus was the only process significantly stressed by the effluent and showed a strong non-linear negative response from the lowest levels of effluent contribution (I:C ratios smaller than 5). Finally, primary production and organic matter decomposition did not show any significant relationship to increasing levels of effluent contribution, which would indicate that other external factors exert stronger influence.

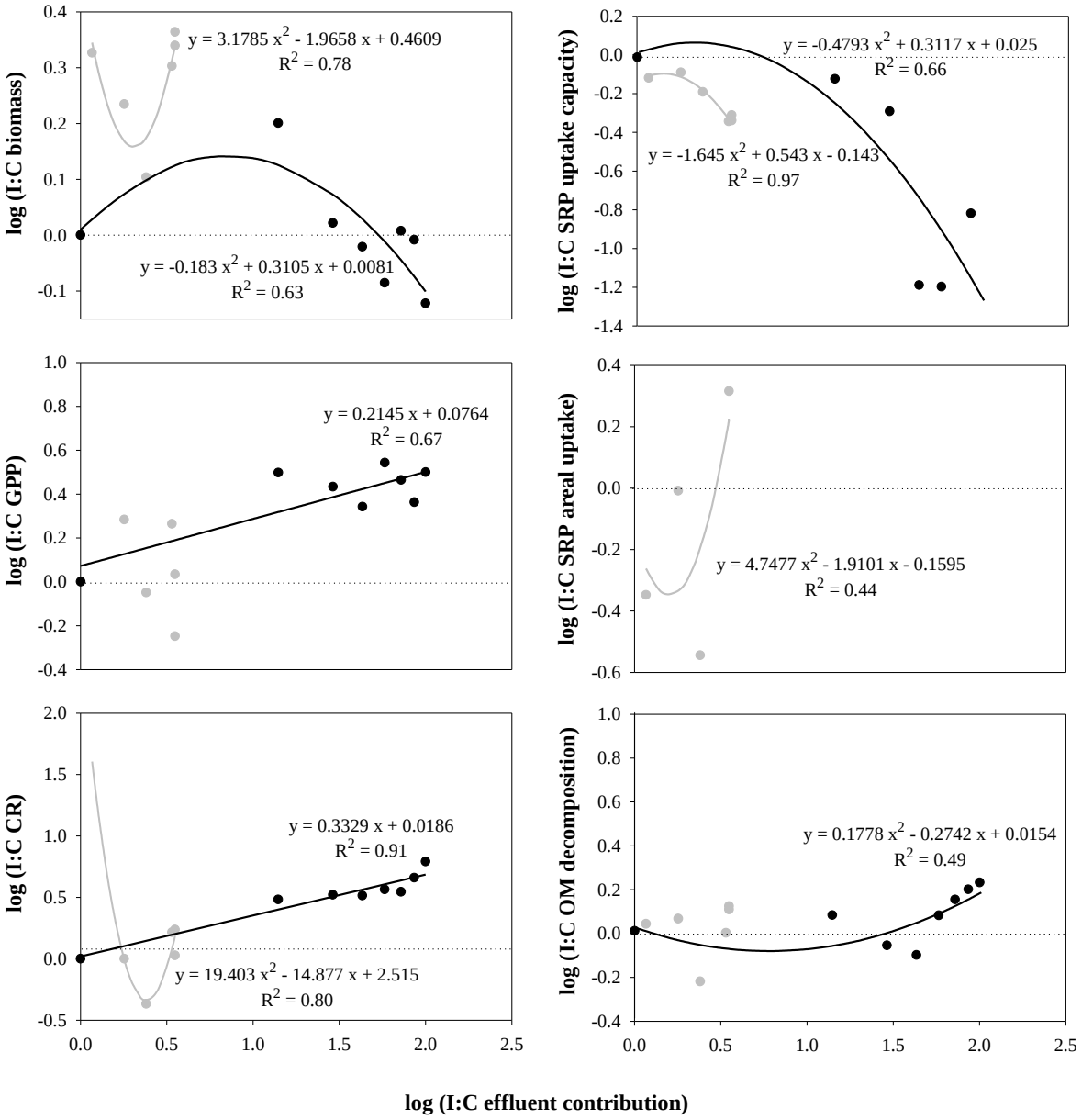

Figure 5. I:C ratios of some of the functional responses measured *vs.* the effluent contribution in the field manipulative (Chapter 3, grey series) and laboratory channel experiments (Chapter 4, black series). Results are shown in logarithmic scale.

Results from the field manipulative (Chapter 3, Fig. 5 grey series) and laboratory experiments (Chapter 4, Fig.5 black series) showed that the effluent exerted also different effects on stream functions. Results from the field manipulative experiment described similar patterns to the ones of the field observational experiment (Fig. 4), such as increased biofilm biomass, community respiration and phosphorus areal uptake, although in this case all showed non-linear responses (from *c.a.* 2% of effluent contribution). Similarly, biofilm phosphorus uptake capacity was the only process significantly stressed by the effluent, showing also a strong non-linear negative response (from smaller effluent contributions than 2%). Finally, gross primary production and organic matter decomposition were again unaffected by increasing levels of effluent contribution. However, these results contrast with the laboratory experiment

(Chapter 4, Fig. 5 black series). In the latter case, effluent contribution promoted not only community respiration but also gross primary production and organic matter decomposition (from 10-30% of effluent contribution). Biofilm phosphorus uptake was again stressed by increasing effluent concentration (effluent below 10%), showing a strong non-linear negative response. Finally, biofilm biomass was the only process showing the expected subsidy-stress pattern, as it increased up to a maximum effluent contribution (around 15%), where it became saturated and stressed below control levels.

Therefore, urban effluents exerted similar effects on both field experiments, but response patterns slightly differed among experiments. Field experiments showed similar results, although relationships were more clear in the observational experiment (Figs. 4 *vs.*5). This can be attributed to the larger gradient of effluent contribution, from nearly unpolluted streams (I:C ratio of SRP concentration = 1) to others in which effluents constituted almost all the stream water flow (I:C ratio of SRP concentration = 129). In addition, in the manipulative experiment, the closed riparian canopy caused strong light limitation, which could probably cause weak functional responses in the biofilm (von Schiller *et al.*, 2007; Bernot *et al.*, 2010). On the other hand, the laboratory experiment yielded clearer response patterns, likely as a consequence of the larger concentration gradient, and the generally better conditions for biofilm growth (higher light and temperature) (Stelzer *et al.*, 2003).

Altogether, these results suggest that urban effluents should be diluted to concentrations below 30% to avoid impairing the functioning of the receiving ecosystems. This threshold could be useful when planning the implementation of WWTPs.

2. Overview of the main results

In this dissertation, I combined observational and manipulative, field and laboratory experiments (Table 1). The field observational experiment (Chapter 2) focused on the joint effects of urban pollution and hydrology on stream ecosystem functioning. I compared the ecosystem functioning in reaches upstream and downstream from sewage inputs, which differed in their dilution capacity. Sewage inputs, even if they were small and did not affect noticeably stream hydrology, caused detrimental consequences on water quality and altered ecosystem functioning. The changes on ecosystem functioning seemed especially related to organic micro-contaminants. None of the measured processes, however, followed the expected Subsidy-Stress pattern, being mostly subsidized instead. The only processes being stressed were those directly regulated by microbial communities, suggesting that some compensatory mechanisms could mitigate ecosystem-level responses. These results emphasise the complexity of effects of urban pollution under multiple stress scenarios.

To eliminate confounding factors, I performed a field manipulative experiment (Chapter 3), unprecedented to my knowledge, following a BACI design. Despite being highly diluted, the effluent subsidized most of the ecosystem processes measured and only a few remained unaffected. As in the previous chapter (Chapter 2), biofilm functioning was the only process stressed by the WWTP effluent.

Altogether, our results indicate that WWTP effluents, even if they are adequately processed and highly diluted by the receiving water body, can exert significant and complex effects on stream ecosystem structure and functioning.

Both field studies failed to detect Subsidy-Stress responses for most variables measured, what raised the question of which is the response to a full gradient of WWTP effluent concentration, a question I addressed in a laboratory experiment (Chapter 4). Biofilm biomass accrual was the only process following the subsidy-stress pattern, whereas most processes showed complex subsidy responses. Here again, biofilm nutrient uptake capacity was the only process stressed by effluent concentration. Most of the processes showed clear legacy effects, although they followed contrasting patterns. These results can help to improve the understanding of the effects of urban pollution on ecosystem functioning, but highlight the complexity of the response.

Table 1. Main results obtained in this dissertation: effects of urban effluents on the response patterns (Subsidy, Stress, Subsidy-Stress or No effect) of each functional process.

	Biofilm accrual Enzymatic activities	Biofilm SRP uptake capacity	Nutrient uptake		Metabolism		Organic matter decomposition
			NH_4^+	SRP	GPP	ER / CR	
Chapter 2	↑ Subsidy AFDM	↓Stress	↑ Subsidy	≈ No effect	≈ No effect	↑ Subsidy	≈ No effect
Chapter 3	↑ Subsidy AFDM ↑ Subsidy Chl-*a* ↑ Subsidy AP ↑ Subsidy BG	↓ Stress	≈ No effect	≈ No effect	≈ No effect	≈ No effect	≈ No effect microbial ↑ Subsidy total
Chapter 4	∩ Subsidy-stress AFDM ↑ Subsidy Chl-*a*	↓ Stress uptake ↓ Stress efficiency	-	-	↑ Subsidy	↑ Subsidy	↑ Subsidy
	Recovery ∩ Subsidy-stress AFDM ↑ Subsidy Chl-*a*	*Recovery* ↓ Stress uptake ↓ Stress efficiency	-	-	*Recovery* ↑ Subsidy	*Recovery* ↑ Subsidy	*Recovery* ≈ No effect

3. Mechanisms responsible for the observed results

Streambed biofilms process and transform nutrients and organic matter through a combination of assimilatory and dissimilatory processes (Romaní, 2010; Battin *et al.*, 2016), what can reduce their concentration, at least temporarily, thus alleviating the environmental problems associated with excess nutrients (Bernhardt *et al.*, 2003; Ribot *et al.*, 2013). I showed that urban effluents subsidized biofilm structure by enhancing total biomass and chlorophyll-*a* (Chapters 2, 3 and 4), which suggests a similar subsidy effect for both heterotrophic and autotrophic communities (Ribot *et al.*, 2015). These results were especially noticeable in field experiments, but less clear in the laboratory experiment, where biofilm biomass followed the subsidy-stress pattern (*i.e.*, increased up to a threshold where the effluent became toxic and decreased below "*normal*" levels), and chlorophyll-*a* followed a saturating subsidy effect. Therefore, although I can only speculate about it, these results lead me to think that urban effluents induced a change in biofilm community, favouring more resistant communities (Drury *et al.*, 2013; Rosi *et al.*, 2018) by developing antibiotic resistance genes (Subirats *et al.*, 2017), in which autotrophs could have prevailed over heterotrophs (Proia *et al.*, 2013). Finally, in the field manipulative experiment the urban effluent also increased biofilm exo-enzymatic activities, although enhanced nutrient availability usually decrease their expression (Romaní *et al.*, 2012). Therefore, it is likely that altered ecosystem functioning is a result of changes in the structure of biofilm community.

Biofilm SRP uptake capacity was consistently stressed in all the experiments of the dissertation (Chapters 2, 3 and 4). Demand for nutrients is affected by the nutrient balance between the biofilm and the water column (Hall *et al.*, 2002). As nutrient ambient concentrations increase, biofilms tend to become less effective in nutrient removal, even reducing uptake to zero in cases of saturation (Earl *et al.*, 2006). Our results showed that biofilm below urban effluents were less effective in removing nutrients from the water column, and even released SRP under the experimental conditions. Urban effluents contribute excessive loads of organic matter and nutrients to freshwater ecosystems (Meyer *et al.*, 2005; Carey & Migliaccio, 2009). In all the experiments of the present dissertation urban effluents significantly increased phosphorus concentration, which could have driven the saturation of the biofilm uptake capacity. Nevertheless, urban effluents are also an important source of organic micro-pollutants (Rosi-Marshall *et al.*, 2015; Sabater *et al.*, 2016; Aymerich *et al.*, 2016), which might also affect biofilm SRP uptake capacity (Chapter 2), as has been previously shown (Proia *et al.*, 2017). As a consequence, this decline in biofilm nutrient uptake could increase the nutrient transport to sensitive receiving waters, such as reservoirs or coastal ecosystems, where they can enhance cause eutrophication (Brack *et al.*, 2007; Alexander *et al.*, 2008).

The response detected at the biofilm level contrasts with nutrient uptake results obtained at the reach scale. It has been reported that the capacity to transform and retain nutrients is high in pristine streams, but it decreases when ambient nutrient concentrations increase (Ensign & Doyle, 2006). Our field experiments, however, contrast with those in the literature. In our observational experiment phosphorus uptake capacity was not significantly influenced by sewage inputs, whereas ammonium uptake increased.

Our field manipulative experiment, on the other hand, showed no significant effects on nutrient uptake, apart from a marginally significant reduction in phosphorus uptake velocity. Probably, the main reason behind these contrasting results is that, even if urban pollution impairs biofilm capacity to retain nutrients, it can be compensated by other ecosystem compartments such as the hyporheic zone, fine sediments or macrophytes (Newbold *et al.*, 1981; Covino *et al.*, 2010; Johnson *et al.*, 2015). Urban effluents are an important source of fine sediments (Miserendino *et al.*, 2008), and in the field observational experiment, in particular, fine sediments could have played a key role retaining nutrients (Withers & Jarvie, 2008), as well as the macrophyte communities growing in the main channel or stream banks (Riis *et al.*, 2012). In the manipulative experiment, stream substrata were dominated by bedrock and cobbles and macrophytes were absent, but the roots from riparian trees could have been determinant mitigating the results observed at the biofilm level.

Ecosystem metabolism also showed contrasting results when compared to the biofilm scale. River metabolism can also be assessed for specific compartments, which allows disentangling their contribution to the whole ecosystem (Acuña *et al.*, 2011). Our field observational experiment showed ecosystem metabolism to increase below sewage inputs, especially ER, and that both ER and GPP increased significantly with pollutant concentrations. These results agree with the ones obtained in the laboratory experiment, where WWTP effluent exposure also increased biofilm metabolic activity. This increase was higher for CR than for GPP, as the latter became saturated at intermediate concentrations of effluent. Nevertheless, both results contrast with those observed in the field manipulative experiment. Our results only showed a weak, statistically non-significant, tendency towards increased CR and GPP. As we mentioned over this third chapter, different reasons could have precluded from seeing clearer effects, such as the lack of enough replication, the low contribution of the effluent and the fact that it was released through pulses. So, results from Chapter 3 could reflect the specific experimental conditions, but results from the other two chapters (2 and 4) agree with the previously observed in the literature. Many studies describe an increase in overall ecosystem metabolism below urban effluents (*e.g.*, Gücker *et al.*, 2006), although most report ER to be especially responsive to pollution (Izagirre *et al.*, 2008; Aristi *et al.*, 2015). In general, ecosystem or community-level GPP responses are less clear, a fact that has been widely attributed to the dependence of GPP on other environmental factors, such as light (Aristi *et al.*, 2015), riparian vegetation (Bernot *et al.*, 2010) or turbidity (Izagirre *et al.*, 2008). As a consequence, these changes in stream metabolism could alter the balance between autotrophic and heterotrophic processes, which could carry the system towards heterotrophy and aggravate the signals of streams stressed by pollution (Brack *et al.*, 2007).

Finally, urban pollution affected differently the microbial and invertebrate contribution to organic matter decomposition. Decomposition of organic matter consists of a series of consecutive processes that are influenced by diverse biotic and biotic factors, which make it highly responsive to environmental factors (Young *et al.*, 2008; Chauvet *et al.*, 2016). The field observational experiment showed that microbial decomposition was not significantly affected below sewage inputs, although it showed a weak

tendency to decrease with increasing concentrations of pollutants. These results agree with those of the field manipulative experiment, in which the effluent addition did not affect microbial decomposition, but increased significantly the one mediated by invertebrates. Both results, however, contrast with the results observed in the laboratory experiment, where microbial decomposition increased significantly with effluent contribution. Several authors showed increased decomposition rates in laboratory experiments (Biasi *et al.*, 2017), although these responses are not always translated into field (Solagaistua *et al.*, 2018). These results suggest that the abiotic factors employed in the present laboratory experiment, which included high nutrient concentrations, absence of light limitation and warm water temperatures, played a key role enhancing decomposition rates (Spänhoff *et al.*, 2007). Thus, as reported by other authors (Ferreira *et al.*, 2015), well-controlled laboratory experiments usually show stronger and clearer response patterns than field experiments, as their simplistic nature offers a better control of other confounding factors that can play a determinant role behind the inconsistencies observed in the field. Finally, in the only chapter where invertebrate organic matter decomposition was assessed (Chapter 3), it was faster with increasing effluent concentration, which could be due to increased leaf palatability (Ferreira *et al.*, 2015) or enhanced invertebrate density (Pascoal *et al.*, 2003). These results agree with previous works that suggest that invertebrates can be more responsive than microbes to pollution (Hieber & Gessner, 2002). WWTP effluents can subsidize microbial activities, such as exo-enzymatic activities and respiration, which can promote organic matter decomposition (Ferreira *et al.*, 2006). As invertebrates depend on microbial communities to condition leaf litter and increase its palatability, invertebrate decomposition can be affected by either direct and indirect effects (Bundschuh *et al.*, 2011, 2013), which could be one of the reasons of being more responsive. Besides, the role of microbial and invertebrate communities can be altered under pollution (Hieber & Gessner, 2002). In fact, some authors report invertebrate communities to attain their highest densities under moderate concentrations of pollution (Friberg *et al.*, 2011), which suggests that the role of invertebrates can prevail over microbes under certain levels of pollution.

4. The future of urban streams

Urban pollution is one of the most common stressors threatening freshwater ecosystems, often operating in concert with other stressors, such as hydromorphological alterations (EC, 2015; Birk *et al.*, 2018). The types and effects of stressors can be classified from an energy-based perspective, taking into account their intensity, frequency and spatial scale (Fig. 6; Stevenson & Sabater, 2010). According to this hierarchy, nutrients and pollutants are classified as a kind of stressor that occurs at high frequency but usually with low intensity and thus, they are considered highly reversible impacts (Fig. 6). According to this classification, these stressors only move biological communities outside their normal physiological state but their adaptive mechanisms usually are enough to cope with stress and thus, they only result in subtle changes (Segner *et al.*, 2014), which allows the recovery of the system (Sabater *et al.*, 2018b).

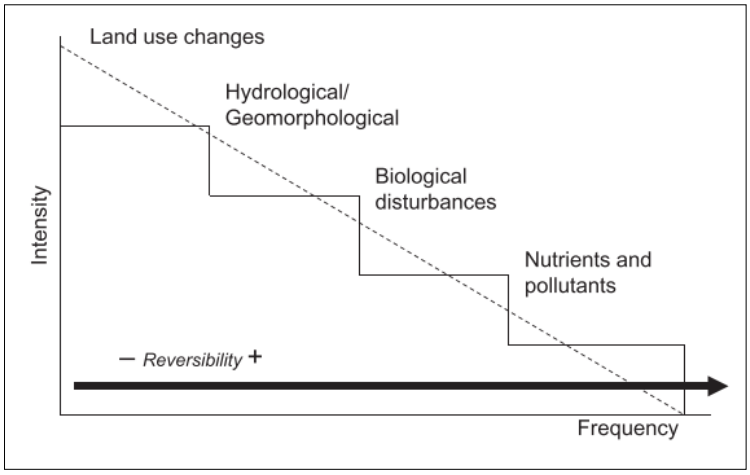

Figure 6. Classification of stressors according to their associated energy, measured by their intensity and frequency. From Sabater *et al.* (2018b).

Nevertheless, results of the present dissertation do not agree with this classification. In fact, in some of the studied streams, we showed that urban effluents greatly impaired water quality, inducing anoxic conditions and nutrient concentrations comparable to the most polluted streams described across Europe (Grizzetti *et al.*, 2017). These changes in water quality lead to detrimental consequences on biological communities, inducing abrupt alterations on invertebrate community composition, as we showed in a parallel work to our field observational experiment (Mor *et al.*, 2019), and on ecosystem functioning, impairing key ecosystem processes such as the biofilm nutrient uptake capacity and ecosystem respiration. Therefore, our results suggest that urban pollution should be classified as a very intense impact that can occur at high frequency in time, causing strong legacy effects and thus, low capacity to recover (Jarvie *et al.*, 2013).

Future predictions do not forecast optimistic scenarios, as these effects can be exacerbated when stressors occur at larger spatial and temporal scales or when they are subjected to novel stressors, including the global expansion of pesticides and pharmaceuticals (Ricart *et al.*, 2010). On the one hand, from the end of the last century the number of urban dwellers is increasing dramatically, and over the next decades the projected size and spatial distribution of the population is expected to continue increasing and concentrating even more around cities (Jones & O´Neill, 2016). As a consequence, effects of urban pollution will be enlarged, increasing the number of streams and rivers subjected to the "*Urban Stream Syndrome"* (*sensu* Walsh *et al.*, 2005). On the other hand, increasing drought is expected to exacerbate the effects of urban pollution, as an increasing number of streams and rivers are expected to become temporary over the next years due to climate change and water abstraction (Acuña *et al.*, 2014). Under these scenarios, the dilution capacity of streams and rivers will be severely reduced and thus, urban effluents are expected to make up a larger proportion of the water flow (Rice & Westerhoff, 2017), leading to a huge increase in the number of effluent-dominated streams (Döll & Schmied, 2012). In addition, associated with the predicted global changes scenarios (Hisdal *et al.*, 2001), rainfall patterns are expected to alter and become more torrential, which involves alternation between drier conditions and extreme rainfall events (Räisänen

et al., 2004). These periods of heavy rainfalls can difficult the functioning of WWTPs, as they can generate uncontrolled spills of untreated wastewater (Corada-Fernández *et al.*, 2017). These accidental releases of untreated sewage can seriously impair stream water quality, as they can significantly increase the concentration of contaminants, including nutrients, heavy metals and other emerging pollutants, such as pharmaceuticals or personal care products (Canobbio *et al.*, 2009; Guasch *et al.*, 2010; Corada-Fernández *et al.*, 2017). On the contrary some other authors classify the huge increase in the production and diversification of new synthetic chemicals as the main drivers of global change (Bernhardt *et al.*, 2017). Although during the last decades they have been ignored when assessing the effects of global change (Stehle & Schulz, 2015), these stressors have greatly increased in quantity, diversity and geographical expansion (Fig. 7). Their production and diversification rates, specifically those of pesticides and pharmaceuticals, have exceeded the increasing rates of most agents previously recognized as the main drivers of global change, such as elevated atmospheric CO_2, nutrient pollution, land-use change or biodiversity loss (Fig. 7a *vs.* Figs. 7b, 7c), and they have been described to create long-term environmental problems. Similarly, massive accumulation of plastic particles in aquatic environments is becoming a concerning environmental problem (Geyer *et al.*, 2017). Most of the studies regarding this issue have focused on marine ecosystems and the risks associated to aquatic biota, whereas freshwater ecosystems have received less attention. However, recent studies have demonstrated not only the presence of plastic particles in freshwater ecosystems, but also that the contamination can be as severe as in the oceans, which can also cause severe toxic effects on freshwater biota (Wagner *et al.*, 2014; Dris *et al.*, 2015; Eerkes-Medranos *et al.*, 2015). Therefore, the massive synthesis of these new chemical compounds, is expected to lead detrimental effects on biological communities, which could ultimately affect ecosystem functioning (Malaj *et al.*, 2014; Bernhardt *et al.*, 2017).

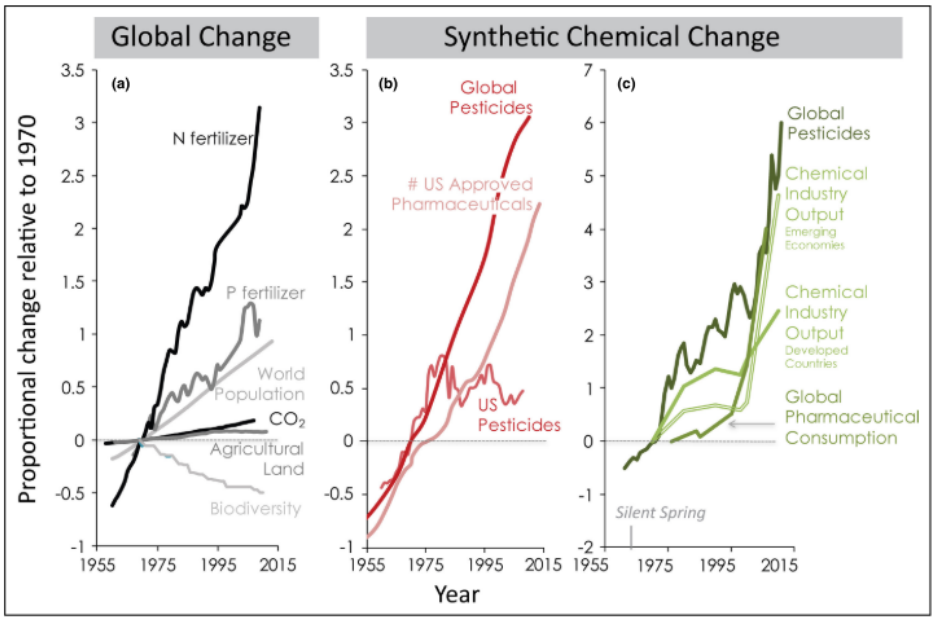

Figure 7. Increasing trends of (a) the agents previously recognized as drivers of global environmental change, (b) the diversity and application of pesticides and pharmaceuticals within the US and globally, and (c) the global trade value of synthetic chemicals as well as for the pesticide and pharmaceutical sectors individually. From Bernhardt *et al.*, (2017).

In order to face and prevent the environmental problems associated with these worrisome scenarios, it is clear that we must develop and improve the existing management strategies. On the one hand, universal sanitation and purification are necessary to establish in developing countries but also in developed countries where direct discharges of polluted water still exist. On the other hand, it is necessary to improve wastewater treatment by the development of more sophisticated tertiary treatment processes, specifically adapted to novel contaminants, such as pharmaceuticals. Although nowadays, this sounds prohibitively expensive, prices will undoubtedly decrease as novel plants become more abundant. Finally, the entire worldwide population should be concerned about individual actions that can reduce the loads of contaminants. Much can be gained by reducing to the strictly necessary the use of potential pollutants, such as pharmaceuticals or plastics. This might sound naive, and often one hears that reducing river pollution even more would be too expensive for today's economy. But the same thing was said in the past of other ambitious programs of environmental stewardship. A few decades ago, Basque rivers were among the most polluted in the world (Fig. 8), their poor status was perceived as the price to live in an advanced society, and investments in environmental improvement as a threat to our economy and well-being. We could not be more wrong. Of course, it took a large amount of money to implement adequate sanitation and treatment procedures at the regional level, but this opened the gate for a more advanced society and more dynamic economy. I would like to believe that worldwide population walks on the same direction, and thus, that important ecological improvements will occur within the next decades, as well.

Figure 8. Urumea River at its way through the city of San Sebastian (NE, Spain) circa 1960 (left) and 2018 (right). Photographs provided by Arturo Elosegi.

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CHAPTER 6

General conclusions

1. Urban effluents studied in the present dissertation had low pH and dissolved oxygen concentration but high electrical conductivity, whereas they showed higher concentrations of nutrients and other micro-pollutants, such as pharmaceuticals and heavy metals.

2. Urban effluents significantly altered the functioning of the studied streams. In general, they subsidized most of the ecosystem processes we measured, although they followed complex and process-specific response patterns.

3. Our observational approximation showed complex response patterns of Mediterranean stream functioning, which were mainly driven by the interaction between urban pollution and water stress. The most significant relationships observed during the experiment were linked to pharmaceutically active compounds, although the complex nature of urban effluents preclude from establishing direct causality.

4. Our field manipulative approximation revealed that even highly diluted urban effluents can trigger complex responses on ecosystem structure and functioning, which could have remained unnoticed under less stringent experimental approaches.

5. Overall, both field experiments showed urban effluents to subsidize most ecosystem processes, with the only exception of biofilm nutrient uptake capacity. Functional responses can differ between biofilm and whole-ecosystem levels.

6. A laboratory experiment across a full gradient of pollution showed no generalized subsidy-stress response, most processes being instead subsidized across all effluent concentrations. We hypothesize that subsidy-stress responses can be found under poorly treated urban effluents.

7. These results highlight that it is crucial to maintain the dilution capacity of streams and rivers, and reveal the need to optimize wastewater treatment processes, in order to reduce the overall ecological impacts of these complex mixtures of pollutants.

CHAPTER 7

Supporting information

Chapter 2 – Joint effects of urban pollution and hydrological alterations on the functioning of Mediterranean streams

Additional supporting information may be found in the online version of this article.

Appendix S1. Supplementary tables and figures.

Appendix S2. R code (Available online upon publication).

Appendix S3. Dataset (Available online upon publication).

Appendix S1

Table S1. Biocide (AbMacrolide, Levamisole, Metronidazole, Ofloxacin, Ronidazole, Sulfamethoxazole, Tiabendazole, and Trimethoprim) and non-biocide drug (Acetaminophen, ARBs, Benzodiazepine, BetaBlocker, Carbamazepine, CCB, Clopidogrel, Fibrate, Glibenclamide, H2Antagonist, Iopromide, LoopDiuretic, Loratadine, NSAID, Opioid, Salbutamol, SSRI, Statin, Tamsulosin, Trazodone, and Venlafaxine) variables derived from the PhACs measured in water samples. All PhACs were measured in nanograms per litre.

Denomination	Variable description
AbMacrolide	Sum of the concentrations of clarithromycin and erythromycin, two macrolide antibiotics that inhibit protein synthesis in gram+ bacteria
Levamisole	Concentration of levamisole, a parasiticide that works as a nicotinic acetylcholine receptor agonist
Metronidazole	Concentration of metronidazole and active metabolites, an antibiotic and parasiticide that inhibits DNA synthesis in anaerobic organisms
Ofloxacin	Concentration of ofloxacin, a quinolone that kills gram +/- bacteria by fragmenting bacterial DNA
Ronidazole	Concentration of ronidazole, antiprotozoal agent used in veterinary medicine
Sulfamethoxazole	Concentration of sulfamethoxazole, a sulfanilamide that interferes with the synthesis of folic acid
Tiabendazole	Concentration of tiabendazole, antifungal and parasiticide that inhibits mitochondrial pathways
Trimethoprim	Concentration of trimethoprim, antibiotic that inhibits the synthesis of tetrahydrofolic acid.
Acetaminophen	Concentration of paracetamol
ARBs	The sum of all ARB (Angiotensin II Receptor Blocker) drugs (irbesartan, losartan, and valsartan)
Benzodiazepine	The sum of the concentrations of all benzodiazepines (alprazolam, diazepam, and lorazepam)
BetaBlocker	The sum of all beta blockers (atenolol, metoprolol, nadolol, propranolol, and sotalol)
Carbamazepine	Carbamazepine, a sodium channel blocker, and active metabolites
CCB	The sum of all CCB (calcium channel blockers) (amlodipine, diltiazem, and verapamil)
Clopidogrel	The only antiplatelet found in wáter samples
Fibrate	The sum of all fibrates, carboxylic acids used as hypolipidemic agents, often together with statins (bezafibrate and gemfibrozil)
Glibenclamide	The only sulfonylurea found in wáter samples
H2Antagonist	The sum of the concentrations of all H2 agonists (cimetidine, famotidine and ranitidine)
Iopromide	A contrast médium, used in computed tomography scan
LoopDiuretic	The sum of the concentrations of all loop diuretics (furosemide and torasemide)
Loratadine	The only histamine H1 inverse agonist found in wáter samples
NSAID	The sum of the concentrations of all NSAIDs (nonsteroideal anti-inflammatory drug) (diclofenac, ibuprofen, indomethacine, ketoprofen, naproxen, piroxicam, salicylic acid, and phenazone)
Opioid	The sum of the concentrations of all opioids found in wáter samples (oxycodone and codeine)
Salbutamol	The only beta 2 agonist found in wáter samples
SSRI	The sum of all SSRIs (selective serotonin reuptake inhibitors) (norfluoxetine = seproxetine, fluoxetine, and citalopram)
Statin	The sum of all statins (3-hydroxy-3-methyl-glutaryl-coenzyme A reductase inhibitors) (atorvastatin and pravastatin)
Tamsulosin	The only alpha blocker found in wáter samples
Trazodone	The only serotonin antagonist found in wáter samples
Venlafaxine	The ony SNRI (serotonin–norepinephrine reuptake inhibitor) found in wáter samples

Table S2. Descriptive statistics of the measured stream water physicochemical properties. IQR = interquartile range; Min = minimum measured value; Max = maximum measured value.

Response	Mean	StDev	Median	IQR	Min	Max	Range
pH	7.8	0.3	7.9	0.4	7.1	8.4	1.3
EC (µS cm^{-1})	1139	730	862	764	454	3020	2566
DO (mg L^{-1})	7.5	3.2	7.8	3.9	0.0	13.5	13.5
NO_3^- (mg N L^{-1})	1.9	2.2	1.1	2.0	0.0	9.4	9.4
NO_2^- (mg N L^{-1})	0.0	0.1	0.0	0.0	0.0	0.3	0.3
NH_4^+ (mg N L^{-1})	1.8	4.4	0.0	2.1	0.0	22.3	22.3
SRP (mg P L^{-1})	0.3	0.5	0.0	0.3	0.0	2.3	2.3
DOC (mg C L^{-1})	6.0	10.3	2.0	5.1	0.6	51.4	50.8

Table S3. Permutational multivariate analysis of variance for stream water physicochemical properties. Multivariate variation in chemical properties was represented by a Euclidean distance matrix. This matrix was partitioned among factors Treatment (two levels: control and impact) and Time (two levels: autumn and spring), and the Treatment x Time interaction, according to the statistical model (split plot anova) that is appropriate for the present experimental design (a repeated measurements Randomized Complete Block design or RCB). The appropriate error term for the above-the-line analysis is the Stream x Treatment interaction, whereas the appropriate error term for the below-the-line analysis is a pooled error term that includes both the Stream x Time and the Stream x Treatment x Time interactions. Two of the sampled streams (Reguers and Senia) were not included in the anova due to too many missing data points (the technique cannot handle unbalance), so only data for eleven streams have been considered (11 streams x 2 stations x 2 sampling occasions = 44 measurements).

Source	df	SS	MS	*F*-value	*p*-value
Stream	10	181.54	18.15	-	
Treatment	1	44.67	44.67	8.92	0.014
Streams x Treatment [= "Whole Plot" Error]	10	50.09	5.01		
Time	1	11.21	11.21	4.10	0.004
Treatment × Time	1	1.83	1.83	0.67	0.637
"Split Plot" [pooled] Error	20	54.68	2.73		
Total	43				

Table S4. Hypothesis testing results, after linear mixed modelling, for the individual water chemical properties measured in thirteen streams. The first two columns report *F*-values together with degrees of freedom (among parentheses); significance (*p*-value < 0.05) is indicated with asterisks. The interaction between Treatment and Time was not significant in any case. Degrees of freedom differ among variables because, in some cases (streams Reguers and Senia), there are missing values. Parameter estimation is reported in Table S5.

Response	Treatment	Time	Fixed factors R^2 (%)
pH	18.2 (1, 34)***	6.6 (1, 34)*	23.5
EC (µS cm^{-1})	0.4 (1, 34)	8.2 (1, 34)**	0.8
DO (mg L^{-1})	28.4 (1, 34)***	10.8 (1, 34)***	36.5
NO_3^- (mg N L^{-1})	1.3 (1, 33)	1.2 (1, 33)	-
NO_2^- (mg N L^{-1})	13.87 (1, 33)**	0.42 (1, 33)	93.5
NH_4^+ (mg N L^{-1})	19.54 (1, 33)***	9.32 (1, 33)**	2.5
SRP (mg P L^{-1})	13.1 (1, 33)**	4.4 (1, 33)*	1.33
DOC (mg C L^{-1})	9.9 (1, 33)**	1.1 (1, 33)	1.8

Table S5. Parameter estimation, after linear mixed modelling, for the individual water chemical properties measured in thirteen streams. The interaction between Treatment and Time was not significant in any case. In the cases of ammonium concentration and nitrite concentration, modelling of error variance using two additional δ parameters was insufficient and it was hence needed to use four additional coefficients, one per Treatment : Time combination, as follows. Ammonium concentration: $\delta_{CA} = 1.00$; $\delta_{CS} = 0.01$; $\delta_{IA} = 358.10$; and $\delta_{IS} = 311.90$. Nitrite concentration: $\delta_{CA} = 1.00$; $\delta_{CS} = 2.47\ 10^{-5}$; $\delta_{IA} = 13.41$; and $\delta_{IS} = 14.22$. Hypothesis testing is reported in Table S4.

Response	Intercept	Impact vs Control	Spring vs Autumn	σ_b	σ	δ_C	δ_I
pH	7.84 (7.70, 7.99)	-0.25 (-0.37, -0.13)	0.15 (0.03, 0.27)	0.17 (0.10, 0.31)	0.21 (0.16, 0.26)	-	-
EC (µS cm^{-1})	1179.4 (768.6, 1590.2)	-	-128.8 (-220.2, -37.5)	713.3 (475.8, 1069.5)	156.8 (123.6, 198.8)	-	-
DO (mg L^{-1})	8.11 (6.72, 9.50)	-3.26 (-4.52, -1.99)	2.02 (0.77, 3.27)	1.42 (0.67, 3.01)	2.15 (1.68, 2.75)	-	-
NO_3^- (mg N L^{-1})	2.17 (0.92, 3.43)	-	-	1.93 (1.25, 2.97)	1.20 (0.85, 1.69)	1.0	0.82
NO_2^- (mg N L^{-1})	0.005 (0.001, 0.009)	0.05 (0.02, 0.08)	-	0.004 (0.003, 0.009)	0.005 (0.002, 0.006)	-	-
NH_4^+ (mg N L^{-1})	1.55 (-1.44, 4.54)	1.66 (0.90, 2.43)	-0.005 (-0.008, -0.002)	5.27 (3.44, 8.08)	0.005 (0.003, 0.008)	-	-
SRP (mg P L^{-1})	0.29 (-0.06, 0.64)	0.14 (0.06, 0.22)	-0.009 (-0.017, -0.0003)	0.62 (0.41, 0.94)	1.01 (0.79, 1.29)	1.0	19.41
DOC (mg C L^{-1})	5.52 (-1.36, 12.40)	3.24 (1.14, 5.34)	-	12.03 (7.80, 18.55)	1.41 (0.90, 2.22)	1.0	3.30

Table S6. Descriptive statistics of the measured stream water antibiotic concentrations. IQR = interquartile range; Min = minimum measured value; Max = maximum measured value.

Response	Mean	StDev	Median	IQR	Min	Max	Range
AbMacrolide (ng L^{-1})	2.7	9.8	0.0	0.0	0.0	65.0	65.0
Levamisole (ng L^{-1})	6.4	21.6	0.0	2.9	0.0	142.1	142.1
Metronidazole (ng L^{-1})	1.22	6.1	0.0	0.9	0.0	44.3	44.3
Ofloxacin (ng L^{-1})	19.9	48.9	1.6	10.5	0.5	281.5	281.0
Ronidazole (ng L^{-1})	0.02	0.1	0.0	0.0	0.0	0.4	0.4
Sulfamethoxazole (ng L^{-1})	3.4	7.4	0.4	3.2	0.0	38.9	38.9
Tiabendazole (ng L^{-1})	0.3	1.8	0.0	0.0	0.0	11.4	11.4
Trimethropin (ng L^{-1})	0.5	2.7	0.0	0.0	0.0	19.0	19.0

Table S7. Permutational multivariate analysis of variance for stream water biocide concentrations. Multivariate variation in water biocide concentrations was represented by a Euclidean distance matrix. This matrix was partitioned among factors Treatment (two levels: control and impact) and Time (two levels: autumn and spring), and the Treatment x Time interaction, according to the statistical model (split plot anova) that is appropriate for the present experimental design (a repeated measurements Randomized Complete Block design or RCB). The appropriate error term for the above-the-line analysis is the Stream x Treatment interaction, whereas the appropriate error term for the below-the-line analysis is a pooled error term that includes both the Stream x Time and the Stream x Treatment x Time interactions. Two of the sampled streams (Reguers and Senia) were not included in the anova due to too many missing data points (the technique cannot handle unbalance), so only data for eleven streams have been considered (11 streams x 2 stations x 2 sampling occasions = 44 measurements).

Source	df	SS	MS	*F*-value	*p*-value
Stream	10	61.62	6.16	----	
Treatment	1	17.87	17.87	3.05	0.110
Streams x Treatment [= "Whole Plot" Error]	10	58.51	5.85		
Time	1	14.54	14.54	3.03	0.001
Treatment × Time	1	9.53	9.53	1.99	0.021
"Split Plot" [pooled] Error	20	95.95	4.80		
Total	43				

Table S8. Hypothesis testing results, after linear mixed modelling, for the individual water biocide concentrations measured in thirteen streams. The first two columns report *F*-values together with degrees of freedom (among parentheses) and significance (p-value < 0.05) is indicated with asterisks. The interaction between Treatment and Time was not significant in any case. Degrees of freedom differ among variables because, in some cases (streams Reguers and Senia), there are missing values. Parameter estimation is reported in Table S9.

Response	Treatment	Time	Fixed factors R^2 (%)
AbMacrolide (ng L^{-1})	4.29 (1, 37)*	2.57 (1, 37)	11.7
Levamisole (ng L^{-1})	4.29 (1, 34)*	1.43 (1, 34)	98.4
Metronidazole (ng L^{-1})	1.18 (1, 37)	27.77 (1, 37)***	91.0
Ofloxacin (ng L^{-1})	6.42 (1, 34)*	8.89 (1, 34)**	96.9
Ronidazole (ng L^{-1})	0.04 (1, 34)	3.95 (1,34)	-
Sulfamethoxazole (ng L^{-1})	3.04 (1, 34)	5.49 (1, 34)*	23.2
Tiabendazole (ng L^{-1})	1.59 (1, 34)	0.23 (1, 34)	-
Trimethropin (ng L^{-1})	1.64 (1, 34)	1.24 (1, 34)	-

Table S9. Parameter estimation, after linear mixed modelling, for the individual water biocide concentrations measured in thirteen streams. The interaction between Treatment and Time was not significant in any case. In this case, due to the structure of the data, only two additional δ parameters were used to model the error variance, being only possible for Levamisole, Metronidazole, Ofloxacin and Sulfamethoxazole. Hypothesis testing is reported in Table S8.

Response	Intercept	Impact vs Control	Spring vs Autumn	σ_b	σ	CoefC	CoefI
AbMacrolide (ng L^{-1})	2.06 (-2.51, 6.63)	5.33 (0.12, 10.54)	-	1.27 (0.004, 458.49)	9.27 (7.42, 11.59)	-	-
Levamisole (ng L^{-1})	0.38 (-0.09, 0.85)	11.67 (0.21, 23.13)	-	0.03 (1.59E-72, 4.47E+68)	0.76 (0.53, 1.09)	1.00	37.71
Metronidazole (ng L^{-1})	-0.002 (-0.17, 0.17)	-	0.63 (0.39, 0.87)	0.04 (7.43E-11, 1.75E+07)	0.30 (0.20, 0.45)	1.00	28.15
Ofloxacin (ng L^{-1})	4.31 (2.60, 6.01)	31.44 (6.17, 56.71)	-2.08 (-3.50, -0.66)	2.31 (1.35, 3.94)	1.65 (1.07, 2.53)	1.00	38.45
Ronidazole (ng L^{-1})	0.01 (-0.04, 0.07)	-	-	0.07 (0.04 - 0.13)	0.07 (0.06 - 0.09)	-	-
Sulfamethoxazole (ng L^{-1})	3.15 (0.86, 5.43)	-	-2.75 (-5.13, -0.37)	2.43 (1.13, 5.23)	2.97 (1.87, 4.70)	1.00	2.92
Tiabendazole (ng L^{-1})	-0.13 (-1.06, 0.81)	-	-	0.0002 (2.92E-171, 1.10E+163)	1.79 (1.46, 2.20)	-	-
Trimethropin (ng L^{-1})	0.47 (-0.94, 1.87)	-	-	-	-	-	-

Table S10. Descriptive statistics of the measured concentrations of stream water non-biocide drugs. IQR = interquartile range; Min = minimum measured value; Max = maximum measured value.

Response	Mean	StDev	Median	IQR	Min	Max	Range
Acetaminophen (ng L^{-1})	701.8	1615.5	17.6	307.2	0.0	6504.6	6504.6
ARBs (ng L^{-1})	293.7	797.5	10.6	192.1	0.0	4081.0	4081.0
Benzodiazepine (ng L^{-1})	6.1	11.4	0.7	5.05	0.0	62.7	62.7
BetaBlocker (ng L^{-1})	51.4	160.1	1.4	15.8	0.0	934.3	934.3
Carbazepamine (ng L^{-1})	39.3	118.6	0.3	13.7	0.0	656.6	656.6
CCB (ng L^{-1})	3.7	9.3	0.0	1.6	0.0	38.2	38.2
Clopidogrel (ng L^{-1})	0.3	0.8	0.0	0.2	0.0	4.3	4.3
Fibrate (ng L^{-1})	50.4	106.4	0.9	33.27	0.0	479.2	479.2
Glibenclamide (ng L^{-1})	1.8	6.3	0.0	0.0	0.0	35.4	35.4
H_2Antagonist (ng L^{-1})	8.1	32.8	0.1	0.8	0.0	225.8	225.8
Iopromide (ng L^{-1})	5.8	14.8	2.7	2.7	0.0	83.9	83.9
LoopDiuretic (ng L^{-1})	114.2	240.3	23.1	88.7	0.0	955.2	955.2
Loratadine (ng L^{-1})	0.02	0.1	0.0	0.0	0.0	0.6	0.6
NSAID (ng L^{-1})	838.7	1892.2	95.8	495.1	0.0	9844.2	9844.2
Opioid (ng L^{-1})	14.3	38.6	0.1	7.2	0.0	238.0	238.0
Salbutamol (ng L^{-1})	1.3	6.5	0.1	0.1	0.0	43.7	43.7
SSRI (ng L^{-1})	16.0	69.9	0.2	3.8	0.0	495.1	495.1
Statin (ng L^{-1})	27.8	102.6	0.0	6.3	0.0	686.0	686.0
Tamsulosin (ng L^{-1})	0.3	1.0	0.0	0.0	0.0	6.6	6.6
Trazodone (ng L^{-1})	1.3	3.6	0.0	0.3	0.0	21.1	21.1
Venlafaxine (ng L^{-1})	25.4	67.3	1.7	19.6	0.0	347.0	347.0

Table S11. Permutational multivariate analysis of variance for stream water non-biocide drug concentrations. Multivariate variation in water non-biocide drug concentrations was represented by a Euclidean distance matrix. This matrix was partitioned among factors Treatment (two levels: control and impact) and Time (two levels: autumn and spring), and the Treatment x Time interaction, according to the statistical model (split plot anova) that is appropriate for the present experimental design (a repeated measurements Randomized Complete Block design or RCB). The appropriate error term for the above-the-line analysis is the Stream x Treatment interaction, whereas the appropriate error term for the below-the-line analysis is a pooled error term that includes both the Stream x Time and the Stream x Treatment x Time interactions. Two of the sampled streams (Reguers and Senia) were not included in the anova due to too many missing data points (the technique cannot handle unbalance), so only data for eleven streams have been considered (11 streams x 2 stations x 2 sampling occasions = 44 measurements).

Source	df	SS	MS	*F*-value	*p*-value
Stream	10	299.10	29.91	----	
Treatment	1	115.17	115.17	4.60	0.058
Streams x Treatment [= "Whole Plot" Error]	10	250.39	25.04		
Time	1	22.19	22.19	2.28	0.041
Treatment × Time	1	21.67	21.67	2.23	0.046
"Split Plot" [pooled] Error	20	194.48	9.72		
Total	43				

Table S12. Hypothesis testing results, after linear mixed modelling, for the individual water non-biocide drug concentrations measured in thirteen streams. The first two columns report *F*-values together with degrees of freedom (among parentheses) and significance (*p*-value < 0.05) is indicated with asterisks. The interaction between Treatment and Time was only significant in the case of Clopidogrel. Degrees of freedom differ among variables because, in some cases (streams Reguers and Senia), there are missing values. Parameter estimation is reported in Table S13.

Response	Treatment	Time	Fixed factors R^2 (%)
Acetaminophen (ng L^{-1})	10.6 (1, 34)**	4.4 (1, 34)*	99.9
ARBs (ng L^{-1})	6.9 (1, 37)**	0.7 (1, 37)	95.2
Benzodiazepine (ng L^{-1})	14.6 (1, 37)***	0.03 (1, 37)	44.4
BetaBlocker (ng L^{-1})	6.2 (1, 37)*	1.2 (1, 37)	99.96
Carbazepamine (ng L^{-1})	4.5 (1, 37)*	1.4 (1, 37)	83.7
CCB (ng L^{-1})	11.1 (1, 37)**	3.1 (1, 37)	19.7
Clopidogrel (ng L^{-1})	8.8 (1, 33)**	6.3 (1, 33)*	98.4
Fibrate (ng L^{-1})	13.5 (1, 37)***	2.6 (1, 37)	97.4
Glibenclamide (ng L^{-1})	3.9 (1, 34)	1.0 (1, 34)	-
H_2Antagonist (ng L^{-1})	3.2 (1, 37)	0.1 (1, 37)	-
Iopromide (ng L^{-1})	8.2 (1, 34)**	8.4 (1, 34)**	80.6
LoopDiuretic (ng L^{-1})	12.1 (1, 37)**	0.4 (1, 37)	90.9
Loratadine (ng L^{-1})	1.3 (1, 34)	0.5 (1, 34)	-
NSAID (ng L^{-1})	10.7 (1, 37)**	3.6 (1, 37)	98.5
Opioid (ng L^{-1})	5.9 (1, 37)*	0.5 (1,37)	97.5
Salbutamol (ng L^{-1})	1.8 (1, 34)	14.3 (1, 34)***	99.9
SSRI (ng L^{-1})	2.7 (1, 37)	1.6 (1, 37)	-
Statin (ng L^{-1})	5.0 (1, 37)*	0.2 (1, 37)	7.5
Tamsulosin (ng L^{-1})	3.8 (1, 34)	1.3 (1, 34)	-
Trazodone (ng L^{-1})	6.9 (1, 34)*	1.3 (1, 34)	95.3
Venlafaxine (ng L^{-1})	6.4 (1, 34)*	3.5 (1, 34)	91.0

Table S13. Parameter estimation, after linear mixed modelling, for the individual water non-biocide drug concentrations measured in thirteen streams. The interaction between Treatment and Time was only significant in the case of Clopidogrel. For some specific substances modelling of error variance using two additional δ parameters was insufficient and hence, it was needed to use four additional coefficients, one per Treatment:Time interaction, as follows. Acetominophen concentration: δ_{CA} = 1.00; δ_{CS} = 1.76; δ_{IA} = 515.76; and δ_{IS} = 478.33. Benzodiazepine concentration: δ_{CA} = 1.00; δ_{CS} = 4.99E+05; δ_{IA} = 2.88E+06; and δ_{IS} = 5.18E+06. BetaBlocker concentration: δ_{CA} = 1.00; δ_{CS} = 1.31; δ_{IA} = 216.89; and δ_{IS} = 313.04. Carbazepamine concentration: δ_{CA} = 1.00; δ_{CS} = 1.13E-04; δ_{IA} = 44.10; and δ_{IS} = 34.98. Fibrate concentration: δ_{CA} = 1.00; δ_{CS} = 2.75E-04; δ_{IA} = 222.19; and δ_{IS} = 220.65. Glibenclamide concentration: δ_{CA} = 1.00; δ_{CS} = 4.00E-05; δ_{IA} = 3.56; and δ_{IS} = 0.14. Iopromide concentration: δ_{CA} = 1.00; δ_{CS} = 3.15; δ_{IA} = 34.77; and δ_{IS} = 6.85. Opioid concentration: δ_{CA} = 1.00; δ_{CS} = 2.38; δ_{IA} = 91.93; and δ_{IS} = 30.22. Hypothesis testing is reported in Table S12.

Response	Intercept	Impact vs Control	Spring vs Autumn	σ_b	σ	δ_C	δ_I
Acetaminophen (ng L^{-1})	5.5 (2.2, 8.7)	1299.6 (487.7, 2111.4)	5.1 (0.2, 10.1)	3.3 (1.0, 11.3)	4.1 (1.7, 9.7)	-	-
ARBs (ng L^{-1})	23.2 (-11.9, 58.4)	549.6 (126.0, 973.3)	-	56.9 (36.5, 88.7)	25.9 (17.4, 38.7)	1.00	41.10
Benzodiazepine (ng L^{-1})	1.7 (-0.9, 4.2)	8.0 (3.7, 12.2)	-	4.5 (3.0, 6.7)	2.90E-06 (9.87E-12, 0.85)	-	-
BetaBlocker (ng L^{-1})	0.5 (-0.1, 1.0)	99.2 (18.8, 179.7)	-	0.6 (0.2, 1.6)	0.8 (0.4, 1.5)	-	-
Carbazepamine (ng L^{-1})	5.2 (-2.9, 13.2)	64.6 (2.7, 126.5)	-	13.8 (9.0, 21.1)	4.0 (2.6, 6.2)	-	-
CCB (ng L^{-1})	2.0 (-2.2, 6.2)	7.4 (2.9, 11.8)	-	2.9 (0.8, 9.9)	8.0 (6.3, 10.0)	-	-
Clopidogrel (ng L^{-1})	0.05 (0.02, 0.09)	1.0 (0.5, 1.5)	-0.05 (-0.10, -0.01)	7.29E-03 (2.05E-10, 2.59E+05)	0.06 (0.04, 0.08)	1.00	16.15
Fibrate (ng L^{-1})	2.5 (-2.0, 7.0)	95.9 (43.0, 148.9)	-	7.9 (5.3, 11.8)	0.6 (0.3, 1.1)	-	-
Glibenclamide (ng L^{-1})	3.1 (-2.4, 8.7)	-	-	9.8 (6.6, 14.6)	1.3 (0.8, 2.0)	-	-
H_2Antagonist (ng L^{-1})	0.1 (-0.01, 0.2)	-	-	0.05 (6.94E-04, 3.3)	0.2 (0.1, 0.3)	1.00	255.41
Iopromide (ng L^{-1})	0.3 (-0.2, 0.8)	2.4 (-1.0, 5.9)	2.3 (0.7, 3.8)	1.01E-04 (6.51E-286, 1.58E+277)	0.8 (0.5, 1.3)	-	-
LoopDiuretic (ng L^{-1})	13.5 (-5.2, 32.2)	208.6 (87.3, 329.9)	-	15.8 (4.6, 55.1)	29.4 (19.9, 43.2)	1.00	10.35
Loratadine (ng L^{-1})	0.014 (-0.005, 0.033)	-	-	0.02 (0.01, 0.06)	0.02 (0.01, 0.04)	1.00	5.09
NSAID (ng L^{-1})	70.9 (15.9, 126.0)	1567.0 (596.6, 2537.3)	-	88.3 (56.4, 138.3)	42.5 (28.5, 63.4)	1.00	57.40
Opioid (ng L^{-1})	0.3 (-0.3, 1.0)	14.5 (2.1, 26.9)	-	0.9 (0.4, 2.1)	0.8 (0.2, 2.4)	-	-
Salbutamol (ng L^{-1})	0.01 (-0.02, 0.03)	-	0.06 (0.03, 0.09)	0.018 (0.003, 0.107)	0.004 (0.002, 0.006)	1.00	237.34

SSRI (ng L^{-1})	0.6 (-0.2, 1.5)	-	-	1.4 (0.9, 2.1)	0.7 (0.4, 1.0)	1.00	145.32
Statin (ng L^{-1})	-6.1 (-56.8, 44.6)	55.6 (5.4, 105.8)	-	46.3 (20.8, 103.2)	89.4 (71.2, 112.3)	-	-
Tamsulosin (ng L^{-1})	0.03 (-0.01, 0.07)	-	-	6.25E-03 (1.28E-15, 3.06E+10)	0.06 (0.04, 0.09)	1.00	21.15
Trazodone (ng L^{-1})	0.12 (-0.04, 0.3)	2.4 (0.5, 4.3)	-	5.37E-05 (1.30E-305, 2.22E+296)	0.27 (0.20, 0.37)	1.00	17.26
Venlafaxine (ng L^{-1})	3.2 (-0.9, 7.2)	43.2 (8.4, 78.0)	-	6.6 (4.3, 10.3)	1.7 (1.1, 2.7)	1.00	49.97

Table S14. Descriptive statistics of the measured stream hydrological properties. IQR = interquartile range; Min = minimum measured value; Max = maximum measured value.

Response	Mean	StDev	Median	IQR	Min	Max	Range
Temperature (ºC)	14.8	1.2	14.6	1.4	12.5	17.9	5.4
Water Flow (m^3 s^{-1})	0.06	0.09	0.03	0.09	0.00	0.34	0.3
Width (m)	4.1	3.2	3.0	4.3	0.0	10.6	10.6
Mean Velocity (m s^{-1})	0.06	0.05	0.06	0.06	0.00	0.17	0.2
Depth (m)	0.22	0.24	0.15	0.19	0.00	0.93	0.93
Hydrological Stress (days)	11.8	20.1	0.0	18.6	0.0	60.0	60.0

Table S15. Permutational multivariate analysis of variance for stream hydrological properties, represented by a Euclidean distance matrix. This matrix was partitioned among the only factor Treatment (two levels: control and impact) according to the statistical model (split plot anova) that is appropriate for the present experimental design (a repeated measurements Randomized Complete Block design or RCB). Two of the sampled streams (Reguers and Bot Canaleta) were not included in the anova due to too many missing data points (the technique cannot handle unbalance), so only data for eleven streams have been considered (11 streams x 2 stations = 22 measurements).

Source	df	SS	MS	*F*-value	*p*-value
Stream	10	5423.6	542.36	----	
Treatment	1	178.6	178.60	1.06	0.331
Streams x Treatment [= "Whole Plot" Error]	10	1680.7	168.07		
Total	21				

Table S16. Hypothesis testing results, after linear mixed modelling, for the individual hydrological properties measured in the thirteen streams. The first two columns report *F*-values together with degrees of freedom (among parentheses) and significance (*p*-value < 0.05) is indicated with asterisks. Degrees of freedom differ among variables because, in some cases (streams Reguers and Bot Canaleta), there are missing values. Parameter estimation is reported in Table S17.

Response	Treatment	Fixed factors R^2 (%)
Temperature (ºC)	0.3 (1,11)	0.4
Water Flow (m^3 s^{-1})	0.01 (1,11)	9.54E-04
Width (m)	3.23E-04 (1,11)	3.69E-04
Mean Velocity (m s^{-1})	0.06 (1,12)	0.1
Depth (m)	0.06 (1,12)	0.02
Hydrological Stress (days)	1.98 (1,12)	2.4

Table S17. Parameter estimation, after linear mixed modelling, for the individual physical properties measured in thirteen streams. In this case, due to the structure of the data, only two additional δ parameters were used to model the error variance. Hypothesis testing is reported in Table S16.

Response	Intercept	Impact vs Control	σ_b	σ	δ_C	δ_I
Temperature (ºC)	14.77 (14.04, 15.50)	-	1.09 (0.64, 1.86)	0.47 (0.10, 2.29)	1.00	1.82
Water Flow (m^3 s^{-1})	0.06 (0.01, 0.11)	-	0.08 (0.05, 0.12)	0.02 (0.01, 0.03)	1.00	3.64E-05
Width (m)	4.00 (1.90, 6.11)	-	2.68 (1.64, 4.39)	2.12 (1.13, 3.97)	1.00	0.61
Mean Velocity (m s^{-1})	0.06 (0.03, 0.09)	-	0.03 (0.02, 0.07)	0.04 (0.02, 0.07)	1.00	0.81
Depth (m)	0.22 (0.07, 0.37)	-	0.23 (0.15, 0.35)	0.11 (0.05, 0.22)	1.00	0.29
Hydrological Stress (days)	15.32 (1.41, 29.24)	-	14.40 (9.64, 21.52)	17.97 (12.06, 26.77)	1.00	8.19E-06

Table S18. Descriptive statistics of the measured stream functional processes. IQR = interquartile range; Min = minimum measured value; Max = maximum measured value.

Response	Mean	StDev	Median	IQR	Min	Max	Range
BAT (g m^{-2} d^{-1})	0.03	0.03	0.03	0.05	0.01	0.10	0.10
SRPUC (µg P h^{-1})	1.93	5.30	1.46	4.77	-17.09	10.70	27.78
SRPAU (µg P m^{-2} min^{-1})	570.4	1598.9	41.1	230.8	-97.4	7556.8	7654.2
NH4AU (µg N m^{-2} min^{-1})	1310.8	2298.2	78.2	1625.1	2.3	9562.7	9560.4
GPP (mg O_2 m^{-2} d^{-1})	4.84	4.68	3.38	4.26	0.58	21.28	20.7
ER (mg O_2 m^{-2} d^{-1})	9.04	10.05	-5.57	11.51	-40.27	-0.28	39.99
OMD ($dd^{-1}.10^{-5}$)	12.24	9.01	12.16	14.69	0.49	29.90	29.42

Table S19. Permutational multivariate analysis of variance for stream functional processes, represented by a Euclidean distance matrix. This matrix was partitioned among the only factor Treatment (two levels: control and impact) according to the statistical model (split plot anova) that is appropriate for the present experimental design (a repeated measurements Randomized Complete Block design or RCB). Two of the sampled streams (Reguers and La Sènia) were not included in the ANOVA due to too many missing data points (the technique cannot handle unbalance), so only data for eleven streams have been considered (11 streams x 2 stations = 22 measurements).

Source	df	SS	*F*-value	*p*-value
Stream	11	62.13	----	
Treatment	1	19.31	4.76	0.002
Streams x Treatment [= "Whole Plot" Error]	9	36.49		
Total	21			

Table S20. Hypothesis testing results, after linear mixed modelling, for the individual functional processes measured in the thirteen streams. The first two columns report *F*-values together with degrees of freedom (among parentheses) and significance (*p*-value < 0.05) is indicated with asterisks. Degrees of freedom differ among variables because, in some cases (streams Reguers and La Sènia), there are missing values. Parameter estimation is reported in Table S21.

Response	Treatment	Fixed factors R^2 (%)
BAT (g m^{-2} d^{-1})	4.3 (1,10)$^{\cdot}$	48.1
SRPUC (µg P h^{-1})	39.0 (1,10)***	38.5
SRPAU (µg P m^{-2} min^{-1})	1.6 (1,9)	0.02
NH4AU (µg N m^{-2} min^{-1})	9.5 (1,9)**	99.9
GPP (mg O_2 m^{-2} d^{-1})	3.4 (1,10)	41.1
ER (mg O_2 m^{-2} d^{-1})	4.8 (1,10)*	34.5
OMD ($dd^{-1}.10^{-5}$)	0.02 (1,12)	0.07

Table S21. Parameter estimation, after linear mixed modelling, for the individual functional processes measured in thirteen streams. In this case, due to the structure of the data, only two additional δ parameters were used to model the error variance. Hypothesis testing is reported in Table S20.

Response	Intercept	Impact vs Control	σ_b	σ	δ_C	δ_I
BAT (g m^{-2} d^{-1})	0.023 (0.016, 0.029)	0.019 (-0.002, 0.040)	7.54E-07 (1.88E-279, 3.03E+266)	0.010 (0.007, 0.016)	1.00	3.12
SRPUC (µg P h^{-1})	4.95 (2.73, 7.17)	-5.50 (-7.46, -3.54)	3.54 (2.36, 5.32)	1.51E-04 (9.57E-17, 2.40E+08)	1.00	2.0E+04
SRPAU (µg P m^{-2} min^{-1})	809.26 (-441.52, 2060.05)	-	2064.54 (1383.63, 3080.52)	129.70 (81.72, 205.87)	1.00	7.8E-04
NH4AU (µg N m^{-2} min^{-1})	19.61 (-1.61, 40.82)	2284.50 (603.70, 3965.30)	30.79 (19.40, 48.87)	0.02 (3.7E-15, 9.2E+10)	1.00	1.4E+05
GPP (mg O_2 m^{-2} d^{-1})	3.15 (1.85, 4.46)	-	1.02 (0.01, 90.56)	1.68 (0.32, 8.73)	1.00	3.46
ER (mg O_2 m^{-2} d^{-1})	-5.21 (-8.55, -1.86)	-7.20 (-14.54, 0.13)	5.02 (1.71, 14.69)	0.75 (3.4E-20, 1.7E+19)	1.00	15.05
OMD ($dd^{-1}.10^{-5}$)	12.01 (6.78, 17.24)	-	3.21 (0.31, 32.82)	8.04 (4.67, 13.81)	1.00	1.14

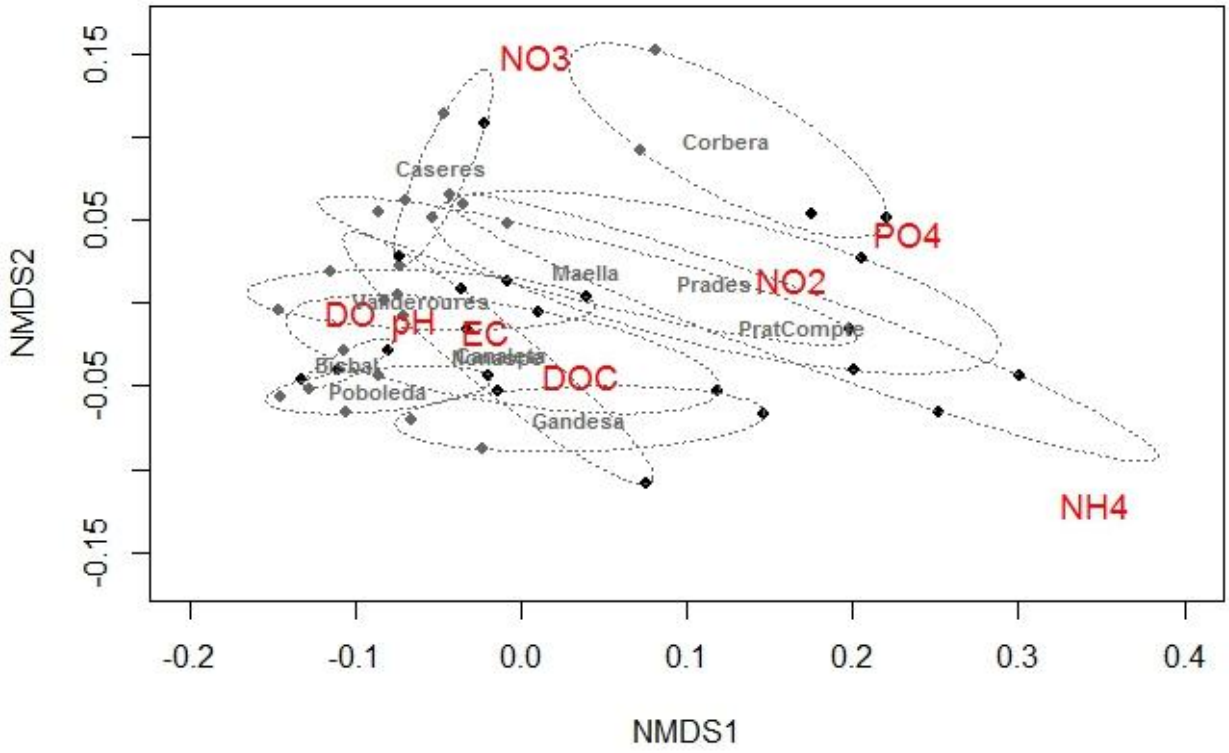


Figure S1. Main physicochemical gradients in stream water described by the unconstrained ordination technique NMDS (non–metric multidimensional scaling) using the Euclidean distance; stress = 0.03. Impact (black dots) and control (grey dots) stations are indicated. The ellipsoid hulls enclose all points in the same stream. Two of the sampled streams (Reguers and Senia) were not included in the analysis due to too many missing data points. See Table S3 for hypothesis testing for the multivariate case and Table S2 for acronym descriptions.

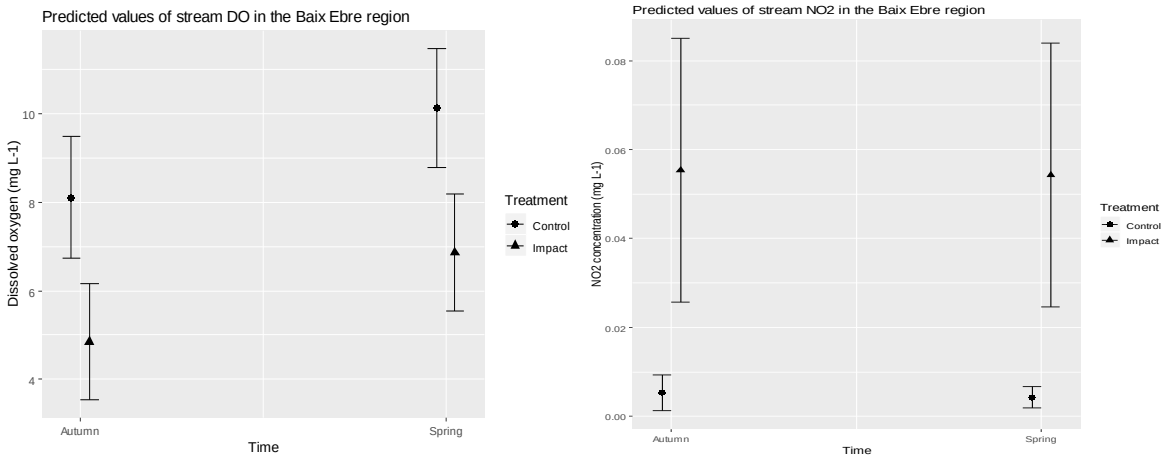


Figure S2. Mean effects of Treatment and Time with 95% confidence intervals on dissolved oxygen (left) and nitrite concentration (right), as fitted via linear mixed modeling using restricted maximum likelihood (see also Tables S4-S5).

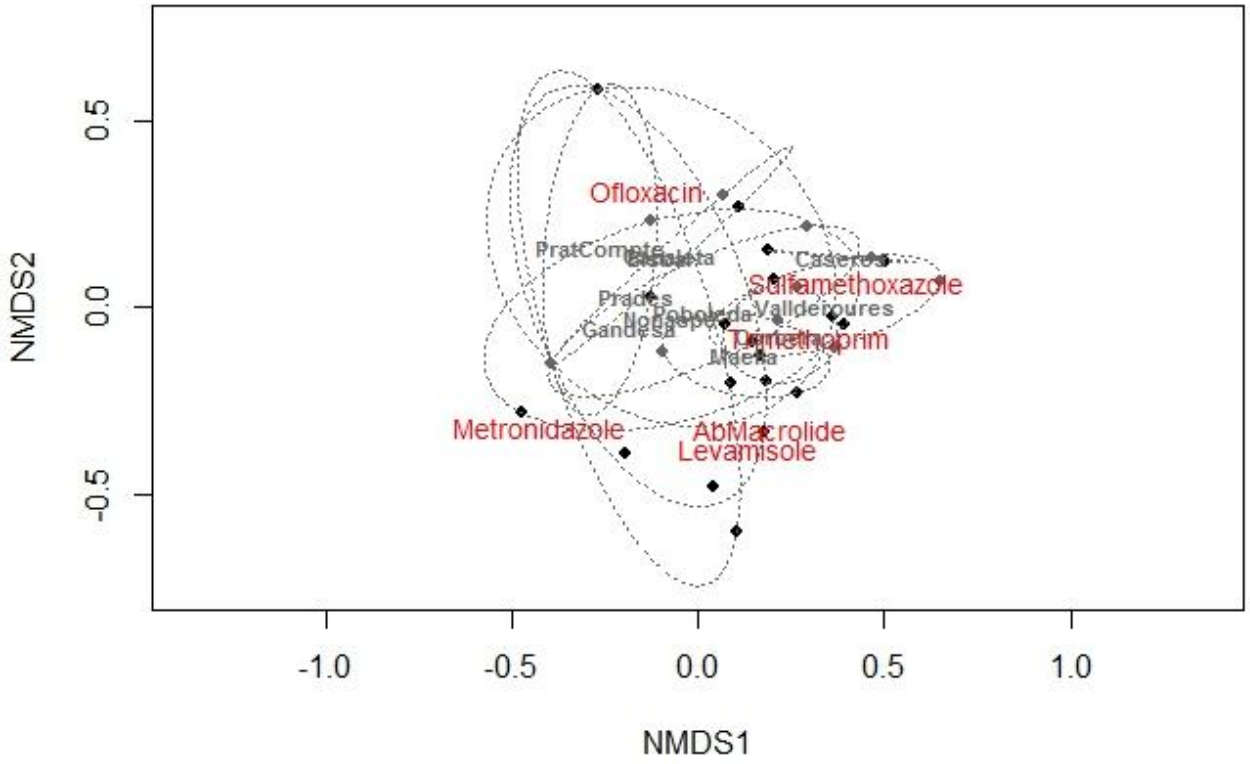


Figure S3. Main gradients of biocide concentrations in stream water described by the unconstrained ordination technique NMDS (non–metric multidimensional scaling) using the Euclidean distance; stress = 0.08. Impact (black dots) and control (grey dots) sampling stations are indicated. The ellipsoid hulls enclose all points in the same stream. Two of the sampled streams (Reguers and Senia) were not included in the analysis due to too many missing data points. See Table S7 for hypothesis testing for the multivariate case and Table S6 for acronym descriptions.

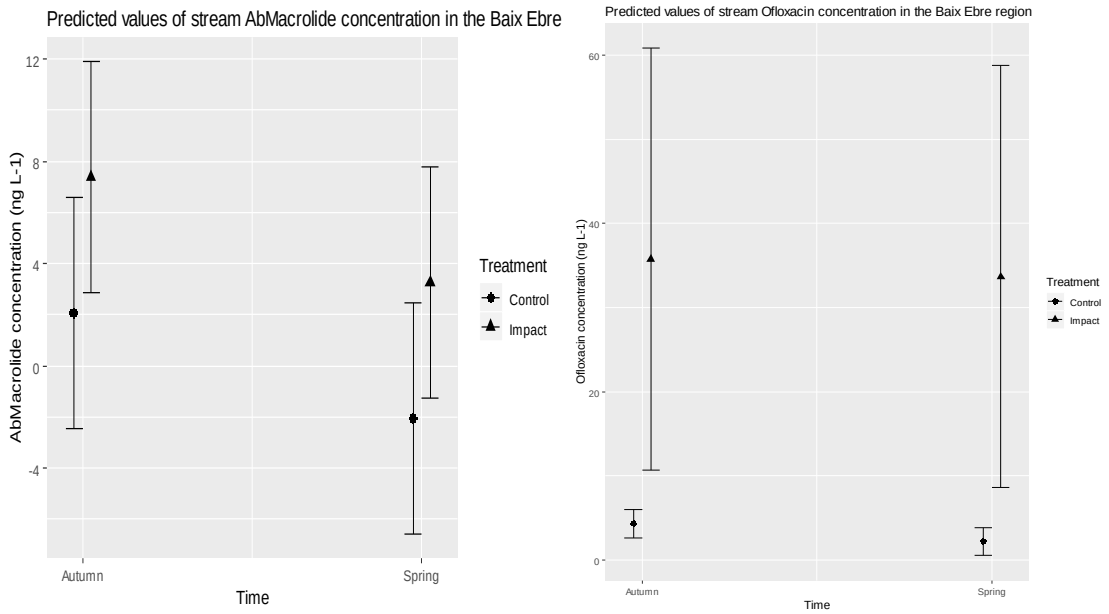


Figure S4. Mean effects of Treatment and Time with 95% confidence intervals on stream AbMacrolide concentration (left) and ofloxacin concentration (right), as fitted via linear mixed modeling using restricted maximum likelihood (see also Tables S8-S9).

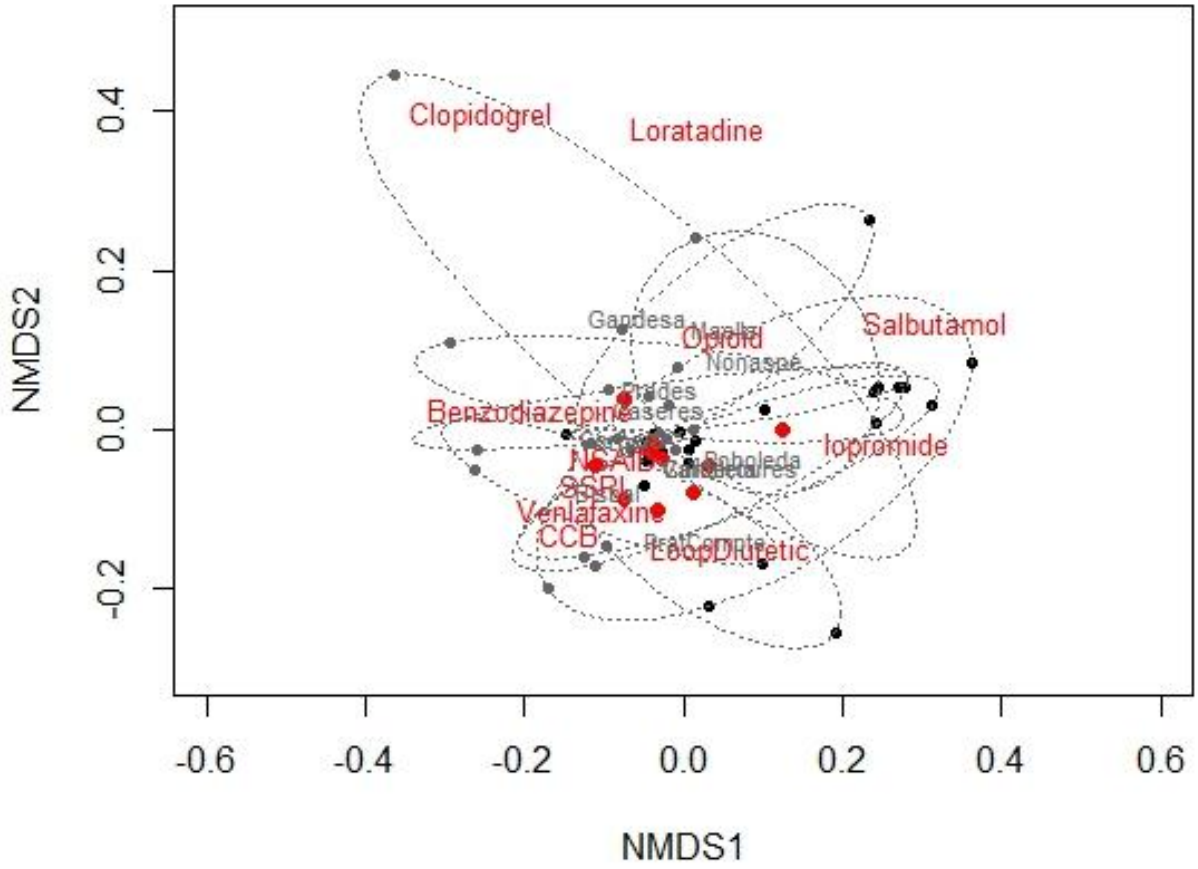


Figure S5. Main gradients of non-biocide drug concentrations in stream water described by the unconstrained ordination technique NMDS (non–metric multidimensional scaling) using the Euclidean distance; stress = 0.06. Impact (black dots) and Control (grey dots) reaches are indicated. The ellipsoid hulls enclose all points in the same stream. Two of the sampled streams (Reguers and Senia) were not included in the analysis due to too many missing data points. See Table S11 for hypothesis testing for the multivariate case and Table S12 for acronym descriptions.

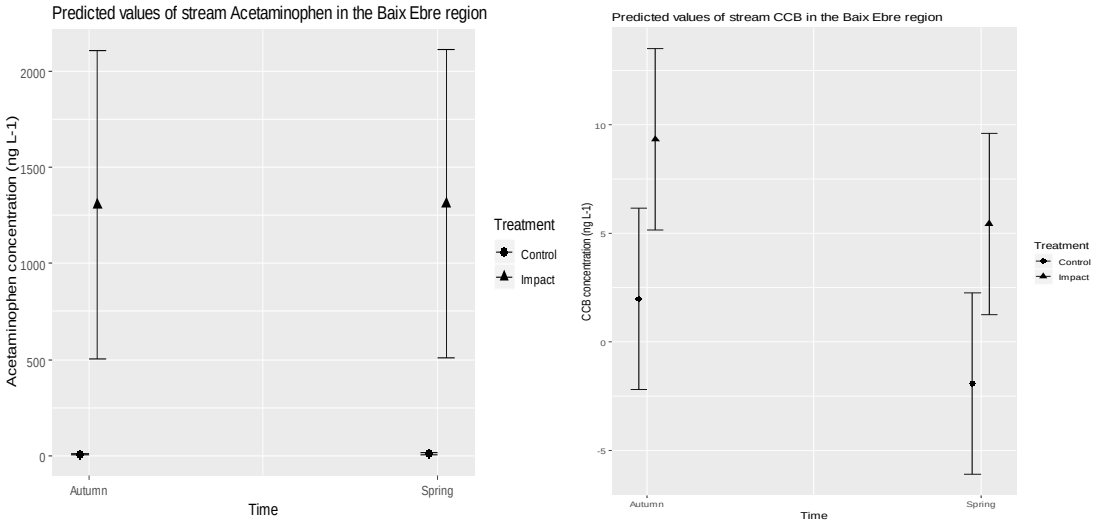


Figure S6. Mean effects of Treatment and Time with 95% confidence intervals on stream Acetaminophen concentration (left) and CCB concentration (right), as fitted via linear mixed modeling using restricted maximum likelihood (see also Tables S12-S13).

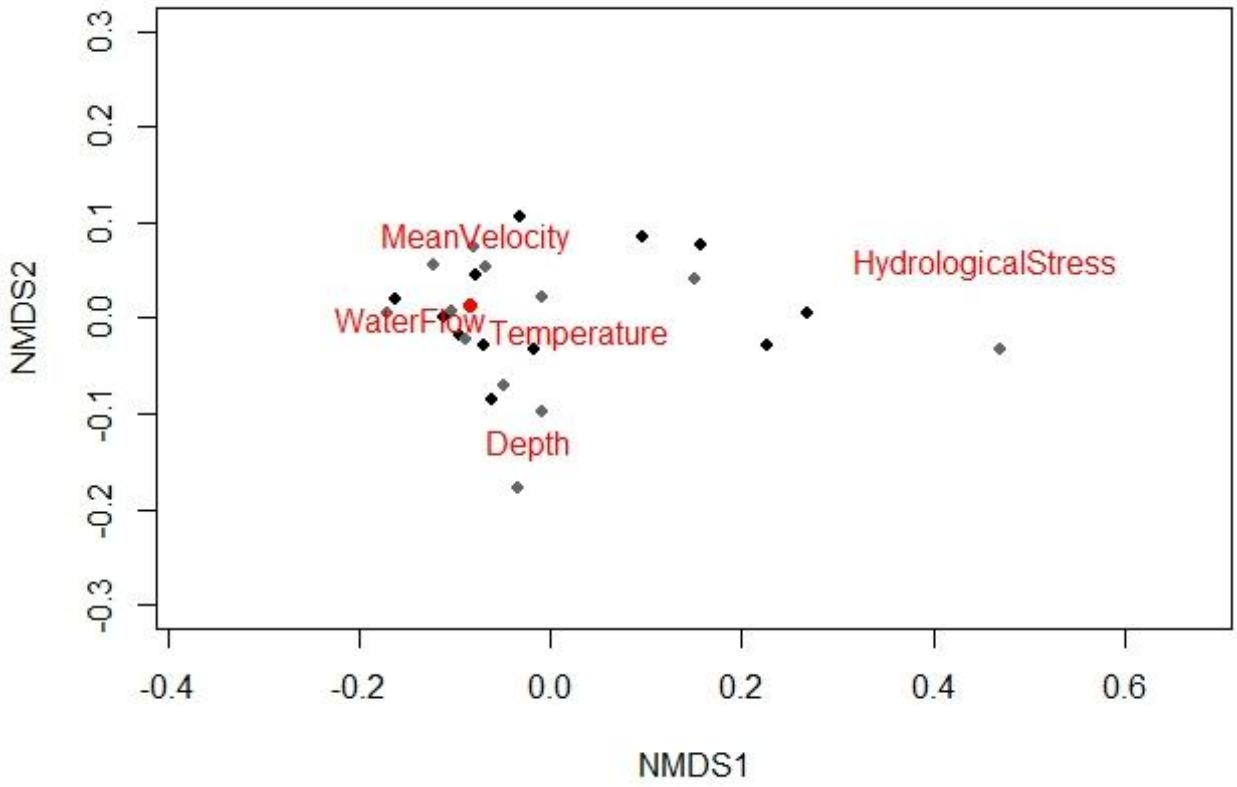


Figure S7. Main gradients of stream hydrological properties described by the unconstrained ordination technique NMDS (non-metric multidimensional scaling) using the Euclidean distance; stress = 0.02. Impact (black dots) and Control (grey dots) reaches are indicated. In this case, as there are not replicated measurements per stream and treatment, ellipsoids cannot be drawn. Two of the sampled streams (Reguers and Bot Canaleta) were not included in the analysis due to too many missing data points. See Table S15 for hypothesis testing for the multivariate case and Table S14 for acronym descriptions.

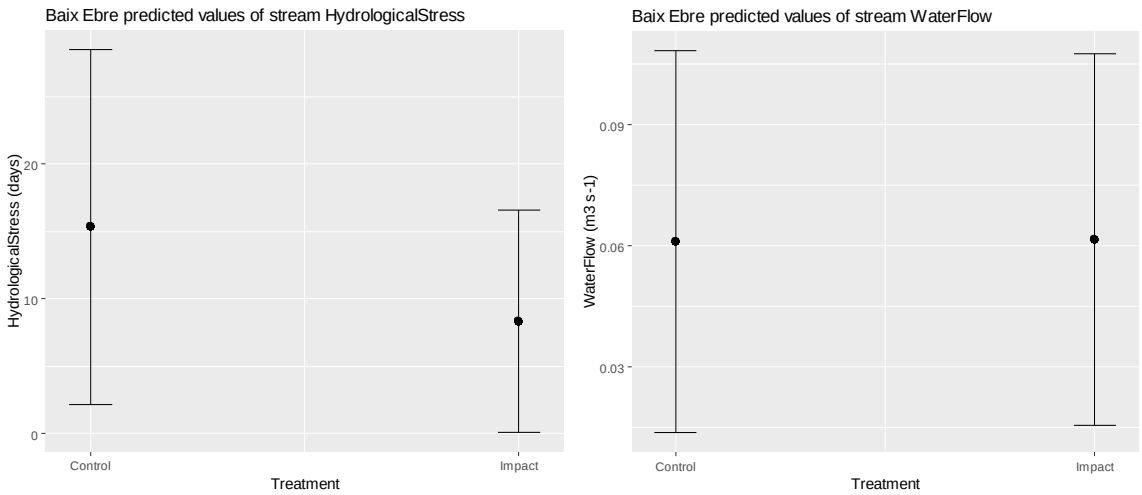


Figure S8. Mean effects of Treatment with 95% confidence intervals on stream Hydrological Stress (left) and Water flow (right), as fitted via linear mixed modeling using restricted maximum likelihood (see also Tables S16-S17).

Appendix S2

Appendix S2. R Code. Joint effects of urban pollution and hydrological alterations on the functioning of Mediterranean streams by Olatz Pereda, Daniel von Schiller, Gonzalo García-Baquero, Jordi-René Mor, Vicenç Acuña, Sergi Sabater and Arturo Elosegi.

```
#########################################################################
# Title: "Joint effects of urban pollution and hydrological           #
#         alterations on the functioning of Mediterranean streams"    #
#                                                                       #
#                                                                       #
#                                                                       #
# Authors: O. Pereda, D. von Schiller, G. García-Baquero, J.-R. Mor,  #
# V. Acuña, S. Sabater & A. Elosegi                                     #
#                                                                       #
# Appendix S2: Supplementary material (R Coding)                        #
#                                                                       #
# Data analysis was carried out by O. Pereda*, G. García-Baquero and  #
# Daniel Von Schiller                                                   #
# *email: olatz.pereda@ehu.eus                                          #
#########################################################################

# Data analysis was carried out using R software v.3.4.0. (R Core Team.
# 2017. R: A language and environment for statistical computing. version
# 3.4.0. R Foundation for Statistical Computing, Vienna, Austria.

# This file was created using Tinn-R Editor [Faria J.C., Grosjean P.
# & Jelihovschi E. (2014). Tinn-R Editor - GUI for R Language and
# Environment. Tinn-R Editor is free software.

# R packages
# **********
# John Fox and Sanford Weisberg (2011). An {R} Companion to Applied
# Regression, Second Edition. Thousand Oaks CA: Sage. URL:
# install.packages("car")                       # run if needed
library(car)

# Maechler, M., Rousseeuw, P., Struyf, A., Hubert, M., Hornik, K.(2018).
# cluster: Cluster Analysis Basics and Extensions. R package version
2.0.7-1.
# install.packages("cluster")                   # run if needed
library(cluster)

# John Fox (2003). Effect Displays in R for Generalised Linear Models.
# Journal of Statistical Software, 8(15), 1-27. URL
# install.packages("effects")                   # run if needed
library(effects)

# Catherine Hurley (2012). gclus: Clustering Graphics. R package
# version 1.3.1. https://CRAN.R-project.org/package=gclus
# install.packages("gclus")                     # run if needed
library(gclus)

# Wickham, H. 2016. ggplot2: Elegant Graphics for Data Analysis.
# New York: Springer-Verlag.
# install.packages("ggplot2")                   # run if needed
library(ggplot2)
```

```
# Pinheiro, J., D. Bates, S. DebRoy, D. Sarkar, and R-Core-Team. 2018.
# nlme: Linear and Nonlinear Mixed Effects Models. R package version 3.1-137.
# install.packages("nlme")                    # run if needed
library(nlme)

# Barton, K. 2018. MuMIn: Multi-Model Inference. R package version 1.40.4.
# install.packages("MuMIn")                   # run if needed
library(MuMIn)

# Lüdecke, D. 2018. sjPlot: Data Visualization for Statistics in Social Science.
# R package version 2.6.2.
# install.packages("sjPlot")                  # run if needed
library(sjPlot)

# J. Oksanen, F.G. Blanchet, M. Friendly, R. Kindt, P. Legendre, D. McGlinn,
# P.R. Minchin, R.B. O'Hara, G.L. Simpson, P. Solymos, M.H.H. Stevens,
# E. Szoecs and H. Wagner (2018). vegan: Community Ecology Package. R package
# install.packages("vegan")                   # run if needed
library(vegan)

# John M Chambers (2013). SoDA: Functions and Examples for "Software for
# Data Analysis". R package version 1.0-6.
# install.packages("SoDA")                    # run if needed
library(SoDA)

# J. Oksanen, F.G. Blanchet, M. Friendly, R. Kindt, P. Legendre,
# D. McGlinn, P.R. Minchin, R.B. O'Hara, G.L. Simpson, P. Solymos,
# M.H.H. Stevens, E. Szoecs and H. Wagner (2018). vegan: Community
# Ecology Package. R package version 2.4-1.
# install.packages("vegan")                   # run if needed
library(vegan)

# CONTENTS OF THIS SCRIPT:
# ************************
# 1. Step 1: Preparing the data. Observed patterns in stream functioning
# 2. Step 2: The relationship between stream functioning and the
#             environment: a multivariate point of view
# 3. Step 3: The relationship between stream functioning and the
#             environment: Generalised Linear Modelling (GLM)
# ********************************************************************** #
# ********************************************************************** #

##########
# Step 1 #
##########

# A Randomized Complete Block (RCB) design
# ****************************************
# We begin by exploring multivariate variation in stream functioning.
# The statistical design used to collect data corresponds to a Randomized
# Complete Block (RCB) design, where the surveyed streams are "blocks"
# and the effluent is the experimental treatment [with two levels:
# Control and Impact].

# 1.1. Load and prepare the dataset
# *********************************
# Set up working directory (change as required)
# setwd(dir)
```

```
# Load the dataset (change as required)
BaixEbre <- read.csv("C:/…/Appendix S3.csv", row.names = 1, sep =";")
str(BaixEbre)
## 'data.frame': 26 obs. of     54 variables (two categorical variables
and
## 52 numerical variables)
## (...)
## Bisbal, Canaleta, Caseres, Corbera, Gandesa, Maella, Nonaspe, Poboleda,
## Prades, PratCompte, Reguers, Senia, and Vallderoures are the names of
## the surveyed streams.

# First construct a geographic matrix (location of the sample units):
names(BaixEbre[,c(3:4)])
## [1] "Latitude"    "Longitude"
loc <- BaixEbre[-c(3, 21:22, 23),c(3:4)]
## Rows 3, 21, 22, and 23 are here deleted because they contain NAs.

# Transform Lat/Lon data into Cartesian coordinates (X and Y)
library(SoDA)
spa <- as.data.frame(geoXY(loc$Latitude, loc$Longitude)) head(spa)
##         X         Y
## 1 49273.989  72641.94
## 2 49301.065  72425.37
## 3 21151.784  42265.74
## 4  9527.528  45749.51
## 5  8808.310  46539.11
## 6 29175.712  50645.92

# Construct a matrix of functional variables:
names(BaixEbre[,c(5:11)])
## [1] "Biomassaccrual" "SRPuptake"          "SRPU"              "NH4U"
## [5] "GPP"                "ER"                 "Decomposition"
## We will consider seven functional variables

# We will construct the matrix of functional variables,
# considering only the following seven functional variables
# and excluding the NA values for some of the streams
funct <- BaixEbre[-c(3, 21:22, 23),c(5:11)] names(funct)
## [1] "Biomassaccrual" "SRPuptake"          "SRPU"              "NH4U"
## [5] "GPP"                "ER"                 "Decomposition"
## Biomassaccrual: Biomass accrual rate on biofilm carriers
##                    (g AFDM m^-2 day^-1)
## SRPuptake:   SRP uptake capacity on biofilm carriers (µg P h^-1)
## SRPU:    Whole-reach SRP areal uptake (µg P m^-2 min^-1)
## NH4U:    Whole-reach NH4 areal uptake (µg N m^-2 min^-1)
## GPP: Whole-reach gross primary production (mg O2 m^-2 day^-1)
## ER:    Whole-reach ecosystem respiration (mg O2 m^-2 day-1)
## Decomposition: Decomposition rate of wooden sticks (dd^-1 10^-5)

# Change the names of the variables
colnames(funct)[1] <- "BAT"              # Biomassaccrual = BAT
colnames(funct)[2] <- "SRPUC"            # SRPuptake = SRPUC
colnames(funct)[3] <- "SRPAU"            # SRPU = SRPAU
colnames(funct)[4] <- "NH4AU"            # NH4U = NH4AU
colnames(funct)[7] <- "OMD"              # Decomposition = OMD
## GPP = GPP
## ER = ER
names(funct)
## [1] "BAT"     "SRPUC" "SRPAU" "NH4AU" "GPP"       "ER"      "OMD"
```

```
# Tranform the negative signs of ER to positive signs
ERp <- (funct$ER)*(-1) funct$ERp <- ERp
funct <- funct[, -c(6)]

# Explore whether transformations are needed
funct.z.boxplot <- as.data.frame(scale(funct)) boxplot(funct.z.boxplot)
## No transformations are needed, since the variables do not deviate much
## from symmetry

# Construct a water chemical characteristics matrix:
names(BaixEbre[,c(12:19)])
## [1] "pH" "EC" "DO" "NH4" "NO2" "NO3" "SRP" "DOC"
chem <- BaixEbre[-c(3, 21:22, 23),c(12:19)]

# Explore whether transformations are needed
chem.z.boxplot <- as.data.frame(scale(chem))
boxplot(chem.z.boxplot)
## NH4, NO2, and SRP are right-skewed

# Square-root transform the following variables
sqrtNH4 <- sqrt(chem$NH4)
chem$sqrtNH4 <- sqrtNH4
sqrtNO2 <- sqrt(chem$NO2)
chem$sqrtNO2 <- sqrtNO2
sqrtSRP <- sqrt(chem$SRP)
chem$sqrtSRP <- sqrtSRP
chem <- chem[,-c(4:5,7)]
boxplot(scale(chem))

# Change the names of some variables
colnames(chem)[6] <- "Ammonium"       # sqrtNH4 = Ammonium
colnames(chem)[7] <- "Nitrite"        # sqrtNO2 = Nitrite
colnames(chem)[8] <- "SRP"            # sqrtSRP = Soluble reactive
phosphorus (SRP)
names(chem)
## [1] "pH"          "EC"          "DO"    "NO3"    "DOC"    "Ammonium"
## [7] "Nitrite"     "SRP"

# Construct a hydrological properties matrix:
names(BaixEbre[,c(20:25)])
## [1] "Temperature"          "WaterFlow"            "Width"
## [4] "MeanVelocity"         "Depth"                "HydrologicalStress"
hydr <- BaixEbre[-c(3, 21:22, 23),c(20:25)]

# Explore whether transformations are needed
hydr.z.boxplot <- as.data.frame(scale(hydr)) boxplot(hydr.z.boxplot)
## HydrologicalStress is right-skewed

# Square-root transform the following variable and change its name
HydrStr <- sqrt(hydr$HydrologicalStress)
hydr$HydrStr <- HydrStr
hydr <- hydr[,-c(6)]
boxplot(scale(hydr))

names(hydr)
## [1] "Temperature"    "WaterFlow"    "Width"    "MeanVelocity"    "Depth"
## [6] "HydrStr"

# Construct a biocides matrix:
names(BaixEbre[,c(26:33)])
```

```
## [1] "AbMacrolide"         "Metronidazole"    "Ofloxacin"       "Ronidazole"
## [5] "Sulfamethoxazole" "Tiabendazole"        "Trimethoprim"    "Levamisole"
bioc <- BaixEbre[-c(3, 21:22, 23),c(26:33)]

# Explore whether transformations are needed
bioc.z.boxplot <- as.data.frame(scale(bioc)) boxplot(bioc.z.boxplot)
## AbMacrolide, Trimethoprim, and Levamisole are right-skewed

# Square-root transform the aforementioned variables
sqrtAbMacrolide <- sqrt(bioc$AbMacrolide)
bioc$sqrtAbMacrolide <- sqrtAbMacrolide
sqrtTrimethoprim <- sqrt(bioc$Trimethoprim)
bioc$sqrtTrimethoprim <- sqrtTrimethoprim
sqrtLevamisole <- sqrt(bioc$Levamisole)
bioc$sqrtLevamisole <- sqrtLevamisole
bioc <- bioc[,-c(1,7,8)]

boxplot(scale(bioc))

names(bioc)
## [1] "Metronidazole"        "Ofloxacin"         "Ronidazole"
## [4] "Sulfamethoxazole"     "Tiabendazole"      "sqrtAbMacrolide"
## [7] "sqrtTrimethoprim"     "sqrtLevamisole"

# Change the names of some variables
colnames(bioc)[6] <- "Macrolide"           # Macrolide = sqrtAbMacrolide
colnames(bioc)[7] <- "Trimethoprim"        # Trimethoprim = sqrtTrimethoprim
colnames(bioc)[8] <- "Levamisole"          # Levamisole = sqrtLevamisole

names(bioc)
## [1] "Metronidazole"       "Ofloxacin"          "Ronidazole"
## [4] "Sulfamethoxazole" "Tiabendazole"          "Macrolide"
## [7] "Trimethoprim"        "Levamisole"

# Construct a non-biocide drugs matrix (pharmaceuticals, except biocides):
names(BaixEbre[,c(34:54)])
#  [1] "Acetaminophen"    "ARBs"    "Benzodiazepine"    "BetaBlocker"
#  [5] "Carbamazepine"    "CCB"              "Clopidogrel"      "Fibrate"
#  [9] "Glibenclamide"    "H2Antagonist"     "Iopromide"       "LoopDiuretic"
# [13] "Loratadine"       "NSAID"           "Opioid"           "Salbutamol"
# [17]   "SSRI"           "Statin"          "Tamsulosin"       "Trazodone"
# [21] "Venlafaxine"

drug <- BaixEbre[-c(3, 21:22, 23),c(34:54)]

# Explore whether transformations are needed
drug.z.boxplot <- as.data.frame(scale(drug))
boxplot(drug.z.boxplot)
## Acetaminophen, BetaBlocker, and other variables are right-skewed

# Square-root transform the aforementioned variables
sqrtAcetaminophen <- sqrt(drug$Acetaminophen)
drug$sqrtAcetaminophen <- sqrtAcetaminophen
sqrtBetaBlocker <- sqrt(drug$BetaBlocker)
drug$sqrtBetaBlocker <- sqrtBetaBlocker
sqrtCarbamazepine <- sqrt(drug$Carbamazepine)
drug$sqrtCarbamazepine <- sqrtCarbamazepine
sqrtCCB <- sqrt(drug$CCB)
drug$sqrtCCB <- sqrtCCB
sqrtClopidogrel <-sqrt(drug$Clopidogrel)
drug$sqrtClopidogrel <- sqrtClopidogrel
```

```
sqrtGlibenclamide <- sqrt(drug$Glibenclamide)
drug$sqrtGlibenclamide <- sqrtGlibenclamide
sqrtH2Antagonist <- sqrt(drug$H2Antagonist)
drug$sqrtH2Antagonist <- sqrtH2Antagonist
sqrtSalbutamol <- sqrt(drug$Salbutamol)
drug$sqrtSalbutamol <- sqrtSalbutamol
sqrtSSRI <- sqrt(drug$SSRI)
drug$sqrtSSRI <- sqrtSSRI
sqrtStatin <- sqrt(drug$Statin)
drug$sqrtStatin <- sqrtStatin
sqrtTamsulosin <- sqrt(drug$Tamsulosin)
drug$sqrtTamsulosin <- sqrtTamsulosin
sqrtTrazodone <- sqrt(drug$Trazodone)
drug$sqrtTrazodone <- sqrtTrazodone
drug <- drug[,-c(1,4,5,6,7,9,10,16,17,18,19,20)]
boxplot(scale(drug))

names(drug)
##  [1] "ARBs"                 "Benzodiazepine"       "Fibrate"
##  [4] "Iopromide"            "LoopDiuretic"         "Loratadine"
##  [7] "NSAID"                "Opioid"               "Venlafaxine"
## [10] "sqrtAcetaminophen" "sqrtBetaBlocker" "sqrtCarbamazepine"
## [13] "sqrtCCB"              "sqrtClopidogrel" "sqrtGlibenclamide"
## [16] "sqrtH2Antagonist" "sqrtSalbutamol"  "sqrtSSRI"
## [19] "sqrtStatin" "sqrtTamsulosin"  "sqrtTrazodone"

# In summary, so far we have loaded the dataset and generated six
# data matrices: <funct> (the response matrix); <chem>, <hydr>, <bioc>,
# and <drug> (four candidate explanatory matrices); and <spa> (a
# geographic matrix). Several candidate explanatory variables were sqrt-
# transformed, so that they are now in a form more amenable to linear
# modelling purposes.

# Finally, create the design matrix: this contains the statistical design
design <- BaixEbre[-c(3, 21:22, 23), c(1:2)] names(design)
## [1] "Stream"      "Treatment"

## We have excluded data from streams Canaleta, Reguers and Senia, which
## contain too many NAs. Hence only ten streams will be here
## considered.

# 1.2. Standardize the variables in the response matrix
# *****************************************************
library(vegan)
funct.stand <- decostand(funct, method = "standardize", MARGIN = 2)
## This is necessary/convenient, since the variables in <funct> are
## expressed in differing scales

# 1.3. Testing for linear spatial depencies
# *****************************************
# Test for linear trend
anova(rda(funct.stand ~ X + Y, data = spa), by = "term")
## Permutation test for rda under reduced model
## Terms added sequentially (first to last)
## Permutation: free
## Number of permutations: 999
## Model: rda(formula = funct.stand ~ X + Y, data = spa)
##           Df Variance      F Pr(>F)
## X          1   0.3284 1.1111  0.338
## Y          1   1.0561 3.5734  0.029 *
## Residual 19   5.6155
```

```
## The test is based in permutations, hence slightly different p-values
## may be obtained each time the analysis is run. Nonetheless, the test
## conclusions will not vary. In this case, we observe that a linear
## trend along the spatial variable Y is present. For this reason, we
## now detrend the response matrix.

# A linear trend is present along Y; therefore detrending is necessary:
funct.stand.det <- resid(lm(as.matrix(funct.stand) ~ ., data = spa))
head(funct.stand.det)
##         BAT       SRPUC      SRPAU       NH4AU         GPP        OMD
## 1 -0.2303217  0.49758446  0.2346490  0.02559649 -0.50141593  0.8547350
## 2 -0.8859398 -0.18648303  0.2214001  0.01965860  0.03675215  0.6247857
## 4 -0.1169370 -0.01173786 -0.5320835 -0.48212607 -0.38923558  1.0650851
## 5 -0.6313876  1.44804093 -0.2380926 -0.48014040 -0.34220177 -1.1844621
## 6  0.6819762  0.41680055 -0.1993361 -0.40292349 -0.99352815  0.5537806
## 7 -0.8162993 -0.95895827  0.6104441 -0.57520951  0.17251374  1.1824363
##         ERp
## 1 -0.51524529
## 2 -0.44349135
## 4 -0.50530436
## 5 -0.53369149
## 6 -1.06746540
## 7  0.09934069

## This is the detrended response matrix.

# Having detrended the response matrix, we are now compelled to detrend
# also the four environment matrices, as follows:
chem.det <- as.data.frame(resid(lm(as.matrix(chem) ~ ., data = spa)))
head(chem.det)

hydr.det <- as.data.frame(resid(lm(as.matrix(hydr) ~ ., data = spa)))
head(hydr.det)

bioc.det <- as.data.frame(resid(lm(as.matrix(bioc) ~ ., data = spa)))
head(bioc.det)

drug.det <- as.data.frame(resid(lm(as.matrix(drug) ~ ., data = spa)))
head(drug.det)
## Hence chem.det, hydr.det, bioc.det, and drug.det are the new
## potentially explanatory matrices.

# 1.4. Nonmetric Multidimensional Scaling (NMDS)
# **********************************************

# ***************************************************************************
# The following steps (1.4, 1.5 and 1.6) have been applied also to each of
# the matrices <chem>, <hydr>, <bioc> and <drug>. However, just for brevity,
# here we demonstrate data analysis for just the functional variables.
# ***************************************************************************

# Create the ordination using function metaMDS {vegan}, which finds a
# stable solution using random starts.

# Create the ordination: notice that only the response matrix is used
# here, using the Euclidean distance. For more on NMDS, see Legendre &
# Legendre (2012). Numerical Ecology. Elsevier.
funct.stand.det.nmds <- metaMDS(funct.stand.det,
                                distance = "euclidean", autotransform = FALSE)
```

```
## 'comm' has negative data: 'autotransform', 'noshare' and 'wascores'
## set to FALSE
## Run 0 stress 0.1178499
## Run 1 stress 0.1156765
## ... New best solution
## (...)
##
## Run 18 stress 0.1198991
## Run 19 stress 0.1178506
## Run 20 stress 0.1156808
## ... Procrustes: rmse 0.001000095      max resid 0.003285652
## ... Similar to previous best
## *** Solution reached

## Run 20 stress is 0.1157, which is perfectly adequate and indicates that
## a more than acceptable ordination has been obtained.

# Now that the ordination has been created, without external reference and
# using only information contained in funct.stand.det, we prepare the data to
# plot these non-constrained ordination results in a manner that includes the
# statistical design information. Hence this information, which is contained
# in matrix design, is "overlaid" onto the ordination a posteriori.

# Check the adequacy of the ordination: Shepard plot and NMDS goodness of fit:
windows(6,6) par(mfrow = c(1,1))
stressplot(funct.stand.det.nmds, main = "Shepard plot")
# Non-metric goodness-of-fit of the ordination: R2 = 0.987.
# Linear goodness-of-fit of the ordination: R2 = 0.961.

# Define colours for the "sites" (to be used in plotting the diagram)
form <- factor(c(1,2,2,1,2,1,2,1,2,1,2,1,2,1,2,1,2,1,2,2,1,2),
                levels = c(1,2),
                labels = c("Control","Impact")) colvec <- c("blue", "red")
stcol = colvec[form]
## Black points indicate impact

# Plot the ordination (full page)
plot(funct.stand.det.nmds, display = "sites", type = "n",
      # xlim = c(-0.15, 0.35),
      ylim = c(-3, 2), cex = 0.7)
points(funct.stand.det.nmds, display = "sites", cex = 0.9, col = stcol,
       pch = 19)

# Lines join points in the group Stream
with(design, ordiellipse(funct.stand.det.nmds, groups = Stream,
                         kind = "se", draw = "line", label = T,
                         cex = 0.8, col = "grey40",
                         font = 1, lwd = 1, lty = 3,
                         show.groups = c("Corbera", "Bisbal", "Senia",
                         "Prades", "Gandesa", "Caseres", "Maella", "Nonaspe",
                         "Canaleta", "Vallderoures", "Reguers")))

## Poboleda (not represented to avoid crowding) is close to Bisbal
## PratCompte (not represented to avoid crowding) is close to Prades
## The graph shows how Impact points (in black) run through
## the whole NMDS1 axis, whereas Control points do not. On the other
## hand, Impact points run through the whole urban-agricultural NMDS2 axis,
## but Control points do not. So Control points appear to exhibit less
## variability than Impact points.
```

```
# 1.5. Permutational Multivariate Analysis of Variance Using Distance Matrices
# ************************************************************************************
# We are now to carry out analysis of variance (that is anova) using distance
# matrices using vegan function adonis2(). This technique partitions distance
# matrices among sources of variation (Treatment and Time, in our case) and
# fitting linear models to distance matrices.

# McArdle, B.H. and M.J. Anderson. 2001. Fitting multivariate models to
# community data: A comment on distance-based redundancy analysis.
# Ecology, 82: 290-297.

# Anderson, M.J. 2001. A new method for non-parametric multivariate analysis
# of variance. Austral Ecology, 26: 32-46.

# Legendre & Legendre (2012). Numerical Ecology. Elsevier.

adonis2(funct.stand.det ~ Stream + Treatment,
         data = design,
         method = "euclidean", permutations = 999)
##            Df  SumOfSqs      R2      F   Pr(>F)
## Stream     11   62.132  0.52687  1.3933  0.162
## Treatment  1    19.308  0.16373  4.7628  0.002 **  # This test is meaningless
## Residual   9    36.485  0.30939
## Total      21  117.925  1.00000
## Hence Treatment is significant.

# Now we need to express correctly the table above:

## Source                          | df |    SS    |    F   | p-value |
## --------------------------------|----|----------|--------|---------
## Stream                          | 11 | 62.132 | ----  |         |
## Treatment                       |  1 | 19.308 | 4.76  |  0.002  |
## Streams x  Treatment            |  9 | 36.485 |       |         |
## [ = "Whole Plot" Error]         |    |        |       |         |
## --------------------------------|----|----------|--------|---------
## Total                           | 21 |
## In summary, we find evidence to reject the null hypotheses of no
## Treatment effect on multivariate variation in stream funcionality.
# ********************************************************************** #

# 1.6. Mixed modelling for functional variables
# *********************************************

# Definition of function for later use
# ************************************
# The next function was provided by Borcard, D., F. Gillet & P. Legendre.
# 2001. Numerical Ecology with R. New York, Springer, as part of the
# electronic material accompanying the book.
# See http://adn.biol.umontreal.ca/~numericalecology/numecolR/.

# panelutils.R
# License: GPL-2
# Author: Francois Gillet, February 2007
## Put Pearson, Spearman or Kendall correlations on the upper panel
panel.cor <- function(x, y, method="pearson", digits=3, cex.cor=1.2)
{
  usr <- par("usr"); on.exit(par(usr)) par(usr = c(0, 1, 0, 1))
  r <- cor(x, y, method=method)
  ra <- cor.test(x, y, method=method)$p.value txt <- round(r, digits)
  sig <- 1 prefix <- ""
```

```
  if(ra <= 0.1) prefix <- "." if(ra <= 0.05) prefix <- "*" if(ra <= 0.01)
  prefix <- "**" if(ra <= 0.001) prefix <- "***" if(ra <= 0.001) sig <- 2
  color <- 2
  if(r < 0) color <- 4
  #          color <- "gray10"
  #          if(r < 0) color <- "gray50"
  txt <- paste(txt, prefix, sep="\n")
  text(0.5, 0.5, txt, cex = cex.cor, font=sig, col=color)
}

## Put histograms on the diagonal
panel.hist <- function(x, ...)
{
  usr <- par("usr"); on.exit(par(usr))
  par(usr = c(usr[1:2], 0, 1.5) ) h <- hist(x, plot = FALSE)
  breaks <- h$breaks; nB <- length(breaks) y <- h$counts; y <- y/max(y)
  rect(breaks[-nB], 0, breaks[-1], y, col="cyan", ...)
  #          rect(breaks[-nB], 0, breaks[-1], y, col="gray", ...)
}

# Usage:
# pairs(num.mat, lower.panel=panel.smooth, upper.panel=panel.cor,
# diag.panel=panel.hist)
# pairs(num.mat, lower.panel=panel.smooth, upper.panel=panel.cor,
# method="kendall")

# Double Check suitability of variables for linear modelling purposes
# ********************************************************************
# Previously, a matrix <func> was generated. It is the matrix that we will
# use here.
str(funct)
## 'data.frame':    22 obs. of   7 variables:
## $ BAT: num 0.027 0.01 0.012 0.033 0.019 0.053 0.014 0.099 0.028 0.017
...
## $ SRPUC: num 4.076 0.335 6.67 1.446 10.695 ...
## $ SRPAU: num 13 9.8 NA 249.6 48.1 ...
## $ NH4AU: num 4.7 15.2 NA 875.4 13.9 ...
## $ GPP: num 1.21 3.77 6.22 2.98 4.45 1.44 5.3 9.48 1.85 1.54 ...
## $ ER: num -2.41 -3.12 -9.56 -3.63 -5.57 ...
## $ OMD: num 24.54 22.53 6.02 22.25 1.89 ...
## funct: the stream functional data. This matrix describes the
## functional responses of streams for each sample unit.

# Explore the variables: box plots of the standardized variables;
funct.z.boxplot <- as.data.frame(scale(funct))
boxplot(funct.z.boxplot)
## Most variables are about symmetrical.

# Investigate the presence of large values: Cleveland dotplots
windows(title = "Cleveland dotplots", 7, 7)
op <- par(mfrow = c(2,4), mar = c(4,3,3,2))
dotchart(funct$BAT, main = "BAT")
dotchart(funct$SRPUC, main = "SRPUC")
dotchart(funct$SRPAU, main = "SRPAU")
dotchart(funct$NH4AU, main = "NH4AU")
dotchart(funct$GPP, main = "GPP")
dotchart(funct$ER, main = "ER") d
otchart(funct$OMD, main = "OMD")
par(op)
## Some relatively large values are present in the cases of SRPAU and NH4AU.
```

```
# Investigate colinearity among the relevant functional variables
# ***************************************************************

# Kendall tau rank correlation among functional variables
library(cluster) library(gclus)
funct.ken <- cor(funct[ , ], method = "kendall",
                 use = "na.or.complete")
round(funct.ken , 2)
##          BAT  SRPUC  SRPAU  NH4AU   GPP   OMD    ERp
## BAT     1.00  -0.10   0.30  0.24   0.00  -0.16   0.02
## SRPUC  -0.10   1.00  -0.44 -0.56  -0.26  -0.08  -0.37
## SRPAU   0.30  -0.44   1.00  0.57   0.15  -0.10   0.24
## NH4AU   0.24  -0.56   0.57  1.00   0.30  -0.24   0.41
## GPP     0.00  -0.26   0.15  0.30   1.00   0.06   0.49
## OMD    -0.16  -0.08  -0.10 -0.24   0.06   1.00  -0.29
## ERp     0.02  -0.37   0.24  0.41   0.49  -0.29   1.00
## The untransformed variables are not, in general, strongly correlated
## among them.

# Bivariate plots with smooth curves, histograms and Kendall correlations
(funct.o <- order.single(funct.ken))
## [1] 2 6 5 7 4 3 1
windows(title = "Rank correlation matrix", 7, 7)
op <- par(mfrow = c(1,1), pty = "s")
pairs(funct[ , ][,funct.o],
      lower.panel = panel.smooth, upper.panel = panel.cor,
      method = "kendall", diag.panel = panel.hist,
      main = "Bivariate plots with smooth curves, histograms and Kendall
              correlations",
       cex.main = 0.9) par(op)
## Correlations are Kendall correlations.

# Summarise the (untransformed) functional variables
# ***************************************************
summary(funct)

##       BAT               SRPUC               SRPAU              NH4AU
##  Min.   :0.00700   Min.   :-17.0890   Min.   : -97.40   Min.   :   2.30
##  1st Qu.:0.01750   1st Qu.:  0.2465   1st Qu.:  11.65   1st Qu.:  13.15
##  Median :0.02850   Median :  1.4550   Median :  38.90   Median :  49.65
##  Mean   :0.03505   Mean   :  1.7605   Mean   : 553.86   Mean   :1232.56
##  3rd Qu.:0.04225   3rd Qu.:  4.1867   3rd Qu.: 223.12   3rd Qu.:1603.97
##  Max.   :0.10400   Max.   : 10.6950   Max.   :7556.80   Max.   :9562.70
##
##       GPP              OMD               ERp
##  Min.   : 0.580   Min.   : 0.487   Min.   : 0.280
##  1st Qu.: 1.640   1st Qu.: 5.811   1st Qu.: 1.880
##  Median : 3.180   Median :14.735   Median : 4.600
##  Mean   : 4.774   Mean   :13.945   Mean   : 9.013
##  3rd Qu.: 5.607   3rd Qu.:21.298   3rd Qu.:13.963
##  Max.   :21.280   Max.   :29.902   Max.   :40.270

# Standard deviations of the (untransformed) environmental variables
sqrt(var(funct$BAT, use = "na.or.complete"))
## [1] 0.02596972
sqrt(var(funct$SRPUC, use = "na.or.complete"))
## [1] 5.440586
sqrt(var(funct$SRPAU, use = "na.or.complete"))
## [1] 1634.51
sqrt(var(funct$NH4AU, use = "na.or.complete"))
## [1] 2320.692
```

```r
sqrt(var(funct$GPP, use = "na.or.complete"))
## [1] 4.780021
sqrt(var(funct$ER, use = "na.or.complete"))
## [1] 10.28797
sqrt(var(funct$OMD, use = "na.or.complete"))
## [1] 8.724429

# 1.6.1. Modelling biomass accrual - BAT (g m-2 d-1)
# ************************************************
Functioning <- cbind(design,funct)
Functioning

# a. Inspect the data
# *******************
names(funct)
## [1] "BAT" "SRPUC" "SRPAU" "NH4AU" "GPP" "OMD" "ERp"

library(ggplot2)
ggplot(Functioning, aes(y = BAT, x = Treatment)) +
       geom_point() + facet_wrap(~Stream)
## Canaleta and Senia have no control data

# Explore normality and homogeneity of variance
ggplot(Functioning) + geom_boxplot(aes(y = BAT, x = Treatment))
## We observe heterogeneity in the group variances control data are less
## variable than impact data

# Interaction: In the case of the functional variables, the field survey
# was not repeated in time, so there cannot be any interaction between
# Treatment and Time. However, this was not the case for explanatory
# variables in <chem>, <phys>, <bioc>, and <drug>.

# b. Fit the model
# ****************
# Fit the model: since there is a single value per Treatment,
# only one random structure is possible ("random = ~1 | Stream");
# "na.action = na.omit" causes NAs to be omitted, but the presence of
# lack of balance does not serioulsy affect estimation.
library(nlme)
BAT.lme.ad.REML <- lme(fixed = BAT ~ Treatment,
                       data = Functioning,
                       random = ~1 | Stream,
                       method = "REML",
                       na.action = na.omit)
plot(BAT.lme.ad.REML)
## The residual plot indicates there is heterogeneity of variance, so
## re-fitting the model with control of heterogeneity is needed.

# We will re-fit the model, this time ncluding a structure for the control
# of variance heterogeneity
BAT.lme.ad.REML.vs <- lme(fixed = BAT ~ Treatment,
                          data = Functioning,
                          random = ~1 | Stream,
                          method = "REML",
                          na.action = na.omit,
                          weights = varIdent(form = ~ 1 | Treatment))

# Before accepting the model above, let us check model assumptions:
# homogeneity of variance
plot(BAT.lme.ad.REML.vs)
## The residual plot now indicates no violation of the homogeneity assumption
```

```r
# Check model assumptions: normality
qqnorm(resid(BAT.lme.ad.REML.vs))
qqline(resid(BAT.lme.ad.REML.vs))
## Overall, model assumptions are about OK.

# Inspect fit
summary(BAT.lme.ad.REML.vs)
## Linear mixed-effects model fit by REML
##  Data: Functioning
##       AIC       BIC   logLik
## -87.43014 -82.45147 48.71507
##
## Random effects:
## Formula: ~1 | Stream
##         (Intercept)    Residual
## StdDev: 6.723985e-07 0.009949316
##
## Variance function:
##  Structure: Different standard deviations per stratum
##  Formula: ~1 | Treatment
##  Parameter estimates:
##  Control    Impact ##  1.000000  3.177616
## Fixed effects: BAT ~ Treatment
##                     Value      Std.Error   DF    t-value   p-value
## (Intercept)       0.02390000 0.003146250  11   7.596345   0.0000
## TreatmentImpact   0.02043333 0.009653591   9   2.116656   0.0634
##  Correlation:
##                   (Intr)
## TreatmentImpact   -0.326
##
## Standardized Within-Group Residuals:
##        Min        Q1        Med        Q3        Max
## -1.1808700 -0.7715482 -0.3426632 0.4092295 2.1207488
##
## Number of Observations: 22
## Number of Groups: 12

# c. Inference
# *************
# Hypothesis testing
anova(BAT.lme.ad.REML.vs)
##               numDF denDF   F-value p-value
## (Intercept)       1    11 76.82100  <.0001
## Treatment         1     9  4.48023  0.0634
## There is weak evidence against the null hypothesis of no treatment effect

# Approximate 95% CI for model parameters
intervals(BAT.lme.ad.REML.vs, which = "fixed")
## Fixed effects:
##                     lower          est.      upper
## (Intercept)      0.016975150 0.02390000  0.03082485
## TreatmentImpact -0.001404607 0.02043333  0.04227127
## The estimates are quite precise. For instance, the effect size of Impact
## (vs. control) is 0.02 with 95% confidence interval (0.00, 0.04).

# Approximate 95% CI for model parameters
intervals(BAT.lme.ad.REML.vs, which = "var-cov")
## In this particular case, confidence intervals on var-cov components can
## not be obtained, since the approximate variance-covariance is non- positive
## definite.
```

```
## Nonetheless, from the summary we know that the variability due to Stream
## is a normal random variable with mean 0 and variance (6.72E-07)^2,
## i.e. b_i ~ N(0, (7.54E-07)^2). Unexplained variability has variance 0.01^2.

# Approximate R^2 explained by the random factor Site, and the fixed factors
# Year and Fencing
library(MuMIn)
r.squaredGLMM(BAT.lme.ad.REML.vs)
##        R2m        R2c
##    0.5227978   0.5227978
## R2m is the 'marginal' R^2 and represents the variance explained by only
## the fixed factors. In this case, R^2_marginal = 52.3%. Hence Treatment
## explains 52.3% of the observed variability in stream BAT.
## R2c is the 'conditional' R^2 and represents the variance explained by both
## fixed and random factors. In this case, R^conditional = 52.3%. Therefore,
## the random factor Stream does not contribute, in this case, to the
## explanation of variation in BAT.

# Plot model: marginal effects
library(sjPlot)
plot_model(BAT.lme.ad.REML.vs, type = "eff", terms = c("Treatment"),
           title = "Baix Ebre predicted values of biofilm biomass accrual rate",
           axis.title = "Biofilm biomass accrual rate -
                         (g AFDM per square m per day)",
           colors = "bw")
## Close plotting device
## As we knew, effluent appears to cause BAT to increase by 0.02 units.
## However, as we also knew, this effect (vs. control) is 0.02 with 95%
## confidence interval (0.00, 0.04) and the corresponding p-value is rather
## marginal (p-value = 0.0634). This is reflected in the confidence intervals
## plotted in this graph, which reflect the uncertainty in the observed
## estimates for the group typical values (estimated via ML, not just by
## observed means)

# The typical values plotted above, but in numbers:
library(effects)
data.eff = as.data.frame(Effect(c("Treatment"), BAT.lme.ad.REML.vs,
                          xlevels = list(Treatment = 2)))
data.eff
##     Treatment      fit          se       lower       upper
## 1    Control  0.02390000 0.003146250 0.01733704 0.03046296
## 2     Impact  0.04433333 0.009126496 0.02529580 0.06337087
# ***********************************************************************


# ***************************************************************************
# This step (1.6.1) has been applied to each functional variable (SRPUC, SRPAU,
# NH4AU, GPP, OMD and ER). However, here, for the sake of brevity, we do not
# write down every procedure.
# ***************************************************************************

##########
# Step 2 #
##########

# In what follows, we apply model selection procedures within the framework
# of Redundancy Analysis (RDA). Prior to this (step 1.3), we tested for linear
# spatial dependencies in the response matrix, which turned out to be
# significant. For this reason, here we use not only standardized variables,
# but also detrended (spatially correlated variables tend to be selected in
# procedures of variable selection, just because they are positively spatially
```

```
# correlated, but the issue disappears if the variables are previously
# detrended.)

# Finally, we also will carry out partitioning of multivariate variation
# in stream functioning to determine the unique contributions of
# environmental properties (chem, hydr, bioc, and drug) to the explanation
# of change in stream functioning.

# 2.1. The relationship between stream functioning and environment
# ****************************************************************

# 2.1.1. Stepwise selection of chem variables using ordistep()
# ************************************************************
model.rda.allchem <- rda(funct.stand.det ~ .,
                         data = chem.det)
step <- ordistep(rda(funct.stand.det ~ 1, data = chem.det),
                 scope = formula(model.rda.allchem), steps = 9999)

## (...)
## Step: funct.stand.det ~ SRP
##
##              Df    AIC      F      Pr(>F)
## - SRP         1   38.938  9.8031  0.005 **
##
##              Df    AIC     F       Pr(>F)
## + DOC         1   31.641  2.3077  0.060 .
## + Nitrite     1   31.711  2.2401  0.085 .
## + Ammonium    1   32.259  1.7171  0.090 .
## + DO          1   32.240  1.7351  0.125
## + EC          1   32.249  1.7266  0.160
## + pH          1   33.399  0.6714  0.625
## + NO3         1   33.586  0.5046  0.805

## Model selection suggests that SRP (sqrtSRP), and perhaps
## either DOC or Nitrite (sqrtNO2), but not both, should provide a
## parsimonious model. The solution is slightly unstable in the sense
## that either DOC or Nitrite are also selected in some runs. This is due
## to the limiting p-values corresponding to these variables.

# Fit the model
funct.rda.chem.model <- rda(funct.stand.det ~ SRP + DOC,
                            data = chem.det)
# Before accepting the model above, we need to examine variance inflation.
# This is always necessary, but even more when managing highly co-linear
# explanatory variables. Let us examine variance inflation factors
vif.cca(funct.rda.chem.model)
##         SRP         DOC
##    1.798093    1.798093
## The variance inflation factors values are well below 5, which
## indicate that the model should be valid.

# Global significance of the model: anova.cca(funct.rda.chem.model,
permutations = 9999)
## Permutation test for rda under reduced model
## Permutation: free
## Number of permutations: 9999
## Model: rda(formula = funct.stand.det ~ SRP + DOC,
## data = chem.det)
##             Df  Variance      F     Pr(>F)
## Model        2   2.2552    6.3759   1e-04 ***
## Residual    19   3.3603
```

```
# Examine significance of terms (same as above, in this case, since
# only one explanatory variable has been included in the model)
anova.cca(funct.rda.chem.model, by = "term", permutations = 9999)
## Model: rda(formula = funct.stand.det ~ SRP + DOC,
## data = chem.det)
##            Df    Variance       F     Pr(>F)
## SRP         1     1.8471    10.4441 0.0001 ***
## DOC         1     0.4081     2.3077 0.0696 .
## Residual   19     3.3603

# Amount of variance explained by the model above:
(R2a.funct.rda.chem.model <-
    RsquareAdj(funct.rda.chem.model)$adj.r.squared)
## [1] 0.3386215
## Water SRP + DOC heterogeneity explains 33.8% of multivariate
## variation in stream functioning. Once that SRP has been
## selected, the inclusion of further variables is a matter of opinion,
## although the inclusion in this (partial) "chemical" model of DOC
## would clearly be within reason. It is important to bear in mind that
## SRP, here, does not represent just the contribution of only
## SRP to the explanation of stream functioning. On the contrary,
## since this is an observational study in which no orthogonality exists,
## it also conveys information about other highly co-linear chemical
## descriptors.

# 2.1.2. Stepwise selection of hydr variables
# ******************************************
model.rda.allhydr <- rda(funct.stand.det ~ .,
                         data = hydr.det)
step <- ordistep(rda(funct.stand.det ~ 1, data = hydr.det),
                 scope = formula(model.rda.allhydr), permutations = 9999)
## The last step of the model selection procedure shows:
## Start: funct.stand.det ~ 1
##                 Df    AIC      F      Pr(>F)
## + Width          1  38.480   2.3644  0.0557 .
## + WaterFlow      1  38.403   2.4426  0.0604 .
## + Temperature    1  39.331   1.5157  0.1939
## + MeanVelocity   1  39.789   1.0729  0.3503
## + Depth          1  40.010   0.8618  0.4179
## + HydrStr        1  40.253   0.6328  0.6317
## Width and WaterFlow have nearly significant p-values in the procedure.
## We will fit a model using these two predictors.

# Fit the model
funct.rda.hydr.model <- rda(funct.stand.det ~ WaterFlow + Width,
                            data = hydr.det)

# Examine variance inflation using a model with WaterFlow and Width:
vif.cca(funct.rda.hydr.model)
##  WaterFlow     Width
##   1.269221    1.269221
## The variance inflation factors values are well below 5, which
## indicate that the model should be valid.

# Global significance of the model:
anova.cca(funct.rda.hydr.model, permutations = 9999)
## Model: rda(formula = funct.stand.det ~ WaterFlow + Width,
## data = hydr.det)
##            Df   Variance     F     Pr(>F)
## Model       2    1.2568   2.7394  0.0203 *
## Residual   19    4.3587
```

```r
# Examine significance of terms
anova.cca(funct.rda.hydr.model, by = "term", permutations = 9999)

## Model: rda(formula = funct.stand.det ~ WaterFlow + Width,
## data = hydr.det)
##              Df   Variance      F     Pr(>F)
## WaterFlow     1    0.6112   2.6642  0.0495 *
## Width         1    0.6457   2.8146  0.0358 *
## Residual     19    4.3587

# Amount of variance explained by the model
(R2a.funct.rda.hydr.model <-
    RsquareAdj(funct.rda.hydr.model)$adj.r.squared)
## [1] 0.1421124
## Heterogeneity in these two variables explains c.14% of multivariate
## variation in stream functioning.

# 2.1.3. Stepwise selection of bioc variables
# ******************************************
model.rda.allbioc <- rda(funct.stand.det ~ .,
                         data = bioc.det)
step <- ordistep(rda(funct.stand.det ~ 1, data = bioc.det),
                 scope = formula(model.rda.allbioc), steps = 9999)

## (...)
## Step: funct.stand.det ~ Macrolide + Tiabendazole +
## Ofloxacin + Sulfamethoxazole
##                         Df     AIC    F      Pr(>F)
## - Sulfamethoxazole       1 27.990  3.2546   0.040 *
## - Ofloxacin              1 28.439  3.6720   0.025 *
## - Tiabendazole           1 30.908  6.1274   0.005 **
## - Macrolide              1 33.231  8.7023   0.005 **
## ---
##                        Df    AIC      F      Pr(>F)
## + Trimethoprim          1 25.875  1.7318   0.160
## + Metronidazole         1 26.987  0.8582   0.490
## + Levamisole            1 27.183  0.7088   0.585
## + Ronidazole            0 26.137
## Model selection repetition suggests that Macrolide, and either
## Tiabendazole or Ronidazole (but not both), together with
## Sulfamethoxazole and Ofloxacin, contribute to explain stream
functioning.

# Fit the model
funct.rda.bioc.model <- rda(funct.stand.det ~
                            Macrolide + Tiabendazole + Ofloxacin +
                            Sulfamethoxazole, data = bioc.det)


# Global significance of the model:
anova.cca(funct.rda.bioc.model, permutations = 9999)
## Model: rda(formula = funct.stand.det ~ Macrolide + Tiabendazole +
## Ofloxacin + Sulfamethoxazole, data = bioc.det)

##             Df   Variance      F     Pr(>F)
## Model        4    3.4340   6.6903  1e-04 ***
## Residual    17    2.1815

# Significance of terms
anova.cca(funct.rda.bioc.model, by = "term", permutations = 9999)
```

```
##                    Df  Variance      F      Pr(>F)
## Macrolide           1  1.88840   14.7161  0.0001 ***
## Tiabendazole        1  0.65701    5.1200  0.0085 **
## Ofloxacin           1  0.47098    3.6703  0.0483 *
## Sulfamethoxazole    1  0.41763    3.2546  0.0297 *
## Residual           17  2.18147
## As usual, since this is a test based on permutations, the p-values
## may vary slightly each time that the analysis is run. The conclusions,
## though, should not vary.

# Amount of variance explained by the bioc model
(R2a.funct.rda.bioc.model <-
    RsquareAdj(funct.rda.bioc.model)$adj.r.squared)
## [1] 0.5201212
## Heterogeneity in water biocides explains c.52% of multivariate
## variation in stream functioning.

# 2.1.4. Stepwise selection of drug variables
# ******************************************
model.rda.alldrug <- rda(funct.stand.det ~ .,
                         data = drug.det)
step <- ordistep(rda(funct.stand.det ~ 1, data = drug.det),
                 scope = formula(model.rda.alldrug), steps = 9999)

## (...)
## Step: funct.stand.det ~ Opioid + NSAID + sqrtSalbutamol +
## Iopromide + sqrtSSRI
##                     Df     AIC       F     Pr(>F)
## - sqrtSSRI           1   24.835   5.0469  0.015 *
## - NSAID              1   25.239   5.4375  0.010 **
## - sqrtSalbutamol     1   27.447   7.7002  0.010 **
## - Iopromide          1   25.189   5.3886  0.005 **
## - Opioid             1   27.312   7.5555  0.005 **
## ---
##                      Df    AIC       F     Pr(>F)
## + sqrtCarbamazepine   1  19.909   2.1093  0.085 .
## + sqrtH2Antagonist    1  19.857   2.1498  0.090 .
## + ARBs                1  20.637   1.5519  0.150
## + Benzodiazepine      1  20.465   1.6819  0.155
## + sqrtGlibenclamide   1  20.689   1.5130  0.160
## + sqrtCCB             1  20.866   1.3810  0.240
## + sqrtTamsulosin      1  20.713   1.4948  0.265
## + Fibrate             1  21.174   1.1533  0.350
## + sqrtBetaBlocker     1  21.322   1.0446  0.415
## + Loratadine          1  21.544   0.8840  0.430
## + sqrtTrazodone       1  21.735   0.7463  0.560
## + Venlafaxine         1  21.720   0.7574  0.590
## + LoopDiuretic        1  21.760   0.7282  0.600
## + sqrtClopidogrel     1  21.787   0.7093  0.675
## + sqrtStatin          1  22.095   0.4908  0.765
## + sqrtAcetaminophen   1  22.185   0.4278  0.825

# Fit the model and examine variance inflation factors
funct.rda.drug.model <- rda(funct.stand.det ~ Opioid + Iopromide +
                            sqrtSalbutamol + NSAID + sqrtSSRI,
                            data = drug.det)

vif.cca(funct.rda.drug.model)
##     Opioid    Iopromide   sqrtSalbutamol    NSAID      sqrtSSRI
##   3.513848    1.538899       4.779396      4.581612    5.601394
## The variance inflation factors values are well below 10, tough
```

```
## some are around or over 5.

# Global significance of the model:
anova.cca(funct.rda.drug.model, permutations = 9999)
##             Df  Variance     F       Pr(>F)
## Model        5   4.0524   8.2961   1e-04 ***
## Residual    16   1.5631

# Examine significance of terms
anova.cca(funct.rda.drug.model, by = "term", permutations = 9999)

## Model: rda(formula = funct.stand.det ~ Opioid + Iopromide +
## sqrtSalbutamol + NSAID + sqrtSSRI, data = drug.det)
##                  Df  Variance     F       Pr(>F)
## Opioid            1  2.30490  23.5931  0.0001 ***
## Iopromide         1  0.32281   3.3043  0.0418 *
## sqrtSalbutamol    1  0.45997   4.7082  0.0160 *
## NSAID             1  0.47167   4.8281  0.0180 *
## sqrtSSRI          1  0.49305   5.0469  0.0027 **
## Residual         16  1.56310

# Amount of variance explained by the model above
(R2a.funct.rda.drug.model <-
    RsquareAdj(funct.rda.drug.model)$adj.r.squared)
## [1] 0.6346591
## Heterogeneity in these variables explains 63.5% of multivariate
## variation in stream functioning.

# 2.1.5. A "saturated" model
# **************************
# One way to proceed further would be building a model including all the
# variables selected in each group, as follows:

# Construct an environmental variable including all selected
# environmental descriptors in the above procedures

# Generate an <env> matrix with only the previously selected variables
env <- BaixEbre[-c(3, 21:22, 23),c(18,19,21:22,26,28,30:31,44,47:50)]
names(env)
## [ 1] "SRP"            "DOC"            "WaterFlow"        "Width"
##  [5] "AbMacrolide"    "Ofloxacin"      "Sulfamethoxazole" "Tiabendazole"
##  [9] "Iopromide"      "NSAID"           "Opioid"           "Salbutamol"
## [13] "SSRI"

# Square-root transform the appropriate variables
sqrtSRP <- sqrt(env$SRP)
env$sqrtSRP <- sqrtSRP
sqrtAbMacrolide <- sqrt(env$AbMacrolide)
env$sqrtAbMacrolide <- sqrtAbMacrolide
sqrtSalbutamol <- sqrt(env$Salbutamol)
env$sqrtSalbutamol <- sqrtSalbutamol
sqrtSSRI <- sqrt(env$SSRI)
env$sqrtSSRI <- sqrtSSRI
# Delete the untransformed variables
env <- env[,-c( 1, 5, 12, 13)]

# Change the names of some variables
colnames(env)[10] <- "SRP"
colnames(env)[11] <- "Macrolide"
colnames(env)[12] <- "Salbutamol"
colnames(env)[13] <- "SSRI"
```

```
# Inspect the new <env> matrix
names(env)
##  [1] "DOC"    "WaterFlow"        "Width"            "Ofloxacin"
##  [5] "Sulfamethoxazole"  "Tiabendazole"   "Iopromide"    "NSAID"
##  [9] "Opioid"    "SRP"   "Macrolide"      "Salbutamol"
## [13] "SSRI"
# Detrend <env>
env.det <- as.data.frame(resid(lm(as.matrix(env) ~ ., data = spa)))

# Fit the "saturated" model
funct.rda.saturated.model <- rda(funct.stand.det ~ SRP + DOC + WaterFlow +
                                   Width + Macrolide + Tiabendazole +
                                   Sulfamethoxazole + Ofloxacin + Salbutamol
                                   + Opioid + NSAID + Iopromide + SSRI,
                                   data = env.det)

# Variance inflation factors
vif.cca(funct.rda.saturated.model)
##  SRP              DOC              WaterFlow          Width
##  11.349494        12.061142        3.749383           4.255335
##  Macrolide        Tiabendazole     Sulfamethoxazole   Ofloxacin
##  13.437680        14.148958        8.017241           11.210461
##  Salbutamol       Opioid           NSAID              Iopromide
##  19.553308        27.504902        14.283877          5.860342
##  SSRI
##  29.389190
## As expected, not all variance inflation factors values are below 5!

# Amount of variance explained by the model above:
(R2a.funct.rda.saturated.model <-
  RsquareAdj(funct.rda.saturated.model)$adj.r.squared)
## [1]   0.6787031

# Examine significance of terms
anova.cca(funct.rda.saturated.model, by = "term", permutations = 9999)
##                   Df   Variance      F      Pr(>F)
## SRP                1   1.84711   21.4989  0.0001 ***
## DOC                1   0.40813    4.7504  0.0164 *
## WaterFlow          1   0.49241    5.7312  0.0045 **
## Width              1   0.44064    5.1287  0.0056 **
## Macrolide          1   0.52227    6.0788  0.0048 **
## Tiabendazole       1   0.41069    4.7801  0.0063 **
## Sulfamethoxazole   1   0.06858    0.7982  0.5211
## Ofloxacin          1   0.10161    1.1826  0.3331
## Salbutamol         1   0.12538    1.4594  0.2399
## Opioid             1   0.27403    3.1895  0.0365 *
## NSAID              1   0.10783    1.2550  0.2747
## Iopromide          1   0.08632    1.0047  0.3904
## SSRI               1   0.04317    0.5025  0.7482
## Residual           8   0.687
## As a result, we obtain a model in which NOT ALL explanatory variables
## are significant. This occurs because, so far, we took into account
## co-linearity among the variables within each matrix (i.e. among the
## variables in chem, hydr...), but not co-linearity between variables
## placed in the different matrices.

# 2.1.6. "Saturated" model: partitioning of variation
# ***************************************************
# So far we have fitted a RDA "saturated" model using four sets of
# variables: chem component, hydr component, drug component, and bioc
```

```
# component. We already know that this "saturated" model is not
# parsimonious and now set ourselves to study the unique contributions
# of each component

# Define the components
hydrcomp <- env.det[, c(2:3)]               # X1: hydrcomp
names(hydrcomp)
## [1] "WaterFlow" "Width"
chemcomp <- env.det[,c(1,10)]               # X2: chemcomp
names(chemcomp)
## [1] "DOC" "SRP"
bioccomp <- env.det[, c(4:6,11)]            # X3: bioccomp
names(bioccomp)
## [1] "Ofloxacin" "Sulfamethoxazole" "Tiabendazole" "Macrolide"
drugcomp <- env.det[, c(7:9,12,13)]         # X4: drugcomp
names(drugcomp)
## [1] "Iopromide" "NSAID" "Opioid" "Salbutamol" "SSRI"

# Variation partitioning using function varpart() of vegan
(funct.varpart <- varpart(funct.stand.det, hydrcomp, chemcomp, bioccomp,
                          drugcomp))
## (...)
## The results are best understood if plotted

plot(funct.varpart, cex = 1.0, digits = 1, cutoff = 0.01, bg = 1:4)
## The model explains about 70% (68%) of multivariate variation in stream
## functioning. Also, we see that the unique contribution of X2 (chem)
## and X3 (bioccomp) in this model is null. This is but another indication
## that we must reduce the number of <explanatory> variables.

# A better graph of the results above:
showvarparts(4, cex = 0.95, bg = 1:4, Xnames = NA,
             labels = c("Hydrological\nproperties\n\n3%",
                        "Water chemical\ncharacteristics",
                        "Biocides",
                        "Non-biocides\ndrugs\n\n6%",
                        "", "2%", "1%", "3%",
                        "", "17%", "5%", "",
                        "31%", "4%", "", "32%"))
text("Response is stream functioning", x = 0.05, y = 1.20, cex = 1.0)
# ********************************************************************** #

# 2.2. Fitting a parsimonious model
# *********************************

# 2.2.1. Backwards procedure
# **************************
# A simple way to build a parsimonious model in which all terms are
# significant is to apply a BACKWARDS procedure to the saturated model
# (the one obtained in step 2.2.6).
funct.rda.saturated.model <- rda(funct.stand.det ~ ., data = env.det)
model.rda.parsimonious.back <- ordistep(funct.rda.saturated.model,
                                        Pin = 0.01, Pout = 0.05, steps = 999)
## In conclusion, even with the stringent conditions set, the solution
## provided is relatively unstable. Nonetheless, Macrolide, Salbutamol,
## NSAID and Iopromide are always in the final solution.

# 2.2.2. Stepwise procedure
# *************************
# Another simple way to build a parsimonious model in which all terms are
# significant is to apply a STEPWISE procedure, considering as candidate
```

```
# explanatory variables only the 13 variables previously selected. This
# procedure is similar to the procedures already applied to obtain the
# partial models.
# Stepwise procedure
funct.rda.saturated.model <- rda(funct.stand.det ~ ., data = env.det)
model.rda.parsimonious.stepwise <-
  ordistep(rda(funct.stand.det ~ 1, data = env.det),
           scope = formula(funct.rda.saturated.model), , steps = 999)

## As shown by the next last step, a model with Iopromide, Macrolide,
## Tiabendazole and NSAID is always selected. Occasionally, also
## Sulfamethoxazole may creep into the model, albeit supported by only
## a marginal p-value:

## (...) [only last step here reported]
##                       Df   AIC     F      Pr(>F)
## - Sulfamethoxazole    1  19.451  2.1928  0.075 .
## - Iopromide           1  20.673  3.2312  0.030 *
## - Macrolide           1  25.026  7.4392  0.005 **
## - Tiabendazole        1  26.898  9.5211  0.005 **
## - NSAID               1  28.009 10.8423  0.005 **
## ---
##                Df    AIC     F      Pr(>F)
## + Salbutamol   1   17.974  1.9220  0.090 .
## + Ofloxacin    1   18.891  1.2309  0.225
## + Width        1   19.007  1.1453  0.325
## + DOC          1   18.991  1.1568  0.355
## + SSRI         1   19.172  1.0247  0.480
## + Phosphate    1   19.598  0.7176  0.535
## + WaterFlow    1   19.437  0.8328  0.555
## + Opioid       1   19.595  0.7193  0.655
## ---

# 2.2.3. A parsimonious model
# ***************************

# Fit the parsimonious model
funct.rda.parsimonious.model <- rda(funct.stand.det ~ NSAID + Tiabendazole
                                    + Macrolide + Iopromide,
                                    data = env.det)

# Variance inflation factors
vif.cca(funct.rda.parsimonious.model)
##       NSAID      Tiabendazole    Macrolide     Iopromide
##    1.403777         1.775565       1.379471    1.558568

# Global significance of the model:
anova.cca(funct.rda.parsimonious.model, permutations = 9999)
##            Df  Variance      F    Pr(>F)
## Model       4    4.0057   10.575  1e-04 ***
## Residual   17    1.6098

# Amount of variance explained by parsimonious model:
(R2a.funct.rda.parsimonious.model <-
    RsquareAdj(funct.rda.parsimonious.model)$adj.r.squared)
## [1] 0.6458682

# Examine significance of terms
anova.cca(funct.rda.parsimonious.model,
          by = "term", permutations = 9999)
```

```
##                Df  Variance      F      Pr(>F)
## NSAID           1   1.70099   17.9626  0.0001 ***
## Tiabendazole    1   0.89583    9.4600  0.0001 ***
## Macrolide       1   0.91733    9.6871  0.0001 ***
## Iopromide       1   0.49151    5.1904  0.0144 *
## Residual       17   1.60984

# Contribution of axes
funct.rda.parsimonious.model
##                  Inertia    Proportion  Rank
## Total             5.6155      1.0000
## Constrained       4.0057      0.7133     4
## Unconstrained     1.6098      0.2867     7
## Inertia is variance

## Eigenvalues for constrained axes:
##    RDA1    RDA2    RDA3    RDA4
##  2.3959  0.8402  0.4742  0.2953

## Eigenvalues for unconstrained axes:
##     PC1     PC2     PC3     PC4     PC5     PC6     PC7
##  0.5942  0.4202  0.2720  0.1980  0.0823 0.0305 0.0127

# First axis
(2.3959/4.0057)*100; (2.3959/5.6155)*100
## [1] 59.81227
## [1] 42.66584
## The first axis accounts for 59.8% of explained variation
## (42.7% of total var.)

# Second axis
(0.8402/4.0057)*100; (0.8402/5.6155)*100
## [1] 20.97511
## [1] 14.96216
## The second axis accounts for 20.9% of explained variation
## (14.9% of total var.)
# Third axis
(0.4742/4.0057)*100; (0.4742/5.6155)*100
## [1] 11.83813
## [1] 8.444484
## The third axis accounts for 11.8% of explained variation
## (8.4% of total var.)

# Plot the model (axis 1 and 2)
windows(title = "RDA - scaling 2 - stream funcionality-environment RDA")
plot(funct.rda.parsimonious.model, type = "none",
      choices = c(1, 2), scaling = "species",
      main = "(a) RDA Parsimonious model: axes 1 and 2", cex.main = 1.4,
      xlab = "RDA1: 59.8% of constrained variance (42.7% of total variance)",
      xlim = c(-2.0, 3.0),
      ylab = "RDA2: 20.9% of constrained variance (15.0% of total variance)",
      ylim = c(-2.0, 2.0))
text(funct.rda.parsimonious.model, dis = "cn", scaling = "species", cex = 1.2,
     col = "red", choices = c(1, 2))
points(funct.rda.parsimonious.model, pch = 21, col = "black", bg = black",
     cex = 0.8, scaling = "species", choices = c(1, 2))
text(funct.rda.parsimonious.model, "species", col = "forestgreen", cex = 1.2,
      scaling = "sites", choices = c(1, 2))

# Plot the model (axis 1 and 3)
windows(title = "RDA - scaling 2 - stream funcionality-environment RDA")
```

```
plot(funct.rda.parsimonious.model, type = "none",
      choices = c(1, 3), scaling = "species",
      main = "(b) RDA Parsimonious model: axes 1 and 3", cex.main = 1.4,
      xlab = "RDA1: 59.8% of constrained variance (42.7% of total variance)",
      xlim = c(-2.0, 3.0),
      ylab = "RDA3: 11.8% of constrained variance (8.4% of total variance)",
      ylim = c(-1.5, 2.0))
text(funct.rda.parsimonious.model, dis = "cn", scaling = "species", cex = 1.2,
     col = "red", choices = c(1, 3))
points(funct.rda.parsimonious.model, pch = 21, col = "black", bg = black",
     cex = 0.8, scaling = "species", choices = c(1, 3))
text(funct.rda.parsimonious.model, "species", col = "forestgreen", cex = 1.2,
     scaling = "sites", choices = c(1, 3),
     select = c("ERp", "SRPUC", "NH4AU", "OMD", "BAT"))
text(funct.rda.parsimonious.model, "species", col = "forestgreen", cex = 1.2,
     scaling = "sites", choices = c(1, 3), select = c("GPP"), adj = c(0.5,-0.5))
text(funct.rda.parsimonious.model, "species", col = "forestgreen", cex = 1.2,
     scaling = "sites", choices = c(1, 3), select = c("SRPAU"), adj = c(0.5,0.5))

## The antifungical Tiabendazole is positively associated with SRPAU
## (strongly) and NH4AU (weakly); Tiabendazole is also negatively
## associated with OMD (weakly).

## The biocide Macrolide is associated with BAT (positively)
## and SRPUC (negatively). It is also positively and weakly associated
## with NH4AU, GPP and ERp.

## NSAID (nonsteroideal anti-inflammatory drugs) are negatively associated
## with SRPUC (strongly) and OMD (weakly). These drugs are also associated
## positively with ERp (strongly) and NH4AU (weakly).

## Iopromide, a contrast medium used in computed tomography scan, is
## positively associated with both SRPAU and NH4AU (weakly).
## Iopromide, NSAID, Tiabendazole, and Macrolide are given in nano grams
## per L.

## SRPAU = SRP areal uptake (µg per m^2 per minute).
## NH4U = NH4 areal uptake (µg per m^2 per minute)
## OMD = Organic Matter Descomposition.
## BAT = Biomass accrual rate (g per m^2 per day)
## SRPUC = SRP uptake capacity (µg P per hour).
## GPP = Gross Primary Production (mg O_2 per m^2 per day)
## ERp = Ecological Respiration "positive" (mg O^2 per m^2 and day)

## Summary of monotonic associations:
##         Macrolide    NSAID      Tiabendazole   Iopromide
##        ------------------------------------------------
## NH4AU  Weak (+)     Weak (+)   Weak (+)       Weak (+)
## SRPUC  Strong (-)   Strong (-)
## ERp    Weak (+)     Strong (+)
## BAT    Strong (+)
## GPP    Weak (+)
## OMD                 Weak (-)
## SRPAU                          Strong (+)     Weak (+)

# Joint graph (a and b together)
# ******************************
windows(title = "Joint graph", 16, 8.9)
par(mfrow = c(1,2))
```

```
plot(funct.rda.parsimonious.model, type = "none",
     choices = c(1, 2), scaling = "species",
      main = "(a) RDA Parsimonious model: axes 1 and 2", cex.main = 1.4,
      xlab = "RDA1: 59.8% of constrained variance (42.7% of total variance)",
      xlim = c(-2.0, 3.0),
      ylab = "RDA2: 20.9% of constrained variance (15.0% of total variance)",
      ylim = c(-2.0, 2.0))
text(funct.rda.parsimonious.model, dis = "cn", scaling = "species", cex = 1.2,
     col = "red", choices = c(1, 2))
points(funct.rda.parsimonious.model, pch = 21, col = "black", bg = "black",
     cex = 0.8, scaling = "species", choices = c(1, 2))
text(funct.rda.parsimonious.model, "species", col = "forestgreen", cex = 1.2,
     scaling = "sites", choices = c(1, 2))

plot(funct.rda.parsimonious.model, type = "none", choices = c(1, 3),
      scaling = "species",
      main = "(b) RDA Parsimonious model: axes 1 and 3", cex.main = 1.4,
      xlab = "RDA1: 59.8% of constrained variance (42.7% of total variance)",
      xlim = c(-2.0, 3.0),
      ylab = "RDA3: 11.8% of constrained variance (8.4% of total variance)",
      ylim = c(-1.5, 2.0))
text(funct.rda.parsimonious.model, dis = "cn", scaling = "species", cex = 1.2,
     col = "red", choices = c(1, 3))
points(funct.rda.parsimonious.model, pch = 21, col = "black", bg = "black",
     cex = 0.8, scaling = "species", choices = c(1, 3))
text(funct.rda.parsimonious.model, "species", col = "forestgreen", cex = 1.2,
     scaling = "sites", choices = c(1, 3),
     select = c("ERp", "SRPUC", "NH4AU", "OMD", "BAT"))
text(funct.rda.parsimonious.model, "species", col = "forestgreen", cex = 1.2,
     scaling = "sites", choices = c(1, 3), select = c("GPP"), adj = c(0.5,-0.5))
text(funct.rda.parsimonious.model, "species", col = "forestgreen", cex = 1.2,
     scaling = "sites", choices = c(1, 3), select = c("SRPAU"), adj = c(0.5,0.5))

# Define components for variation partitioning
bioccomp <- env.det[, c(6,11)]        # X1: Tiabendazole and Macrolide
drugcomp <- env.det[, c(7:8)]         # X2: NSAID and Iopromide

# Variation partitioning using function varpart() of vegan
(funct.varpart <- varpart(funct.stand.det, bioccomp, drugcomp))
## Partition of variance in RDA
##
## Call: varpart(Y = funct.stand.det, X = bioccomp, drugcomp) ##
## Explanatory tables:
## X1: bioccomp ## X2: drugcomp ##
## No. of explanatory tables: 2
## Total variation (SS): 117.93
##              Variance:   5.6155
##  No. of observations:  22
##
## Partition table:
##                       Df  R.squared   Adj.R.squared  Testable
## [a+b] = X1             2   0.45328       0.39573        TRUE
## [b+c] = X2             2   0.37133       0.30515        TRUE
## [a+b+c] = X1+X2        4   0.71332       0.64587        TRUE
## Individual fractions
## [a] = X1|X2            2                 0.34072        TRUE
## [b]                    0                 0.05502       FALSE
## [c] = X2|X1            2                 0.25013        TRUE
## [d] = Residuals                          0.35413       FALSE
```

```r
# Plot the results
plot(funct.varpart, cex = 1.0, bg = 2:3)

# A better graph of the results above:
showvarparts(2, cex = 0.87, bg = c("green", "blue"), Xnames = NA,
             labels = c("Biocide component\n(Tiabendazole \nand Macrolide)
                        \n= 34%","Shared\nfraction\n= 6%", "Drug
                        component\n(NSAID \nand Iopromide)\n= 25%",
                        "35%"))
text("Response is stream functioning", x = 0.5, y = 0.95, cex = 1.0)
## The values indicated in the Venn diagram are proportions of variation
## in stream functioning explained by model components (adj.-R2).

# Test the unique contribution of the fraction corresponding to the pure
# bioccomp
anova(rda(funct.stand.det, bioccomp, drugcomp))
## Model: rda(X = funct.stand.det, Y = bioccomp, Z = drugcomp)
##           Df  Variance     F      Pr(>F)
## Model      2   1.9205   10.14  0.001 ***
## Residual  17   1.6098
# Test the unique contribution of the fraction corresponding to the pure
# drugcomp
anova(rda(funct.stand.det, drugcomp, bioccomp))
## Model: rda(X = funct.stand.det, Y = drugcomp, Z = bioccomp)
##           Df  Variance     F       Pr(>F)
## Model      2   1.4603   7.7101  0.001 ***
## Residual  17   1.6098
# ************************************************************************ #
# ************************************************************************ #

##########
# Step 3 #
##########

# Finally, we will test the relationship between the individual functional
# variables and the selected environmental predictors via Generalised
# Linear Models (GLM). The goal is to visualize the form of the relationships,
# and quantify the variability explained, rather than testing for the
# relationships themselves. This is so because we already know that the
# following relationships are statistically significant:

## Summary of monotonic associations:
##         Macrolide     NSAID       Tiabendazole     Iopromide
##         ------------------------------------------------------
## NH4AU   Weak (+)      Weak (+)    Weak (+)         Weak (+)
## SRPUC   Strong (-)    Strong (-)
## ERp     Weak (+)      Strong (+) ## BAT    Strong (+)
## GPP     Weak (+)
## OMD                   Weak (-)
## SRPAU                             Strong (+)       Weak (+)

# 3.1. Generalised Linear Modelling (GLM)
# ***************************************
# The variables in <funct.stand.det> have been standardized and detrended
names(as.data.frame(funct.stand.det))
## [1] "BAT" "SRPUC" "SRPAU" "NH4AU" "GPP" "OMD" "ERp"
## SRPAU = SRP areal uptake (µg per m^2 per minute).
## NH4U = NH4 areal uptake (µg per m^2 per minute)
## OMD = Organic Matter Descomposition.
## BAT = Biomass accrual rate (g per m^2 per day)
```

```
## SRPUC = SRP uptake capacity (µg P per hour).
## GPP = Gross Primary Production (mg O_2 per m^2 per day)
## ERp = Ecological Respiration "positive" (mg O^2 per m^2 and day)
## These variables have been standardized and detrended, hence negative
## values are possible.

# The variables in <env.det> have been detrended
names(env.det)
##  [1] "DOC"      "WaterFlow"      "Width"          "Ofloxacin"
##  [5] "Sulfamethoxazole"   "Tiabendazole"  "Iopromide"     "NSAID"
##  [9] "Opioid"     "SRP"      "Macrolide"      "Salbutamol"
## [13] "SSRI"
## Iopromide, NSAID, Tiabendazole, and Macrolide were measured in nanog
## per L. These variables have been detrended; for this reason, negative
## values are possible.

# Merge the funct with env to generate the data frame <glm,data>
glm.data <- merge(funct.stand.det, env.det, by = "row.names")
head(glm.data)
## This dataframe <glm.data> is the dataframe that we will use to test
## for relationships between individual functional variables and the
## selected environmental predictors (Macrolide, Tiabendazole, NSAID,
## and Iopromide). We will limit ourselves to relationships identified
## in step 2.

# 3.3.1. NH4AU
# ************
# Macrolide
# *********
model.NH4AU.Macrolide <- glm(NH4AU ~ Macrolide,
                             family = gaussian, data = glm.data)
summary(model.NH4AU.Macrolide)
##      Null deviance:   11.3316   on 21   degrees of freedom ## Residual
deviance:                 7.4379   on 20   degrees of freedom

# R^2_GLM (%): 1 - (Residual Deviance/Null Deviance)
round((1 - (7.4379/11.3316))*100, 1)
## [1] 34.4

# Analysis of deviance table
anova(model.NH4AU.Macrolide, test = "Chisq")
##            Df  Deviance Resid.  Df   Resid. Dev   Pr(>Chi)
## NULL                            21     11.3316
## Macrolide   1     3.8937        20      7.4379   0.001213 **

# Peruse whether (spatial) autocorrelation is present in model residuals
acf(residuals(model.NH4AU.Macrolide), main = "Raw residual ACF")
## No significant autocorrelation is present in model residuals.

# Obtain the values predicted by the fitted model
model.NH4AU.Macrolide.pred <- predict(model.NH4AU.Macrolide,
                                      se = TRUE, type = "response")

# Draw the base plot with the observed data points
plot(glm.data$Macrolide, glm.data$NH4AU, type = "p",
     xlab = "Macrolide concentration (nanograms per litre)",
     ylab = "Ammonium areal uptake")

# Draw model fit with approximate 95% confidence bands
I <- order(glm.data$Macrolide)
```

```
lines(glm.data$Macrolide[I], model.NH4AU.Macrolide.pred$fit[I], lwd = 1)
lines(glm.data$Macrolide[I],model.NH4AU.Macrolide.pred$fit[I] +
      1.96 * model.NH4AU.Macrolide.pred$se.fit[I], lty = 2, lwd = 1)
lines(glm.data$Macrolide[I], model.NH4AU.Macrolide.pred$fit[I] -
      1.96 * model.NH4AU.Macrolide.pred$se.fit[I], lty = 2, lwd = 1)

# NSAID
# *****
model.NH4AU.NSAID <- glm(NH4AU ~ NSAID,
                         family = gaussian, data = glm.data)
summary(model.NH4AU.NSAID)
##     Null deviance:   11.3316   on 21   degrees of freedom
## Residual deviance:    8.2806   on 20   degrees of freedom

# R^2_GLM (%): 1 - (Residual Deviance/Null Deviance)
round((1 - (8.2806/11.3316))*100, 1)
## [1] 26.9

# Analysis of deviance table
anova(model.NH4AU.NSAID, test = "Chisq")
##         Df  Deviance Resid. Df   Resid. Dev   Pr(>Chi)
## NULL                        21      11.3316
## NSAID   1       3.051        20       8.2806   0.006636 **

# Peruse whether (spatial) autocorrelation is present in model residuals
acf(residuals(model.NH4AU.NSAID), main = "Raw residual ACF")
## No significant autocorrelation is present in model residuals.

# Obtain the values predicted by the fitted model
model.NH4AU.NSAID.pred <- predict(model.NH4AU.NSAID,
                                  se = TRUE, type = "response")

# Draw the base plot with the observed data points
plot(glm.data$NSAID, glm.data$NH4AU,type = "p",
     xlab = "NSAIDs concentration (nanograms per litre)",
     ylab = "Ammonium areal uptake")

# Draw model fit with approximate 95% confidence bands
I <- order(glm.data$NSAID)
lines(glm.data$NSAID[I], model.NH4AU.NSAID.pred$fit[I], lwd = 1)
lines(glm.data$NSAID[I],model.NH4AU.NSAID.pred$fit[I] +
      1.96 * model.NH4AU.NSAID.pred$se.fit[I], lty = 2, lwd = 1)
lines(glm.data$NSAID[I],model.NH4AU.NSAID.pred$fit[I] -
      1.96 * model.NH4AU.NSAID.pred$se.fit[I], lty = 2, lwd = 1)

# Tiabendazole
# ************
model.NH4AU.Tiabendazole <- glm(NH4AU ~ Tiabendazole,
                                family = gaussian, data = glm.data)
summary(model.NH4AU.Tiabendazole)
##     Null deviance:   11.3316   on 21   degrees of freedom
## Residual deviance:    6.9577   on 20   degrees of freedom

# R^2_GLM (%): 1 - (Residual Deviance/Null Deviance)
round((1 - (6.9577/11.3316))*100, 1)
## [1] 38.6

# Analysis of deviance table
anova(model.NH4AU.Tiabendazole, test = "Chisq")
```

```
##                Df  Deviance Resid. Df     Resid. Dev    Pr(>Chi)
## NULL                                21      11.3316
## Tiabendazole    1      4.3739        20       6.9577    0.0003914 ***

# Peruse whether (spatial) autocorrelation is present in model residuals
acf(residuals(model.NH4AU.Tiabendazole), main = "Raw residual ACF")
## No significant autocorrelation is present in model residuals.

# Obtain the values predicted by the fitted model
model.NH4AU.Tiabendazole.pred <- predict(model.NH4AU.Tiabendazole,
                                         se = TRUE, type = "response")
# Draw the base plot with the observed data points
plot(glm.data$Tiabendazole, glm.data$NH4AU,type = "p",
     xlab = "Tiabendazole concentration (nanograms per litre)",
     ylab = "Ammonium areal uptake")

# Draw model fit with approximate 95% confidence bands
I <- order(glm.data$Tiabendazole)
lines(glm.data$Tiabendazole[I], model.NH4AU.Tiabendazole.pred$fit[I], lwd = 1)
lines(glm.data$Tiabendazole[I], model.NH4AU.Tiabendazole.pred$fit[I] +
      1.96 * model.NH4AU.Tiabendazole.pred$se.fit[I], lty = 2, lwd = 1)
lines(glm.data$Tiabendazole[I],model.NH4AU.Tiabendazole.pred$fit[I] -
      1.96 * model.NH4AU.Tiabendazole.pred$se.fit[I], lty = 2, lwd = 1)

# Iopromide
# *********
model.NH4AU.Iopromide <- glm(NH4AU ~ Iopromide,
                             family = gaussian, data = glm.data)
summary(model.NH4AU.Iopromide)
##     Null deviance:   11.3316   on 21   degrees of freedom
## Residual deviance:    8.8536   on 20   degrees of freedom

# R^2_GLM (%): 1 - (Residual Deviance/Null Deviance)
round((1 - (8.8536/11.3316))*100, 1)
## [1] 21.9

anova(model.NH4AU.Iopromide, test = "Chisq")
##           Df Deviance Resid. Df   Resid. Dev  Pr(>Chi)
## NULL                         21     11.3316
## Iopromide  1    2.478        20      8.8536   0.01798 *

# Peruse whether (spatial) autocorrelation is present in model residuals
acf(residuals(model.NH4AU.Iopromide), main = "Raw residual ACF")
## No significant autocorrelation is present in model residuals.

# Obtain the values predicted by the fitted model
model.NH4AU.Iopromide.pred <- predict(model.NH4AU.Iopromide,
                                      se = TRUE, type = "response")

# Draw the base plot with the observed data points
plot(glm.data$Iopromide, glm.data$NH4AU, type = "p",
     xlab = "Iopromide concentration (nanograms per litre)",
     ylab = "Ammonium areal uptake")

# Draw model fit with approximate 95% confidence bands
I <- order(glm.data$Iopromide)
lines(glm.data$Iopromide[I], model.NH4AU.Iopromide.pred$fit[I], lwd = 1)
lines(glm.data$Iopromide[I], model.NH4AU.Iopromide.pred$fit[I] +
      1.96 * model.NH4AU.Iopromide.pred$se.fit[I], lty = 2, lwd = 1)
lines(glm.data$Iopromide[I],model.NH4AU.Iopromide.pred$fit[I] -
      1.96 * model.NH4AU.Iopromide.pred$se.fit[I], lty = 2, lwd = 1)
```

```
# ************************************************************************ #

# 3.3.2. SRPUC
# ************
# Macrolide
# *********
model.SRPUC.Macrolide <- glm(SRPUC ~ Macrolide + I(Macrolide^2),
                             family = gaussian, data = glm.data)
summary(model.SRPUC.Macrolide)
##     Null deviance:   19.2075   on 21   degrees of freedom
## Residual deviance:    6.2721   on 19   degrees of freedom

# R^2_GLM (%): 1 - (Residual Deviance/Null Deviance)
round((1 - (6.2721/19.2075))*100, 1)
## [1] 67.3

anova(model.SRPUC.Macrolide, test = "Chisq")
##                  Df Deviance Resid.   Df    Resid. Dev    Pr(>Chi)
## NULL                                  21      19.2075
## Macrolide         1    10.3112        20      8.8963   2.285e-08 ***
## I(Macrolide^2)    1     2.6242        19      6.2721     0.00481 **

# Peruse whether (spatial) autocorrelation is present in model residuals
acf(residuals(model.SRPUC.Macrolide), main = "Raw residual ACF")
## No significant autocorrelation is present in model residuals.

# Obtain the values predicted by the fitted model
model.SRPUC.Macrolide.pred <- predict(model.SRPUC.Macrolide,
                                      se = TRUE, type = "response")

# Draw the base plot with the observed data points
plot(glm.data$Macrolide, glm.data$SRPUC, type = "p",
     xlab = "Detrended macrolide (nanograms per litre)",
     ylab = "Standardized and detrended SRP uptake capacity")

# Draw model fit with approximate 95% confidence bands
I <- order(glm.data$Macrolide)
lines(glm.data$Macrolide[I], model.SRPUC.Macrolide.pred$fit[I], lwd = 1)
lines(glm.data$Macrolide[I],model.SRPUC.Macrolide.pred$fit[I] +
      1.96 * model.SRPUC.Macrolide.pred$se.fit[I], lty = 2, lwd = 1)
lines(glm.data$Macrolide[I],model.SRPUC.Macrolide.pred$fit[I] -
      1.96 * model.SRPUC.Macrolide.pred$se.fit[I], lty = 2, lwd = 1)

# NSAID
# *****
model.SRPUC.NSAID <- glm(SRPUC ~ NSAID,
                         family = gaussian, data = glm.data)
summary(model.SRPUC.NSAID)
##     Null deviance:   19.2075   on 21   degrees of freedom
## Residual deviance:    9.8192   on 20   degrees of freedom

# R^2_GLM (%): 1 - (Residual Deviance/Null Deviance)
round((1 - (9.8192/19.2075))*100, 1)
## [1] 48.9

anova(model.SRPUC.NSAID, test = "Chisq")
##        Df  Deviance Resid. Df  Resid. Dev    Pr(>Chi)
## NULL                      21     19.2075
## NSAID   1      9.3883       20      9.8192   1.226e-05 ***
```

```
# Peruse whether (spatial) autocorrelation is present in model residuals
acf(residuals(model.SRPUC.NSAID), main = "Raw residual ACF")
## No significant autocorrelation is present in model residuals.

# Obtain the values predicted by the fitted model
model.SRPUC.NSAID.pred <- predict(model.SRPUC.NSAID,
                                  se = TRUE, type = "response")

# Draw the base plot with the observed data points
plot(glm.data$NSAID, glm.data$SRPUC, type = "p",
     xlab = "Detrended NSAIDs (nanograms per litre)",
     ylab = "Standardized and detrended SRP uptake capacity")
# Draw model fit with approximate 95% confidence bands
I <- order(glm.data$NSAID)
lines(glm.data$NSAID[I], model.SRPUC.NSAID.pred$fit[I], lwd = 1)
lines(glm.data$NSAID[I],model.SRPUC.NSAID.pred$fit[I] +
      1.96 * model.SRPUC.NSAID.pred$se.fit[I], lty = 2, lwd = 1)
lines(glm.data$NSAID[I],model.SRPUC.NSAID.pred$fit[I] -
      1.96 * model.SRPUC.NSAID.pred$se.fit[I], lty = 2, lwd = 1)
# ************************************************************************ #

# 3.3.3. ERp
# **********
# Macrolide
# *********
model.ERp.Macrolide <- glm(ERp ~ Macrolide + I(Macrolide^2),
                           family = gaussian, data = glm.data)
summary(model.ERp.Macrolide)
##      Null deviance:   19.4029   on 21   degrees of freedom ## Residual
deviance:                 9.5399   on 19   degrees of freedom

# R^2_GLM (%): 1 - (Residual Deviance/Null Deviance)
round((1 - (9.5399/19.4029 ))*100, 1)
## [1] 50.8

anova(model.ERp.Macrolide, test = "Chisq")
##                  Df  Deviance Resid.   Df    Resid. Dev  Pr(>Chi)
## NULL                                    21    19.4029
## Macrolide         1     5.5065         20    13.8964  0.0009276 ***
## I(Macrolide^2)    1     4.3565         19     9.5399  0.0032233 **

# Peruse whether (spatial) autocorrelation is present in model residuals
acf(residuals(model.ERp.Macrolide), main = "Raw residual ACF")
## No significant autocorrelation is present in model residuals.

# Obtain the values predicted by the fitted model
model.ERp.Macrolide.pred <- predict(model.ERp.Macrolide,
                                    se = TRUE, type = "response")

# Draw the base plot with the observed data points
plot(glm.data$Macrolide, glm.data$ERp, type = "p",
     xlab = "Detrended macrolide (nanograms per litre)",
     ylab = "Standardized and detrended ecological respiration")

# Draw model fit with approximate 95% confidence bands
I <- order(glm.data$Macrolide)
lines(glm.data$Macrolide[I], model.ERp.Macrolide.pred$fit[I], lwd = 1)
lines(glm.data$Macrolide[I], model.ERp.Macrolide.pred$fit[I] +
      1.96 * model.ERp.Macrolide.pred$se.fit[I], lty = 2, lwd = 1)
lines(glm.data$Macrolide[I], model.ERp.Macrolide.pred$fit[I] -
      1.96 * model.ERp.Macrolide.pred$se.fit[I], lty = 2, lwd = 1)
```

```
# NSAID
# *****
model.ERp.NSAID <- glm(ERp ~ NSAID,
                       family = gaussian, data = glm.data)
summary(model.ERp.NSAID)
##     Null deviance:   19.4029   on 21   degrees of freedom
## Residual deviance:    3.4986   on 20   degrees of freedom

# R^2_GLM (%): 1 - (Residual Deviance/Null Deviance)
round((1 - (3.4986/19.4029 ))*100, 1)
## [1] 82.0

anova(model.ERp.NSAID, test = "Chisq")
##        Df Deviance Resid.   Df    Resid. Dev   Pr(>Chi)
## NULL                        21      19.4029
## NSAID   1     15.904        20       3.4986   < 2.2e-16 ***

# Peruse whether (spatial) autocorrelation is present in model residuals
acf(residuals(model.ERp.NSAID), main = "Raw residual ACF")
## No significant autocorrelation is present in model residuals.

# Obtain the values predicted by the fitted model
model.ERp.NSAID.pred <- predict(model.ERp.NSAID,
                                se = TRUE, type = "response")

# Draw the base plot with the observed data points
plot(glm.data$NSAID, glm.data$ERp, type = "p",
     xlab = "Detrended NSAIDs (nanograms per litre)",
     ylab = "Standardized and detrended ecological respiration")

# Draw model fit with approximate 95% confidence bands
I <- order(glm.data$NSAID)
lines(glm.data$NSAID[I], model.ERp.NSAID.pred$fit[I], lwd = 1)
lines(glm.data$NSAID[I], model.ERp.NSAID.pred$fit[I] +
      1.96 * model.ERp.NSAID.pred$se.fit[I], lty = 2, lwd = 1)
lines(glm.data$NSAID[I], model.ERp.NSAID.pred$fit[I] -
      1.96 * model.ERp.NSAID.pred$se.fit[I], lty = 2, lwd = 1)
# ********************************************************************** #

# 3.3.4. BAT
# **********
# Macrolide
# *********
model.BAT.Macrolide <- glm(BAT ~ Macrolide,
                           family = gaussian, data = glm.data)
summary(model.BAT.Macrolide)
##     Null deviance: 20.8784     on 21   degrees of freedom
## Residual deviance:    6.8544   on 20   degrees of freedom

# R^2_GLM (%): 1 - (Residual Deviance/Null Deviance)
round((1 - (6.8544/20.8784))*100, 1)
## [1] 67.2

anova(model.BAT.Macrolide, test = "Chisq")
##             Df Deviance Resid.  Df    Resid. Dev    Pr(>Chi)
## NULL                            21      20.8784
## Macrolide   1      14.024       20       6.8544   1.586e-10 ***

# Peruse whether (spatial) autocorrelation is present in model residuals
acf(residuals(model.BAT.Macrolide), main = "Raw residual ACF")
## No significant autocorrelation is present in model residuals.
```

```
# Obtain the values predicted by the fitted model
model.BAT.Macrolide.pred <- predict(model.BAT.Macrolide,
                                    se = TRUE, type = "response")

# Draw the base plot with the observed data points
plot(glm.data$Macrolide, glm.data$BAT, type = "p",
     xlab = "Detrended macrolide (nanograms per litre)",
     ylab = "Standardized and detrended biomass accrual rate")

# Draw model fit with approximate 95% confidence bands
I <- order(glm.data$Macrolide)
lines(glm.data$Macrolide[I], model.BAT.Macrolide.pred$fit[I], lwd = 1)
lines(glm.data$Macrolide[I], model.BAT.Macrolide.pred$fit[I] +
      1.96 * model.BAT.Macrolide.pred$se.fit[I], lty = 2, lwd = 1)
lines(glm.data$Macrolide[I], model.BAT.Macrolide.pred$fit[I] -
      1.96 * model.BAT.Macrolide.pred$se.fit[I], lty = 2, lwd = 1)
# ********************************************************************* #

# 3.3.5. GPP
# **********
# Macrolide
# *********
model.GPP.Macrolide <- glm(GPP ~ Macrolide,
                           family = gaussian, data = glm.data)
summary(model.GPP.Macrolide)
##     Null deviance: 18.221    on 21   degrees of freedom
## Residual deviance:  15.042    on 20   degrees of freedom

# R^2_GLM (%): 1 - (Residual Deviance/Null Deviance)
round((1 - (15.042/18.221))*100, 1)
## [1] 17.4

anova(model.GPP.Macrolide, test = "Chisq")
##             Df Deviance Resid.  Df    Resid. Dev  Pr(>Chi)
## NULL                             21      18.221
## Macrolide    1     3.1788       20      15.043    0.0398 *

# Peruse whether (spatial) autocorrelation is present in model residuals
acf(residuals(model.GPP.Macrolide), main = "Raw residual ACF")
## No significant autocorrelation is present in model residuals.

# Obtain the values predicted by the fitted model
model.GPP.Macrolide.pred <- predict(model.GPP.Macrolide,
                                    se = TRUE, type = "response")

# Draw the base plot with the observed data points
plot(glm.data$Macrolide, glm.data$GPP, type = "p",
     xlab = "Detrended macrolide (nanograms per litre)",
     ylab = "Standardized and detrended gross primary production")

# Draw model fit with approximate 95% confidence bands
I <- order(glm.data$Macrolide)
lines(glm.data$Macrolide[I], model.GPP.Macrolide.pred$fit[I], lwd = 1)
lines(glm.data$Macrolide[I], model.GPP.Macrolide.pred$fit[I] +
      1.96 * model.GPP.Macrolide.pred$se.fit[I], lty = 2, lwd = 1)
lines(glm.data$Macrolide[I], model.GPP.Macrolide.pred$fit[I] -
      1.96 * model.GPP.Macrolide.pred$se.fit[I], lty = 2, lwd = 1)
# ********************************************************************* #
```

```
# 3.3.6. OMD
# **********
# NSAID
# *****
model.OMD.NSAID <- glm(OMD ~ NSAID,
                       family = gaussian, data = glm.data)
summary(model.OMD.NSAID)
##     Null deviance: 18.905    on 21   degrees of freedom
## Residual deviance:  14.302    on 20   degrees of freedom

# R^2_GLM (%): 1 - (Residual Deviance/Null Deviance)
round((1 - (14.302/18.905))*100, 1)
## [1] 24.3

anova(model.OMD.NSAID, test = "Chisq")

##        Df Deviance Resid.  Df   Resid. Dev   Pr(>Chi)
## NULL                        21     18.904
## NSAID   1     4.6029        20     14.302    0.01118 *

# Peruse whether (spatial) autocorrelation is present in model residuals
acf(residuals(model.OMD.NSAID), main = "Raw residual ACF")
## No significant autocorrelation is present in model residuals.

# Obtain the values predicted by the fitted model
model.OMD.NSAID.pred <- predict(model.OMD.NSAID,
                                se = TRUE, type = "response")

# Draw the base plot with the observed data points
plot(glm.data$NSAID, glm.data$OMD, type = "p",
     xlab = "Detrended NSAIDs (nanograms per litre)",
     ylab = "Standardized and detrended organic matter decomposition")

# Draw model fit with approximate 95% confidence bands
I <- order(glm.data$NSAID)
lines(glm.data$NSAID[I], model.OMD.NSAID.pred$fit[I], lwd = 1)
lines(glm.data$NSAID[I], model.OMD.NSAID.pred$fit[I] +
      1.96 * model.OMD.NSAID.pred$se.fit[I], lty = 2, lwd = 1)
lines(glm.data$NSAID[I], model.OMD.NSAID.pred$fit[I] -
      1.96 * model.OMD.NSAID.pred$se.fit[I], lty = 2, lwd = 1)
# ************************************************************************ #

# 3.3.7. SRPAU
# ************
# Tiabendazole
# ************
model.SRPAU.Tiabendazole <- glm(SRPAU ~ Tiabendazole,
                                family = gaussian, data = glm.data)
summary(model.SRPAU.Tiabendazole)
##     Null deviance: 9.9793    on 21   degrees of freedom
## Residual deviance:  1.6590    on 20   degrees of freedom

# R^2_GLM (%): 1 - (Residual Deviance/Null Deviance)
round((1 - (1.6590/9.9793))*100, 1)
## [1] 83.4

anova(model.SRPAU.Tiabendazole, test = "Chisq")
##                Df Deviance Resid.  Df   Resid. Dev    Pr(>Chi)
## NULL                               21      9.9793
## Tiabendazole    1     8.3203       20      1.6590   < 2.2e-16 ***
```

```
# Peruse whether (spatial) autocorrelation is present in model residuals
acf(residuals(model.SRPAU.Tiabendazole), main = "Raw residual ACF")
## No significant autocorrelation is present in model residuals.

# Obtain the values predicted by the fitted model
model.SRPAU.Tiabendazole.pred <- predict(model.SRPAU.Tiabendazole,
                                         se = TRUE, type = "response")

# Draw the base plot with the observed data points
plot(glm.data$Tiabendazole, glm.data$SRPAU, type = "p",
     xlab = "Detrended tiabendazole (nanograms per litre)",
     ylab = "Standardized and detrended SRP areal uptake")

# Draw model fit with approximate 95% confidence bands
I <- order(glm.data$Tiabendazole)
lines(glm.data$Tiabendazole[I], model.SRPAU.Tiabendazole.pred$fit[I], lwd = 1)
lines(glm.data$Tiabendazole[I], model.SRPAU.Tiabendazole.pred$fit[I] +
      1.96 * model.SRPAU.Tiabendazole.pred$se.fit[I], lty = 2, lwd = 1)
lines(glm.data$Tiabendazole[I], model.SRPAU.Tiabendazole.pred$fit[I] -
      1.96 * model.SRPAU.Tiabendazole.pred$se.fit[I], lty = 2, lwd = 1)

# Iopromide
# *********
model.SRPAU.Iopromide <- glm(SRPAU ~ Iopromide,
                             family = gaussian, data = glm.data)
summary(model.SRPAU.Iopromide)
##     Null deviance: 9.9793    on 21   degrees of freedom
## Residual deviance:  7.6026    on 20   degrees of freedom

# R^2_GLM (%): 1 - (Residual Deviance/Null Deviance)
round((1 - (7.6026/9.9793))*100, 1)
## [1] 23.8

anova(model.SRPAU.Iopromide, test = "Chisq")
##           Df  Deviance Resid. Df R  esid. Dev  Pr(>Chi)
## NULL                         21     9.9793
## Iopromide  1    2.3767        20     7.6026    0.0124 *

# Peruse whether (spatial) autocorrelation is present in model residuals
acf(residuals(model.SRPAU.Iopromide), main = "Raw residual ACF")
## No significant autocorrelation is present in model residuals.
# Obtain the values predicted by the fitted model
model.SRPAU.Iopromide.pred <- predict(model.SRPAU.Iopromide,
                                      se = TRUE, type = "response")
# Draw the base plot with the observed data points
plot(glm.data$Iopromide, glm.data$SRPAU, type = "p",
     xlab = "Detrended iopromide (nanograms per litre)",
     ylab = "Standardized and detrended SRP areal uptake")

# Draw model fit with approximate 95% confidence bands
I <- order(glm.data$Iopromide)
lines(glm.data$Iopromide[I], model.SRPAU.Iopromide.pred$fit[I], lwd = 1)
lines(glm.data$Iopromide[I], model.SRPAU.Iopromide.pred$fit[I] +
      1.96 * model.SRPAU.Iopromide.pred$se.fit[I], lty = 2, lwd = 1)
lines(glm.data$Iopromide[I], model.SRPAU.Iopromide.pred$fit[I] -
      1.96 * model.SRPAU.Iopromide.pred$se.fit[I], lty = 2, lwd = 1)
# ********************************************************************* #
```

```
## So we have learned that all associations are monotonic, as follows:
##         Macrolide    NSAID        Tiabendazole   Iopromide
##         ---------------------------------------------------
## NH4AU   34.4% (L+)    26.9% (L+)   38.6% (L+)     21.9% (L+)
## SRPUC   67.3% (CL-)   48.9% (L-)
## ERp     50.8% (CL+)   82.0% (L+)
## BAT     67.2% (L+)
## GPP     17.4% (L+)
## OMD                   24.3% (L-)
## SRPAU                              83.4% (L+)        23.8% (L+)

## CODE: L = linear; CL = curvilinear; "+" = increasing; "-" = decreasing
## The numbers indicate the R^2_GLM corresponding to each relationship.
## Hence it seems clear that the single most important explanatory
## variable is the sum of the concentrations in stream water of the
## macrolide biocides, followed by the sum of the concentrations in
## stream water of the NSAIDs (non-steroids anti-inflamatory drugs).Most
## relationships are linear and positive, though negative and curvilinear
## relationships do arise.

# 3.2. Joint graph
# ****************
windows(title = "Joint graph", 8.3, 9.7) par(mfrow = c(3,3))

# 1. SRPAU ~ Tiabendazole
# ***********************
plot(glm.data$Tiabendazole, glm.data$SRPAU, type = "p", pch = 20,
     xlab = "Tiabendazole concentration", ylab = "SRPAU")
I <- order(glm.data$Tiabendazole)
lines(glm.data$Tiabendazole[I], model.SRPAU.Tiabendazole.pred$fit[I], lwd = 1)
lines(glm.data$Tiabendazole[I], model.SRPAU.Tiabendazole.pred$fit[I] +
      1.96 * model.SRPAU.Tiabendazole.pred$se.fit[I], lty = 2, lwd = 1)
lines(glm.data$Tiabendazole[I], model.SRPAU.Tiabendazole.pred$fit[I] -
      1.96 * model.SRPAU.Tiabendazole.pred$se.fit[I], lty = 2, lwd = 1)

# 2. ER ~ NSAIDs
# ***************
plot(glm.data$NSAID, glm.data$ERp, type = "p", pch = 20,
     xlab = "NSAIDs concentration", ylab = "ER")
I <- order(glm.data$NSAID)
lines(glm.data$NSAID[I], model.ERp.NSAID.pred$fit[I], lwd = 1)
lines(glm.data$NSAID[I], model.ERp.NSAID.pred$fit[I] +
      1.96 * model.ERp.NSAID.pred$se.fit[I], lty = 2, lwd = 1)
lines(glm.data$NSAID[I], model.ERp.NSAID.pred$fit[I] -
      1.96 * model.ERp.NSAID.pred$se.fit[I], lty = 2, lwd = 1)

# 3. SRPUC ~ AbMacrolides
# ***********************
plot(glm.data$Macrolide, glm.data$SRPUC, type = "p", pch = 20,
     xlab = "Macrolide concentration", ylab = "SRPUC")
I <- order(glm.data$Macrolide)
lines(glm.data$Macrolide[I], model.SRPUC.Macrolide.pred$fit[I], lwd = 1)
lines(glm.data$Macrolide[I], model.SRPUC.Macrolide.pred$fit[I] +
      1.96 * model.SRPUC.Macrolide.pred$se.fit[I], lty = 2, lwd = 1)
lines(glm.data$Macrolide[I], model.SRPUC.Macrolide.pred$fit[I] -
      1.96 * model.SRPUC.Macrolide.pred$se.fit[I], lty = 2, lwd = 1)

# 4. BAT ~ AbMacrolides
# *********************
plot(glm.data$Macrolide, glm.data$BAT, type = "p", pch = 20,
```

```
      xlab = "Macrolide concentration", ylab = "BAT")
I <- order(glm.data$Macrolide)
lines(glm.data$Macrolide[I], model.BAT.Macrolide.pred$fit[I], lwd = 1)
lines(glm.data$Macrolide[I], model.BAT.Macrolide.pred$fit[I] +
      1.96 * model.BAT.Macrolide.pred$se.fit[I], lty = 2, lwd = 1)
lines(glm.data$Macrolide[I], model.BAT.Macrolide.pred$fit[I] -
      1.96 * model.BAT.Macrolide.pred$se.fit[I], lty = 2, lwd = 1)

# 5. ER ~ AbMacrolides
# ********************
plot(glm.data$Macrolide, glm.data$ERp, type = "p", pch = 20,
      xlab = "Macrolide concentration", ylab = "ER")
I <- order(glm.data$Macrolide)
lines(glm.data$Macrolide[I], model.ERp.Macrolide.pred$fit[I], lwd = 1)
lines(glm.data$Macrolide[I], model.ERp.Macrolide.pred$fit[I] +
      1.96 * model.ERp.Macrolide.pred$se.fit[I], lty = 2, lwd = 1)
lines(glm.data$Macrolide[I], model.ERp.Macrolide.pred$fit[I] -
      1.96 * model.ERp.Macrolide.pred$se.fit[I], lty = 2, lwd = 1)

# 6. SRPUC ~ NSAIDs
# ******************
plot(glm.data$NSAID, glm.data$SRPUC, type = "p", pch = 20,
      xlab = "NSAIDs concentration", ylab = "SRPUC")
I <- order(glm.data$NSAID)
lines(glm.data$NSAID[I], model.SRPUC.NSAID.pred$fit[I], lwd = 1)
lines(glm.data$NSAID[I], model.SRPUC.NSAID.pred$fit[I] +
      1.96 * model.SRPUC.NSAID.pred$se.fit[I], lty = 2, lwd = 1)
lines(glm.data$NSAID[I], model.SRPUC.NSAID.pred$fit[I] -
      1.96 * model.SRPUC.NSAID.pred$se.fit[I], lty = 2, lwd = 1)

# 7. NH4AU ~ Tiabendazole
# ***********************
plot(glm.data$Tiabendazole, glm.data$NH4AU, type = "p", pch = 20,
      xlab = "Tiabendazole concentration", ylab = "NH4AU")
I <- order(glm.data$Tiabendazole)
lines(glm.data$Tiabendazole[I], model.NH4AU.Tiabendazole.pred$fit[I], lwd = 1)
lines(glm.data$Tiabendazole[I], model.NH4AU.Tiabendazole.pred$fit[I] +
      1.96 * model.NH4AU.Tiabendazole.pred$se.fit[I], lty = 2, lwd = 1)
lines(glm.data$Tiabendazole[I], model.NH4AU.Tiabendazole.pred$fit[I] -
      1.96 * model.NH4AU.Tiabendazole.pred$se.fit[I], lty = 2, lwd = 1)

# 8. OMD ~ NSAIDs
# ***************
plot(glm.data$NSAID, glm.data$OMD, type = "p", pch = 20,
      xlab = "NSAIDs concentration", ylab = "OMD")
I <- order(glm.data$NSAID)
lines(glm.data$NSAID[I], model.OMD.NSAID.pred$fit[I], lwd = 1)
lines(glm.data$NSAID[I], model.OMD.NSAID.pred$fit[I] +
      1.96 * model.OMD.NSAID.pred$se.fit[I], lty = 2, lwd = 1)
lines(glm.data$NSAID[I], model.OMD.NSAID.pred$fit[I] -
      1.96 * model.OMD.NSAID.pred$se.fit[I], lty = 2, lwd = 1)

# 9. GPP ~ AbMacrolides
# *********************
plot(glm.data$Macrolide, glm.data$GPP, type = "p", pch = 20,
      xlab = "Macrolide concentration", ylab = "GPP")
I <- order(glm.data$Macrolide)
lines(glm.data$Macrolide[I], model.GPP.Macrolide.pred$fit[I], lwd = 1)
lines(glm.data$Macrolide[I], model.GPP.Macrolide.pred$fit[I] +
      1.96 * model.GPP.Macrolide.pred$se.fit[I], lty = 2, lwd = 1)
lines(glm.data$Macrolide[I], model.GPP.Macrolide.pred$fit[I] -
```

```
        1.96 * model.GPP.Macrolide.pred$se.fit[I], lty = 2, lwd = 1)

# ************************************************************************ #
# ************************ END OF THE SCRIPT ************************** #
```

Chapter 3 - Ecosystem manipulation reveals effects of a highly diluted WWTP effluent on stream ecosystem structure and functioning

Appendix S1. Statistical results obtained for all the structural and functional variables measured.

Appendix S2. Dataset of the water characteristics (Available online upon publication).

Appendix S3. Dataset of the response variables (Available online upon publication).

Appendix S4. R code (Available online upon publication).

Appendix S1

Table S1. Effect size and the interaction between period and reach (BA:CI) obtained for all the variables. Statistical results are obtained from linear mixed-effects models and linear models for the variables with or without replicates, respectively. Note that distinction between effluent discharge and no discharge periods is also made for the variables related with water characteristics. *p* values and *d.f.* were obtained by means of likelihood ratio tests. Bold values indicate statistically significant results with $p < 0.05$.

Variable	Effect size	d.f.	$F_{BA:CI}$	*p*-value
pH - Effluent release period	0.92	1,7	14.40	**0.007**
pH - During no effluent release	0.99	1,8	0.27	0.619
T (ºC) - Effluent release period	1.09	1,7	2.00	0.201
T (ºC) - During no effluent release	0.97	1,9	0.70	0.426
Conductivity (µS cm^{-1}) - Effluent release period	1.52	1,5	66.66	**<0.001**
Conductivity (µS cm-1) - During no effluent release	0.97	1,8	0.02	0.899
DO (%) Effluent release period	0.94	1,7	6.27	**0.041**
DO (%) - During no effluent release	0.99	1,9	0.09	0.768
DO (mg L^{-1}) - Effluent release period	0.92	1,7	6.13	**0.042**
DO (mg L-1) - During no effluent release	1.00	1,9	0.01	0.914
Cl^- (mg L^{-1}) - Effluent release period	1.24	1,9	1.61	0.237
Cl^- (mg L^{-1}) - During no effluent release	1.00	1,10	0.02	0.886
SO_4^{2-} (mg L^{-1}) - Effluent release period	0.83	1,10	0.64	0.443
SO_4^{2-} (mg L^{-1}) - During no effluent release	1.14	1,10	0.49	0.500
NO_2^- (mg N L^{-1}) - Effluent release period	16.97	1,10	2.12	0.176
NO_2^- (mg N L^{-1}) - During no effluent release	3.01	1,10	0.86	0.375
NO_3^- (mg N L^{-1}) - Effluent release period	1.67	1,10	2.21	0.168
NO_3^- (mg N L^{-1}) - During no effluent release	0.93	1,10	0.04	0.838
NH_4^+ (mg N L^{-1}) - Effluent release period	5.18	1,9	4.71	**0.058**
NH_4^+ (mg N L^{-1}) - During no effluent release	2.34	1,9	5.61	**0.042**
DIN (mg N L^{-1}) - Effluent release period	2.38	1,10	8.27	**0.017**
DIN (mg N L^{-1}) - During no effluent release	1.03	1,10	0.02	0.893
TDN (mg N L^{-1}) - Effluent release period	1.55	1,9	6.16	**0.035**
TDN (mg N L^{-1}) - During no effluent release	0.89	1,9	1.64	0.233
DOC (mg C L^{-1}) - Effluent release period	1.13	1,10	0.33	0.578
DOC (mg C L^{-1}) - During no effluent release	0.79	1,10	1.78	0.212
SRP (mg P L^{-1}) - Effluent release period	2.39	1,9	14.24	**0.004**
SRP (mg P L^{-1}) - During no effluent release	0.60	1,9	2.52	0.147
Biofilm biomass (g m^{-2})	2.07	1,102	16.77	**<0.001**
Chlorophyll-*a* (mg m^{-2})	2.32	1,102	8.34	**0.005**
Alkaline phosphatase activity (µmol h^{-1} m^{-2})	2.23	1,96	6.12	**0.015**
β-glucosidase activity (µmol h^{-1} m^{-2})	4.15	1,96	9.82	**0.002**

Biofilm SRP uptake capacity (μg P h^{-1})	0.47	1,102	51.07	**<0.0001**
Gross primary production (mg O_2 h^{-1} m^{-2})	1.12	1,51	0.55	0.460
Community respiration (mg O_2 h^{-1} m^{-2})	1.08	1,52	0.16	0.688
Production-to-respiration ratio (GPP:CR)	1.28	1,51	1.18	0.283
Phosphate uptake length (m)	1.34	1,8	0.41	0.542
Phosphate uptake velocity (mm min^{-1})	0.52	1,8	4.96	0.057
Phosphate areal uptake (μg P min^{-1} m^{-2})	0.28	1,8	2.87	0.129
Ammonium uptake length (m)	1.40	1,10	2.27	0.163
Ammonium uptake velocity (mm min-1)	0.44	1,10	1.97	0.191
Ammonium areal uptake (μg N min^{-1} m^{-2})	1.04	1,10	0.01	0.921
Microbial decomposition (day^{-1})	0.96	1,100	0.09	0.761
Total decomposition (day^{-1})	1.41	1,103	9.15	**0.003**

Appendix S4

Appendix S4. R Code. Ecosystem manipulation reveals effects of a highly diluted WWTP effluent on stream ecosystem structure and functioning by Olatz Pereda, Libe Solagaistua, Miren Atristain, Ioar de Guzmán, Aitor Larrañaga, Daniel von Schiller and Arturo Elosegi.

```
#########################################################################
# Title: "Ecosystem manipulation reveals effects of a highly diluted  #
#         wastewater treatment plant (WWTP) effluent on stream        #
#         ecosystem structure and functioning"                        #
#                                                                     #
# Authors: O. Pereda, L. Solagaistua, M. Atristain, I. de Guzmán,     #
#          A. Larrañaga, D. von Schiller & A. Elosegi                 #
#                                                                     #
# Appendix S4: Supplementary material (R Coding)                      #
#                                                                     #
# Data analysis was carried out by O. Pereda* and Libe Solagaistua    #
# *email: olatz.pereda@ehu.eus                                        #
#########################################################################

# Data analysis was carried out using R software v.3.4.0. (R Core Team.
# 2017. R: A language and environment for statistical computing. Version
# 3.4.0. R Foundation for Statistical Computing, Vienna, Austria.
# URL http://www.R-project.org/).

# This file was created using Tinn-R Editor [Faria J.C., Grosjean P.
# & Jelihovschi E. (2014). Tinn-R Editor - GUI for R Language and
# Environment. Tinn-R Editor is free software.

# References
# ***********
# The analysis presented here was written with the fundamental aid of the
# next:

# Madsen H. & Thyregod P. (2010).
# Introduction to General and Generalized Linear Models. CRP press.
# ISBN: 978-1420091557

# Pinheiro J. & Bates D. (2000).
# Mixed-effects models in S and S-PLUS. Springer-Verlag, New York.
# ISBN: 978-0-387-98957-0. doi: 10.1007/b98882

# Pinheiro J. & Bates D. (2016).
# nlme: Linear and Nonlinear Mixed Effects Models.

# Pinheiro J. & Bates D. (2017).
# nlme: Linear and Nonlinear Mixed Effect Models.
# R package version 3.1-131. https://CRAN.R-project.org/package=nlme.

# Zuur A.F., Ieno E.N., Walker N., Saveliev A.A. & Smith G.M. (2010).
# Mixed Effects Models and Extensions in Ecology with R. Springer-Verlag,
# New York. ISBN: 978-0-387-87457-9. doi: 10.1007/978-0-387-87458-6
# ***********************************************************************

# Contents of the script:
# ************************
# This R code exemplifies the analysis described in the main body of the
# paper.
```

```r
# The code uses a dataset from an ecosystem-level manipulation experiment
# following a BACI (Before-After/Control-Impact) design, in which we
# diverted part of the effluent of a large tertiary urban WWTP effluent
# into a small nearly unpolluted stream.

# 1. Step 1: The effects of the effluent addition are studied on Water
# Characteristics.
# This data includes: - Water physico-chemical characteristics:
#                           - pH
#                           - Temperature
#                           - Conductivity
#                           - Dissolved oxygen saturation and concentration
#                     - Main nutrient concentrations:
#                           - Chloride (Cl)
#                           - Sulfate (SO4)
#                           - Nitrite (N-NO2)
#                           - Nitrate (N-NO3)
#                           - Ammonium (N-NH4)
#                           - Dissolved inorganic nitrogen (DIN)
#                           - Total dissolved nitrogen (TDN)
#                           - Dissolved organic carbon (DOC)
#                           - Soluble reactive phosphorus (SRP)
# ***This data is provided in the Appendix S2***

# 2. Step 2: After this exploration, effects of the effluent addition are
# studied on ecosystem structure and functioning.
#  2.1. Biofilm structure: - Chlorophyll-a concentration
#                          - Biofilm biomass
#
#  2.2. Ecosystem functioning: - Biofilm exo-enzymatic activities:
#                                  - Alkaline phosphatase (AP)
#                                  - ß-glucosidase (BG)
#                              - Biofilm capacity to uptake SRP
#                              - Biofilm metabolism:
#                                  - Gross primary production (GPP)
#                                  - Community respiration (CR)
#                              - Whole-reach nutrient uptake (SRP, NH4):
#                                  - Uptake length
#                                  - Uptake velocity
#                                  - Areal uptake
#                              - Organic matter decomposition:
#                                  - Microbial
#                                  - Total
# ***This data is provided in the Appendix S3***
##########################################################################
# In all cases, the effect of the effluent addition is given by the
# interaction between period (Before/After) and Reach (Control/Impact)
# BA*CI
##########################################################################

# Ecosystem-level manipulation experiment: BACI design
# ****************************************************

# R packages:
# ***********
# Pinheiro, J., D. Bates, S. DebRoy, D. Sarkar, and R-Core-Team. 2018.
# nlme: Linear and Nonlinear Mixed Effects Models.
# R package version 3.1-137.
# install.packages("nlme")                    # run if needed
library(nlme)
```

```
# Wickham, H. 2016. ggplot2: Elegant Graphics for Data Analysis.
# New York: Springer-Verlag.
# install.packages("ggplot2")                    # run if needed
library(ggplot2)

# Wickham H., François R., Henry L. & Müller K. 2018.
# dplyr: a grammar of data manipulation.
# install.packages("dplyr")                    # run if needed
library(dplyr)

# Wickham H., Henry L. & and R-Core-Team. 2019.
# Tidyr: Easily tidy data with "spread()" and "gather()" functions.
# install.packages("tidyr")                    # run if needed
library(tidyr)

# Hope R.M. 2013. Rmisc: Ryan Miscellaneous.
# install.packages("Rmisc")                    # run if needed
library(Rmisc)
#########################################################################

# ************************************************************
# 1. Effects of effluent addition on Water Characteristics.
#    1.1.: Physico-chemical characteristics.
# ************************************************************

#########################################
#     EFFLUENT RELEASE PERIOD - ER      #
#########################################

# *************
# Conductivity
# *************
# Load the data (Change the directory as required):
data<-read.csv("C:/.../Pereda et al.
               Ecosphere_Data.WaterCharacteristics.csv",
               h=T,sep=";",dec=".")
# We will only work with "Effluent release period" data
data <- subset(data,data$Period!="NR")
data<- na.omit(data)
str(data)
summary(data)
names(data) data

# We will define the model
model<- lme(Conductivity ~ 1+B.A*C.I, random= ~1|Day, na.action=na.omit,
            data=data)

anova(model)
## Analysis of Variance Table
## Response: Conductivity
##                 numDF denDF   F-value    p-value
 #   (Intercept)       1     9   231.35101   <.0001
 #   B.A               1     9     2.22214   0.1702
 #   C.I               1     7     8.71176   0.0214
 #   B.A:C.I           1     7    10.82559   0.0133

# Before accepting the model above, check model assumptions:
plot(resid(model))
hist(resid(model))
shapiro.test(resid(model)) # p-value = 0.01467
```

```r
## Overall, model assumptions do not look good.
## There could be some influent large observation.

# Check if there is some large observation:
# ****************************************
lowerq = quantile(resid(model))[2]
upperq = quantile(resid(model))[4]
iqr = upperq - lowerq
extreme.threshold.upper = (iqr * 3) + upperq
extreme.threshold.upper      #75%: 78.12594
extreme.threshold.lower = lowerq - (iqr * 3)
extreme.threshold.lower      #25%: -78.03266
# Give residual values to each data of the data.frame
data$resid<- resid(model)
# Order residual values from the lowest to the largest
data[order(data$resid),]
# 34, 38 data seem to be large observations.

# If we do not consider these large observations:
data<-read.csv("C:/.../Pereda et al.
               Ecosphere_Data.WaterCharacteristics.csv",
               h=T,sep=";",dec=".")
data <-data [-c(34,38),]

# We will only work with "Effluent release period" data
data <- subset(data,data$Period!="NR")
data<- na.omit(data)

# Re-define the model
model<- lme(Conductivity ~ 1+B.A*C.I, random= ~1|Day, na.action=na.omit,
            data=data)
anova(model)
## Analysis of Variance Table
## Response: Conductivity
##                numDF denDF   F-value    p-value
 #   (Intercept)      1     9  461.8757    <.0001
 #   B.A              1     9    1.4505    0.2592
 #   C.I              1     5   24.7170    0.0042
 #   B.A:C.I          1     5   66.6609    0.0004

# Before accepting the model above, check model assumptions:
plot(resid(model))
hist(resid(model))
shapiro.test(resid(model)) # p-value = 0.271
## Overall, model assumptions look better.

# But before accepting it, check again if there is still any influent
# large observation
# ************************************************************************
lowerq = quantile(resid(model))[2]
upperq = quantile(resid(model))[4]
iqr = upperq - lowerq
extreme.threshold.upper = (iqr * 3) + upperq
extreme.threshold.upper      #75%: 20.7689
extreme.threshold.lower = lowerq - (iqr * 3)
extreme.threshold.lower      #25%: -20.54186
# Give residual values to each data of the data.frame
data$resid<- resid(model)
# Order residual values from the lowest to the largest
data[order(data$resid),]
## There is not any influent large observation.
```

```
## So, we will select the latter model for the results.

# Effect size:
# ************
Treatment <- unite(data, Treatment, c(B.A, C.I), remove=FALSE)
dataSE<- summarySE(Treatment, measurevar="Conductivity",
                   groupvars=c("Treatment"))
Effect.size <- ((dataSE$Conductivity[2])/(dataSE$Conductivity[1]))/
               ((dataSE$Conductivity[4])/(dataSE$Conductivity[3]))
Effect.size # 1.516933

# ***********************************************************
# This same step has been repeated for each of the variables:
#    - pH
#    - Water mean temperature
#    - Dissolved oxygen saturation
#    - Dissolved oxygen concentration
# However, just for brevity, we demonstrate a single analysis.
# ***********************************************************

##########################################
#      DURING NO EFFLUENT RELEASE - NR     #
##########################################
# The same step followed in 1.1. section has been repeated for all
# variables during periods of no effluent release:
#    - Conductivity
#    - pH
#    - Water mean temperature
#    - Dissolved oxygen saturation
#    - Dissolved oxygen concentration
# Although, for brevity, we do not demonstrate all the analyses.
# ************************************************************

# ********************* END OF STEP 1.1 **************************#
#####################################################################

# ***********************************************************
# 1. Effects of effluent addition on Water Characteristics.
#    1.2.: Water chemistry - Nutrient concentrations.
# ***********************************************************

########################################
#      EFFLUENT RELEASE PERIOD - ER    #
########################################

# *************
# Chloride (Cl)
# *************
# Load the data (Change the directory as required):
data<-read.csv("C:/.../Pereda et al.
               Ecosphere_Data.WaterCharacteristics.csv",
               h=T,sep=";",dec=".")
# We will only work with "Effluent release period" data
data <- subset(data,data$Period!="NR")
str(data)
summary(data)
names(data)
data
```

```
# We will define the model
model<- lme(Cl ~ 1+B.A*C.I, random= ~1|Day, na.action=na.omit,
            data=data)
anova(model)
## Analysis of Variance Table

 #  Response: Cl
 #                numDF   denDF   F-value      p-value
 #  (Intercept)      1      10     79.01936     <.0001
 #  B.A              1      10     5.03658      0.0487
 #  C.I              1      10     4.12016      0.0698
 #  B.A:C.I          1      10     2.40063      0.1523

# Before accepting the model above, check model assumptions:
plot(resid(model))
hist(resid(model))
shapiro.test(resid(model)) # p-value = 9.985e-05
## Overall, model assumptions do not look good.
## There could be some influent large observation.

# Check if there is some large observation:
#****************************************
lowerq = quantile(resid(model))[2]
upperq = quantile(resid(model))[4]
iqr = upperq - lowerq
extreme.threshold.upper = (iqr * 3) + upperq
extreme.threshold.upper      #75%: 12.65418
extreme.threshold.lower = lowerq - (iqr * 3)
extreme.threshold.lower      #25%: -14.85002
# Give residual values to each data of the data.frame
data$resid<- resid(model)
# Order residual values from the lowest to the largest
data[order(data$resid),]
# 34 data seem to be large observation.

# If we do not consider this large observation:
data<-read.csv("C:/.../Pereda et al.
               Ecosphere_Data.WaterCharacteristics.csv",
               h=T,sep=";",dec=".")
data <-data [-c(34),]
# We will only work with "Effluent release period" data
data <- subset(data,data$Period!="NR")

# Re-define the model
model<- lme(Cl ~ 1+B.A*C.I, random= ~1|Day, na.action=na.omit,
            data=data)
anova(model)
## Analysis of Variance Table
 #  Response: Cl
 #                numDF  denDF   F-value      p-value
 #  (Intercept)    1      10      219.42109    <.0001
 #  B.A            1      10        6.17686    0.0322
 #  C.I            1         9      4.56911    0.0613
 #  B.A:C.I        1         9      1.60572    0.2369

# Before accepting the model above, check model assumptions:
plot(resid(model))
hist(resid(model))
shapiro.test(resid(model)) # p-value = 0.1365
## Overall, model assumptions looks better.
```

```
# Before accepting, check again the presence of influent large observations
# ***************************************************************************
lowerq = quantile(resid(model))[2]
upperq = quantile(resid(model))[4]
iqr = upperq - lowerq
extreme.threshold.upper = (iqr * 3) + upperq
extreme.threshold.upper      #75%: 13.43141
extreme.threshold.lower = lowerq - (iqr * 3)
extreme.threshold.lower      #25%: -13.29397
# Give residual values to each data of the data.frame
data$resid<- resid(model)
# Order residual values from the lowest to the largest
data[order(data$resid),]
# There is not any influent large observation.

## So, we will select the latter model for the results.

# Effect size:
# ************
Treatment <- unite(data, Treatment, c(B.A, C.I), remove=FALSE)
dataSE<- summarySE(Treatment, measurevar="Cl", groupvars=c("Treatment"))

Effect.size <- ((dataSE$Cl[2])/(dataSE$Cl[1]))/
                 ((dataSE$Cl[4])/(dataSE$Cl[3]))
Effect.size # 1.241563

# ***********************************************************
# This same step has been repeated for each of the variables:
#    - Sulfate (SO4)
#    - Nitrite (N-NO2)
#    - Nitrate (N-NO3)
#    - Ammonium (N-NH4)
#    - Dissolved inorganic nitrogen (DIN)
#    - Total dissolved nitrogen (TDN)
#    - Dissolved organic carbon (DOC)
#    - Soluble reactive phosphorus (SRP)
# However, just for brevity, we demonstrate a single analysis.
# ***********************************************************

##########################################
#      DURING NO EFFLUENT RELEASE - NR     #
##########################################
# The same step followed in 1.2. section has been repeated for all
# variables during periods of no effluent release:
#    - Sulfate (SO4)
#    - Nitrite (N-NO2)
#    - Nitrate (N-NO3)
#    - Ammonium (N-NH4)
#    - Dissolved inorganic nitrogen (DIN)
#    - Total dissolved nitrogen (TDN)
#    - Dissolved organic carbon (DOC)
#    - Soluble reactive phosphorus (SRP)
# Although, for brevity, we do not demonstrate all the analyses.
# ***********************************************************

# ******************** END OF STEP 1.2 ***************************#
#########################################################################
```

```
# ************************************************************************
# 2. Effects of effluent addition on Response Variables.
#   2.1.: Biofilm structure:  - Chlorophyll-a concentration
#                             - Biofilm biomass
#
# * Note that there is no distinction between effluent release periods
# (ER) and periods of no effluent release (NR) for response variables.
# ************************************************************************

#  ************************************************
# Biofilm chlorophyll-a concentration (mg Chl-a m-2)
# ************************************************
# Load the data (Change the directory as required):
data<-read.csv("C:/.../Pereda et al.
               Ecosphere_Data.ResponseVariables.csv",
               h=T,sep=";",dec=".")
str(data)
summary(data)
names(data)
data

# We will define the model
# On a first step, we will explore the need to add a variance structure
# to deal with heteroscedasticity (Only applicable in the case of
# variables with replicates per sampling day) and allow having
# different variances per sampling day.
var.str <-varIdent(form= ~ 1|Date)
model1<- lme(Chl.a_m2 ~ 1+B.A*C.I, random=~1|Date, weight= var.str,
             na.action=na.omit, data=data)
model2<- lme(Chl.a_m2 ~ 1+B.A*C.I, random=~1|Date,
             na.action=na.omit, data=data)
AIC(model1, model2)
##            df       AIC
## model1     17     603.6298
## model2     6      650.9037
## As it had lower value of AIC, we will choose the first model.

anova(model1)
## Analysis of Variance Table
## Response: Biofilm chlorophyll-a concentration

 ##                numDF  denDF   F-value      p-value
 ## (Intercept)        1   106    24.212884   <.0001
 ## B.A                1    10     0.000206   0.9888
 ## C.I                1   106     0.014598   0.9041
 ## B.A:C.I            1   106    10.132288   0.0019

# Before accepting the model above, check model assumptions:
plot(resid(model1))
hist(resid(model1))
shapiro.test(resid(model1)) # p-value = 2.453e-10
## Overall, model assumptions do not look good.
## There could be some influent large observations.

options(max.print = 99999)
# Check if there is some large observation:
# ***************************************
lowerq = quantile(resid(model1))[2]
upperq = quantile(resid(model1))[4]
iqr = upperq - lowerq
extreme.threshold.upper = (iqr * 3) + upperq
```

```
extreme.threshold.upper      #75%: 8.092743
extreme.threshold.lower = lowerq - (iqr * 3)
extreme.threshold.lower      #25%: -8.593109
# Give residual values to each data of the data.frame
data$resid<- resid(model1)
# Order residual values from the lowest to the largest
data[order(data$resid),]
# 96, 40, 107 data seem to be influent large observations

# If we do not consider these large observations
data<-read.csv("C:/.../Pereda et al.
               Ecosphere_Data.ResponseVariables.csv",
               h=T,sep=";",dec=".")
data <-data [-c(96, 40, 107),]

# Re-define the model
model1<- lme(Chl.a_m2 ~ 1+B.A*C.I, random=~1|Date, weight= var.str,
             na.action=na.omit, data=data)
anova(model1)
## Analysis of Variance Table
## Response: Biofilm chlorophyll-a concentration
 ##               numDF   denDF    F-value      p-value
 ## (Intercept)      1     103       23.483828    <.0001
 ## B.A              1     10         0.061625    0.8090
 ## C.I              1     103        0.294258    0.5887
 ## B.A:C.I          1     103        8.165571    0.0052

# Before accepting the model above, check model assumptions:
plot(resid(model1))
hist(resid(model1))
shapiro.test(resid(model1)) # p-value = 0.002054
## Overall, model assumptions look better.

options(max.print = 99999)
# Before accepting, check again the presence of influent large observations
# ***************************************************************************
lowerq = quantile(resid(model1))[2]
upperq = quantile(resid(model1))[4]
iqr = upperq - lowerq
extreme.threshold.upper = (iqr * 3) + upperq
extreme.threshold.upper      #75%: 8.699495
extreme.threshold.lower = lowerq - (iqr * 3)
extreme.threshold.lower      #25%: -8.802737
# Give residual values to each data of the data.frame
data$resid<- resid(model1)
# Order residual values from the lowest to the largest
data[order(data$resid),]
# 99 data continue being influent large observation.

# If we do not consider this large observation
data<-read.csv("C:/.../Pereda et al.
               Ecosphere_Data.ResponseVariables.csv",
               h=T,sep=";",dec=".")
data <-data [-c(96, 40, 107, 99),]

# Re-define the model
model1<- lme(Chl.a_m2 ~ 1+B.A*C.I, random=~1|Date, weight= var.str,
             na.action=na.omit, data=data)
anova(model1)
## Analysis of Variance Table
## Response: Biofilm chlorophyll-a concentration
```

```
 #                  numDF  denDF   F-value      p-value
 #  (Intercept)       1    102      23.267484    <.0001
 #  B.A               1    10        0.124159    0.7319
 #  C.I               1    102       0.216022    0.6431
 #  B.A:C.I           1    102       8.339581    0.0047

# Before accepting the model above, check model assumptions:
plot(resid(model1))
hist(resid(model1))
shapiro.test(resid(model1)) # p-value = 0.08627
## Overall, model assumptions look much better.

options(max.print = 99999)
# Before accepting, check again the presence of influent large observations
# ***************************************************************************
lowerq = quantile(resid(model1))[2]
upperq = quantile(resid(model1))[4]
iqr = upperq - lowerq
extreme.threshold.upper = (iqr * 3) + upperq
extreme.threshold.upper      #75%: 7.864986
extreme.threshold.lower = lowerq - (iqr * 3)
extreme.threshold.lower      #25%: -7.698185
# Give residual values to each data of the data.frame
data$resid<- resid(model1)
# Order residual values from the lowest to the largest
data[order(data$resid),]
# There are no more influent large observations.
## So, we will select the latter model for the results.

# Effect size:
# ************
Treatment <- unite(data, Treatment, c(B.A, C.I), remove=FALSE)
dataSE<- summarySE(Treatment, measurevar="Chl.a_m2",
                   groupvars=c("Treatment"))
Effect.size <- ((dataSE$Chl.a_m2[2])/(dataSE$Chl.a_m2[1]))/
               ((dataSE$Chl.a_m2[4])/(dataSE$Chl.a_m2[3]))
Effect.size # 2.314528

# ***************************************************************************
# This same step has been repeated for all response variables:
#  2.1. Biofilm structure: - Chlorophyll-a concentration
#                          - Biofilm biomass
#  2.2. Ecosystem functioning: - Biofilm exo-enzymatic activities:
#                                  - Alkaline phosphatase (AP)
#                                  - ß-glucosidase (BG)
#                              - Biofilm capacity to uptake (SRP)
#                              - Biofilm metabolism:
#                                  - Gross primary production (GPP)
#                                  - Community respiration (CR)
#                              - Whole-reach nutrient uptake (SRP,NH4):
#                                  - Uptake length
#                                  - Uptake velocity
#                                  - Areal uptake
#                              - Organic matter decomposition:
#                                  - Microbial
#                                  - Total
# However, just for brevity, we demonstrate a single analysis.
# ***************************************************************
# ********************** END OF STEPs 2.1 and 2.2 ********************

#                            END OF THE ANALYSIS                          #
```

Chapter 4 - Immediate and legacy effects of urban pollution on river ecosystem functioning: a mesocosm experiment

Appendix S1. Supplementary tables and figures.

Appendix S2. R code.

Appendix S1

Table S1. Characterization of the WWTP effluent water used during the experiment (100% treatment). Mean values ± standard error (SE) of the physico-chemical conditions (n = 30), concentrations of nutrients, major anions and cations, dissolved organic carbon (DOC) (n = 36, except DOC where n = 30) and heavy metals (n = 3 after 32 days of exposure).

Physico-chemical conditions		*Nutrient concentration (mg L^{-1})*		*Heavy metals (µg L^{-1})*	
pH	7.9 ± 0.1	$N\text{-}NO_3^-$	16.1 ± 4.2	Mn	6.8 ± 0.8
Temperature (º C)	21.2 ± 0.2	$N\text{-}NO_2^-$	5.8 ± 2.0	Fe	51.9 ± 3.9
Conductivity (µS cm^{-1})	1348 ± 60.5	$N\text{-}NH_4^+$	16.1 ± 3.2	Co	1.46 ± 0.3
DO concentration (mg L^{-1})	6.9 ± 0.4	SRP	0.7 ± 0.1	Ni	5.39 ± 1.1
DO saturation (% Sat.)	78 ± 4.6	$S\text{-}SO_4^{2-}$	21.1 ± 0.7	Cu	81.9 ± 1.6
		Br^-	0.1 ± 0.0	Zn	71.8 ± 4.8
		Na^+	154.2 ± 4.6	As	7.3 ± 0.2
		Cl^-	198.8 ± 5.89	Cd	0.06 ± 0.0
		K^+	22.4 ± 0.7	Hg	0.06 ± 0.0
		Mg^{2+}	12.9 ± 0.4	Pb	0.08 ± 0.0
		Ca^{2+}	60.0 ± 2.9		
		DOC	14.3 ± 0.5		

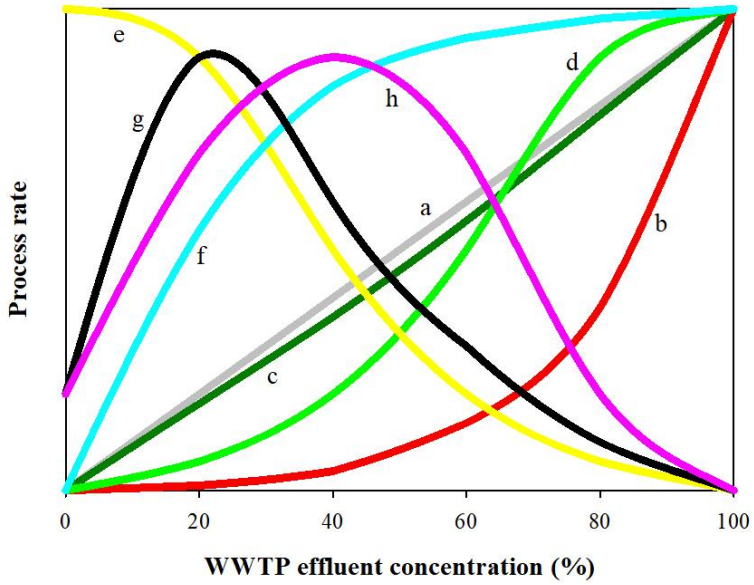


Figure S1. Representation of the models potentially fitting the different process rates measured along the gradient of WWTP effluent concentration: a) Linear, b) Exponential, c) Power, d) Logistic, e) Logit, f) Monod, g) Haldane, h) Quadratic. The Haldane and Quadratic models were used as a proxy of the subsidy-stress scheme. See Table 1 for a detailed description of the models.

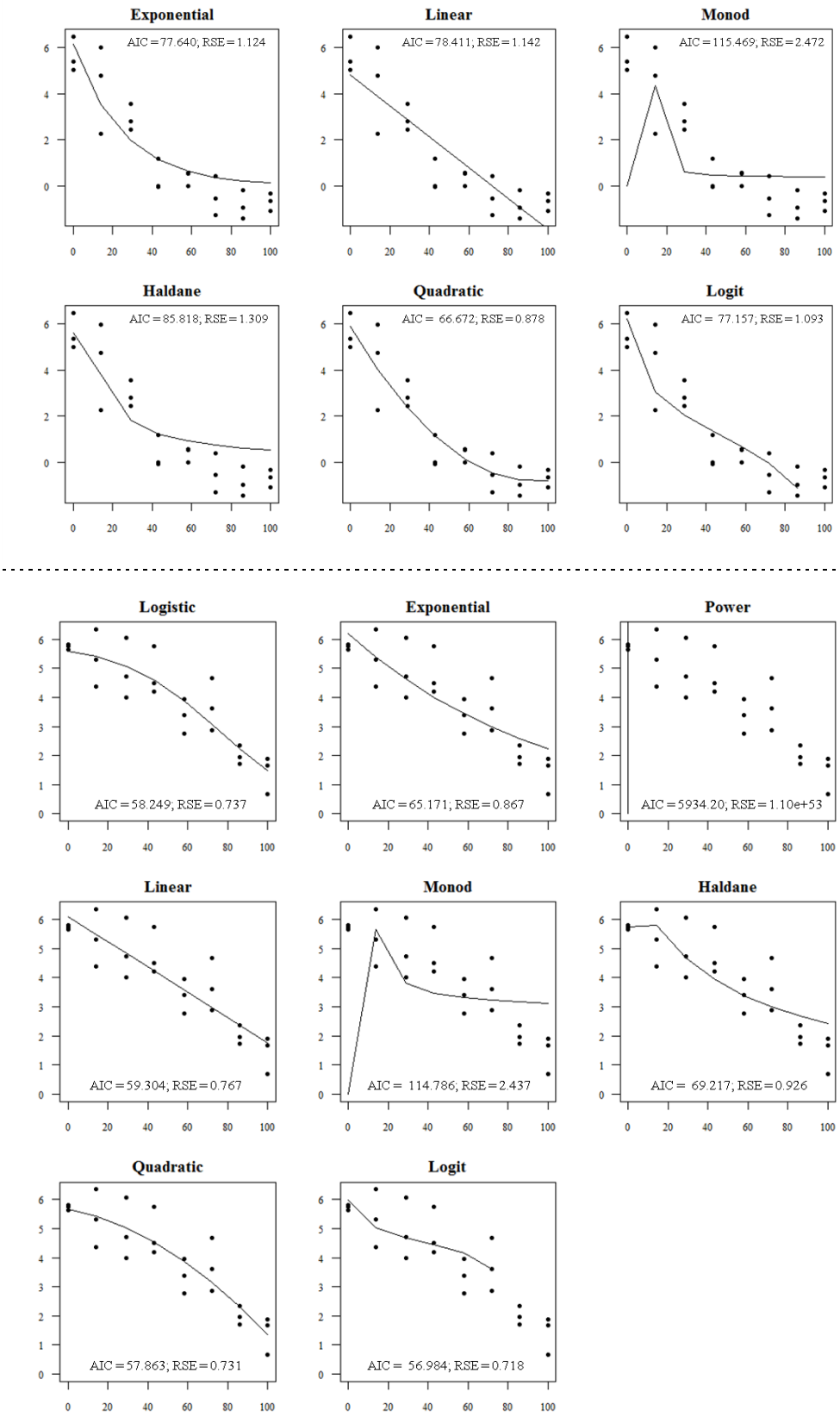


Figure S2. Fitted models for soluble reactive phosphorus (SRP) uptake capacity on biofilm carriers during the exposure phase (6 figures above dotted line) and the recovery phase (8 figures below dotted line). Labels on X axis denote the SRP uptake capacity of the biofilm (μg P h^{-1}) and the labels on Y axis denote the gradient of effluent dilution (%). Each figure is computed with its corresponding Akaike Information Criterion (AIC) and Relative Standard Error (RSE) values.

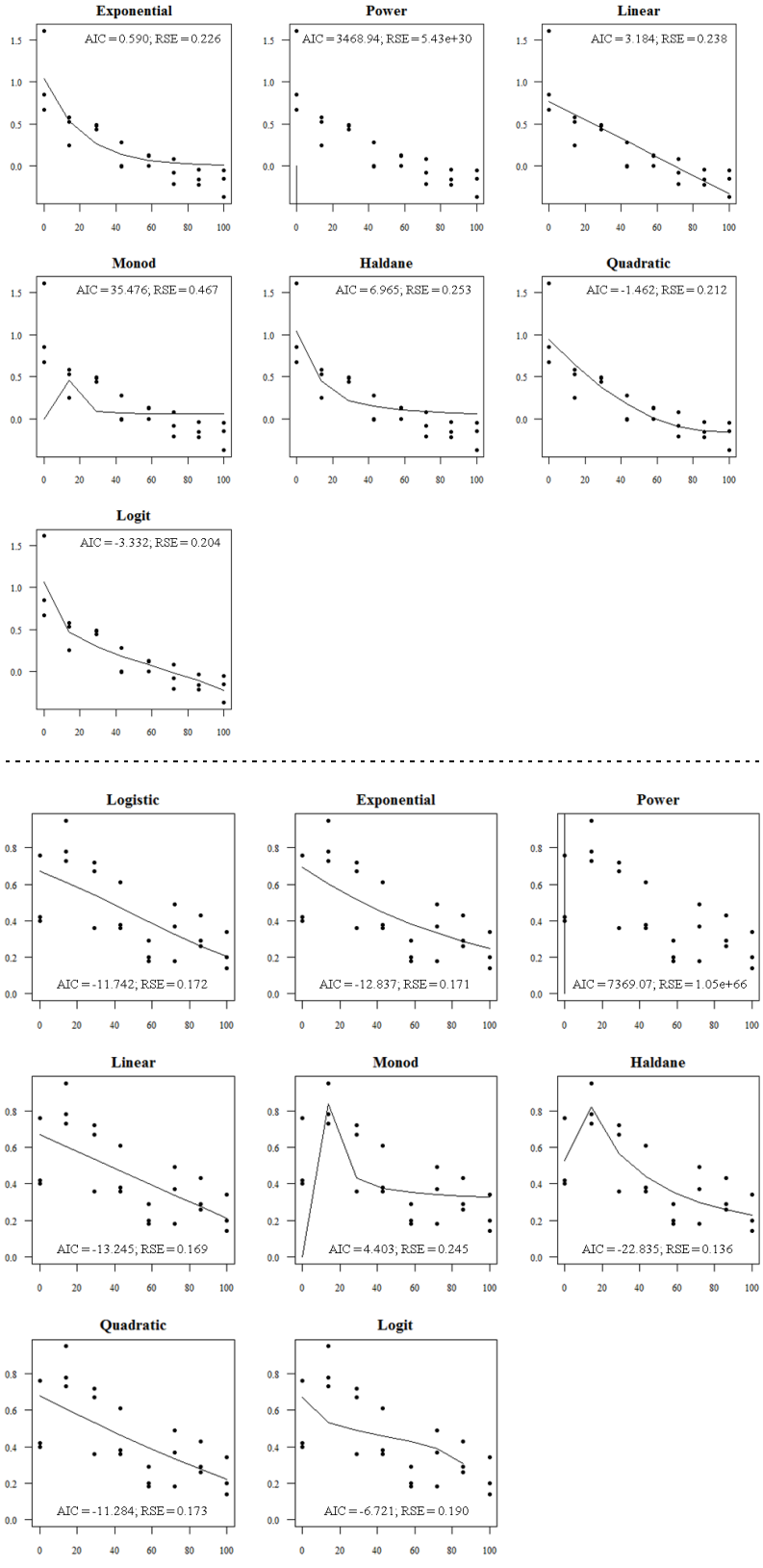


Figure S3. Fitted models for soluble reactive phosphorus (SRP) uptake efficiency on biofilm carriers during the exposure phase (7 figures above dotted line) and the recovery phase (8 figures below dotted line). Labels on X axis denote the SRP uptake efficiency of the biofilm (μg P mg $AFDM^{-1}$ h^{-1}) and the labels on Y axis denote the gradient of effluent dilution (%). Each figure is computed with its corresponding Akaike Information Criterion (AIC) and Relative Standard Error (RSE) values.

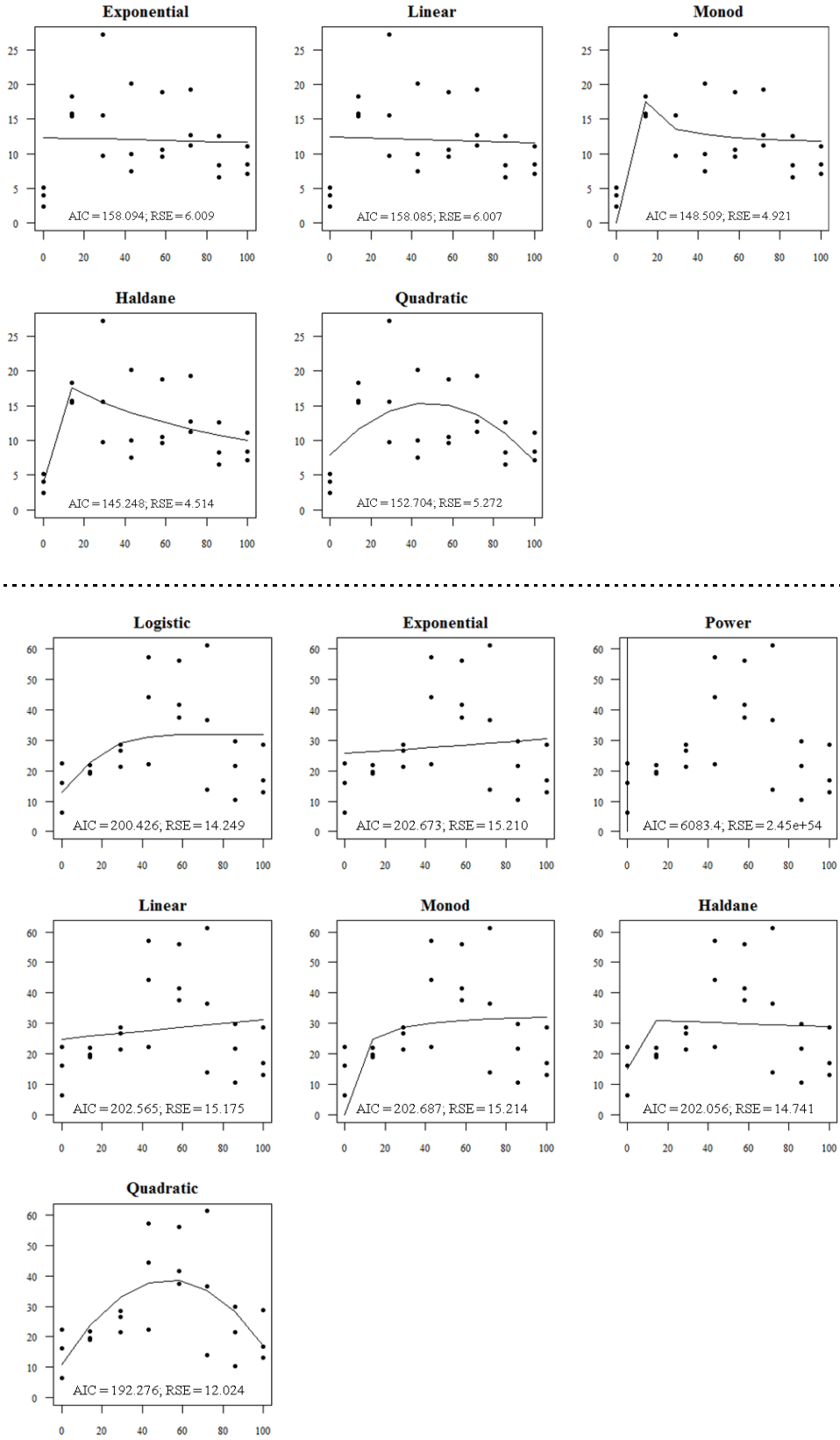


Figure S4. Fitted models for Chlorophyll-*a* (Chl-*a*) concentration on biofilm carriers during the exposure phase (5 figures above dotted line) and the recovery phase (7 figures below dotted line). Labels on X axis denote the Chl-*a* concentration of the biofilm (µg cm^{-2}) and the labels on Y axis denote the gradient of effluent dilution (%). Each figure is computed with its corresponding Akaike Information Criterion (AIC) and Relative Standard Error (RSE) values.

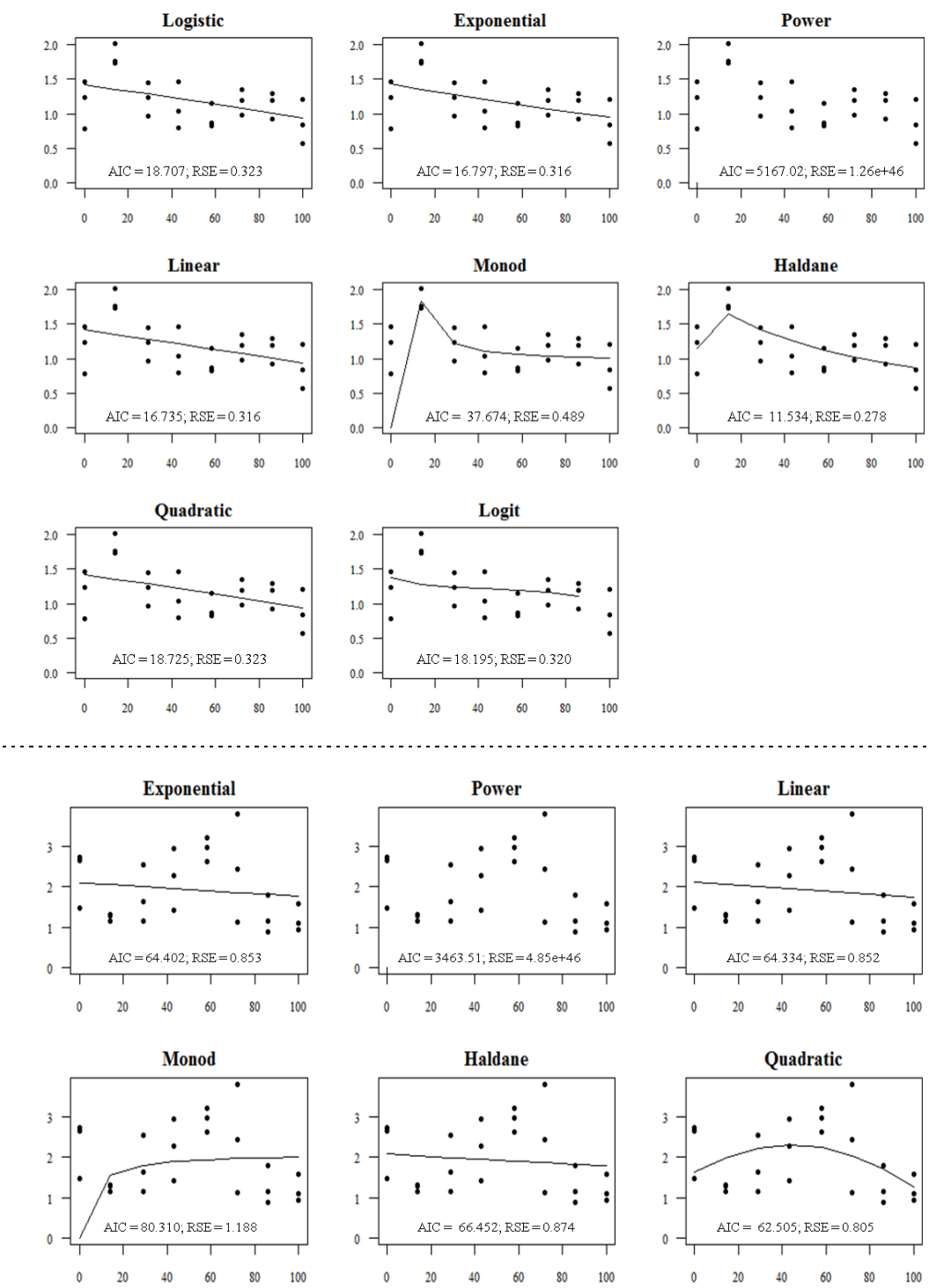


Figure S5. Fitted models for total biomass on biofilm carriers during the exposure phase (8 figures above dotted line) and the recovery phase (6 figures below dotted line). Labels on X axis denote the total biomass (mg cm^{-2}) and the labels on Y axis denote the gradient of effluent dilution (%). Each figure is computed with its corresponding Akaike Information Criterion (AIC) and Relative Standard Error (RSE) values.

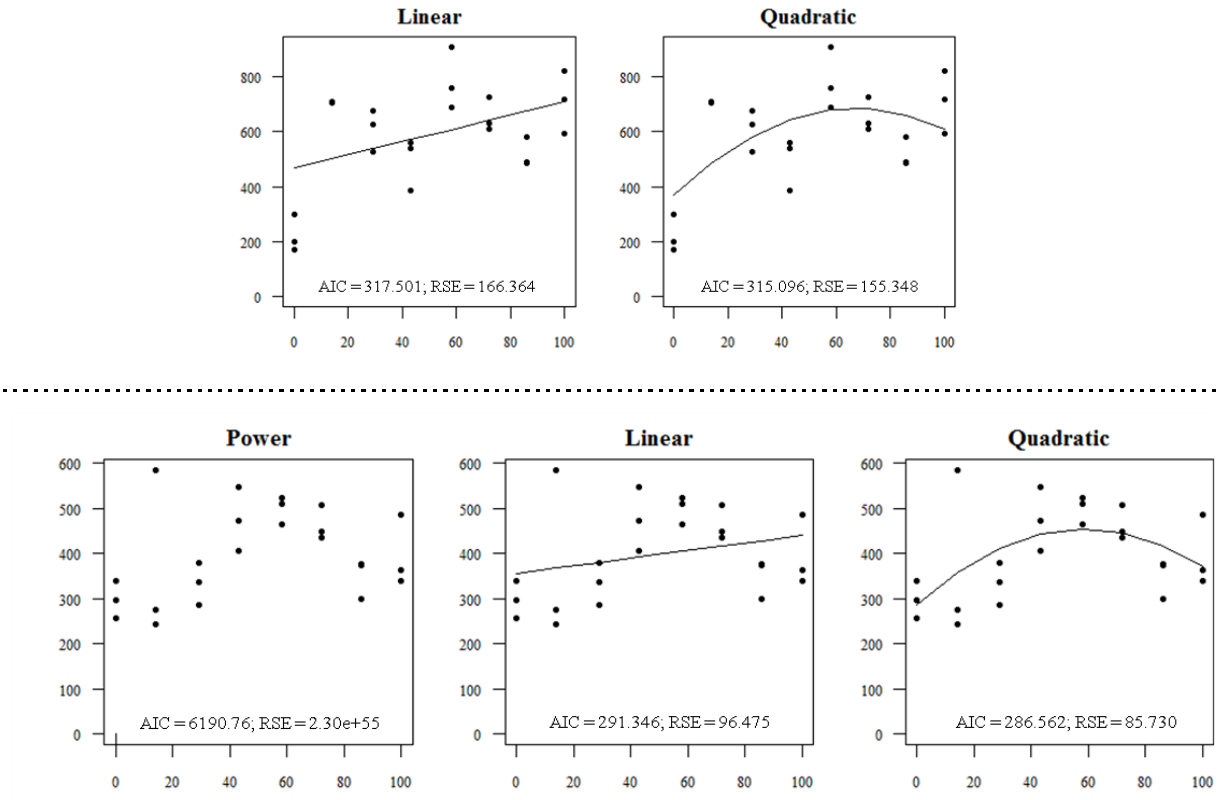


Figure S6. Fitted models for benthic gross primary production (GPP) during the exposure phase (2 figures above dotted line) and the recovery phase (3 figures below dotted line). Labels on X axis denote the GPP (mg O_2 m^{-2} h^{-1}) and the labels on Y axis denote the gradient of effluent dilution (%). Each figure is computed with its corresponding Akaike Information Criterion (AIC) and Relative Standard Error (RSE) values.

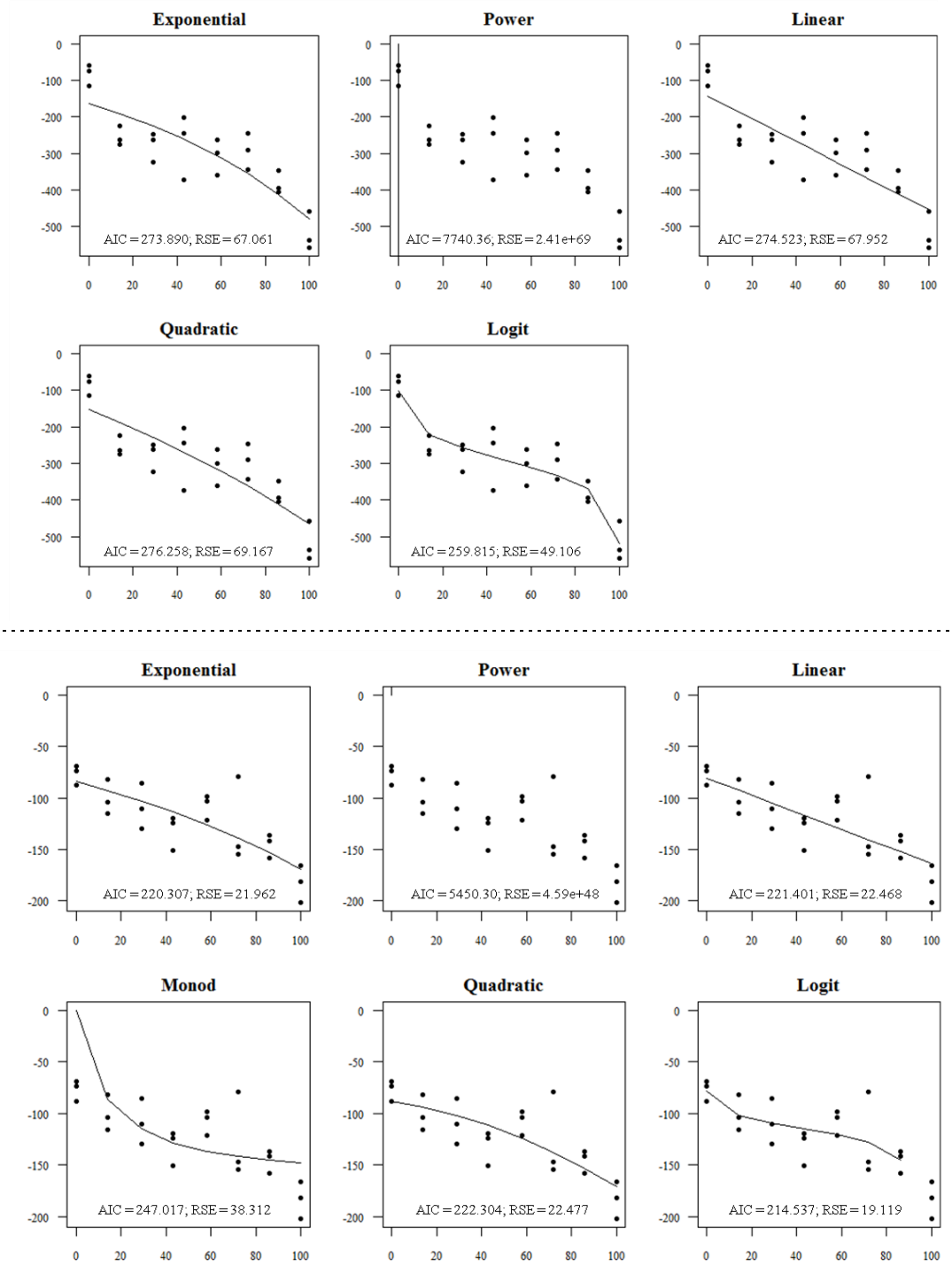
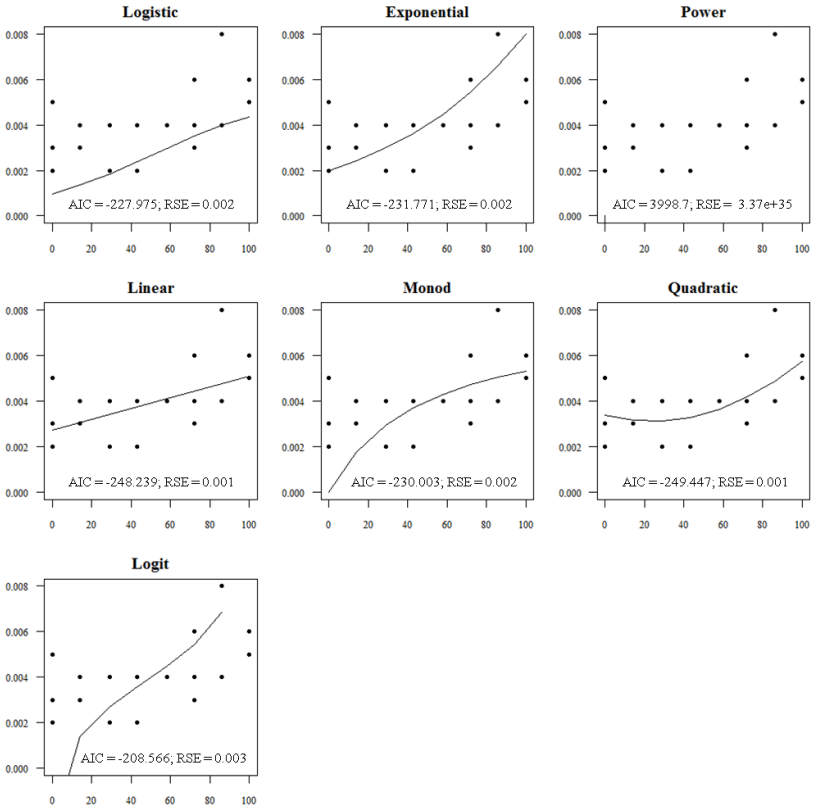


Figure S7. Fitted models for benthic community respiration (CR) during the exposure phase (5 figures above dotted line) and the recovery phase (6 figures below dotted line). Labels on X axis denote the CR (mg O_2 m^{-2} h^{-1}) and the labels on Y axis denote the gradient of effluent dilution (%). Each figure is computed with its corresponding Akaike Information Criterion (AIC) and Relative Standard Error (RSE) values.



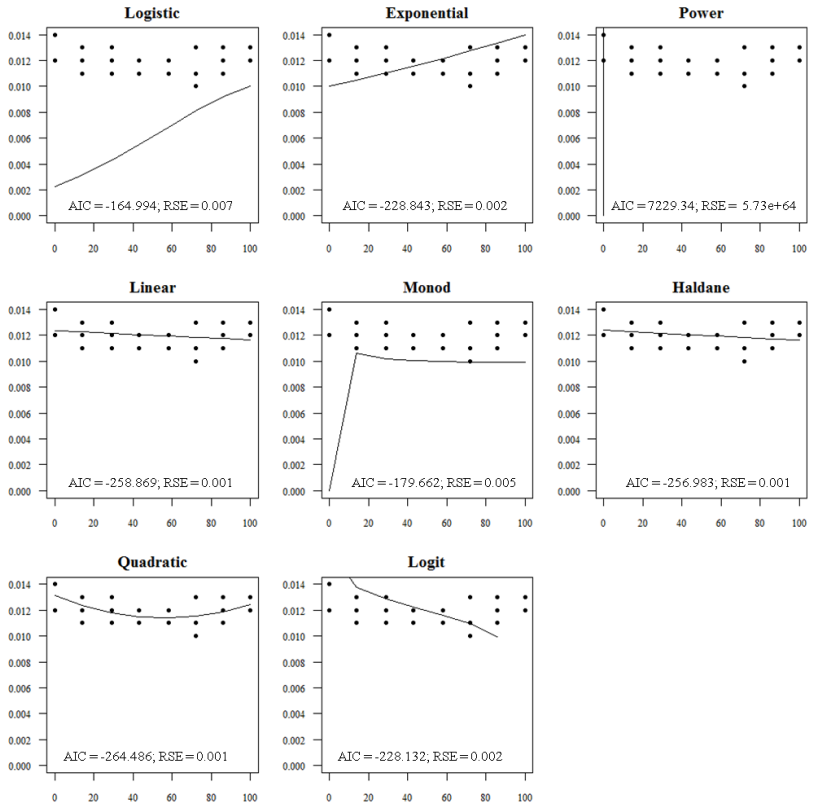


Figure S8. Fitted models for organic matter decomposition (OMD) during the exposure phase (7 figures above dotted line) and the recovery phase (8 figures below dotted line). Labels on X axis denote the rates of OMD (d^{-1}) and the labels on Y axis denote the gradient of effluent dilution (%). Each figure is computed with its corresponding Akaike Information Criterion (AIC) and Relative Standard Error (RSE) values.

Appendix S2

Appendix S2. R Code. Immediate and legacy effects of urban pollution on river ecosystem functioning: a mesocosm experiment by Olatz Pereda, Vicenç Acuña, Daniel von Schiller, Sergi Sabater and Arturo Elosegi.

```
#########################################################################
#                                                                       #
# Title: "Immediate and legacy effects of urban pollution on river      #
#          ecosystem functioning: A mesocosm experiment"                #
#                                                                        #
#                                                                        #
# Authors: O. Pereda, V. Acuña, D. von Schiller, S. Sabater &           #
#          A. Elosegi                                                   #
#                                                                        #
# Appendix S2: Supplementary material (R Coding)                        #
#                                                                       #
# Data analysis was carried out by O. Pereda*, Vicenç Acuña and         #
# Carme Font                                                            #
# *email: olatz.pereda@ehu.eus                                          #
#########################################################################

# Data analysis was carried out using R software v.3.4.0. (R Core Team.
# 2017. R: A language and environment for statistical computing. version
# 3.4.0. R Foundation for Statistical Computing, Vienna, Austria.

# This file was created using Tinn-R Editor [Faria J.C., Grosjean P.
# & Jelihovschi E. (2014). Tinn-R Editor - GUI for R Language and
# Environment. Tinn-R Editor is free software.

# R packages
# **********

# Pinheiro, J., D. Bates, S. DebRoy, D. Sarkar, and R-Core-Team. 2018.
# nlme: Linear and Nonlinear Mixed Effects Models. R package version 3.1-137.
# install.packages("nlme")                    # run if needed
library(nlme)

# CONTENTS OF THIS SCRIPT:
# ************************
# 1. Step 1: Selection of the most appropriate model to explain the
#             observed patterns in ecosystem functions measured in
#             artificial stream channels #
# ********************************************************************** #
# ********************************************************************** #

##########
# Step 1 #
##########
selection_model<-function(){ # Load package 'nlme' library("nlme")

  # 1.1. Load and prepare the dataset
  # *********************************
  # Set up working directory (change as required)
  # Setwd("C:/.../DataAnalysis")
  # Load the dataset (change the mane as required)
  name_file<-"SRP_BioboxCubes.csv"
```

```
# ************************************************************
# Herein, just for brevity, we demonstrate data analysis
# for just one group of the functional variables.
# The same steps were repeated for the other groups of variables:
# "Metabolism.csv" and "LeafLitter_Decomposition.csv".
# ************************************************************

# Include names of the variables names
if(name_file == "SRP_BioboxCubes.csv")
  var_names <- c("USRP",  "Chla", "AFDM", "USRPAFDM", "ELAPA", "ESAPA")
if(name_file == "Metabolism.csv")
  var_names <- c("CR",  "GPP",  "NM", "PR", "CRAFDM", "GPPChla",
                 "ChlaES", "ChlaEL",  "AFDMES", "AFDMEL")
if(name_file == "LeafLitter_Decomposition.csv")
  var_names <- c("k_day", "ConsumedAFDM")
# ************************************************************

# Read data from file
data<-read.csv(name_file, header=TRUE, sep=";")

if(name_file=="SRP_BioboxCubes.csv"){ i=which(data$Date=="T22")
  j=which(data$Date=="T56")
  data<-data[c(i,j),]
}

n<-length(var_names) # Number of variables
phase<-c("Exposure", "Recovery")

for(l in 1:length(phase)){
  file<-subset(data, data$Phase==phase[l]) X<-file$Treatment # Define X
  values
  N<-length(X) # Sample size

  for(i in 1:n){ # For each variable
    # Column in file corresponding to var i
    j<-which(colnames(file)==var_names[i])
    # Define variable Y
    Y<-file[,j]
    # Total sum of squares
    SST<-sum((Y-mean(Y))**2)

    # File name with the results of each model
    if(name_file == "Metabolism.csv")
      file_name <- paste0("results_Metabolism/", phase[l], "_model_",
                          var_names[i], ".txt")
    if(name_file == "SRP_BioboxCubes.csv")
      file_name <-paste0("results_SRP/", phase[l], "_model_",
                          var_names[i], ".txt")
    if(name_file == "LeafLitter_Decomposition.csv")
      file_name <- paste0("results_Decomposition/", phase[l],
                          "_model_",
                          var_names[i], ".txt")
    # File name for the figures of the models
    if(name_file == "Metabolism.csv")
      plot_name <- paste0("results_Metabolism/", phase[l],
                          "_regressions_plot_",
                         var_names[i], ".png")
    if(name_file == "SRP_BioboxCubes.csv")
      plot_name <- paste0("results_SRP/", phase[l],
                          "_regressions_plot_",
                          var_names[i], ".png")
```

```
if(name_file == "LeafLitter_Decomposition.csv")
   plot_name<-paste0("results_Decomposition/", phase[l],
                     "_regressions_plot_",
                     var_names[i], ".png")
# From now, all plots are saved in this file
# It can also be pdf, jpeg, tiff or bmp
png(plot_name, width=1000, height = 1000, res=100)
if(name_file == "SRP_BioboxCubes.csv" & var_names[i] == "AFDM")
   par(mfrow=c(4,3),mar=c(3,4,2,0), las=1, cex=1, cex.lab=1,
       font.lab=1,
       cex.axis=0.8, family="serif")
else
   par(mfrow=c(3,3),mar=c(3,4,2,0), las=1, cex=1, cex.lab=1,
       font.lab=1,
      cex.axis=0.8, family="serif")

# Writes first line in output file
cat(" Phase: ", phase[l], " Variable: ", var_names[i], "\n\n",
    file = file_name)
mod=NULL

# 1.2. Selection of the most appropriate model
# ********************************************

# a. Logistic
# ************
mod <- tryCatch(gnls(Y~a/(1+exp(-(X-X0)*b)), start=c(a=mean(c(Y[N-
2],
                           Y[N-1], Y[N])), b=1/sd(X), X0=mean(X))),
                           error=function(e) NULL )
if(!is.null(mod)) out<-func(mod, X, Y, "Logistic", k=3, SST, N,
file_name)
mod=NULL

# b. Exponential
# ***************
mod <- tryCatch(gnls(Y~a*exp(b*X), start=c(a=abs(min(Y)),
                     b=log(max(abs(Y))/abs(min(Y)))/max(X))),
                     error=function(e) NULL )
if(!is.null(mod)) out<-func(mod, X, Y, "Exponential", k=2, SST, N,
file_name)
mod=NULL

# c. Power
# *********
mod <- tryCatch(gnls(Y~a*X**b, start=c(a=mean(c(Y[N-2],
                     Y[N-1], Y[N])),
                     b=sd(X))), error=function(e) NULL )
if(!is.null(mod)) out<-func(mod, X, Y, "Power", k=2, SST, N,
   file_name)
mod=NULL

# d. Linear
# **********
mod <- lm(Y~X)

if(!is.null(mod)) out<-func(mod, X, Y, "Linear", k=2, SST, N,
   file_name)
mod=NULL
```

```
        # e. Monod
        # *********
        mod <- tryCatch(gnls(Y~a*X/(b+X),
                             start=c(a=sign(Y[which.max(abs(Y))])*max(abs(Y)),
                             b=mean(X))), error=function(e) NULL )
        if(!is.null(mod)) out<-func(mod, X, Y, "Monod", k=2, SST, N,
           file_name)
        mod=NULL

        # f. Haldane
        # ***********
        X[1:3]<-0.001 X2<-X**2

        mod <- tryCatch(nls(Y~a*X/(b+c*X+X2),

        start=list(a=sign(Y[which.max(abs(Y))])*mean(abs(Y[1:3])),
                         b=X[1]*X[1], c=mean(Y)), algorithm="port",
        lower=c(0,0,0)),
                         error=function(e) NULL )
        if(!is.null(mod)) out<-func(mod, X, Y, "Haldane", k=3, SST, N,
           file_name)
        mod=NULL
        X[1:3]<-0

        # g. Quadratic
        # *************
        mod <- lm(Y~X+X2)
        if(!is.null(mod)) out<-func(mod, X, Y, "Quadratic", k=3, SST, N,
           file_name)
        mod=NULL

        # h. logit
        # *********
        b = sign(mean(Y[(N-2):N])-mean(Y[1:3]))*1/sd(Y)
                  if(n>4 && var_names[i]=="CRAFDM") b=-2000
        mod <- suppressWarnings(tryCatch(gnls(Y~X0+1/b*log((X+1)/(a-(X+1))),
                                 start=start=list(X0=mean(Y),
                                 a=mean(c(X[N-2], X[N-1],
                                 X[N]))-1, b=b)),
                                 error=function(e) NULL ))
       if(!is.null(mod)) out<-func(mod, X, Y, "Logit", k=3, SST, N,
          file_name)
        mod=NULL

        dev.off() # Stop saving plots
      }
    }
}

func <- function(mod, X, Y, name_mod, k=2, SST, N, file_name){
  plot(X, Y, pch=20, main=name_mod, ylab="", xlab="", xlim=c(min(0,
       min(X)), max(0,max(X))), ylim=c(min(0, min(Y)), max(0,max(Y))))
    if(is.null(mod)==FALSE){
         lines(X, predict(mod)) # Regression line

     # Compute the fitness index for each model
     AIC_val <- AIC(mod)
     SSE <- sum((predict(mod)-Y)**2, na.rm=TRUE) # Sum of squared errors
     RSE_val <- sqrt(SSE/(N-k)) # Root (mean) squared errors
```

```
    SSR<-sum((mod$fitted-mean(Y))**2) #
    R2_val <-1-SSE/SST #linear and quadratic models

    # Writing the result in file
    cat("\n \n model: ", name_mod," \n", file=file_name, sep='',
        append=TRUE)
    a=mod$coefficients[1]
    if(is.null(a))
      a=summary(mod)$coefficients[1,1]
      b=mod$coefficients[2]
     if(is.null(b))
      b=summary(mod)$coefficients[2,1]
     if(name_mod=="Haldane"){
      c=summary(mod)$coefficients[3,1]
     if(is.null(c))
       c=summary(mod)$coefficients[3,1]

    #   a=a/c
    #   b=b/c
    }
    if(name_mod=="Quadratic")
    c=mod$coefficients[3]
    cat(" parameters: a = ", a, "; b = ", b, file=file_name, sep='',
          append=TRUE)
    if(name_mod=="Logistic" || name_mod=="Logit")
      cat(" ; X0 = ", mod$coefficients[3], file=file_name, sep='',
          append=TRUE)
    if(name_mod=="Haldane" || name_mod=="Quadratic")
      cat(" ; c = ", c, file=file_name, sep='', append=TRUE)
      cat("\n AIC = ", AIC_val, "; RSE = ", RSE_val,
        file=file_name, sep='', append=TRUE)
    if(name_mod=="Linear" || name_mod=="Quadratic")
       cat("; R^2 = ", R2_val,
        file=file_name, sep='', append=TRUE)
        list(AIC_val=AIC_val, RSE_val=RSE_val, R2_val=R2_val)

    residuals=predict(mod)-Y
    residuals=residuals[which(!is.na(residuals))]
    a="no-test"
    p=-1
    if(!is.na(var(residuals)) & var(residuals)>0){
      p=shapiro.test(residuals)$p.value if(p>=0.05) {
        a="norm"
      }
      if(p<0.05)    a="no-norm"
    }
    cat("\n residuals distr: ", a, ", p-value = ",p, " \n",
         file=file_name,
        sep='', append=TRUE)

  }
}

#  ************************************************************************ #
#  ************************ END OF THE SCRIPT ************************** #
```