

This PhD dissertation explored the effects of global change on the functioning of Mediterranean river ecosystems, which are severely impacted by human activities. For a broad spatial perspective, we measured the breakdown rate of sticks placed at 66 sites in 4 Iberian river basins (Ebro, Llobregat, Júcar and Guadalquivir), and related it with physico-chemical, biological and geomorphological characteristics of the sites. Breakdown showed high variability, especially in downstream sites, the peak breakdown rate being driven by temperature, phosphorus, land use, pollution, invertebrate communities, and riparian vegetation. We also studied flow regulation by reservoirs, one prevalent impact in the Mediterranean region. In a field experiment we measured the effects of flow regulation on organic matter accumulation and on ecosystem metabolism in 3 rivers. Reservoirs reduced flood events and promoted the accumulation of more benthic organic matter, thus fostering river metabolism. In another paper we studied the effects of chemical pollution from a wastewater treatment plant by measuring river ecosystem functioning upstream and downstream from the effluent. Most ecological variables measured showed a subsidy effect by the effluent, likely reflecting increased concentrations of nutrients and organic matter, but detailed analyses of the production-irradiance curves yielded evidence for some subtle stress effects on primary producers. Finally, we analyzed the interactions between assimilable and toxic pollutants in a manipulative experiment in artificial streams. Nutrients subsidized biological activity at all assessed concentrations, whereas emerging pollutants produced some toxic stress only after 4 weeks of exposure, indicating long-term toxic effects. Interestingly, high nutrient concentrations alleviated the stress effects of emerging pollutants. Overall, our results provide evidence of the important ecological effects of global change on Mediterranean rivers, and suggest important consequences for the society, in terms of ecosystem services.



Ibon Aristi de la Hoz · PhD dissertation 2015 Effects of global change on the functioning of Mediterranean Rivers

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Ibon Aristi 2015



eman ta zabal zazu



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Effects of global change on the functioning of Mediterranean Rivers

by

Ibon Aristi de la Hoz

2015



*Ibartxotik iberorako saltoa
hiretzat dek Pello*

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Summary

This PhD dissertation explored the effects of global change on the functioning of Mediterranean river ecosystems, which are severely impacted by human activities. For a broad spatial perspective, we measured the breakdown rate of sticks placed at 66 sites in 4 Iberian river basins (Ebro, Llobregat, Jucar and Guadalquivir), and related it with physico-chemical, biological and geomorphological characteristics of the sites. Breakdown showed high variability, especially in downstream sites, the peak breakdown rate being driven by temperature, phosphorus, land use, pollution, invertebrate communities, and riparian vegetation. We also studied flow regulation by reservoirs, one prevalent impact in the Mediterranean region. In a field experiment we measured the effects of flow regulation on organic matter accumulation and on ecosystem metabolism in 3 rivers. Reservoirs reduced flood events and promoted the accumulation of more benthic organic matter, thus fostering river metabolism. In another paper we studied the effects of chemical pollution from a wastewater treatment plant by measuring river ecosystem functioning upstream and downstream from the effluent. Most ecological variables measured showed a subsidy effect by the effluent, likely reflecting increased concentrations of nutrients and organic matter, but detailed analyses of the production-irradiance curves yielded evidence for some subtle stress effects on primary producers. Finally, we analyzed the interactions between assimilable and toxic pollutants in a manipulative experiment in artificial streams. Nutrients subsidized biological activity at all assessed concentrations, whereas emerging pollutants produced some toxic stress only after 4 weeks of exposure, indicating long-term toxic effects. Interestingly, high nutrient concentrations alleviated the stress effects of emerging pollutants.

Overall, our results provide evidence of the important ecological effects of global change on Mediterranean rivers, and suggest important consequences for the society, in terms of ecosystem services.

Laburpena

Tesi honetan ibai-ekosistema Mediterranearetako funtzionamenduan aldaketa globalaren efektuak ikertzen dira, ibai hauek giza-jardueren ondorioz zuzenki erasanak baitaude. Ikuspegi espazial zabal batean, 4 ibai iberiarretako (Ebro, Llobregat, Jucar eta Guadalquivir) 66 puntutan materia organikoaren deskonposiziotasak neurtu genituen, eta lekuan lekuko aldagai fisiko-kimikoekin, biologikoekin, eta geomorfologikorekin erlazionatu genituen. Deskonposizio tasek aldakortasun handia azaldu zuten oro har, ibaian beherako tokietan batik bat, tasa maximoa tenperaturak, fosforoak, arroaren areak, toxikotasunak, ornogabeek, ibar-basoen zabalerak eta diatomeoek azalduz. Era berean, urtegien bidezko emarien erregulazioa aztertu genuen, zeina inpaktu nagusienetako bat baita eskualde mediterranean. Landa-esperimentu batean erregulazioak materia organikoaren metaketen eta ekosistemen metabolismoaren gaineko eragina ikertu genuen hiru ibaitan. Urtegiek uhaldien maiztasuna gutxitu zuten, eta honek materia organikoaren metaketa bultzatu zuen, azkenik ibaiako metabolismoa emendatuz. Beste lan batean hondakin-uren arazketa-estazio batek sortutako kutsadura kimikoaren eragina aztertu genuen, ekosistemaren funtzionamendua efluetaren gaintetik eta azpitik neurtuta. Neurturiko aldagai ekologiko gehienek erakutsi zuten kutsadura kimikoak jarduera biologikoa areagotzen zuela, ziurrenik mantengai eta materia organikoaren gorakadak eragina, baina eginiko ekoizpen-irradiantzi kurben analisi zehatzagoek bestelako estres erantzun apal bat ere erakutsi zuten ekoizle primarioengan. Azkenik, mesokosmotan eginiko manipulazio-esperimentu batean, kutsatzaile asimilagarrien eta toxikoen elkarrekintzak neurtu genituen. Mantenuagiek jarduera biologikoa areagotu zuten neurturiko kontzentrazioa gutzietan, aldiz kutsatzaile emergenteek estres efektuak sortu zituzten 4. astean. Kontzentrazio altuetan mantenguaiek kutsatzaile emergenteen efektu toxikoak arindu zituzten.

Oro har, gure emaitzek aldaketa globalak ibai mediterranean dituen efektu ekologiko garrantzitsuak azpimarratzen dituzte, eta bide batez, honek gizartean, zerbitzu ekosistemikoei dagokienean behintzat, izan ditzakeen ondorioen garrantzia iradoki.

Resumen

Esta tesis explora los efectos del cambio global en el funcionamiento de los ecosistemas de ríos mediterráneos, que se ven severamente impactados por las actividades de los humanos. Para tener una perspectiva espacial amplia, medimos la tasa de descomposición de palitos colocados en 66 puntos de 4 ríos ibéricos (Ebro, Llobregat, Júcar y Guadalquivir), relacionándola con características físico-químicas, biológicas y geomorfológicas de cada sitio. La descomposición mostró gran variabilidad, especialmente en puntos bajos de los ríos, estando la tasa máxima relacionada con la temperatura, el fósforo, el área de cuenca, la toxicidad, los invertebrados, la anchura de la zona riparia y las diatomeas. También estudiamos el efecto de la regulación del caudal por embalses, un impacto común en la región mediterránea. En un estudio de campo medimos los efectos de la regulación en la acumulación de materia orgánica y en el metabolismo del ecosistema en 3 ríos. Los embalses redujeron la frecuencia de las crecidas y promovieron la acumulación de materia orgánica, que potenció el metabolismo fluvial. En otro trabajo estudiamos los efectos de la contaminación química producida por una estación depuradora de aguas residuales, midiendo el funcionamiento de los ecosistemas riparios aguas arriba y abajo del efluente. La mayoría de variables analizadas mostraron un efecto de subsidio, que probablemente reflejaba el incremento en la concentración de nutrientes y materia orgánica, mientras que análisis detallados de las curvas de producción-irradiación mostraron evidencias de ligero estrés en productores primarios. Finalmente, analizamos la interacción entre contaminantes asimilables y tóxicos en un experimento manipulativo en mesocosmos. Los nutrientes incrementaron la actividad biológica en todas las concentraciones medidas, mientras que los contaminantes emergentes produjeron algunos efectos de estrés tras 4 semanas de exposición. Los nutrientes a concentraciones altas aliviaron el efecto tóxico de estos emergentes. En general, nuestros resultados proporcionan evidencias de los importantes efectos ecológicos del cambio global en los ríos mediterráneos y sugieren también consecuencias en la sociedad, en términos de servicios ecosistémicos.

Contents

Chapter 1: General introduction.....	25
Chapter 2: Assessing the effects of multiple stressors on the functioning of Mediterranean rivers using poplar wood breakdown.....	35
Chapter 3: Flow regulation by dams affects ecosystem metabolism in Mediterranean rivers.....	51
Chapter 4: Mixed effects of effluents from a wastewater treatment plant on river ecosystem metabolism: subsidy or stress?	71
Chapter 5: Does subsidy alleviate stress? The effects of emerging pollutants on stream biofilm communities depend on nutrient concentration.....	91
Chapter 6: General discussion.....	107
Chapter 7: General conclusions.....	113
Chapter 8: References.....	117

CHAPTER 1

General introduction



Human population and per capita use of resources have been rising dramatically in the last centuries, resulting in environmental change of global proportions (Vitousek, 1994; United Nations Environment Programme, 2007) and affecting every ecosystem on earth (Millennium Ecosystem Assessment, 2005). This change is so deep that Crutzen (2002) coined the term *Anthropocene* to refer to it, meaning that humans are now the dominant force determining the sediment record. As a result of this global environmental change, large effects are expected in river ecosystem functioning (Rockström *et al.*, 2009), which is the base of many ecosystem services essential for humans (Sweeney *et al.*, 2004; Millennium Ecosystem Assessment, 2005).

Streams and rivers are among the most affected ecosystems (Dudgeon, 2010), especially those in highly populated areas with a shortage of water availability

(Vörösmarty *et al.*, 2010), such as the Mediterranean region (Fig. 1), where they are subject to multiple pressures including flow regulation, water abstraction, pollution, changes in channel form, modification of riparian areas, and invasive exotic species (Sabater, 2008; Ricart *et al.*, 2010). These pressures can be seen as stressors *sensu* Auerbach (1981), as they result from human activity, exceed the range of normal variation of rivers, and adversely affect individual taxa, community composition or ecosystem functioning. Many ecosystems are influenced simultaneously by multiple stressors (Munns, 2006; Niyogi *et al.*, 2007; Couillard *et al.*, 2008), and Mediterranean rivers are a paradigmatic example. Unfortunately, their complex interactions make the joint consequences of multiple stressors unpredictable on the basis of knowledge of single effects (Preston, 2002; Townsend, Uhlmann & Matthaei, 2008).

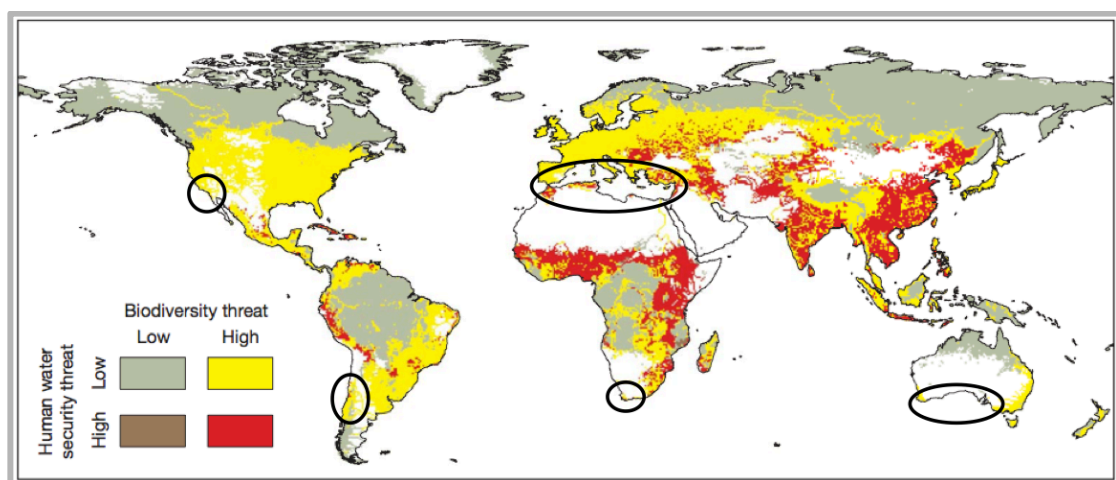


Figure 1. Patterns of threat to human water security and river biodiversity. Mediterranean climate regions are located within the black circles. Adapted from Vörösmarty *et al.* (2010).

Mediterranean climate is characterized by highly seasonal precipitation and temperature, with hot, dry summers, and cool, wet winters, which results in rivers subject to severe summer drought and strong winter floods (Gasith & Resh, 1999). In a sense, they resemble temperate rivers during wet periods but desert rivers during summer (Romaní & Sabater, 1997; Bernal *et al.*, 2013). At the same time, they show unique ecological patterns and produced by a well-defined and predictable seasonality on water availability (Hershkovitz & Gasith, 2013). These streams and rivers are ecologically unique, as Mediterranean climate regions are hotspots of biodiversity with high rates of endemism (Myers *et al.*, 2000; Smith & Darwall, 2006; Bonada, Rieradevall & Prat, 2007).

Future climate scenarios for Mediterranean climate regions predict an increase of drought conditions and of the occurrence of extreme events (International Panel on Climate Change, 2007). Warming will be larger than the global average for Mediterranean regions and annual precipitation will decrease (Cayan *et al.*, 2006; Christensen *et al.*, 2007; Giorgi & Lionello, 2008; Giannakopoulos *et al.*, 2009). Annual runoff is expected to decrease too (Milly, Dunne & Vecchia, 2005; Milliman *et al.*, 2008), mainly as a consequence of increased evapotranspiration (Tague, Seaby & Hope, 2009; Buendía *et al.*,

2015). Besides, strong seasonal variability in flow makes Mediterranean rivers especially vulnerable to pollution and water extraction during the dry seasons (Cooper *et al.*, 2013; López-Doval *et al.*, 2013).

Flow regime is far from natural in most rivers, as dams modify the quantity, timing and variability of water discharge (Poff & Allan, 1995). Today, 15% of the world's total runoff ($40,000 \text{ km}^3 \text{ y}^{-1}$) is retained in *ca.* 45,000 large dams (>15 m in height, (Nilsson *et al.*, 2005)), which also retain 20% of the global sediment flux (Syvitski *et al.*, 2005). Dams usually reduce the magnitude and frequency of extreme flow events (Richter *et al.*, 2003), therefore affecting hydraulics and physical habitat, and exert strong effects on aquatic organisms (Poff & Allan, 1995; Allan & Castillo, 2007). The regions with a Mediterranean climate are strongly affected by flow regulation (Nilsson *et al.*, 2005) because of the large abundance of dams, and because their biota is adapted to their highly seasonal regime, which is disrupted by regulation (Gasith & Resh, 1999; Bernal *et al.*, 2013; Bonada & Resh, 2013). In particular, the Iberian Peninsula hosts one fifth of all European reservoirs (Avakyan & Iakovleva, 2006), and has the largest number of dams per inhabitant and per land area in the world (García de Jalón, 2003). The effects of flow regulation on river biota are relatively well known (Ward, Tockner & Schiemer, 1999;

Jansson, Nilsson & Renöfält, 2000; Nilsson & Svedmark, 2002; Santos *et al.*, 2004; Mueller, Pander & Geist, 2011; Martínez *et al.*, 2013; Ponsati *et al.*, 2014), but little is known on its effects on river ecosystem functioning.

In addition to flow regulation, many stressors increasingly impair Mediterranean rivers, where strong droughts increase the effects of pollution (López-Doval *et al.*, 2013). Pollution can reach rivers from both point and diffuse sources (Carter, 2000), and often appear in complex mixtures whose joint effects can have contrasting effects (*e.g.* Culp, Podemski & Cash, 2000). The effluents of wastewater treatment plants (WWTP) are clear examples of complex mixtures of pollutants reaching river ecosystems, as they include nutrients, organic matter, metals, pesticides, and other contaminants such as emerging pollutants (Ternes, 1998; Petrovic *et al.*, 2002; Gros, Petrović & Barceló, 2007), and are another common impact on river ecosystems (Bernhardt & Palmer, 2007; Grant *et al.*, 2012), especially in conurbations (United Nations Population Division, 2006).

River ecosystems have multiple functions, which can be measured with a varied array of techniques, change at different spatial and temporal scales, and respond to environmental stressors in specific manners (*e.g.* Bunn & Davies, 2000; Young, Matthaei & Townsend, 2008; Elosegi, Díez & Mutz, 2010;

Elosegi & Sabater, 2013). Commonly measured ecosystem functions include decomposition of organic matter, nutrient retention and river metabolism (*e.g.* von Schiller *et al.*, 2008), which are integrative measures of river ecosystem functioning (Graça, 2001; Hieber & Gessner, 2002; Enquist *et al.*, 2003), and respond to human activities (Fellows *et al.*, 2006; Newbold *et al.*, 2006) at different scales (Houser *et al.*, 2005; von Schiller *et al.*, 2007).

Organic matter decomposition is an important process, as allochthonous detritus are the main energy source supporting many stream food webs (Fishers & Likens, 1973; Webster *et al.*, 1999). Decomposition is a complex process involving many stages (Gessner, Chauvet & Dobson, 1999; Tank *et al.*, 2010). Immediately after immersion, and mainly during the first 24 h, many soluble compounds are leached from leaves, which can loss up to 30% of their mass (Petersen & Cummins, 1974). Later on, leaves are colonized and conditioned by microbes, what increases their palatability for detritivores, the chemistry and the structure of the material is modified as a consequence of enzymatic and mechanical activity (Bärlocher, 1985). It is especially important the fact that microorganisms can use dissolved nutrients from the water column whereas they use the organic matter as a source of carbon (Stelzer, Heffernan & Likens, 2003). Therefore, the

leaves that are colonized by microbes have a higher overall content of nutrients, especially nitrogen and phosphorus, which is one of the main reasons for their enhanced palatability (Danger *et al.*, 2012). Finally, shredders fragment the material enhancing the surface-to-volume ratio, and thus, promoting further colonization by microbes (Cummins, 1974). Therefore, there is a positive feedback loop between microbial and invertebrate activities (Bergfur *et al.*, 2007; Greenwood *et al.*, 2007). Thus, litter decomposition can be affected by many factors such as temperature (Chauvet & Suberkropp, 1998), nutrients (Suberkropp & Chauvet, 1995), pH (Dangles *et al.*, 2004) or physical abrasion (Ferreira *et al.*, 2006).

Decomposition plays a pivotal role in river ecosystems, as it is the initial step for detritic pathways, what led Gessner and Chauvet (2002) to advocate the use of leaf litter to measure ecosystem functioning. Nevertheless, preparing leaf litter bags is very time-consuming (Arroita *et al.*, 2012), and variable litter quality can mask environmental patterns (Sariyildiz & Anderson, 2003). This situation led several authors to propose the use standardized substrates instead, such as cellulose bands (Rulik, Zavrelova & Duchoslav, 2001), cotton strips (Tiegs *et al.*, 2007), calico (Imberger, Thompson & Grace, 2010), or wooden sticks (Arroita *et al.*, 2012). Besides, wood is also a common material

in freshwater ecosystems, and its breakdown responds to water temperature, eutrophication and pollution in the same way as leaf breakdown (Díez *et al.*, 2002; Stelzer *et al.*, 2003; Tank & Dodds, 2003; Gulis *et al.*, 2004; Arroita *et al.*, 2012).

Ecosystem metabolism is another integrative measure of river ecosystem functioning since it summarizes the outcome of energy and material fluxes through ecosystems (Enquist *et al.*, 2003). The combination of gross primary production (GPP) and ecosystem respiration (ER) can be measured fairly accurately and is meaningful across all types of streams and rivers (Tank *et al.*, 2010). GPP is controlled by light availability (Bunn, Davies & Mosisch, 1999; Hill, Mulholland & Marzolf, 2001), temperature (Gillooly *et al.*, 2001), and nutrient concentration (Hill *et al.*, 2000; Mulholland *et al.*, 2001), whereas ER is mainly controlled by temperature (Gillooly *et al.*, 2001; Acuña *et al.*, 2008; Yvon-Durocher *et al.*, 2012) and availability of organic matter (Young & Huryn, 1999; Acuña *et al.*, 2004). Besides, the changes in ecosystem metabolism are important, as they reflect changes in energy transfer and organic carbon fluxes along fluvial networks (Battin *et al.*, 2008).

River ecosystem metabolism can be measured using changes in dissolved oxygen concentration in closed chambers or in open channels (Odum, 1956;

Reichert, Uehlinger & Acuña, 2009). For the chamber method, a portion of the stream community (*i.e.* a cobble covered by biofilm) has to be placed inside a chamber, which often makes it impossible to obtain accurate measurements of reach-scale metabolism. On the other hand, open-channel measurements are strongly dependent on reliably estimating the exchange of gas between water and air (which depends on the reaeration coefficient). There are a variety of methods to estimate the reaeration coefficient, but can be summarized in empirical methods (Edwards & Owens, 1965; Tsivoglou & Neal, 1976; Generoux & Hemond, 1992), in the night-time regression method (Hornberger & Kelly, 1975) or in gas-injection methods (Wanninkhof, Mulholland & Elwood, 1990; Jin *et al.*, 2012). Some studies compared chambers with open systems and have found that open-system methods generally provide higher estimates (Bott *et al.*, 1978, Marzolf *et al.*, 1994; Aristegi, Izagirre & Elozegi, 2010). Chambers can limit the availability of nutrients and thus affect primary production estimates (Bott *et al.*, 1997), whereas underestimation of respiration can be due to the exclusion of macrofauna and hyporheos (Hendricks, 1993; Marzolf *et al.*, 1994). Some other studies have compared measurements of both single and two-station methods showing strong concordance among them (*e.g.* Young & Huryn, 1999).

All these functional indicators give us an integrated view of ecosystem functioning, and thus, complement structural metrics to assess ecosystem integrity (Minshall, 1996; Matthews *et al.*, 1982; Bunn & Davies, 2000; Gessner & Chauvet, 2002). Besides, ecosystem functioning can be affected by multiple stressors, like changes in nutrient concentration (Gulis and Suberkropp, 2003), pollution (Niyogi *et al.*, 2001), altered hydrology (Datry *et al.*, 2011) or changes in land uses (Hladyz *et al.*, 2011), thus giving a useful tool to question and understand the human induced perturbations and their effects on the freshwater ecosystems.

According to the “Subsidy-Stress hypothesis” (Odum, Finn & Franz, 1979) some environmental factors can promote biological activity at low levels but reduce it beyond a threshold, whereas others are always detrimental to organisms, their impact rising with their intensity or concentration (Fig. 2). In this framework, assimilable contaminants such as nutrients would be classified in the first group, as they promote biological activity up to a threshold (Sutton *et al.*, 2011), whereas toxic contaminants (*e.g.* heavy metals, pesticides) would be classified in the second group, as they are always detrimental. Both, assimilable and toxic contaminants affect river ecosystem functioning (Woodcock & Huryn, 2005; Izagirre *et al.*, 2008; Bundschuh *et al.*,

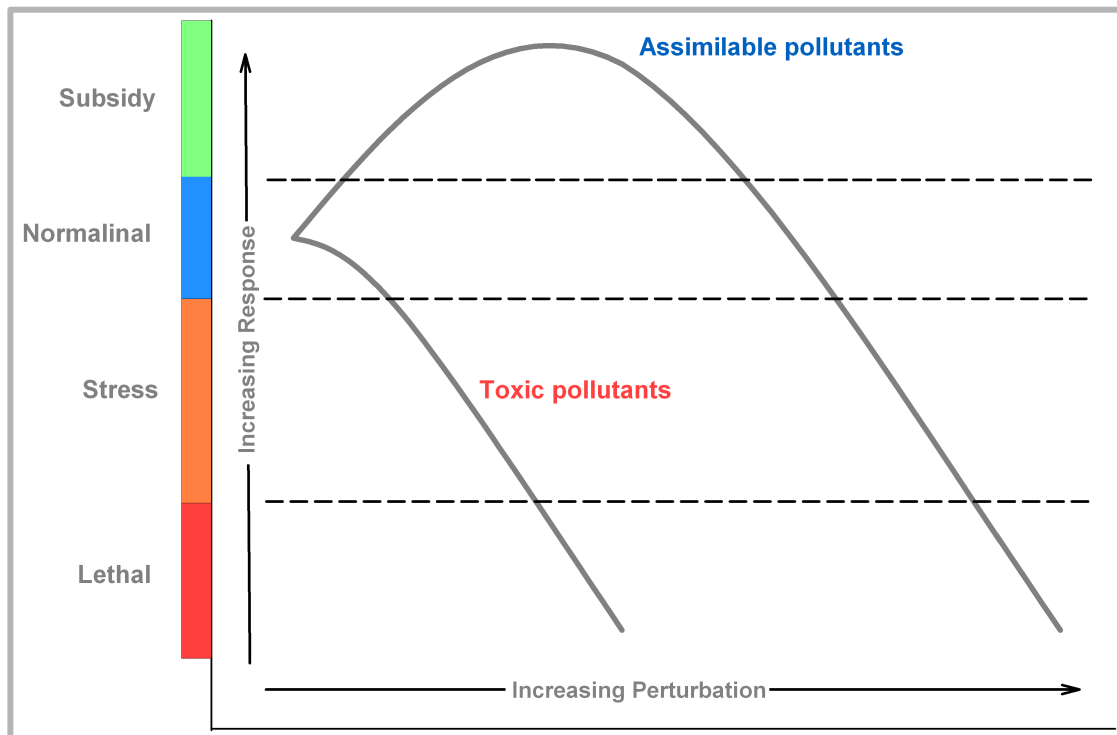


Figure 2. Hypothetical performance curve for a disturbed ecosystem subjected to two kinds of inputs. Adapted from Odum, Finn & Franz, 1979.

2009; Bernot *et al.*, 2010; Moreirinha *et al.*, 2011; Cabrini *et al.*, 2013; Rosi-Marshall *et al.*, 2013). Therefore, and depending of their mixed composition and the resulting concentrations on rivers, WWTP effluents can act either as a subsidy or a stress for the receiving ecosystems (Cardinale, Bier & Kwan, 2012), in ways that are difficult to predict.

Currently, most information available on freshwater ecosystems focuses on the environmental effects of single stressors (Maltby, 1999), and ignores the interactions that can occur in the complex mixtures of contaminants often found in nature. Contaminants can interact in a

synergistic or antagonistic way, either directly (when acting on the same target) or indirectly (when acting on different targets) (Coors & Meester, 2008). It has been suggested that moderate nutrient concentrations can improve the physiological status of organisms, and thus, reduce their sensitivity to toxic contaminants (Guasch *et al.*, 2004; Morin *et al.*, 2010a), although there is still little empirical evidence for this hypothesis. Clearly, more manipulative experiments are needed to disentangle individual from combined effects of stressors (Townsend, Uhlmann & Matthaei, 2008; Friberg, 2010).

This dissertation is focused on how anthropogenic stressors affect the functioning of Mediterranean rivers, by combining field and laboratory experiments and by measuring the response at different levels, from the biofilm to the whole ecosystem. We try to answer to the following questions:

1. How does organic matter processing vary across Mediterranean rivers of the Iberian Peninsula, and what are the main drivers for these changes?
2. Does flow regulation affect the storage of organic matter and river ecosystem metabolism?
3. What is the effect of WWTP effluents on the functioning of river ecosystems?
4. Does nutrient concentration alleviate the toxicity of emerging contaminants to biofilm?

CHAPTER 2

Assessing the effects of multiple stressors on the functioning of Mediterranean rivers using poplar wood breakdown

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Damià Barceló & Arturo Elosegi

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Abstract

Mediterranean rivers in the Iberian Peninsula are being increasingly affected by human activities, which threaten their ecological status. A clear picture of how do these multiple stressors affect river ecosystem functioning is still lacking. We addressed this question by measuring a key ecosystem process, namely breakdown of organic matter, at 66 sites distributed across Mediterranean Spain. We performed breakdown experiments by measuring the mass lost by wood sticks for 54 to 106 days. Additionally, we gathered data on physico-chemical, biological and geomorphological characteristics of study sites.

Study sites spanned a broad range of environmental characteristics and breakdown rates varied fiftyfold across sites. No clear geographic patterns were found between or within basins. 90th quantile regressions performed to link breakdown rates with environmental characteristics included the following 7 variables in the model, in decreasing order of importance: altitude, water content in phosphorus, catchment area, toxicity, invertebrate-based biotic index, riparian buffer width, and diatom-based quality index. Breakdown rate was systematically low in high-altitude rivers with few human impacts, but showed a high variability in areas affected by human activity. This increase in variability is the result of the influence of multiple stressors acting simultaneously, as some of these can promote whereas others slow down the breakdown of organic matter. Therefore, stick breakdown gives information on the intensity of a key ecosystem process, which would otherwise be very difficult to predict based on environmental variables.

Keywords: organic matter breakdown, wood, multiple stressors, Mediterranean rivers

Introduction

Human population and per capita use of resources have been rising dramatically in the last centuries, resulting in environmental change of global proportions (Vitousek, 1994; IPCC, 2007; UNEP, 2007), which is affecting every ecosystem on earth (Millennium Ecosystem Assessment, 2005). Streams and rivers are among the most affected ecosystems (Dudgeon, 2010), especially those in highly populated areas with a shortage of water availability (Vörösmarty *et al.*, 2010), like the Mediterranean region. Mediterranean rivers are subject to multiple stressors including regulation, pollution, changes in channel form, modification of riparian areas, and invasive exotic species (Sabater, 2008;

Ricart *et al.*, 2010). The effects of these stressors are seldom additive, but usually interact in complex ways. As a result of global environmental change, large effects are expected in river ecosystem functioning (Rockström *et al.*, 2009), which is the base of many ecosystem services (Sweeney *et al.*, 2004; Millennium Ecosystem Assessment, 2005). Nevertheless, most of the information available on the response of river ecosystem functioning to environmental changes derives from studies of limited spatial extent. This fact occurs because measuring ecosystem functioning simultaneously at multiple sites needs either large teams (*e.g.* Bernot *et al.*, 2010) or large investments in monitoring stations (*e.g.* Izagirre *et al.*, 2008). Therefore, there are still large

uncertainties on the effect of multiple stressors on river ecosystem functioning.

River ecosystem functioning is not a simple variable. Ecosystems have multiple functions, which can be measured with a varied array of techniques, change at different spatial and temporal scales, and respond to environmental stressors in specific manners (*e.g.*, Bunn & Davies, 2000; Young, Matthaei & Townsend, 2008; Eloisegi *et al.*, 2010; Eloisegi & Sabater, 2013). Commonly measured ecosystem functions include nutrient retention and river metabolism (*e.g.* von Schiller *et al.*, 2008), processes which respond to human activities (Fellows *et al.*, 2006; Newbold *et al.*, 2006) at different scales (Houser *et al.*, 2005; von Schiller *et al.*, 2007), but which are time consuming and expensive to measure. Decomposition, usually measured as breakdown of particulate organic matter (mostly leaf litter), is another process commonly measured to assess the effects of environmental changes on ecosystem functioning (Robinson & Jolidon, 2005; Lecerf *et al.*, 2006; McKie & Malmqvist, 2009). Breakdown of organic matter is a complex process involving leaching of soluble compounds, physical abrasion, colonization by microbial bacteria and fungi, and fragmentation by invertebrate shredders (Tank *et al.*, 2010). Therefore, it can be affected by multiple stressors, like

changes in water nutrient concentration (Gulis & Suberkropp, 2003), pollution (Niyogi *et al.*, 2001), altered hydrology (Datry *et al.*, 2011) or changes in land uses (Hladyz *et al.*, 2011). Some of these stressors, like increased temperatures, tend to promote breakdown (Dang *et al.*, 2009), whereas others, like acidification, can slow it down (Dangles *et al.*, 2004), therefore making it difficult to predict breakdown at sites subject to multiple stressors.

Breakdown plays a pivotal role in river ecosystems, as it is the initial step for detritic pathways, what led Gessner & Chauvet (2002) to advocate the use of leaf litter to measure ecosystem functioning. Nevertheless, preparing leaf litter bags is time-consuming, litter quality can be quite variable (Sariyildiz & Anderson, 2003), and physical abrasion during floods can confound breakdown dynamics (Ferreira *et al.*, 2006). Thus, several authors proposed to use standardized substrates instead, such as cellulose bands (Rulik *et al.*, 2001), cotton strips (Tiegs *et al.*, 2007) or calico (Imberger *et al.*, 2010), as these materials are much less variable in their chemical composition and less prone to fragmentation than leaves (Egglisshaw, 1972; Tiegs *et al.*, 2007; Imberger *et al.*, 2010). Nevertheless, some of these alternative materials are alien to river ecosystems, and thus, the rate of their degradation cannot be easily translated

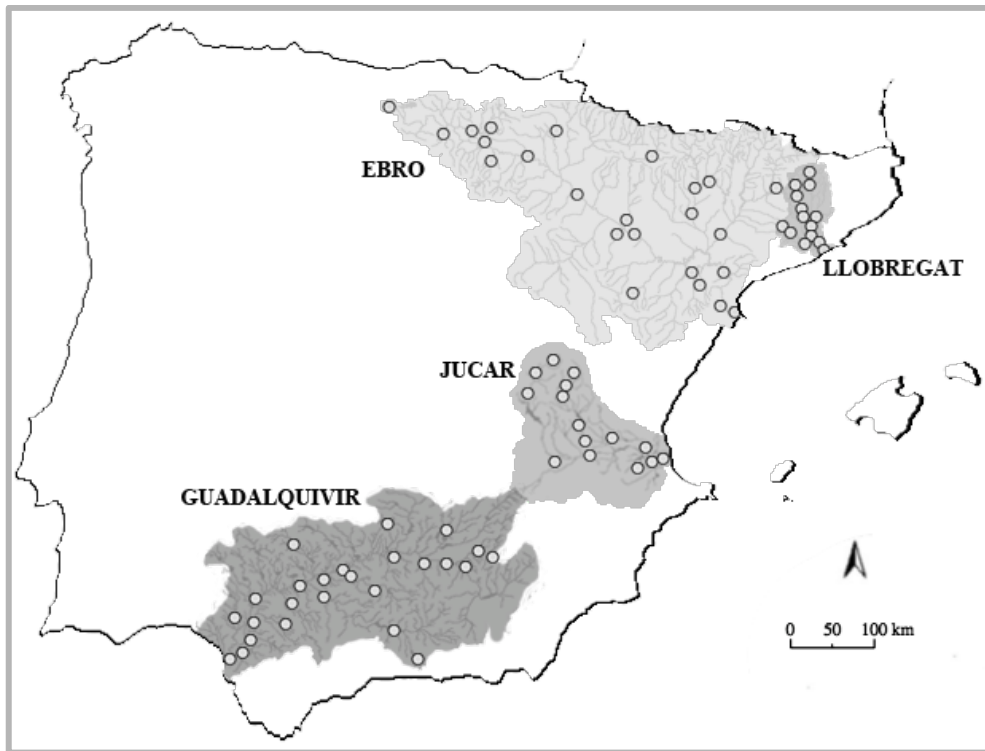


Fig. 1. Study sites and river basins in the Iberian Peninsula.

into the natural functioning of rivers.

A material that is common in most rivers, easy to handle, and useful to measure ecosystem functioning, is dead wood (Díez *et al.*, 2002). Therefore, authors have measured the breakdown of wood, in the form of entire logs (Ellis *et al.*, 1999), in the form of branches (Tank & Webster, 1998), or in the form of different types of sticks (Young *et al.*, 2008). Breakdown of wood, like that of leaf litter, is influenced by many factors, including physical and chemical properties of wood (Díez *et al.*, 2002), nutrients in water (Gulis *et al.*, 2004), and water temperature (Spänhoff & Meyer, 2004). Wood breakdown is considered a slow process (Hyatt & Naiman, 2001), but small pieces with high surface-to-volume ratio can suffer important breakdown in

short periods (Spänhoff & Meyer, 2004). Therefore, small wooden sticks can provide researchers a fast, easy, cheap and standardized tool to measure one important river ecosystem function (Young *et al.*, 2008; Arroita *et al.*, 2012).

Here we show results of one of the most extensive studies of breakdown published so far. We analysed the breakdown of wood sticks in 66 rivers spread across most of the Mediterranean Iberian Peninsula, thus representing a broad array of environmental conditions and degrees of anthropogenic stressors. Our hypothesis is that multiple stressors will affect breakdown rates in diverging directions, and thus, variance of breakdown rate will be higher at rivers most affected by human actions.

Material and Methods

Study area

This experiment was conducted in 4 river basins in the Iberian Peninsula: Ebro, Llobregat, Júcar and Guadalquivir, which together drain a large part of the Mediterranean Iberian Peninsula (Fig. 1). Climate in these basins is typically Mediterranean, with warm, dry summers and mild, humid winters, continentality increasing from east to west, and aridity from north to south. The long history of human settlements has created a highly heterogeneous mosaic of human land uses, ranging from near natural areas in the mountains to intensively cultivated agricultural lands or to areas with intensive industry and severe pollution. A total of 76 sites were selected for the SCARCE-Consolider project (www.idaea.csic.es/scarceconsolider): 24 in the Ebro, 14 in the Llobregat, 15 in the Júcar and 23 in the Guadalquivir. Most sites coincided with river reaches monitored by the Water Agencies for physico-chemical and biological characteristics, to have as much information as possible on each river reach.

Breakdown experiment

We measured the breakdown of tongue depressors (15 x 1.8 x 0.2 cm) made of

untreated Canadian poplar wood (*Populus nigra x canadiensis*, Moench). Sticks were individually tagged with a pirographer, dried (70 °C, 72 h) and weighed. Bunches of five depressors were tied with nylon filament to a coded plastic ring, and two weights were included to make bunches sink. Three bunches (totalling 15 sticks) were placed in each site in summer (June-July) of 2010 tied to metal bars, roots or boulders, and they were recovered after 54 to 106 days. In the laboratory, depressors were washed with tap water and brushed, dried (70 °C, 72 h) and ashed (500 °C, 5h) to get ash free dry mass (AFDM). Leaching of sticks was simulated in the laboratory, and initial ash content determined to correct initial dry masses. 11 sticks were recovered with missing tips that were not consumed but broken, probably by some boulder transported by the water. To estimate the loss we extrapolated the total mass of the stick from the bits recovered using the area as a reference.

Data treatment

Breakdown rates were calculated according to the negative exponential model (Petersen & Cummins, 1974). Variables that describe the physico-chemical, biological and geomorphological characteristics of the sites were acquired from different sources. Average channel width was calculated

from 5 transversal sections measured along 1 km of channel length from aerial photographs. Aerial photographs were also used to measure the width of the riparian vegetation in 1 km reaches. Altitude, channel slope, catchment area and land uses were determined from GIS layers with Quantum GIS. Water Agencies provided hydrological (discharge and/or water level), physical (temperature, pH, conductivity, suspended solids) and chemical parameters (water content in dissolved oxygen, ammonium and, phosphorus), measured following standard procedures (APHA, 1992). Water Agencies also provided data on biological quality, namely the diatom based IPS (Cemagref, 1982) and the macroinvertebrate-based IBMWP (Alba-Tercedor & Sánchez-Ortega, 1988) for each water mass. Average values were calculated from data available in the incubation period. When there were no available data for the incubation period (19% of the Ebro data, and 48% of the Guadalquivir), we used data from previous years as a proxy.

Additionally, coinciding with our experiments, the SCARCE-Consolider consortium analysed the concentrations of 111 priority or emerging organic pollutants in water, which included endocrine disruptors compounds, pesticides, perfluorinated compounds, UV filters and pharmaceuticals. According to criteria from the EU Directive 2009/90,

concentrations below the limit of quantification (LOQ) were considered half the LOQ value for each pollutant. Total toxicity of the pollutants was determined as Toxic Units (TU). Half maximal effective concentrations (EC_{50}) and median lethal doses (LC_{50}) for *Daphnia magna* (Cladocera, Crustacea) for 48h were collected from literature (mainly gathered from <http://sitem.herts.ac.uk/aeru/footprint/en/index.htm>; <http://cfpub.epa.gov/ecotox>). Data for EC_{50} were more abundant than data for LC_{50} , so we used the former as a toxicity reference in our study, as both variables were highly correlated ($r > 0.75$, $P < 0.001$). We assumed an additive toxicity of all pollutants and thus, estimated the maximal expected effect of the mixture (TU_{sum}) with the following formula (modified from Sprague, 1970):

$$Toxic\ Units\ Sum = TU_{sum} = \sum_{i=1}^n TU_i = \sum_{i=1}^n \frac{C_i}{EC_{50i}}$$

$$\log TU_{sum} = \log \sum_{i=1}^n TU_i$$

where C_i is the concentration of the compound i and EC_{50i} is the half maximal effective concentration of that pollutant for *D. magna*. When more than one EC_{50} values was found, we used the arithmetic mean of all found values. When no EC_{50} value was found for a specific pollutant, we did not take the pollutant into account.

Normality of all variables was tested by means of Shapiro tests, and departures corrected with transformations (log and

log+1) for statistical analyses. Linear relationships among variables were tested by Pearson correlations. For ordination analyses and multiple regressions we considered only 63 sites where there were no missing data. We performed a non-metric multidimensional scaling (NMDS) with normalized data to ordinate sites according to physical (2 variables), chemical (4 variables), geomorphological (9 variables) and biological (2 variables) characteristics, together with wood breakdown rates. Site descriptors could model the central tendency of the breakdown rate by means of multiple linear regressions. Nevertheless, we were concerned about the spurious results that

could emerge given the large number of variables used for modeling and the use of second-order equations, necessary as non-linear relationships have been reported for the relations rates and environmental variables (Lecerf *et al.*, 2006; Woodward *et al.*, 2012). These reasons, as well as the fact that measured variables might not explain the central tendency of breakdown rates but might instead limit their maximum values (see Cade & Noon, 2003; Woodward *et al.*, 2012), led us to model the breakdown rate with quantile regressions (Koenker, 2005). We considered the 90th quantile regression (the one that covers 90% of the data). Model selection was performed using Bayesian

Table 1. Average, minimum and maximum for the geomorphological, physical, chemical and biological descriptors of the study sites. The symbol "*" denotes variables that not used for multivariate analyses because of missing values.

<i>Variable</i>	<i>Unit</i>	<i>Average</i>	<i>Minimum</i>	<i>Maximum</i>
Area	<i>Km²</i>	9 437	11.3	84 474
Altitude	<i>m</i>	371.8	1	1179
Width	<i>m</i>	36.4	1.6	385
Slope	<i>m m⁻¹</i>	0.007	0.0001	0.085
Riparian Buffer	<i>m</i>	62.5	3.7	387.1
Urban	<i>%</i>	1.09	0	5.2
Agricultural	<i>%</i>	41.14	0	99.4
Forested	<i>%</i>	57.77	0	100
Discharge*	<i>m³ s⁻¹</i>	17.38	0.05	193
Depth*	<i>cm</i>	83.94	4.8	405.9
Temperature*	<i>°C</i>	19.87	10	26.8
Conductivity	<i>μS cm⁻¹</i>	1061	170	3194
pH		7.93	6	8.5
Turbidity*	<i>NTU</i>	35.13	4.87	35.13
Suspended solids*	<i>mg L⁻¹</i>	35	2	100
Dissolved oxygen	<i>mg L⁻¹</i>	7.54	3.16	14.47
Ammonium	<i>mg L⁻¹</i>	0.5	0.024	9.05
Phosphorus	<i>mg L⁻¹</i>	0.34	0.015	3.475
IBMWP		100.06	4	245
IPS		13	4.5	20
Pollutants	<i>ng L⁻¹</i>	1470	166.7	5 959
TUsum pollutants	<i>log TU</i>	-0.9	-3.6	0.3

Information Criteria (BIC) as a reference (Logan, 2010) and applying a stepwise procedure. First, the variable that showed the best relationship by means of a quantile regression with the processing rates (lowest BIC) was taken. This first variable was retained in the model, and was continued until the minimum second one that most reduced BIC was selected, and so on. No interaction among variables was considered in the model. This stepwise addition of new variables BIC was obtained (Logan, 2010). Temperature, turbidity and suspended solids were not used for this quantile regression, because of missing values.

Results

Site characteristics

Hydrological, physical and chemical characteristics varied enormously among sites (Table 1). Catchment area spanned 4 orders of magnitude, altitude ranged from sea level to 1179 meters, and channel width from 1.6 to 385 m. Slope also spanned 3 orders of magnitude, and riparian vegetation buffer width ranged from 3.7 m to 387 m. Dominance of land uses was very variable among catchments. Urban cover varied from 0 to 5.3%, agricultural cover from 0 to 99.2%, and the cover of forest and seminatural areas from 0 to 100%. Water chemistry of the study sites also displayed a large variation.

Discharge and water depth spanned 3 orders of magnitude. Temperature differed in more than 15 °C from the coldest to the warmest site, and conductivity ranged from 170 to 3194 $\mu\text{S cm}^{-1}$. pH ranged from 6 to 8.5, turbidity from 4.87 to 35.13 NTUs, and suspended solids from 2 to 100 mg L^{-1} . Dissolved oxygen changed from hypoxic sites with 3.16 mg L^{-1} to sites with 14.47 mg L^{-1} . Dissolved nutrients (nitrogen and phosphorous) spanned over 2 orders of magnitude. Biological indices also reflected the great variation of ecological conditions of the sites. IBMWP ranged from 4 (very poor water quality) to 245 (excellent), and IPS from 4.5 (poor) to 20 (excellent). Finally, total pollutant concentrations ranged from 116.7 to 5955.8 ng L^{-1} . The additive maximal expected effect of these pollutants, the TU_{sum} , also ranged from -3.6 to 0.3 in all sites.

Wood breakdown

Although tongue depressors were set in 76 sites, some were vandalized, some scoured by floods, and some simply lost. Therefore, we were able to recover the depressors only in 66 sites. The capacity of rivers to break down wood spanned a large range (Table 2): after the incubation period some sticks had lost 83% of the initial mass, whereas others only lost 1%. Consequently, breakdown rates ranged from 0.00034 to 0.01647 day^{-1} , and the

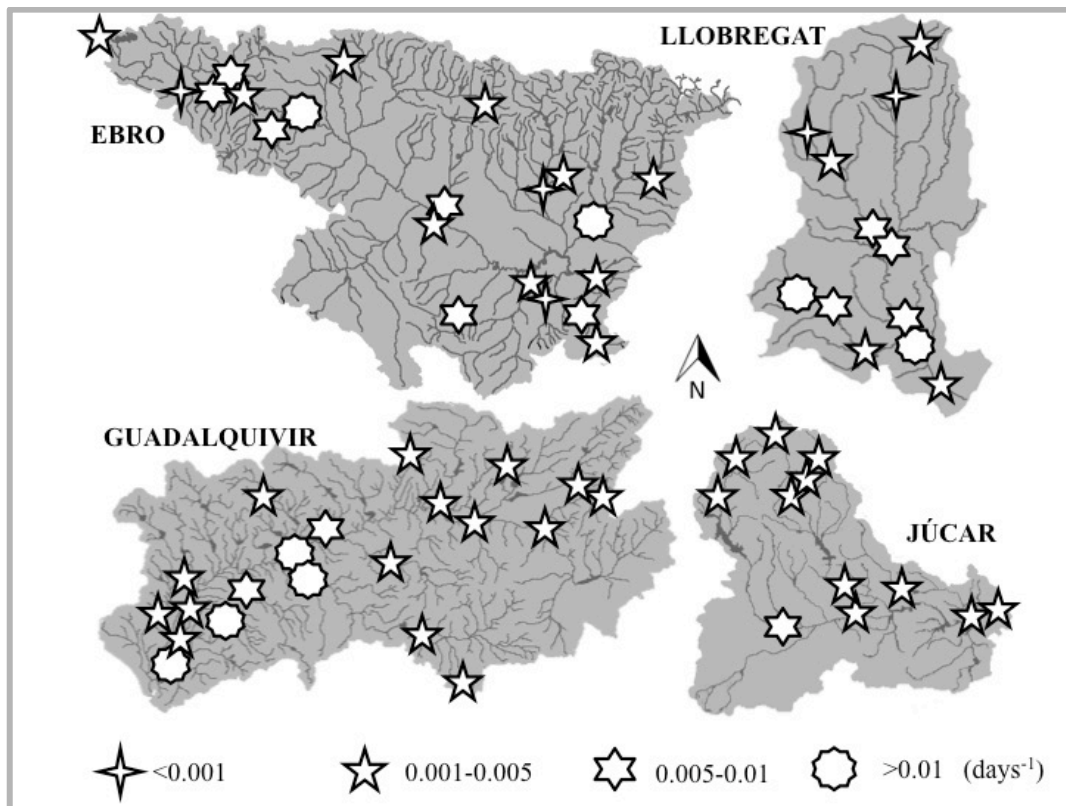
Table 2. Range of remaining mass (RM), decomposition rates (k) and time to lose 50% of the initial mass (T_{50}) for each basin.

	RM (%)	k (days ⁻¹)	T_{50} (days)
Ebro	17-99	0.00034-0.01457	48-2029
Llobregat	24-98	0.00087-0.01647	42-801
Jucar	38-94	0.00105-0.00511	136-661
Guadalquivir	17-99	0.00105-0.01672	41-663

time needed to lose 50% of the initial mass ranged from 42 to 2028 days. It is worth noting that with a few evident exceptions depressors were not fragmented. They retained their form, and those in most advanced decay state were thinner and softer, but not fragmented.

The distribution of breakdown rates showed no clear geographical pattern, neither between nor within basins (Fig. 2),

although some of the highest breakdown rates were measured in tributaries in the southern part of the Guadalquivir basin. NMDS ordination considering site characteristics revealed a high degree of overlap among the four basins, with a slight differentiation between the Guadalquivir and the Júcar and Ebro (Fig. 3). Breakdown rate was located close to agricultural and urban cover and to

**Fig. 2.** Breakdown rates (days⁻¹) of poplar wooden sticks incubated in the study sites. Ranges of breakdown rates were created according to Petersen and Cummins (1974), to which a new category (<math><0.001</math>) was added. Note maps are not in scale.

(Table 3). In general we observed a strong correlation among variables related to human impacts. As one moved toward the lowlands there was a clear increase in agricultural and urban soil uses, a decrease in water quality, and an increase in water temperature. Breakdown rate showed a significant relationship with some hydrological, physical and chemical variables (Table 3). Specifically, it was positively correlated to temperature, conductivity, turbidity, suspended solids, and total concentration of pollutants. On the contrary, breakdown was negatively related to altitude and IPS, but showed no significant relationship with IBMWP. Besides, breakdown rate was significantly related to land uses too, positively with agricultural cover and negatively to forested and semi natural cover.

The 90th quantile regression model included 7 variables, in the following order of importance: altitude, phosphorus, catchment area, TU_{sum}, IBMWP, riparian buffer width, and IPS (Table 4). Both linear and quadratic equations were included in the model. The variable that

was most significantly related to the breakdown rate, altitude, had a negative relationship: breakdown was consistently slow at high altitude sites whereas it showed the entire range of values at low altitude sites. Phosphorus showed a hump-shape relationship, with highest breakdown rates at intermediate concentrations, whereas catchment area and IPS were negatively related to breakdown rate. Finally, positive relationships with breakdown rate were observed for TU_{sum} and riparian buffer width. This model improved BIC value from -478.53 using one variable to -504.87 with 7 variables (Fig. 4).

Discussion

Dead organic matter is one of the main food resources in many streams and rivers (Wallace *et al.*, 1997; Abelho, 2001; Tank *et al.*, 2010). The river continuum concept (Vannote *et al.*, 1980) predicts that the contribution of terrestrial inputs of dead organic matter relative to the autochthonous production of organic

Table 4. Multiple quantile regression explaining the upper threshold (90th quantile) of the breakdown rate of poplar wooden sticks created by forward selection of variables taking BIC as a reference.

	Tendency	x	x ²	BIC
(Intercept -> 1.87 e ⁻²)				
Altitude	Negative	-2.64 e ⁻⁵	1.27 e ⁻⁸	-478.5
Phosphorus	Hump-shape	-4.95 e ⁻³	-6.41 e ⁻³	-488.5
Area	Negative	-2.40 e ⁻³	-8.89 e ⁻⁵	-489.9
TUsum pollutants	Positive	2.77 e ⁻³		-498.8
IBMWP	Hump-shape	8.37 e ⁻⁵	-3.14 e ⁷	-502.1
Riparian buffer	Positive	8.82 e ⁻³	-1.70 e ⁻³	-502.5
IPS	Negative	-3.03 e ⁻⁴		-504.9

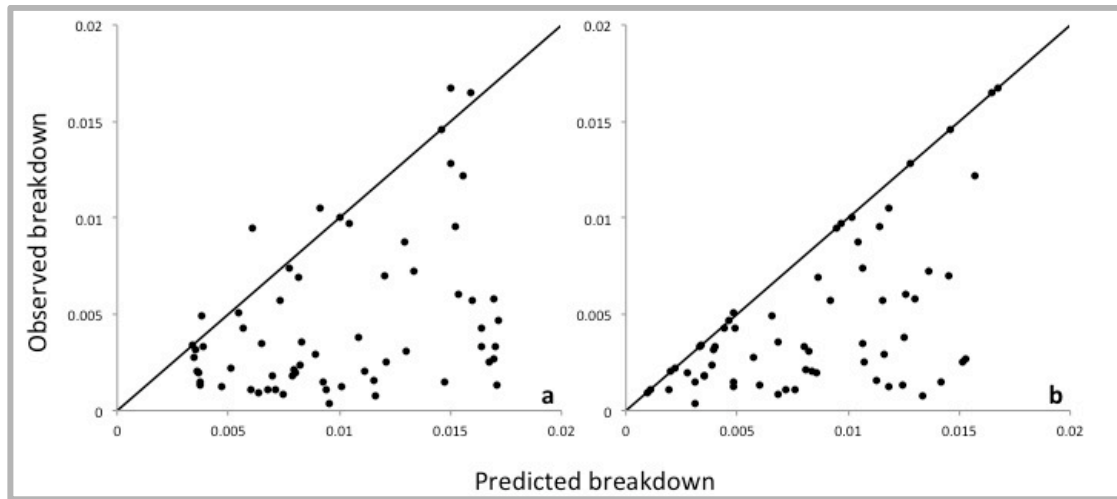


Fig. 4. Relationships between observed and predicted breakdown rates for 90th quantile regression models using the most significantly related variable (Altitude; a) and the variables accepted in the final model (listed in Table 3, b). 1:1 lines are displayed.

matter decreases downstream as the river channel widens, but even in large rivers dead organic matter coming from upstream sections can be an important food resource (Wipfli *et al.*, 2007). Therefore, the rate at which dead organic material is broken down can have important consequences for the flux of energy in food webs, and give insight on river ecosystem functioning (Gessner & Chauvet, 2002).

As mentioned in the introduction, breakdown of organic matter is usually measured by the leaf bag method. This method is expensive and time consuming, and bags incubated in the stream are negatively affected by floods, that cause increased physical fragmentation (Arroita *et al.*, 2012). The present experiment was performed in the worst conditions one could imagine for a breakdown experiment: sticks were deployed in the field when most rivers had a fairly high

discharge, were kept in place for 2 months, and recovered in many cases by a different team of people, sometimes also during high flows and with turbid water. Even so, we only lost all sticks at 10 of the 76 (13%), and were able to calculate breakdown rates at 66 sites. Thus, stick breakdown seem to be a robust method to assess functioning of river ecosystems, especially suitable for extensive surveys where more time-consuming variables like metabolism can hardly be measured (Young *et al.*, 2008). Also, stick breakdown integrates the effect of environmental conditions in the river for a period of one to several months, and thus, can give results that are more reliable than single-day measurements of metabolism, which are very variable (Izaguirre *et al.*, 2008).

Wood breakdown rates in Iberian streams spanned 4 orders of magnitude, thus showing large differences in

ecosystem functioning as a result of different environmental conditions. Iberian rivers, especially those draining to the Mediterranean, are among the most affected by environmental change, as they are subject to multiple stressors such as regulation, abstraction and pollution (Sabater *et al.*, 2009). Therefore, one should expect large differences in ecosystem functioning, as is the case for wood breakdown. Just for comparison, Pozo *et al.* (2011) recently published a study on breakdown of alder leaves on 22 small unpolluted streams spread in 4 regions over the Iberian Peninsula. They found that alder breakdown rates changed sevenfold across their study sites, whereas wood breakdown rates in our sites varied almost fiftyfold. Hence, the larger range found in our study is without doubt a consequence of the more diverse environmental conditions studied, which included very different sizes, river types and levels of ecological quality.

A number of factors could account for the large range in breakdown rates. Very slow breakdown can be caused by nutrient limitation (Woodward *et al.*, 2012), and by strong pollution, that can stop the action of microbes and other organisms associated to litter breakdown, as has been reported, for instance, for very acidic streams (Dangles *et al.*, 2004). More difficult is to explain some cases of extremely high breakdown rate. Nutrient concentrations are known to enhance

breakdown of leaves (Gulis & Suberkropp, 2003) and wood (Díez *et al.*, 2002), but the highest rates measured in the present study, of 0.0167 day^{-1} , are high even for leaves (Petersen & Cummins, 1974). We can rule out abrasion and physical fragmentation as drivers of wood breakdown in our sites, as they were not in fast-flowing sections, and as most recovered pieces were intact albeit soft and thin. The only exception was in some sites where sticks were tied to boulders, which moved and fragmented some stick, and even in these cases we corrected mass loss for the size of the remaining pieces. Whatever the reason, and corroborating our hypothesis, breakdown of organic matter showed a small variability in near natural Iberian rivers, but its variability increases with human impacts, reaching in some cases extremely high values.

The multiple quantile regressions identified altitude, phosphorus, catchment area, TU_{sum} of pollutants, macroinvertebrate-based biotic index, width of the riparian buffer, and diatom index, in this order, as related to wood breakdown. Altitude, the first variable entering the model, was negatively related to breakdown rate. Breakdown was consistently slow in high elevation reaches, whereas in the lowlands both high and low rates could be measured, reflecting the complex character of multiple stressors. It is likely that the effect of altitude mainly reflects changes

in water temperature, which has a strong effect on breakdown (Friberg *et al.*, 2009). Indeed, altitude and water temperature were strongly correlated. We avoided using actual temperature data to construct the models as information of some sites was missing, and the quality of temperature data varied from site to site.

The second variable entering the model was phosphorus, which showed a hump-shaped relationship with breakdown rate, as has been reported elsewhere (Menendez *et al.*, 2011). This hump-shape is derived from the fact that moderate nutrient enrichment promotes the breakdown of organic matter, especially of materials like wood, with a high C:N:P ratio, as microbes can use nutrients dissolved in water to compensate for nutrient deficit in the detritus (Ferreira, Gulis & Graça, 2006), whereas at high nutrient concentrations pollution limits the effects of detritivores (Lecerf *et al.*, 2006; Woodward *et al.*, 2012). It is worth noting that peak breakdown at our sites occurred at a phosphorus concentration around 0.4 mg L⁻¹, which is a bit larger but in the same order of magnitude than the concentration for peak leaf breakdown according to Woodward *et al.* (2012). This again suggests that the same environmental factors are governing the breakdown of both leaves and wood, and perhaps suggests that an external source of nutrients is especially important for wood, which is more nutrient-deficient than most

leaves (Arroita *et al.*, 2012).

More difficult to explain are the contrasting effects of altitude and catchment area on wood breakdown, the positive effect of toxicity, or the contrasting relationship between breakdown rate and the biological indices based on invertebrates and diatoms. Lowland Mediterranean rivers are a paradigm of ecosystems under multiple stressors. Ricart *et al.* (2010) and Muñoz *et al.* (2009) reported high levels of analgesics, anti-inflammatories, lipid regulators, antibiotics and pesticides in the Llobregat River. The implications of all these new compounds for organic matter breakdown are little known, especially when in complex mixtures. Therefore, although we are starting to get a picture of regional variations in ecosystem functioning on rivers subject to multiple stressors, we are still far from being able to explain the values found at each site.

Conclusions

Breakdown of organic matter, an important ecosystem process, showed very large variability across Iberian rivers as a result of multiple stressors. Wood breakdown was consistently slow in high elevation rivers with low levels of human impact, whereas variance increased in lowland areas subject to multiple stressors. The main factors controlling maximum breakdown rate seem to be temperature

and phosphorus concentration, the latter showing a hump-shaped relationship, with low breakdown rates at both low and high phosphorus concentrations, and high rate at medium phosphorus concentration. Whatever the factors governing spatial variations in wood breakdown, tongue depressors offer a simple but powerful method to measure river ecosystem functioning. Ecosystem functioning is the basis of crucial ecosystem services. Therefore, it is important to have direct measures of ecosystem functioning across entire river basins, and to identify the main factors governing it.

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CHAPTER 3

Flow regulation by dams affects ecosystem metabolism in Mediterranean rivers

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Abstract

Large dams regulate river hydrology, and influence water chemistry, sediment dynamics, channel form and biotic communities. These effects may translate into important changes in river ecosystem processes, especially in rivers naturally subject to strong seasonality, such as those under Mediterranean climate. The effects of flow regulation on ecosystem metabolism (*i.e.* gross primary production and ecosystem respiration) were analysed by means of open-stream measurements. Organic matter accrual and metabolism were measured in reaches upstream and downstream from large reservoirs in three tributaries of the Ebro River (NE Iberian Peninsula) during three sampling campaigns. Dams reduced downstream hydrological variability, dampened floods and increased the duration of inter-flood periods. Benthic organic matter increased two-fold and chlorophyll-*a* eight-fold. Gross primary production increased by 59% on average, whereas ecosystem respiration increased by 75%. In general, flow regulation intensified the capacity of downstream river reaches to store and process materials and energy, therefore increasing the amount of organic carbon processed and altering the whole flux of materials and energy along the river continuum.

Keywords: dams, flow regulation, ecosystem metabolism, organic carbon dynamics, Mediterranean rivers

Introduction

The natural flow regime of rivers is determined largely by catchment size and by regional variations in climate, geology, topography and plant cover (Poff *et al.*, 1997). Flow regime affects river ecosystems across a broad range of spatial and temporal scales (Sparks, 1995), and the interaction between flow and physical habitat is a major determinant of the distribution, abundance and diversity of river organisms (Ward, Tockner & Schiemer, 1999; Nilsson & Svedmark, 2002). In most rivers affected by dams, flow regime is far from natural, as dams modify the quantity, timing and variability of water discharge (Poff & Allan, 1995). Today, 15% of the world's total runoff (40,000 km³ y⁻¹) is retained in *ca.* 45,000 large dams (>15 m in height, (Nilsson *et al.*, 2005)), which also retain 20% of the

global sediment flux (Syvitski *et al.*, 2005). The regions with a Mediterranean climate are strongly affected by flow regulation (Nilsson *et al.*, 2005) because of the large abundance of dams, and because their biota is adapted to their highly seasonal regime, which is disrupted by regulation (Gasith & Resh, 1999; Bernal *et al.*, 2013; Bonada & Resh, 2013). In particular, the Iberian Peninsula hosts one fifth of all European reservoirs (Avakyan & Iakovleva, 2006), and has the largest number of dams per inhabitant and per land area in the world (Garcia de Jalón, 2003).

Dams affect the general properties of river sections downstream and have substantial effects on water physical and chemical characteristics (Ward & Stanford, 1983). In most cases, and independently of their operation, they retain all bedload as well as a large

fraction of the suspended load (Vericat & Batalla, 2006; Tena *et al.*, 2011), thus promoting bed armoring and channel incision downstream (Brandt, 2000). Depending on whether dams release surface or deep water, they can trap or release nutrients (Puig *et al.*, 1987; Ahearn, Sheibley & Dahlgren, 2005), and affect thermal regimes (Byren & Davies, 1989; Voelz & Ward, 1989). Depending upon reservoir operation, downstream flow can either rapidly fluctuate or be unnaturally stable, and strongly impact river productivity and biodiversity (Poff *et al.*, 1997; Cortes *et al.*, 1998).

Dams usually reduce the magnitude and frequency of extreme flow events (Richter *et al.*, 2003), therefore affecting hydraulics and physical habitat, and exert strong effects on aquatic organisms (Poff & Allan, 1995; Allan & Castillo, 2007). Natural high flows promote the export of organic resources and trigger community succession, thus allowing many species with fast life cycles and good colonizing ability to re-establish (Fisher, 1983). High flows also mobilize fine sediments that could clog gravel habitats (Beschta & Jackson, 1979), and transport and rearrange woody debris, a key element structuring habitats (Keller & Swanson, 1979). Furthermore, in semi-arid regions such as the Mediterranean, dams transform intermittent river sections to permanent (Sabater & Tockner, 2010). This alteration of the patterns of extreme flow events

(both floods and droughts) is predicted to have important consequences for river ecosystem processes (Elosegi & Sabater, 2013).

The effects of flow regulation on river biota are relatively well known. Regulation affects the growth, survival, dispersal capability and reproduction of native plants, macroinvertebrates and fish, which are adapted to the natural conditions (Jansson, Nilsson & Renöfält, 2000; Santos *et al.*, 2004; Mueller, Pander & Geist, 2011). Therefore, the structure of biological communities downstream from dams can be strongly affected by flow regulation (Ward *et al.*, 1999; Nilsson & Svedmark, 2002; Martínez *et al.*, 2013; Ponsati *et al.*, 2014.). However, much less is known on the effects of flow regulation on river ecosystem processes, despite their relevance for the ecosystem services that rivers provide (Lü, Liu & Fu, 2012). Most of the information currently available concerns processes occurring within the reservoirs, such as nutrient dynamics or primary production (Stanley & Doyle, 2002; Doyle *et al.*, 2005; Bosch, 2008). On the other hand, some studies have reported effects of regulation on organic matter decomposition (Mendoza-Lera *et al.*, 2010; Marcarelli, Van Kirk & Baxter, 2010) or food web interactions (Cross *et al.*, 2011). Regarding river metabolism (*i.e.* gross primary production and ecosystem respiration), it is likely that the effects of regulation depend, among

others, on the details of dam operation. For instance, the depth from which water is released can affect nutrient levels and water temperature, key factors for river metabolism (Hill *et al.*, 2000; Yvon-Durocher *et al.*, 2010). Also, the timing and volume of water released will affect the frequency of extreme flow events, and may therefore affect metabolism (Acuña *et al.*, 2004). For instance, hydropeaking reduces downstream metabolism (Uehlinger, Kawecka & Robinson, 2003), whereas dams that reduce flood frequency can enhance river metabolism (Munn & Brusven, 2004). These changes in ecosystem metabolism are important, as

they reflect changes in energy transfer and organic carbon fluxes along fluvial networks (Battin *et al.*, 2008).

To study the effects of flow regulation on ecosystem metabolism in Mediterranean rivers, we compared reaches located upstream and downstream from three reservoir systems in the Ebro River basin. We predicted that 1) dams would reduce extreme flow events and thus enhance storage of organic carbon (both autochthonous and allochthonous) downstream; and 2) this in turn would enhance ecosystem metabolism (primary production and respiration) with respect to unregulated conditions.

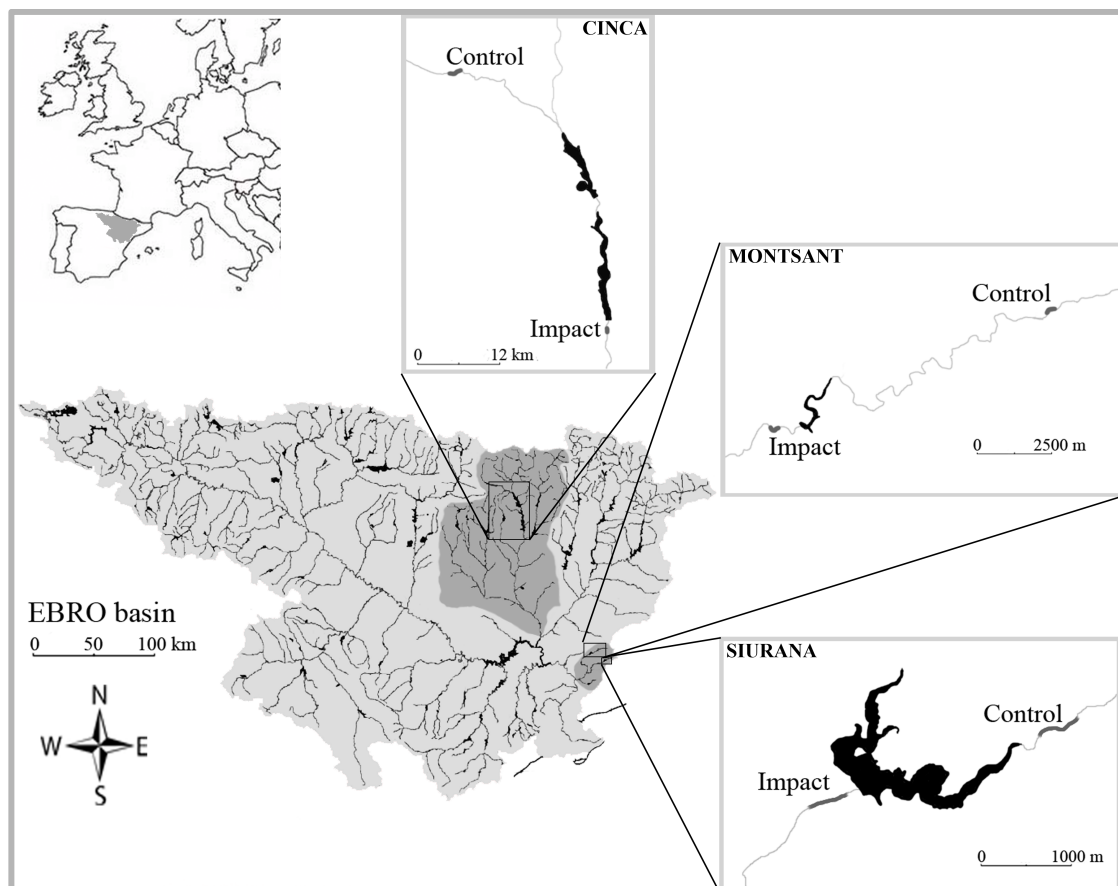


Figure 1. Study sites in the Ebro basin.

Methods

Study sites

The study was carried out in reaches upstream (Control, C) and downstream (Impact, I) from reservoirs in three rivers within the Ebro basin (NE, Spain) (Fig. 1). The Cinca River is 170 km long and drains a basin of 9,000 km² in the Central Pyrenees, which is dominated by sedimentary materials, mostly limestone. Precipitation averages 800 mm (Batalla, Gómez & Kondolf, 2004) and tends to be greater in winter, although discharge peaks in late spring and early summer with the thaw (Beguiría *et al.*, 2003). The Cinca River is regulated by two consecutive large reservoirs, Mediano and El Grado (Table 1), with a storage capacity of 436 and 399 hm³, respectively, which together create an almost continuous 27.5 km-long section of stagnant water. The C reach was set in Ara's River, one unregulated tributary joining the Cinca shortly before it enters Mediano reservoir, whereas the I reach was set in the Cinca below El Grado. The drainage basin is mainly covered by forests (C 51.3%, I 48.4%) and by grassland or bare rocks (C 47%, I 40.2%).

The Montsant River drains a basin of 170 km² and the Siurana River a basin of 347 km², both dominated by limestone substrata. Their climate is strongly Mediterranean, with an average annual precipitation of 589 mm, 80% of it falling from October to April, and a dry period in summer (Candela *et al.*, 2012). The Montsant River is temporary and dries out during summer, whereas the Siurana River is permanent. The Montsant River is regulated by the Margalef reservoir (3 hm³) and the Siurana River by the Siurana reservoir (12 hm³) (Table 1). Their basins are mostly forested (Montsant: C 53.2%, I 45.7%; Siurana: C 78.5%, I 69.5%), cropland being the second most important land use (Montsant: C 29.6%, I 26.6%; Siurana: C 13.8%, I 19.8%). The studied reservoirs differ in their hydrological operation (Table 1). Those in the Cinca River are subject to important water abstraction, which is diverted for irrigation and hydropower use, whereas no significant abstraction occurs either in the Margalef or in the Siurana reservoirs. Studied reservoirs release deep waters, which depending on the period (stratification or mixing) vary from epilimnetic to hypolimnetic. All have set

Table 1. Details of the reservoirs and their operation.

River	Reservoir	Year	Capacity (hm ³)	Drainage basin (km ²)	Abstraction	Hydropower use	Regulation capacity (y ⁻¹)
Cinca	Mediano	1959	436	2375	+	+	1.75
	El grado	1969	399				
Monstant	Margalef	1995	3	97	-	-	3.64
Siurana	Siurana	1972	12	60.4	-	-	0.46

environmental flows, defined as the 10% of the seasonal average. The regulation capacity, *i.e.* the ratio between river annual discharge and the reservoir storage capacity, changes from 0.46 y^{-1} in the Siurana River, to 1.75 y^{-1} in the Cinca River and 3.64 y^{-1} in the Montsant River.

Sampling design

We made sure that the study reaches were at least one third as long as the length integrated by diel oxygen curves, calculated as three times the ratio between water velocity ($m\ s^{-1}$) and reaeration rate (s^{-1}) (McCutchan, Lewis & Saunders, 1998). Thus, the selected reaches ranged from 250 to 2400 m in length, and drained areas from 40 to 2193 km^2 . The I reaches were placed as close as possible to the dams, and making sure that hydraulic conditions were homogenous. Along each reach five sampling sites were established equidistantly, and three sampling campaigns were performed at different hydrologic periods: summer and autumn of 2011, and winter of 2012. Because the C reach of the Montsant River was dry in summer 2011, the sampling campaign was performed in May 2012, just before the summer drought.

Flow regime

To determine the effect of the reservoirs on river flow regimes, we analysed

historic daily mean river flow (1990-2010) at gauging stations close to C and I reaches in the Cinca and Siurana Rivers. There was no gauging station available in the Montsant River, and therefore the long-term hydrologic alteration could not be estimated for that river. Flow variability was characterized using the frequency and duration of extreme flow events (Richter *et al.* 1996), defined as the 25th (low-flow events) and 75th percentiles (high-flow events) of daily discharge for each reach during the entire period. We used the Indicators of Hydrologic Alteration (IHA, v. 7.1) software to calculate these hydrological parameters.

Disturbance by extreme flow events was assessed on the basis of incipient movement of streambed particles (Leopold, Wolman & Miller, 1964; Parker, Klingeman & McLean, 1982), a more reliable estimate than measures based on discharge variation (Townsend, Scarsbrook & Dolédec, 1997). The size distribution of 150 stones in the wet channel was determined once at each site along a straight line on the river bed (Wolman 1954), and the mean grain diameter (GD) calculated from measurements of the B diameter of each particle (a total of 1648) with a gravel-meter; bedrock was given a value of 10 m (10 times the largest diameter measured). The discharge thresholds for initiation of sediment motion and for disruption of riverbed were predicted based on a

comparison between actual and critical dimensionless shear stress (Meyer-Peter & Müller, 1948), calculated from water depth, channel slope, riverbed material density and GD (Acuña *et al.*, 2007). Two thresholds were distinguished: 1) initiation of sediment motion, and 2) disruption of the streambed. In each reach we determined stability time (ST) as the time elapsed since the last flood event that involved sediment motion (ST-1), and the time elapsed since the last flow event that disrupted the river bed (severe flood) (ST-2).

Physicochemical characteristics

Physicochemical characteristics were measured at the five equidistant sites along each reach. Channel width and depth (n = 83) were measured with a measuring rod and tape, water velocity with a flow tracker (Handheld-ADV®), and discharge calculated after multiplying the wet section by the water velocity. Riparian cover was calculated from zenithal photographs taken with a fisheye lens (E171-A Nikon-8mm, Nikon D3000), analysed by means of HemiView 2.1 software. Hourly radiation values ($W m^{-2}$) recorded in the closest meteorological station were then converted to received radiation per site and day ($MJ m^{-2} d^{-1}$) (n = 90).

Water temperature, conductivity, dissolved oxygen (DO) and pH were

measured with hand-held probes (WTW multiline 3310; YSI ProODO handled, Yellow Springs, OH, USA) two times per day (noon and midnight) at each site (n = 90). Water samples were collected in parallel, filtered through fiberglass filters (Whatman GF/F 0.7 μm of mean pore) and frozen at $-20^{\circ}C$ until analysis. The concentration of nitrate was analysed by ion chromatography using a DIONEX C5000 (Dionex Corporation, Sunnyvale, USA). The concentrations of ammonium and phosphate were determined colorimetrically using an Alliance-AMS Smartchem 140 spectrophotometer (AMS, Frepillon, France) (n = 90). Alkalinity was determined on a Metrohm 855 Titrosampler (Metrohm AG, Herisau, Switzerland) (n = 54).

Organic matter

Concentration of dissolved organic carbon (DOC) was measured with a Shimadzu TOC-V CSH analyzer (Shimadzu Corporation, Kyoto, Japan) from the same water samples collected for the nutrients (n = 90). From each site, three water samples (2 L) were taken and filtered through pre-ashed and pre-weighed filters (Whatman GF/F) to determine the suspended particulate organic matter (SPOM) concentration (n = 54). In the same sites, five replicates of benthic organic matter (BOM) were taken with Surber nets (0.09 m^2 of sampling surface,

0.2 mm mesh size). Filters and benthic samples were frozen for transport, and once in the laboratory they were dried (70 °C, 72 h) weighed, ashed (500 °C, 5 h) and re-weighed to estimate ash-free dry mass (AFDM) (n = 90). Additionally, at each site four cobbles were taken for chlorophyll-*a* (Chl-*a*) measurements. Their light-exposed sides were scraped and pooled together; aliquots were frozen at -20 °C until analysis. The scraped surface was estimated by covering stones with aluminium foil of known density and later converting from aluminium weight to surface area. In the laboratory, Chl-*a* was extracted with acetone 90% v/v overnight at 4 °C and quantified spectrophotometrically (Shimadzu UV1800, Kyoto, Japan) after filtration (Whatman GF/C fiberglass filters) of the extract (Jeffrey & Humphrey, 1975) (n = 72).

River ecosystem metabolism

Temperature and DO were recorded at 10-min intervals at the upstream and downstream ends of each reach with optical oxygen probes (YSI 6150 connected to YSI 600 OMS, YSI Inc., Yellow Springs, Ohio, USA). Care was taken to place the probes in places where the flow was funnelled through a single channel. Metabolism was calculated from diel DO changes by the two-station method (Odum, 1956; Reichert, Uehlinger

& Acuña, 2009), except in the Siurana C in summer and winter, where unreliable results forced us to use the single-station method (Reichert *et al.*, 2009). Exchange of DO with the atmosphere was calculated with the night-time method (Hornberger & Kelly, 1975) using the first five hours after sunset. Nominal travel time of water (τ , min) was calculated measuring the time between the peaks of the two breakthrough curves at the upstream and downstream stations after a slug addition of bromide (Hubbard *et al.*, 1982). The following metabolic parameters were obtained from DO variations: ecosystem respiration (ER), gross primary production (GPP), net ecosystem metabolism (NEM) and ecosystem flux (EF). ER was calculated as the sum of net DO production rate during the dark period and respiration values during the light period, these being calculated as the linear interpolation between the net metabolism rate values of sunrise and sunset of the nights before and after the day of interest. GPP was the sum of net metabolism rate during the light period and respiration rates during the light period, as explained above. NEM was calculated as the sum of GPP and ER, and EF as the sum of GPP and ER in absolute values.

Data analysis

Differences between reaches in geomorphological and physicochemical

characteristics (*i.e.* GD, width, depth, velocity, discharge, light, temperature, conductivity, DO, pH, alkalinity and nutrients) were tested using linear mixed models. River, Reach (C vs. I) and Season were used as fixed factors, and Site within Reach as a random factor. Linear mixed models were also used to test differences in the variables related to organic matter availability (*i.e.* DOC, SPOM, BOM and Chl-*a*) with River, Reach and Season as fixed factors, and Site within Reach as a random factor. In the case of variables related to metabolism (*i.e.* GPP, ER, NEM and EF), the same kind of linear models as for organic matter availability were used, but interactions were not considered due to the low number of values. The effect of the reservoirs on the variables was represented by I/C ratios, values above 1 meaning higher measurements at the I reaches.

Finally, we performed two sets of stepwise multiple regressions to discern the environmental factors controlling our response variables ($n = 18$). First, the

variability of organic matter variables (DOC, SPOM, BOM and Chl-*a*) was modelled with all geomorphological and physicochemical variables as independent predictors. Secondly, metabolic variables (GPP, ER and EF) were modelled with all geomorphological, physicochemical and organic matter as independent variables. IHA parameters and GD were not included in the models because only one value was obtained per reach. The Pearson correlation detected multicollinearity among some variables, and we thus decided not to use width, depth, velocity, ST-2, conductivity, DO, alkalinity and pH for multiple regression. All stepwise regressions were performed twice: first with only the continuous variables, an models that give the possibility to extrapolate values of the variable of interest to other situations. The second approach includes explanatory power not expressed in the continuous variables (*i.e.* latent variables) and ranks the factors and variables altogether in terms of importance. Parametric linear models were

Table 2. Frequency and duration (in days) of extreme flow events for the two rivers with gauging stations for the analysed period (1990-2010). Medians and the Impact/Control ratios are also shown.

	Cinca			Siurana		
	Discharge ($\text{m}^3 \text{s}^{-1}$)			C	I	Ratio
	C	I	Ratio			
Frequency and duration of extreme flow events						
Low-flow event count	6	7	1.15	27	0	0
Low-flow event duration (days)	8.5	3	0.35	1	0	0
High-flow event count	11	6	0.55	15	7	0.47
High-flow event duration (days)	4	4	0	1	4.5	4.5

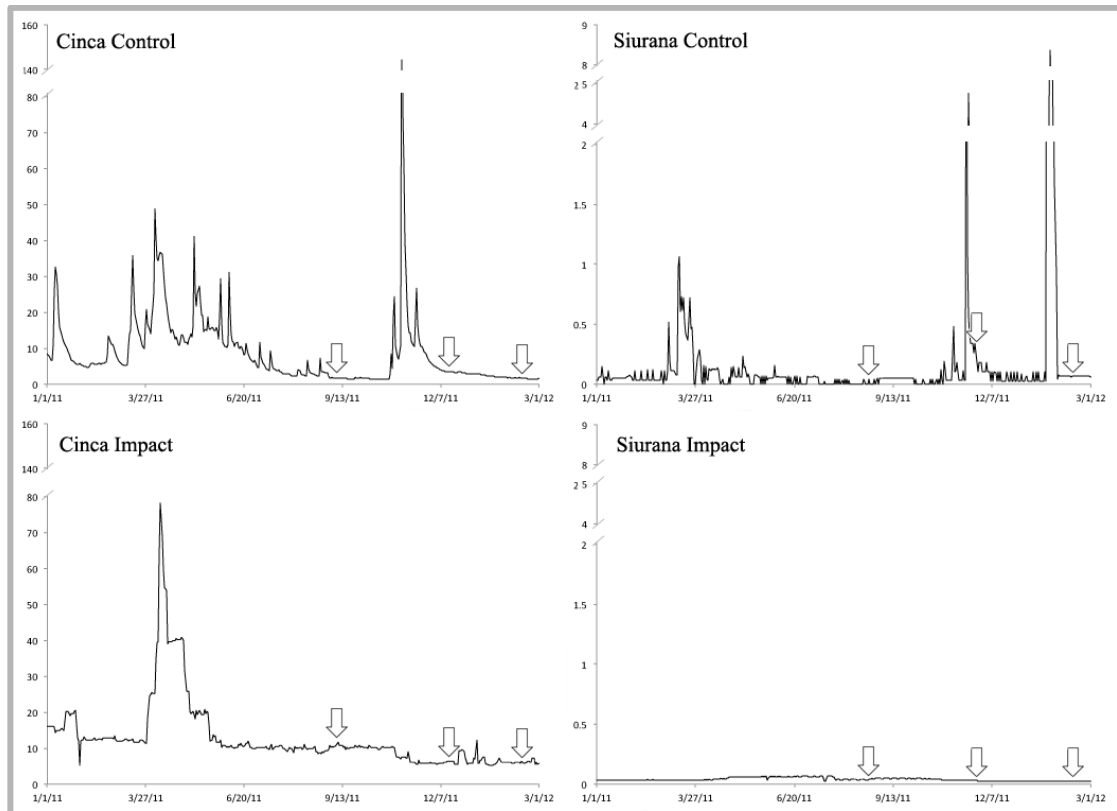


Figure 2. Hydrograph of the Cinca and Siurana river reaches. Sampling campaigns are marked with arrows.

used for the analyses, and variables were included in the model in a forward direction only if Akaike Information Criterion (AIC) values were smaller and the factors or variables included continued to be significant (tested with ANOVA) in the subsequent models. We tested if a significant spatial autocorrelation was present among the sites of the same reach by fitting models with linear, exponential, spherical, Gaussian and rational quadratic correlation structures. The explanatory power was not enhanced by the inclusion of these correlation structures, and so we did not consider them in the stepwise regressions.

Results

Flow regime

Dams affected the frequency and duration of extreme flow events in both the Cinca and the Siurana, the two rivers with available data (Table 2). Overall, dams had a severe effect on river hydrology, reducing flow variability and dampening floods (Table 2; Fig. 2). In the Cinca River, the number of low-flow events increased by 15% in the I reach, but their duration decreased by 65%. In the Siurana, low-flow events completely disappeared in the I reach. On the other hand, the frequency of high-flow events, decreased by 50% below dams in both rivers; their

duration did not change in the Cinca, but increased by 350% in the Siurana. The thresholds for sediment motion and riverbed disruptions were 29 to 550% higher in the I than in the C reaches (Table 3). Also the time of hydrological stability was longer in the I than in the C reaches, but the differences between reaches were not large for Montsant in summer, and for Siurana in summer and autumn.

Physicochemical characteristics

Flow regulation affected channel characteristics: upstream from the reservoirs the rivers followed a braided pattern among bars and prostrate willows, whereas below dams they were single-channel, incised reaches, with frequent outcrops of bedrock and extensive reed

growth on the banks. Nevertheless, the variables measured in the study reaches yielded results less contrasting than expected (Table 4, and Supporting Information Table S1). GD was reduced in the Cinca, increased in the Montsant, and was not affected in the Siurana. No clear pattern was observed in GD as fine sediments abounded disparately among the reeds. Further, the channel width was narrower in the I reaches of the Cinca and Siurana rivers, but not in the Montsant, reflecting the physical constraints of canyon-like channels. Average discharge during the sampling campaigns showed different responses below the dams: it was reduced in the Cinca, increased in the Montsant, and was not affected in the Siurana. In general, the riparian cover was larger at the I reaches, resulting in lower

Table 3. Sediment motion and riverbed disruption thresholds of water depth (m) and stability time (days) before each sampling period. Stability is defined after the time elapsed since the last moderate flood (ST-1) and time elapsed since the last severe flood (ST-2).

	Reach	Sediment motion (m)	Riverbed disruption (m)	Summer		Autumn		Winter	
				ST-1	ST-2	ST-1	ST-2	ST-1	ST-2
Cinca	C	0.4	0.6	14	38	1	5	73	90
	I	2.2	3.9	452	1200	519	1267	612	1360
Montsant	C	0.4	0.7	238	243	98	343	28	47
	I	0.6	0.9	246	246	346	346	421	421
Siurana	C	0.3	0.4	134	138	233	237	96	340
	I	0.7	0.9	142	142	241	241	344	344

Table 4. Results from the comparison (linear mixed models) of physicochemical characteristics between C and I reaches.

Variable	Cinca	Montsant	Siurana	All rivers	Variable	Cinca	Montsant	Siurana	All rivers
GD	C > I	C < I	=	C < I	Conductivity	C < I	C > I	C > I	C > I
Width	C > I	=	C > I	C > I	DO	=	C < I	C < I	=
Depth	=	=	=	=	Alkalinity	C > I	=	=	C > I
Discharge	C > I	C < I	=	C > I	pH	C > I	=	=	=
Velocity	C > I	=	=	=	Nitrate	=	=	C > I	=
Temperature	C < I	=	=	=	Ammonium	=	=	C < I	=
Light	C < I	C > I	C > I	C > I	Phosphate	C > I	C > I	=	C > I

light availability, although this was compensated in the Cinca by the wider active channel in the I reach. Statistically significant but biologically irrelevant differences were detected in water conductivity, DO, alkalinity or pH between the rivers (Table 4, Table S1). In contrast, some of the physicochemical variables showed differences in the I reaches in just one river; water temperature increased in the Cinca, nitrate decreased and ammonium increased in the Siurana. Phosphate decreased in the I reaches in the Cinca and Montsant rivers.

Organic matter

DOC concentration in the C reaches was higher in the Montsant River (average \pm SD, 3.36 ± 2.57 mg L⁻¹) than in the Cinca (0.16 ± 0.06 mg L⁻¹) and Siurana (0.94 ± 0.44 mg L⁻¹). SPOM, on the other hand, showed little differences among C reaches (Cinca 0.62 ± 0.18 mg L⁻¹, Montsant 0.60 ± 0.18 , Siurana 0.69 ± 0.11). BOM in the C reaches was higher in the Montsant River (200.21 ± 200.39 g m⁻²) than in the Siurana (19.15 ± 12.24 g m⁻²) and Cinca (16.36 ± 12.68 g m⁻²). Chl-*a* was higher in Siurana C (4.09 ± 5.61 μ g cm⁻²), followed by the Montsant C (2.01 ± 1.28 μ g cm⁻²) and Cinca C (1.56 ± 1.21 μ g cm⁻²). All organic matter categories were significantly more abundant in the I than in the C reaches (Fig. 3a). Average increases were of 22.3% for DOC, 93.7%

for SPOM, 109.9% for BOM and 713.7% for Chl-*a*. DOC and SPOM were the only organic components that differed among sampling campaigns, showing a significant increase in autumn (Linear mixed model, DOC: $F_{2,68} = 28.9$, $P < 0.0001$; SPOM: $F_{2,68} = 10.2$, $P = 0.0001$). BOM showed no significant differences among reaches in the Montsant River (Linear mixed model, $F_{1,20}=1.69$, $P=0.209$).

River ecosystem metabolism

GPP in the C reaches was higher in the Montsant (2.29 ± 1.75 g O₂ m⁻² d⁻¹) than in the Cinca (1.93 ± 1.20 g O₂ m⁻² d⁻¹) or Siurana (0.55 ± 0.12 g O₂ m⁻² d⁻¹). ER in the C reaches followed the same pattern (Montsant 4.81 ± 2.69 ; Cinca 3.47 ± 1.56 ; Siurana 0.91 ± 0.65 g O₂ m⁻² d⁻¹). EF in the C reaches was the highest in the Montsant (7.10 ± 4.22 g O₂ m⁻² d⁻¹), followed by the Cinca (5.41 ± 0.40 g O₂ m⁻² d⁻¹) and Siurana (1.45 ± 0.74 g O₂ m⁻² d⁻¹). All the C reaches were heterotrophic, except the Cinca C in autumn, where NEM was 1.57 g O₂ m⁻² d⁻¹. NEM values in the C reaches averaged -2.52 ± 1.65 g O₂ m⁻² d⁻¹ in the Montsant, -1.54 ± 2.76 g O₂ m⁻² d⁻¹ in the Cinca, and -0.36 ± 0.56 g O₂ m⁻² d⁻¹ in the Siurana. All metabolic variables showed higher values in the I than in the C reaches (Fig. 3b), showing average increases of 59.1% for GPP, 75.2% for ER, 34.6% for NEM, and 69.7% for EF. Differences for GPP between C and I were only

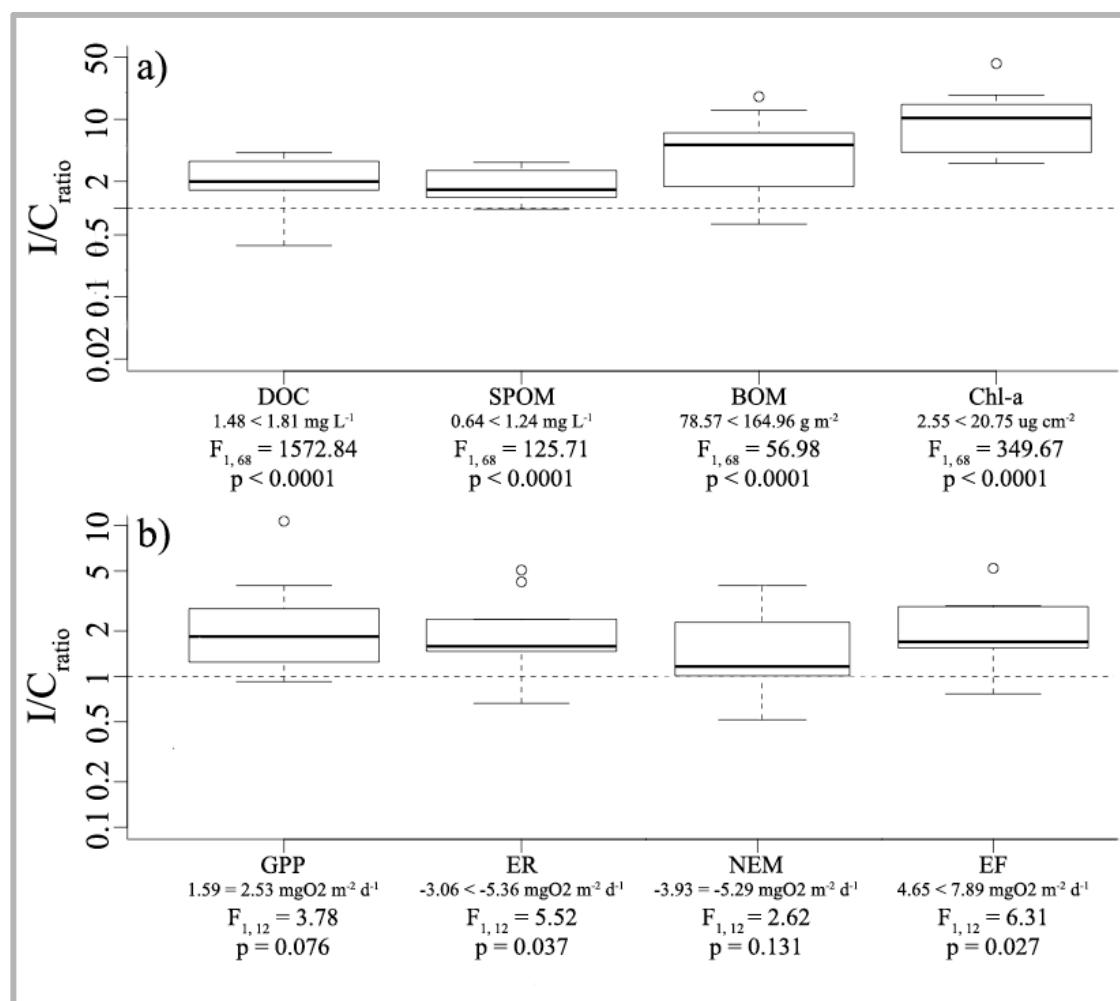


Figure 3. Boxplots of Impact/Control ratios of organic matter (a) and metabolism (b) descriptors. Average values in C and I reaches and the output for the *Reach* source of variation in the linear mixed models are shown. Analyses have been computed with average values per reach ($n = 18$), but values of all five sites per reach ($n = 90$ in total) are included in the statistical analyses for organic matter variables (*Site* within *Reach* have been included as a random factor in the analyses). NEM boxplot excludes the negative I/C ratio for Cinca in autumn.

marginally significant, even when combining all data in a single analysis ($n = 18$). Differences for NEM between reaches were not significant ($n = 18$). The differences were statistically significant for ER and EF (Fig 3b) when combining all data ($n = 18$), although results became non-significant for individual rivers ($n = 6$).

Stepwise regressions performed without the factors River, Reach and Season showed that DOC was related to nitrate and discharge ($R^2 = 0.73$), BOM

was related to DOC and to ST-1 ($R^2 = 0.6$) and Chl-*a* to ST-1 ($R^2 = 0.48$). GPP was explained by BOM, light and discharge ($R^2 = 0.42$), ER by BOM, discharge, ST-1 and light ($R^2 = 0.62$), and EF by BOM, discharge, light and ST-1 ($R^2 = 0.70$). In most cases, stepwise regressions improved when the factors River, Reach and Season were included (Table 5). In this case, variability of DOC was explained by nitrate and River ($R^2 = 0.82$), SPOM by Reach ($R^2 = 0.4$), BOM by DOC, Reach

and phosphate ($R^2 = 0.79$), and Chl-*a* by Reach ($R^2 = 0.77$). GPP was explained by Season, BOM and River ($R^2 = 0.56$), ER by River and BOM ($R^2 = 0.46$), and EF by River and BOM ($R^2 = 0.50$). Including the factors River, Reach and Season improved the variability explained by stepwise regressions in the case of GPP, but not in the case of ER or EF. NEM was not explained by any measured variable in any of the stepwise regressions.

Discussion

Flow regulation by dams enhanced the accumulation of organic carbon (both allochthonous and autochthonous), and shaped the ecosystem metabolism of downstream reaches. Flow regime in the studied rivers was highly variable as is common in Mediterranean rivers (Gasith & Resh, 1999), but dams reduced the hydrologic variability and the magnitude and frequency of extreme flow events downstream. As a result, extreme flow events that could cause sediment motion or riverbed disruption occurred much less frequently in the impact reaches. The effects of these changes in the channel characteristics seemed not to depend on reservoir use and operation. Williams & Wolman (1984) reported that dams resulted in an increased downstream grain size, and that the impact decreased with the age of the reservoir and the distance downstream. In spite of the pervasive

impact of dams on bedload transport, our results did not confirm the effect of the reservoirs on the grain size. The absence of differences in mean grain size seems to be related to the extensive reed banks in the I reaches, which can trap fine sediment and thus reduce the average grain size. In addition to channel form and substratum stability, reservoirs can also affect downstream water quality, depending on their size and location in the river network, on their age and on the retention time of water (Hannan, 1979; Petts, 1984; Bergkamp *et al.*, 2000). Depending on the depth from which water is released they can affect water temperature (Byren & Davies, 1989; Voelz & Ward, 1989), and act as sink or source of nutrients (Puig *et al.*, 1987; Ahearn *et al.*, 2005). In our case, the influence on water physicochemical characteristics was rather small and inconsistent, except on nitrate concentrations, which were significantly correlated to DOC, and phosphate concentrations, which were correlated to BOM. These small effects on water chemistry suggest that any changes in organic matter or in metabolism must be caused by other factors, such as hydrological variability or substrate stability.

Contrasting with the dissimilar response of physicochemical variables, all organic matter variables showed consistent increases below dams, even though causes

Table 5. Stepwise multiple regressions for the organic matter and metabolic components excluding or considering the factors *River*, *Reach* and *Season*. Variables and factors are selected forwardly based on reductions of the AIC of the model. They are listed as in the order of inclusion in the model. ANOVA output of the final model is shown. Signs of the relationship between the variables are also shown.

Response variable	Factors excluded					Factors included				
	Predictive variables	df	F-value	P-value	Relation	Predictive variables	df	F-value	P-value	Relation
DOC	Nitrate	1	40.58	<0.0001	+	Nitrate	1	61.36	<0.0001	+
	Discharge	1	5.42	0.035	-	River	2	9.08	0.003	
	R ² = 0.73					R ² = 0.82				
Log₁₀SPOM	No significant model					Reach	1	12.24	0.003	
	-					R ² = 0.40				
Log₁₀BOM	DOC	1	21.07	0.0004	+	DOC	1	40.99	<0.0001	+
	ST-1	1	6.3	0.024	+	Reach	1	21.21	0.001	
						Phosphate	1	6.22	0.026	+
	R ² = 0.60					R ² = 0.79				
Log₁₀Chl-<i>a</i>	ST-1	1	16.82	0.0008	+	Reach	1	58.46	<0.0001	
	R ² = 0.48					R ² = 0.77				

Table 5. Continuation

	Factors excluded					Factors included				
Log₁₀GPP	Log ₁₀ BOM	1	4.71	0.048	+	Season	2	5.47	0.020	
	Light	1	4.91	0.044	+	Log ₁₀ BOM	1	7.31	0.019	+
	Discharge	1	5.75	0.031	+	River	2	4.21	0.041	
	R ² = 0.42					R ² = 0.56				
Log₁₀ER	Log ₁₀ BOM	1	9.62	0.008	+	River	2	4.86	0.025	+
	Discharge	1	9.43	0.009	+	Log ₁₀ BOM	1	8.06	0.013	+
	ST-1	1	6.98	0.020	+					
	Light	1	5.94	0.030	-					
R ² = 0.62					R ² = 0.46					
NEM	No significant model					No significant model				
Log₁₀EF	Log ₁₀ BOM	1	11.93	0.004	+	River	2	5.55	0.017	
	Discharge	1	12.43	0.004	+	Log ₁₀ BOM	1	9.23	0.009	+
	Light	1	8.16	0.014	+					
	ST-1	1	11.09	0.005	+					
R ² = 0.70					R ² = 0.50					

did not seem to be the same for all. Because the studied I reaches were located close to the dams, it is likely that most of the increase in DOC and SPOM could be related to materials from the reservoir, either from re-suspended organic matter or from outflowed plankton. Reservoirs may be sources for both DOC and SPOM (Wei *et al.*, 2008; Becker *et al.*, 2010), and this seems to be the case in our study sites. Although DOC can be produced in rivers by cell exudates or decaying cells (Nakano, 1996), since the concentrations of DOC and SPOM tended to decrease along our impact reaches (von Schiller, *unpublished data*), it seems that these reaches consumed more organic matter than they produced.

The increase in benthic components (BOM and Chl-*a*) seems to be related to the higher hydrological stability, that reduced scouring benthic organic matter and primary producers (Brookshire & Dwire, 2003; Dewson, James & Death, 2007). It has been observed elsewhere that inter-flood periods promote accumulation of benthic organic matter (Speaker, Moore & Gregory, 1984; Biggs, 1995; Uehlinger, Bühler & Reichert, 1996; Pozo *et al.*, 1997). In our case, when excluding Reach as a factor, ST-1 (*i.e.* time elapsed without sediment-moving flood) was the main factor accounting for BOM and Chl-*a*, thus showing the importance of flood regulation on these organic components. Interestingly, the Montsant River, the one

with similar ST-1 between C and I reaches, showed no significant differences in BOM. In addition to increased hydrological stability, substratum stability below dams can promote algal proliferation (Morley *et al.*, 2008), and encroachment of vegetation increases inputs of organic matter (Webster, Wallace & Benfield, 1995).

Flow regulation by dams was also reflected in ecosystem metabolism. Stepwise regressions selected almost the same explanatory variables for both GPP and ER: BOM, discharge, ST-1 and light. Reach was not included as explanatory variable, but BOM (which responded significantly to the impact of the reservoirs) was related to all the metabolism variables. Both ER and GPP were higher at the impact reaches, especially the former (+75% *vs.* +59%). The differences of these metabolic rates between the C and I reaches seem small when compared with those observed for BOM (+110%) and especially for Chl-*a* (+700%). Given the likely coupling between BOM and ER (Acuña *et al.*, 2004) and between Chl-*a* and GPP (Hill, Mulholland & Marzolf, 2001), one would expect a more pronounced response for GPP since higher inter-flood periods (*i.e.* higher ST-1) increases the epilithic biomass and metabolism (Munn & Brusven, 2004). Nevertheless, GPP, a process depending on the amounts of light reaching the stream (Bunn, Davies &

Mosisch, 1999; Hill *et al.*, 2001), was reduced because vegetation encroachment resulted in lower light reaching the stream in the I reaches of two of the rivers. Moreover, low metabolic activity of benthic algae has also been reported after long inter-flood periods (Izagirre *et al.*, 2008). Besides, ER obviously does not depend on light, but it is strongly dependent on temperature (Acuña *et al.*, 2008), and it was correlated ($R^2 = 0.43$, $P = 0.003$) to it.

Overall, flow regulation by dams did not change NEM but showed a trend to a higher degree of heterotrophy. EF increased in the impact reaches revealing that higher amounts of organic carbon accumulate and are processed in these sections. The effect of regulation on river metabolism is larger than other human disturbances, such as land-use changes, which have been shown to increase EF by 25% in the case of agriculture and 35% in the case of urban use (Bernot *et al.*, 2010), but smaller than those of wastewater treatment plant effluents, which increase EF up to two-fold (Gücker, Brauns & Pusch, 2006). As such, the effects of dams on river metabolism can be considered as intermediate. It needs to be stressed that our results were obtained immediately below dams, and that we would expect a downstream decrease of the impact, eventually recovering normal values (Munn & Brusven, 2004). Whatever the case, the increase in EF of all studied

reaches suggest that the carbon turnover length (Newbold *et al.*, 1982) will be reduced, more carbon will be transformed into inorganic form, and less organic carbon will be transported downstream. Similarly, the nutrient retention, which is a function of hydrological retention and biological activity (Valett *et al.*, 1996; Battin *et al.*, 2008), might increase as a result of the decreased water velocity and the higher metabolic rates (von Schiller *et al.*, 2008). Hence, the impact reaches might have a higher self-purification capacity owing to higher metabolic rates (Acuña *et al.*, 2013). Overall, the effects of regulation on ecosystem metabolism, and especially on EF and NEM, indicate that dams create a discontinuity in carbon fluxes along the river by the combined effects of the reservoirs themselves, and their effect on downstream reaches.

In conclusion, flow regulation had strong effects on the ecosystem processes of the Mediterranean rivers. Dams reduced hydrological variability and dampened floods. Longer inter-flood periods allowed large downstream increases in benthic organic matter and primary producers, which in turn fostered ER and GPP. River reaches below dams had a higher capacity to process organic carbon than those above dams. Higher processing capacities below dams could lead to a shorter organic carbon turnover length, less carbon exported to downstream systems and a higher self-purification capacity.

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CHAPTER 4

Mixed effects of effluents from a wastewater treatment plant on river ecosystem metabolism: subsidy or stress?

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Abstract

The effluents of wastewater treatment plants (WWTP) include a complex mixture of nutrients and pollutants. Nutrients can subsidize autotrophic and heterotrophic organisms, while toxic pollutants can act as stressors, depending, for instance, on their concentration and interactions in the environment. Hence it is difficult to predict the overall effect of WWTP effluents on river ecosystem functioning. We assessed the effects of WWTP effluents on river biofilms and ecosystem metabolism in one river segment upstream from a WWTP and three segments downstream from the WWTP and following a pollution gradient. The photosynthetic capacity and enzymatic activity of biofilms showed no change, with the exception of leucine-amino-peptidase, which followed the pollution gradient most likely driven by changes in organic matter availability. The effluent produced mixed effects on ecosystem-scale metabolism. It promoted respiration (subsidy effect), probably as a consequence of enhanced availability of organic matter. On the other hand, and despite enhanced nutrient concentrations, photosynthesis-irradiance relationships showed that the effluent partly decoupled primary production from light availability, thus suggesting a stress effect. Overall, WWTP effluents can alter the balance between autotrophic and heterotrophic processes and produce spatial discontinuities in ecosystem functioning along rivers as a consequence of the mixed contribution of stressors and subsidizers.

Keywords: ecosystem functioning, metabolism, pollution, subsidy-stress effect, photosynthesis vs. irradiance curve.

Introduction

Pollution from point sources such as wastewater treatment plants (WWTP) is a common impact on river ecosystems (Bernhardt & Palmer, 2007; Grant *et al.*, 2012), especially in conurbations (United Nations Population Division, 2006). For example, more than 2,500 WWTPs have been put into operation over the last three decades in Spain (Serrano, 2007). As WWTP do not remove all contaminants from sewage waters (Rodríguez-Mozaz *et al.*, 2015), their effluents contribute a complex mixture of contaminants to freshwater ecosystems (Ternes, 1998; Petrovic *et al.*, 2002; Kolpin *et al.*, 2004; Gros, Petrovic & Barceló, 2007; Merseburger *et al.*, 2009). WWTPs release nutrients and organic matter (Martí *et al.*,

2004). Together with emerging contaminants such as pharmaceuticals and personal care products (Kuster *et al.*, 2008; Ginebreda *et al.*, 2010). Therefore, WWTPs contribute both assimilable contaminants such as dissolved nutrients and organic matter, which subsidize biological activity (at least up to a threshold beyond which they can suppress it), and toxic contaminants, which are deleterious to organisms and tend to suppress biological activity (Odum, Finn & Franz, 1979). However, most previous studies of the effects of WWTP effluents on ecosystem processes have only considered their subsidy effects (Martí *et al.*, 2004; Merseburger, Martí & Sabater, 2005; Gücker, Brauns & Pusch, 2006; Ribot *et al.*, 2012).

When in excess, assimilable substances entering freshwaters via WWTP effluents can impair water quality, alter the structure of biological communities, cause harmful algal blooms and affect ecosystem functioning (Smith, 2003; Sutton *et al.*, 2011). These substances promote the biomass and activity of both primary producers (algae, macrophytes) and microbial heterotrophs (bacteria, fungi), which are able to use dissolved nutrients and organic matter (Stelzer, Heffernan & Likens, 2003). Moreover, their effects can transmit upwards to other trophic levels (Hart & Robinson, 1990), and eventually affect the entire ecosystem (Woodcock & Huryn, 2005; Izagirre *et al.*, 2008; Bernot *et al.*, 2010; Cabrini *et al.*, 2013). Functioning of freshwater ecosystems can respond linearly to the concentration of assimilable contaminants such as nutrients (Yates *et al.*, 2013; Silva-Junior *et al.*, 2014), but hump-shaped responses have also been observed (Clapcott *et al.*, 2011; Woodward *et al.*, 2012). The toxic contaminants entering freshwaters via WWTP effluents can have direct detrimental effects on aquatic life (Hernando *et al.*, 2006; de Castro-Catala *et al.*, 2014), especially when they occur in mixtures (Cleavers, 2003). Toxic contaminants reduce the abundance and affect the composition of biofilms (Wilson *et al.*, 2003; Ponsatí *et al.*, *In revision*) and invertebrate communities (Muñoz *et al.*,

2009; Alexander *et al.*, 2013; Clements, Cadmus & Brinkman, 2013), and can also affect the rates of ecosystem processes (Bundschuh *et al.*, 2009; Moreirinha *et al.*, 2011; Rosi-Marshall *et al.*, 2013). Autotrophic processes seem to be more sensitive to WWTP pollutants than heterotrophic processes (Proia *et al.*, 2013; Corcoll *et al.*, 2014), but the reasons behind these differences are still far from clear.

Consequently, and depending of their mixed composition and the resulting concentrations on rivers, WWTP effluents can act either as a subsidy or a stress for the receiving ecosystem (Cardinale, Bier & Kwan, 2012). Furthermore, the potential response to contaminants differs between groups of organisms, and ecological interactions add a level of complexity (Segner, Schmitt-Jansen & Sabater, 2014) as, for instance, when the detrimental effects on some organisms promote the activity of others by releasing them from competition or predation (*e.g.* Alexander *et al.*, 2013). Therefore, the response to pollution can differ from the scale of individual components such as biofilm to the scale of the whole ecosystem, as already shown for other environmental pressures such as flow regulation (Aristi *et al.*, 2014; Ponsatí *et al.*, 2014).

We examined whether WWTP effluents were a subsidy or a stress for river ecosystem functioning by comparing one upstream river segment with three

downstream segments in a gradient of nutrient and toxic concentrations. We hypothesized: 1) that WWTP effluents affect autotrophic and heterotrophic metabolism differently; 2) that effects decrease downstream as contaminants such as nutrients and toxic pollutants (of which we used pharmaceuticals as a proxy) decrease following natural attenuation processes; and 3) that the downstream trajectories differ between autotrophic and heterotrophic metabolism because of their different responses to the subsidy/stress effects of WWTP effluents.

Methods

Study design

The study was conducted in the Segre River, a tributary of the Ebro River in the Oriental Pyrenees (NE Iberian Peninsula). At the study site (UTM X: 411856 and UTM Y: 4698346, 31N/ETRS 89), the Segre drains an area of 287 km², with a rain-snow fed flow regime. The river runs through a gravel bed meandering channel across a broad valley mainly covered with native forests but also with some pastures and small agricultural fields. Near the town of Puigcerdà it receives the effluent from a WWTP that treats sewage from *ca.* 30,000 population equivalents.

We compared a control reach (CR) upstream from the WWTP effluent with a 4,000 m-long impact reach downstream

(IR). In the latter, we selected three impact segments at increasing distances from the WWTP effluent: 500 - 1500 m (IR1), 1500 - 2500 m (IR2) and 2500 - 4500 m (IR3). Hereafter we refer to all of them (control plus impacts) as segments for simplicity, and use the term reach only when making overall comparisons between conditions upstream and downstream from the WWTP. Acuña *et al.* (2015) showed that dilution and self-purification reduce the total concentration of pharmaceuticals by 37% along the impact segments.

Environmental measurements

Above-canopy global radiation (GLR) data was obtained from the meteorological station of the Catalan Meteorological Service (Das, Catalan Meteorological Service, located at *ca.* 5 km from the studied reach). Radiation reaching the streambed was estimated by filtering the series of data of global radiation by light interception coefficients calculated by the Hemiview canopy analysis software (version 2.1, Dynamax Inc., Houston, TX, USA). Hemiview was used to perform image analysis of hemispherical photography determining the gap fraction, contributions of direct and diffuse solar radiation from each sky direction, site factors and leaf area index (LAI). Hemispherical photographs of the canopy were taken during the study period (9-10 October 2012), and every 50 m in all the

study reaches, with a high resolution digital camera (Nikon D-70s, NIKON Corporation, Tokyo, Japan) fitted to a 180° fisheye (Fisheye-NIKKOR 8 mm, NIKON Corporation, Tokyo, Japan). Water velocity and discharge were measured at the end of each river reach, according to the methods of Gore and Hamilton (1996) using an acoustic Doppler velocity meter (FlowTracker Handheld-ADV®, SonTek, San Diego, CA, U.S.A.)

Water temperature, conductivity and pH were measured with hand-held probes (WTW multiline 3310, YSI ProODO handled; YSI Inc., Yellow Springs, OH, USA) at the end of each river segment at noon and midnight. Water samples were collected in parallel, filtered through fibreglass filters (Whatman GF/F 0.7 µm nominal pore size, Whatman International Ltd., Maidstone, England) and frozen at -20 °C until analysis. Ammonium concentration was analyzed by ion chromatography using a DIONEXI C5000 (Dionex Corporation, Sunnyvale, USA), phosphate by colorimetry using an Alliance-AMS Smartchem 140 spectrophotometer (AMS, Frepillon, France), and DOC by a Shimadzu TOC-V CSH analyzer (Shimadzu Corporation, Kyoto, Japan). For suspended particulate organic matter (SPOM) three water samples (each 2 L) were filtered through pre-ashed and pre-weighed Whatman GF/F filters. Filters were frozen for

transport, and once in the laboratory they were dried (70 °C, 72 h) weighed, ashed (500 °C, 5 h) and re-weighed to estimate ash-free dry mass (AFDM).

Ten pharmaceuticals belonging to different therapeutic families were measured as a proxy of the concentration of other contaminants within each river segment, from samples collected in parallel to those for nutrients, filtered through nylon filters (0.2 µm mesh, Whatman, Maidstone, UK) and kept at -20 °C until analysis. Analysis of pharmaceuticals was performed following the fully automated on-line methodology described in detail by García-Galán *et al.* (unpublished manuscript available from the author on request). Briefly, 5 mL of surface water were loaded on the on-line chromatographic system (Thermo Scientific EQuan™, Franklin, MA, US) consisting of 2 quaternary pumps and 2 LC columns, one for pre-concentration of the sample and the second for chromatographic separation. The sample was further eluted by means of the mobile phase into the coupled mass spectrometer (TSQ Vantage triple quadrupole; Thermo Scientific, Franklin, MA, US). Chromatographic separation was achieved using a Thermo Scientific Hypersil Gold™ (50 x 2.1 mm, 1.9 µm particle size) column. Target compounds were analyzed under dual negative/positive electro-spray ionization in multiple reaction monitoring (MRM) mode,

monitoring two transitions between the precursor ion and the most abundant fragment ions for each compound. Recoveries of the compounds ranged between 62% and 183% (sulfamethoxazole and ibuprofen, respectively), whereas limits of detection ranged from 0.81 ng L⁻¹ to 7.86 ng L⁻¹ (sulfamethoxazole and venlafaxine, respectively).

Benthic organic matter and biofilm characteristics

Five Surber net (0.09 m², 0.2 mm mesh size) samples for benthic organic matter (BOM) were taken at random from each segment, the material was frozen for transport, and once in the laboratory it was dried (70 °C, 72 h) and ashed (500 °C, 5 h) to calculate AFDM. Chlorophyll-*a* (Chl-*a*) samples were obtained from the upper exposed part of cobbles. From each cobble a surface of 2-3 cm² was scraped with a knife and pooled together to obtain a mixed sampling area of 9 to 18 cm² according to the available biomass. Five replicates were taken in each river segment. Then, samples were immediately frozen (-20 °C) until analysis. In the laboratory, Chl-*a* was extracted with 90% v/v acetone overnight at 4 °C and quantified spectrophotometrically (Shimadzu UV1800) after filtration (Whatman GF/C 1.2 µm) following Jeffrey & Humphrey (1975).

Biofilm functioning was measured on colonized artificial substrata. Unglazed ceramic tiles of 1.25 x 1.25 cm were glued in groups of 110 units onto flat 20 x 20 cm bricks, and 3 flat bricks per segment incubated at a depth of 30 cm in the field during six weeks (30 August 2012 to 10 October 2012) to allow for biofilm colonization. On 9-10 October ceramic tiles from each of three flat bricks were sampled to measure photosynthetic and respiration capacity and enzymatic activities.

Photosynthetic capacity measurements (effective quantum yield (Y_{eff}), maximum photosynthetic capacity (Y_{max}), photochemical quenching (PQ) and non-photochemical quenching (NPQ) were determined in the field by Diving PAM (Pulse Amplitude Modulated) underwater fluorometer (Heinz, Walz, Effeltrich, Germany). Ceramic tiles were placed in individual glass vials, filled with 4 mL of stream water and kept for 20 min in the dark at river temperature to obtain the maximum Chl-*a* fluorescence (F_0), and later exposed to natural light to measure the fluorescence yield (Y_{eff} and Y_{max}) and quenching (PQ and NPQ) (Genty, Briantais & Baker, 1989). Y_{eff} and Y_{max} were respectively used as indicators of photosynthetic efficiency and maximal photosynthetic capacity of algal community. NPQ was used as an indicator of the algal capacity to dissipate the excess

light during stress conditions (Corcoll *et al.*, 2011).

The respiratory capacity (electron transport system, ETS) of the biofilm was determined by the reduction of the electron transport acceptor INT (2- (p-iodopheny1)- 3- (p-nitropheny1)- 5-phenyl tetrazolium chloride) to INT- formazan (iodonitrotetrazoliumformazan) (Blenkinsopp & Lock, 1990). Ceramic tiles were placed in individual glass vials with 4 mL of filtered stream water (Whatman Nylon Membrane 0.2 μm mesh) and kept in the dark at 20 °C. For an INT solution blank, an additional tile was taken and fixed with 4% formaldehyde. Incubations were carried out with the addition of 3mL of 0.02% INT solution for 8 h in the dark with continuous shaking. Samples were frozen at -20 °C after solution removal. Once in the laboratory, INT was extracted with cold methanol for 1 h at 4 °C in the dark. The extract was filtered (Whatman GF/C) and quantified spectrophotometrically at 480 nm with a standard solution of 0-60 $\mu\text{g L}^{-1}$ of INT-Formazan (Sigma Aldrich, St Louis, MO, USA).

We measured activities of three selected extracellular enzymes: alkaline phosphatase (AP, an enzyme linked to phosphorus acquisition), β -glucosidase (BG, involved in the degradation of small organic compounds) and leucine-amino-peptidase (LAP, linked to the use of peptides and proteins as a source of

nitrogen). Activities were determined using substrate analogues of MUF (methylumbelliferyl) and AMC (aminomethylcoumarin), [4-MUF-phosphatase (αP); 4-MUF-B-D-Glucosidase (βG); and L-leucinaminomethylcoumarin (LAP) from Sigma Aldrich]. Ceramic tiles and MUF/AMC substrate blank were placed in individual glass vials with 4 mL of filtered stream water (Whatman Nylon Membrane 0.2 μm mesh) and incubated with 0.120 mL of each substrate (0.3 mmol L^{-1} to ensure substrate saturation (Romaní & Sabater, 1999). Incubation was done in the dark with continuous shaking for 1 h at 20 °C. Two blanks of filtered stream water were also incubated. After addition of 4 mL of 0.05 M glycine buffer, pH 10.4, samples were frozen at -20 °C. Once in the laboratory samples and standard calibrating solutions of MUF and AMC were thawed and quantified by spectrofluorometry (Fluorescence Spectrophotometer F-7000, Hitachi, Tokyo, Japan) (Romaní & Sabater, 1999).

River ecosystem metabolism

Metabolism was calculated from diel dissolved oxygen (DO) changes by the open-system method with either one or two stations (Odum, 1956; Reichert, Uehlinger & Acuña, 2009). We chose the best method (single-station or two-station) to estimate ecosystem metabolism in each

segment following Reichert *et al.* (2009): we compared the ratio of flow velocity to reaeration coefficient ($v:k$) with segment length, and used the single-station method in reaches longer than 3 times the $v:k$ ratio and the two-stations method in shorter reaches. Thus, we used the single-station method for segments CR and IR1, and the two-station method for IR2 and IR3. DO was measured at 10-min intervals for 20 d (from 21 September to 10 October 2012) at the upstream and downstream ends of each river segment with optical oxygen probes (YSI 6150 connected to YSI 600 OMS, YSI Inc., Yellow Springs, USA) from which 10 d under base flow conditions were used. The reaeration coefficient was determined using slug additions of mixed tracer solutions (Jin *et al.*, 2012). Solutions of propane-saturated water were prepared in the laboratory by filling hermetic 20 L plastic tanks with 10 L of distilled water and 10 L of 99%-pure propane gas (Linde Industrial Gases, Barcelona, Spain). The solutions were prepared a few days before the additions and shaken to allow sufficient time for propane to dissolve into the water. A total of 3 slug additions were performed: the first covering IR3, the second covering IR1 and IR2, and the third covering CR. For each slug addition, two of the propane-saturated water solutions were added *in situ* to 60 L containers filled with a solution of 40 L of stream water with a measured amount of conservative solute

tracer (chloride as NaCl). Immediately after mixing, the solutions were added into the stream channel at *ca.* 400 m upstream from the first sampling point to allow for complete lateral mixing. The breakthrough curves of chloride were followed at each station using a hand-held conductivity meter (WTW, Weilheim in Oberbayern, Germany). Five replicate water samples were collected at the conductivity peak using 60 mL plastic syringes fitted with stopcocks. After adding 30 mL of air to each syringe, these were shaken for ~10 min to allow equilibration of the propane gas into the air space. The air space was then collected in pre-evacuated 20 mL glass vials, which were stored at 4 °C until analysis on a gas chromatograph (ThermoFisher Scientific, San Jose, CA, USA). The reaeration coefficient was calculated using the decline in conductivity-corrected propane concentrations between sampling stations as described by Jin *et al.* (2012). Nominal travel time of water was calculated measuring the time between the peaks of the breakthrough curves at the upstream and downstream stations (Hubbard *et al.*, 1982). Ecosystem respiration (ER) was calculated as the sum of net metabolism rate during the dark period and respiration values during the light period, these being calculated as the linear interpolation between the net metabolism rate values of sunrise and sunset of the nights before and after the day of interest. Net ecosystem

metabolism (NEM) was calculated as sum of net metabolism rates during the whole day, and gross primary production (GPP) as the difference between NEM and ER.

Photosynthesis-Irradiance relationships

To evaluate the possible subsidy or stress effect at the ecosystem level, we analyzed the relationship between primary production and irradiance reaching the streambed (P-I). For each river segment, GPP and GLR values from 6 days were fitted to linear and hyperbolic tangent functions by non-linear regression (STATISTICA, version 8; StatSoft Inc., Tulsa, OK, USA), the hyperbolic tangent function including or excluding temperature dependence:

$$GPP = P_{MAX} \cdot \tanh\left(\frac{\alpha \cdot I}{P_{MAX}}\right) \cdot \sigma^{T-20}$$

where P_{MAX} is light saturated photosynthesis, α is the initial slope of the P-I curve, I is the GLR reaching the streambed, σ is the temperature dependence coefficient and T is temperature. The half-saturation light intensity (I_k) was calculated as P_{MAX}/α (Henley, 1993). Selection of the best model (linear or hyperbolic) for each one of the river segments and days was based on the r square value of the fitted models.

Data analysis

Load of transported nutrients and pharmaceutical compounds was calculated by multiplying concentration by discharge, and attenuation was calculated per unit of distance by calculating the reduction of concentrations in the studied reach. Normality of all variables was initially checked with the Kolmogorov-Smirnov test, and variables were log-transformed when necessary. Differences of measured variables among sites were analyzed by means of generalized least-squares (GLS) models that incorporate spatial structure directly into model residuals (N = 8 for physical and chemical measurements; N = 12 - 20 for biofilm measurements; and N = 40 for metabolic measurements). Pearson moment correlation analysis was used with the averaged values of each segment to identify the direction and strength of the relationships between variables (N = 4), and between variables and distance at the end of the river segments. This last type of correlation was performed in two ways, either including CR reach values or excluding them. Normality was tested with the residuals of the models by the Shapiro test. The significance of different comparisons was tested by ANOVA. All analyses were considered significant at $P < 0.05$, and were performed with the R software (version 3.1.1; R Development Core Team, Vienna, Austria).

Results

Table 1. Water physicochemical characteristics for each river segment (mean \pm SD). The * symbol indicates significant difference ($p < 0.05$) in comparison to CR site. GLR: Global radiation reaching the streambed; LAI: Leaf Area index; K_{20} : Reaeration coefficients corrected with temperature; DOC: Dissolved organic carbon; SPOM: Suspended particulate organic carbon.

	CR	IR1	IR2	IR3
Discharge ($\text{m}^3 \text{s}^{-1}$)	0.29 \pm 0.03	0.50 \pm 0.17	0.64 \pm 0.03 *	0.83 \pm 0.24 *
Velocity (m s^{-1})	0.18 \pm 0.06	0.20 \pm 0.08	0.38 \pm 0.14	0.33 \pm 0.08
Depth (m)	0.14 \pm 0.02	0.15 \pm 0.01	0.19 \pm 0.05	0.23 \pm 0.01 *
Width (m)	11.90 \pm 0.85	10.25 \pm 2.47	9.45 \pm 1.34	10.70 \pm 0.42
GLR ($\text{MJ m}^{-2} \text{d}^{-1}$)	4.62 \pm 0.82	9.52 \pm 1.69 *	11.93 \pm 2.12 *	14.34 \pm 2.54 *
LAI	2.52 \pm 0.83	1.76 \pm 0.55	0.71 \pm 0.42 *	0.72 \pm 0.16 *
K_{20} (day^{-1})	32.67	28.79	29.76	34.45
Temperature ($^{\circ}\text{C}$)	13.58 \pm 1.41	13.80 \pm 1.10	13.49 \pm 0.87	13.60 \pm 0.86
pH	8.54 \pm 0.39	8.63 \pm 0.01	8.55 \pm 0.12	8.65 \pm 0.25
Conductivity ($\mu\text{S cm}^{-1}$)	180.90 \pm 0.85	225.75 \pm 13.79 *	214.5 \pm 2.12 *	207.75 \pm 7.42 *
Ammonium (mg L^{-1})	0.012 \pm 0.001	1.92 \pm 1.03 *	0.90 \pm 0.41 *	0.37 \pm 0.33 *
Phosphate (mg L^{-1})	0.039 \pm 0.001	0.292 \pm 0.111 *	0.200 \pm 0.020 *	0.182 \pm 0.004 *
DOC (mg L^{-1})	2.54 \pm 0.15	3.67 \pm 0.41 *	3.14 \pm 0.34 *	2.79 \pm 0.16
SPOM (mg L^{-1})	2.90 \pm 0.08	4.48 \pm 0.51 *	3.04 \pm 0.08	3.02 \pm 0.34

Environmental measurements

Discharge and irradiance increased and LAI decreased along the study reaches (Table 1), but water velocity, depth, channel width, water temperature and pH did not change significantly. Conductivity increased 25% from CR to IR1, while ammonium increased 160-fold (0.01 mg L^{-1} to 1.9 mg L^{-1}) and phosphate 7.5-fold (0.04 mg L^{-1} to 0.3 mg L^{-1} ; Table 1). These three variables decreased further downstream (Table 1). The decrease in ammonium was a result of attenuation processes and not only of dilution or dispersion, as its load increased from 3.48 mg s^{-1} in CR to 960 mg s^{-1} in IR1, and then decreased to 576 and 307 mg s^{-1} in IR2 and IR3, respectively. On the other hand, the WWTP effluent increased the phosphate load from 11.3 mg s^{-1} in CR to 146 mg s^{-1}

in IR1; however, it remained steady further downstream (128 mg s^{-1} and 151 mg s^{-1}), indicating no phosphate attenuation along the impact reach.

Carbamazepine (2.49 ng L^{-1}), ibuprofen (14.42 ng L^{-1}) and sulfamethoxazole (0.95 ng L^{-1}) were the only pharmaceuticals found in CR. All pharmaceuticals analyzed showed significant increases from CR to IR1 (Fig. 1), as well as a progressive decrease from IR1 to IR3. In fact, ibuprofen and sulfamethoxazole returned to values not significantly different than those in CR. The decrease of diclofenac, ibuprofen, sulfadiazine and venlafaxine concentrations was the result of natural attenuation, as shown by reduced loads along the impact reach. For example, diclofenac load reduction from IR1 to IR3 was of 0.59% km^{-1} , whereas venlafaxine

load reduction was of 0.41% km⁻¹. In contrast, the loads of carbamazepine, diazepam, sulfamethoxazole, sulfapyridine and venlafaxine remained steady, and that of sulfamethazine increased downstream. DOC values averaged 2.5 mg L⁻¹ in the CR river segment, increased to 3.7 mg L⁻¹ at IR1, and decreased to 2.8 mg L⁻¹ at IR3. As in the case of phosphate, no clear attenuation could be detected, as the loads transported by the river were 736 mg s⁻¹ in CR, increased to 1835 mg s⁻¹ in IR1, and to 2010 mg s⁻¹ in IR2 and 2316 mg s⁻¹ in IR3. Similarly, SPOM values averaged 2.9 mg L⁻¹ in the CR river segment, increased to 4.5 mg L⁻¹ at IR1 and decreased to 3.0 mg L⁻¹ at IR3, although there were no clear changes in SPOM loads along the impact reach. Both DOC and SPOM concentrations increased significantly from CR to IR1, and then decreased linearly with distance from the WWTP ($R^2 > 0.51$, $P < 0.05$), until they approached pre-disturbance values (Table 1).

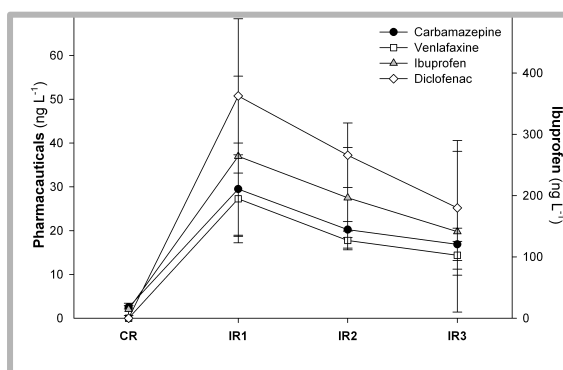


Figure 1. Measured concentrations of some pharmaceuticals along the studied river segments.

Benthic organic matter and biofilm characteristics

BOM and Chl-*a* concentration showed contrasting responses to the WWTP effluent. BOM values averaged 26.9 g AFDM m⁻² at the CR river segment, 139.0 g AFDM m⁻² at IR1, 68.6 g AFDM m⁻² at IR2 and 72.8 g AFDM m⁻² at IR3 (Table 2), but values were not statistically significantly different from those at CR. Chl-*a* values in the CR segment averaged 1.2 µg cm⁻², and showed a progressive increase downstream up to 9.6 µg cm⁻² at IR3 (linear regression with distance, $R^2 = 0.62$, $P < 0.0001$). BOM was positively correlated with conductivity, ammonium and phosphate, and Chl-*a* with discharge and GLR ($R^2 > 0.90$, $P < 0.05$).

Y_{max} and Y_{eff} averaged 0.6 in CR and did not change downstream (Table 2). PQ showed high values (> 0.8) in all segments with no significant changes, while the NPQ increased *ca.* 50% from CR to IR1, with a subsequent decrease until IR3. The ETS showed almost no spatial changes, with values around 20 µg cm⁻¹ h⁻¹ in all river segments. AP activity averaged 65.8 nmol MUF cm⁻² h⁻¹ in CR and decreased in the impact reach from 51.3 nmol MUF cm⁻² h⁻¹ at IR1 to 46.5 nmol MUF cm⁻² h⁻¹ at IR3. BG activity values averaged 59.9 nmol MUF cm⁻² h⁻¹ in CR and reached 116.8 nmol cm⁻² h⁻¹ in IR2. Finally, the LAP activity averaged 66.0 nmol cm⁻² h⁻¹ in CR, increased significantly to 106.9

Table 2. Benthic organic matter and biofilm characteristics in each river segment (mean \pm SD). The * symbol indicates significant difference ($p < 0.05$) in comparison to CR site. BOM: Benthic organic matter; Chl-a: Chlorophyll-a; Y_{max} : Maximum photosynthetic capacity; Y_{eff} : Effective quantum yield; PQ: Photochemical quenching; NPQ: Non-photochemical quenching; ETS: Electron transport system; AP: alkaline phosphatase; BG: β -Glucosidase; LAP: Leucine-amino-peptidase.

	CR	IR1	IR2	IR3
BOM (g m ⁻²)	26.95 \pm 11.99	138.99 \pm 202.36	68.56 \pm 48.51	72.79 \pm 55.85
Chl-a (μ g cm ⁻²)	1.24 \pm 0.24	4.20 \pm 1.89 *	6.16 \pm 1.71 *	9.61 \pm 5.83 *
Y_{max}	0.65 \pm 0.05	0.64 \pm 0.06	0.57 \pm 0.12	0.57 \pm 0.08
Y_{eff}	0.62 \pm 0.01	0.56 \pm 0.03	0.53 \pm 0.11	0.53 \pm 0.10
PQ	0.83 \pm 0.08	0.89 \pm 0.05	0.87 \pm 0.06	0.89 \pm 0.02
NPQ	0.13 \pm 0.01	0.20 \pm 0.05	0.19 \pm 0.09	0.15 \pm 0.06
ETS (μ g cm ⁻² h ⁻¹)	22.48 \pm 2.61	18.95 \pm 4.62	17.65 \pm 4.61	18.00 \pm 1.77
AP (nmol cm ⁻² h ⁻¹)	65.85 \pm 10.99	51.28 \pm 17.49	45.83 \pm 19.83	46.45 \pm 16.23
BG (nmol cm ⁻² h ⁻¹)	59.88 \pm 6.27	50.31 \pm 20.82	116.76 \pm 62.58	48.83 \pm 32.11
LAP (nmol cm ⁻² h ⁻¹)	66.00 \pm 19.34	106.92 \pm 10.77 *	87.73 \pm 10.83	84.25 \pm 11.35

nmol cm⁻² h⁻¹ at IR1, and decreased downstream reaching 84.3 nmol cm⁻² h⁻¹ at IR3. NPQ was positively correlated with conductivity and ammonium, whereas LAP was positively correlated with conductivity, ammonium, DOC and BOM ($R^2 > 0.75$, $P < 0.05$).

River ecosystem metabolism

Ecosystem metabolism followed contrasting longitudinal patterns. There was an almost 3-fold increase in ER from CR to IR1 (from 3.1 to 8.8 g O₂ m⁻² d⁻¹; Fig. 2, Table 3), and a decrease along the impact reach down to 6.6 g O₂ m⁻² d⁻¹ at IR3, a value still two times higher than the

control. Overall, ER was significantly higher in the impact reach than in the control reach, and the decrease downstream of the WWTP was also significant (linear regression with distance, $R^2 = 0.29$, $P = 0.002$). ER was not correlated to DOC or SPOM, but it was to ammonium ($R^2 = 0.99$, $P = 0.001$), phosphates ($R^2 = 0.98$, $P = 0.003$), pharmaceuticals ($R^2 = 0.99$, $P = 0.002$) and to BOM ($R^2 = 0.91$, $P = 0.043$). GPP averaged 0.5 g O₂ m⁻² d⁻¹ in CR (Table 3), it did not differ between CR and IR1, but then increased significantly to 1.24 in IR2 and 2.3 in IR3 (Fig. 2) following the increase

Table 3. Metabolism parameters (mean \pm SD) for each river segment. The * symbol indicates significant difference ($p < 0.05$) in comparison to CR site. GPP: Gross primary production; ER: Ecosystem respiration; NEM: Net ecosystem metabolism; P/R: production to respiration ratio.

	CR	IR1	IR2	IR3
GPP (g O ₂ m ⁻² d ⁻¹)	0.54 \pm 0.15	0.70 \pm 0.25	1.24 \pm 0.38 *	2.30 \pm 0.64 *
ER (g O ₂ m ⁻² d ⁻¹)	-3.11 \pm 0.16	-8.79 \pm 1.11 *	-7.46 \pm 1.85 *	-6.56 \pm 0.98 *
NEM (g O ₂ m ⁻² d ⁻¹)	-2.57 \pm 0.23	-8.09 \pm 0.87 *	-6.22 \pm 1.87 *	-4.6 \pm 1.41 *
P/R	0.17 \pm 0.05	0.08 \pm 0.02 *	0.18 \pm 0.06	0.36 \pm 0.14 *

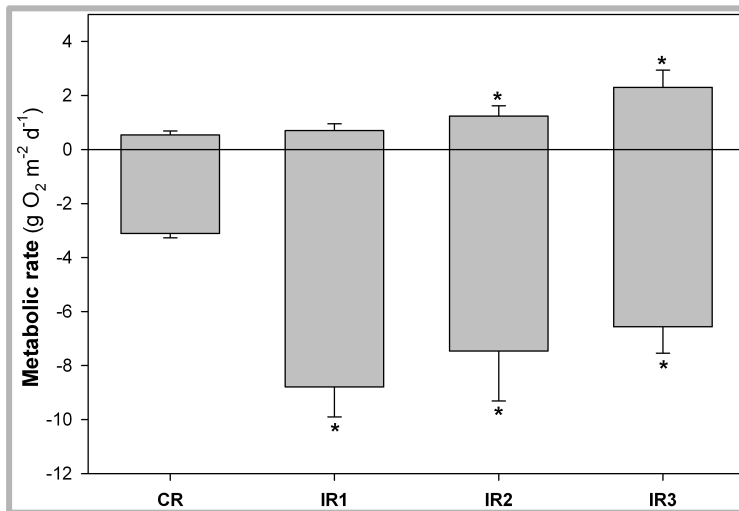


Figure 2. Daily metabolic rates (mean \pm SD) in each river segment. Positive values represent gross primary production (GPP) and negative values ecosystem respiration (ER). The * symbol indicates significant difference ($p < 0.05$) in comparison to CR site.

on the light availability ($R^2 = 0.51$, $P < 0.0001$) (Fig. 3). All river segments were heterotrophic, with NEM values averaging $-2.6 \text{ g O}_2 \text{ m}^{-2} \text{ d}^{-1}$ in CR, increasing to $-8.1 \text{ g O}_2 \text{ m}^{-2} \text{ d}^{-1}$ in IR1, and then decreasing downstream to $-4.3 \text{ g O}_2 \text{ m}^{-2} \text{ d}^{-1}$ in IR3. NEM was significantly higher in all impact segments than in CR. The P/R ratio averaged 0.17 in CR, it decreased significantly in IR1 with values

averaging 0.08, then returned to 0.18 in IR2, and finally increased significantly to 0.36 in IR3. NEM was positively correlated to ammonium ($R^2 = 0.94$, $P = 0.032$) and DOC ($R^2 = 0.94$, $P = 0.032$), whereas P/R showed no significant correlation with any measured variable. No significant correlations were found for measurements at biofilm and ecosystem level.

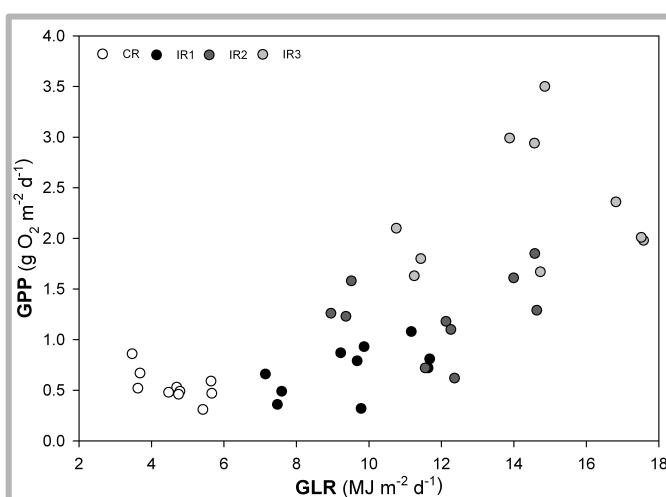


Figure 3. Daily gross primary production (GPP) in relation to the received total GLR. Values from 10 days are shown for each river segment.

Photosynthesis-Irradiance relationship

P-I relationships were strongly affected by the discharge of the WWTP effluent (Fig. 4). The initial slope was lowest at IR1, but by IR2 it returned to values similar to CR, and by IR3 the initial slope was even higher (Table 4). The shape of the P-I curves also changed, following a linear equation at IR1, whereas the hyperbolic equation offered a better fit at the rest of the segments (Table 4). I_K increased in the impact reach, but the difference was only statistically significant in IR3. The hyperbolic equations showing a better fit to the data of CR, IR2, IR3 included temperature as explanatory variable, which improved the fit to the data showing hysteresis; thus, for the same light availability, GPP was lower during the morning than during the afternoon.

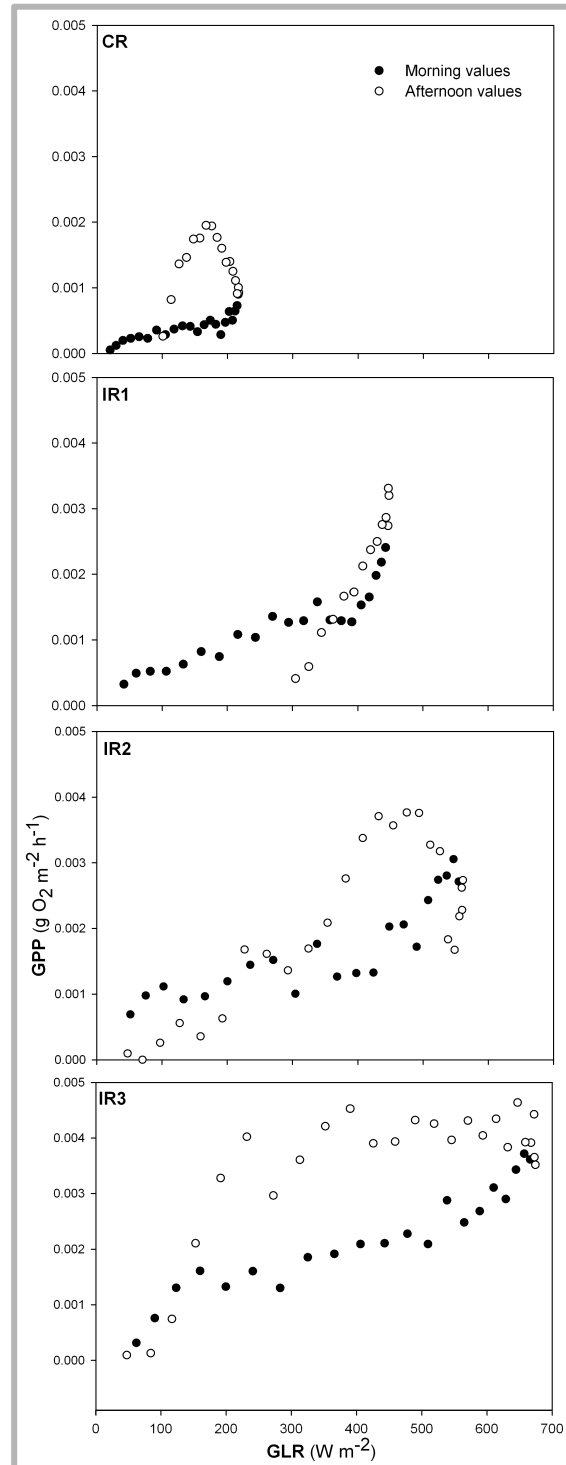


Figure 4. Gross primary production (GPP) versus GLR for the first day of measurement for each reach. Measurements in the morning are indicated by black circles and those in the afternoon by white circles.

Table 4. Production-Irradiance (P-I) relationships and calculated parameters (mean \pm SD) for each river segment. The * symbol indicates significant difference ($p < 0.05$) in comparison to CR site.

	Selected Model	r^2	Initial slopes	Light saturation (I_K) ($W m^{-2}$)
CR	Hyperbolic + Temperature	0.85 ± 0.15	$5.72 \cdot 10^{-5} \pm 4.08 \cdot 10^{-5}$	113.39 ± 62.09
IR1	Linear	0.69 ± 0.10	$5.17 \cdot 10^{-6} \pm 7.53 \cdot 10^{-7} *$	-
IR2	Hyperbolic + Temperature	0.60 ± 0.25	$5.70 \cdot 10^{-5} \pm 4.87 \cdot 10^{-5}$	260.30 ± 211.89
IR3	Hyperbolic + Temperature	0.82 ± 0.10	$6.25 \cdot 10^{-5} \pm 4.74 \cdot 10^{-5}$	$245.78 \pm 65.83 *$

Discussion

The discharge of the WWTP effluent caused a large increase in the concentration of all measured contaminants: nutrients, dissolved and suspended organic matter, and pharmaceutical products. The contaminants below the effluent did not produce evident signs of eutrophication such as anoxia or algal blooms, common in highly polluted rivers (Smith, 2003; Brack *et al.*, 2007). Nevertheless, the ammonium concentration in IR1 was high enough to cause potential toxic effects on stream invertebrates and to impair litter decomposition rates (Baldy *et al.*, 2002; Maltby *et al.*, 2002). On the other hand, the concentration of pharmaceutical compounds such as diclofenac was similar to levels commonly found downstream of WWTP effluent discharges, which may approach 100 ng L⁻¹ (Vieno & Sillanpää, 2014). The lowest concentrations of diclofenac producing toxic effects seem to range between 10 and 1000 ng L⁻¹, depending on the species, exposure duration and endpoints used (Vieno & Sillanpää, 2014). As the observed concentrations in our study near the WWTP effluent discharge (50 ng L⁻¹) are within this range of toxic concentrations, we could expect some toxic effects. Furthermore, toxic effects have been reported in Mediterranean rivers at concentrations just four times higher (220 ng L⁻¹ for diclofenac in average) than those

measured in this study, resulting in changes in algal and macroinvertebrate communities (Muñoz *et al.*, 2009; Ginebreda *et al.*, 2010). Finally, similar effects on NPQ from pharmaceuticals have been reported in the Mediterranean basins (Ponsatí *et al.*, *in revision*), with diclofenac values ranging from 1 to 61 ng L⁻¹.

The concentration of both, assimilable and toxic contaminants decreased downstream of the WWTP effluent discharge. The decrease in ammonium concentration was a consequence of attenuation, not simple dilution, as shown by reduced loads. Ammonium is a highly reactive nutrient that is readily nitrified or taken up by the biota (Martí *et al.*, 2004), and thus often shows downstream attenuation (von Schiller *et al.*, 2008). In contrast, attenuation of phosphate and organic matter (both dissolved and suspended) was less intense. The rate at which different nutrients are retained seems to be highly variable and depends, among others factors, on which is the limiting nutrient in each system (Newbold *et al.*, 1982). For instance, Elósegui *et al.*, (1995) showed the load of phosphate and ammonium to decrease at a similar rate below a point input of raw sewage, whereas Merseburger *et al.* (2005) reported a higher decrease in ammonium than in phosphate concentration downstream of a WWTP effluent. Pharmaceutical compounds

showed contrasting trends: attenuation was observed for diclofenac, ibuprofen, sulfadiazine and verapamil, but not for carbamazepine, diazepam, sulfamethoxazole, sulfapyridine, venlafaxine and sulfamethazine. The observed attenuations in terms of load reduction were similar to that reported at the same site (Acuña *et al.*, 2015) and those from other systems (Writer *et al.*, 2012). Mean relative attenuation for ibuprofen was of $61 \pm 10\%$, and for diclofenac of $12 \pm 26\%$ (Corominas *et al.*, *in revision*).

The differences in biofilm variables between the study reaches suggested that the WWTP effluent was acting more as a subsidy than as a stressor. In general, toxicants and other stressors reduce biofilm biomass and photosynthetic efficiency (Tlili & Montuelle, 2011; Corcoll *et al.*, 2015). Nevertheless, patterns are often complicated by non-linear responses such as hormesis (Calabrese, 2005), reduced sensitivity to toxics under enhanced nutrient concentrations (Guasch *et al.*, 2004), adaptation of communities to past toxicity (Pesce *et al.*, 2011) or interaction between light history and sensitivity to toxicity (Bonnineanu *et al.*, 2012). In our case, Chl-*a* concentrations were largely unaffected by the WWTP effluent, and showed instead a progressive downstream increase, most likely caused by the higher light availability as a consequence of

reduced shading (Roberts, Sabater & Beardall, 2004). BOM, on the other hand, showed a 5-fold increase after the WWTP effluent input, followed by a reduction downstream to values 3 times higher than the control in IR3. Photosynthetic efficiency and enzyme activities also showed little effect of the WWTP. A clear exception was NPQ, which was 54% higher at IR1 than at CR. NPQ has been reported to increase as a response to toxicity in order to protect the photosynthetic apparatus from excess light that cannot be used for photosynthesis (Juneau *et al.*, 2001; Geoffroy *et al.*, 2003). Similarly, LAP activity increased below the discharge of the WWTP effluent and decreased further downstream closely matching the pollution pattern, probably as a result of higher abundance of organic nitrogen along this gradient (Proia *et al.*, 2013). Overall, WWTP effluents seem to have promoted biological activity of the biofilm, rather than reducing it.

At the ecosystem level, respiration was also subsidized, following a pattern similar to that of organic matter. Although the low number of river segments analyzed limits the statistical power of correlation analyses, ER was mostly related to BOM, indicating the likely coupling between both variables along the river, as has been described elsewhere (*e.g.* Young & Huryn, 1999; Acuña *et al.*, 2004). ER has been directly related to anthropogenic inputs of nutrients and

organic matter (Silva-junior *et al.*, 2014; Yates *et al.*, 2013), thereby overriding the negative effects of toxic contaminants such as pharmaceuticals (*e.g.* Rosi-Marshall *et al.*, 2013). GPP was also affected by the WWTP effluent, but showed a constant increase further downstream, which suggests that light was the primary driver of this variable in the studied river. Although GPP has often been linked to nutrient status (*e.g.* Gücker *et al.*, 2006), this relationship only holds when irradiance is not limiting (Artigas *et al.*, 2013). Nevertheless, just below the WWTP effluent (IR1) GPP was depressed with respect to the values expected according to the available irradiance, as shown by the slope and shape of P-I curves, therefore suggesting a stress. As a result of the relative suppression of GPP and the enhancement of ER, there was also a strong decrease in NEM below the WWTP effluent, which recovered downstream because of the reduction of the relative suppression of GPP by toxic pollutants, the increase in light availability, and the decrease of ER along the river segment. Overall, stress effects were only observed for autotrophic processes at both ecosystem and biofilm scales, but only one of the measured biofilm metrics (NPQ) actually reflected the stress effects. This lack of coherence among biofilm metrics on autotrophic processes might be caused by acquired tolerances of the autotrophic community,

as reported by Corcoll *et al.* (2014) in reaches I1 and I2. In regards to heterotrophic processes, subsidy effects were observed at both biofilm and ecosystem scales.

In conclusion, we found ample evidence of WWTP effluents acting as a subsidy, but more limited evidence of them acting as a stressor. Measurements at the biofilm and at the ecosystem level are complementary and mainly differ in their response to subsidy and stress. Most biofilm variables suggested the WWTP effluents acted as a subsidy, whereas at the ecosystem level ER was subsidized, but GPP showed some stress effects as it became partially decoupled from the available light. The complementary response detected at the biofilm and the ecosystem scales stresses the need to study both in order to fully understand the impact of WWTP effluents on river ecosystems.

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CHAPTER 5

Does subsidy alleviate stress? The effects of emerging pollutants on stream biofilm communities depend on nutrient concentration

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Abstract

River ecosystems are threatened by multiple chemicals of anthropogenic origin, including nutrients and pollutants. Some of them have a subsidy effect on ecosystems, whereas others have a toxic effect. In some cases, the prevalence of the subsidy or toxic effect for a given chemical depends on its concentration, and on its interactions with other chemicals in the environment. Given that nutrients can have both a subsidy and a toxic effect depending on their concentration and that are always present in freshwaters, we performed an experiment with artificial streams to assess the effects of nutrients at different concentrations and of emerging pollutants at environmentally relevant concentrations. Our initial hypothesis were that moderate concentrations would have a subsidy effect and might alleviate the effect of emerging pollutants, whereas high concentrations would have a toxic effect and might exacerbate the effect of emerging pollutants. The experimental setting consisted on 18 indoor channels, which were colonized by biofilm for 3 weeks, and then subject to 4 weeks of experimental conditions on a 3×2 factorial structure with three nutrient levels (low, medium or high) and either presence or absence of emerging pollutants (ciprofloxacin, diclofenac, erythromycin, methylparaben, sulfamethoxazole and triclosan), with 3 replicates per treatment. Algal biomass, basal fluorescence, β-glucosidase and metabolic activities were measured during the experiment, and most of them responded linearly to the subsidy effect of nutrients, whereas the toxic effect of emerging pollutants was only detected after 4 weeks of exposure. Therefore, contrary to our hypothesis, no toxic effect was detected for nutrients at the assessed high nutrient concentrations, and only the high nutrient concentrations alleviated the toxic effect of emerging pollutants. In conclusion, our results stress that chemicals with a subsidy effect like nutrients can alleviate the toxic effect of chemicals such as emerging pollutants, and that long-term experiments are required to detect toxic effects of emerging pollutants when assessing their effects at relevant environmental concentrations.

Keywords: emerging pollutants, ecotoxicology, artificial streams, subsidy-stress effects, stream metabolism

Introduction

River ecosystems are commonly threatened by multiple stressors, such as chemical pollution, flow regulation, geomorphological alterations, climate change and invasive species (Vörösmarty *et al.*, 2010). Even chemical pollution is a complex stressor, as many pollutants can reach rivers from both point and diffuse sources, and often appear in complex mixtures whose joint effects can have contrasting effects (*e.g.* Culp, Podemski & Cash, 2000). The effluents of wastewater treatment plants (WWTP) are clear examples of complex mixtures of

pollutants reaching river ecosystems, as they include nutrients, organic matter, metals, pesticides, and other substances such as emerging pollutants (Ternes, 1998; Petrovic *et al.*, 2002; Gros, Petrovic & Barceló, 2007).

The contrasting effects of environmental stressors led Odum, Finn and Franz (1979) to propose the "Subsidy-Stress Hypothesis" (SSH), according to which pollutants can be classified in two groups depending on their ecological effects: assimilable pollutants (*e.g.* dissolved nutrients and organic matter) subsidize biological activity at least up to a threshold beyond which they can suppress

it, whereas toxic pollutants (*e.g.* heavy metals, pesticides) are always deleterious to organisms and tend to suppress biological activity. The SSH is supported by some empirical evidence. Moderated nutrient concentrations have been shown to enhance the biomass and activity of biofilm (Stelzer, Heffernan & Likens, 2003), and to promote ecosystem functioning (Woodcock & Hury, 2005; Bernot *et al.*, 2010; Cabrini *et al.*, 2013). Although, hump-shape responses of ecosystem processes have also been reported, most of them in correlative studies measured in the field (Izagirre *et al.*, 2008; Wagenhoff *et al.*, 2011; Woodward *et al.*, 2012; Dunck *et al.*, 2015), but there are also some manipulative ones (Wagenhoff *et al.*, 2012; 2013). Measured variables of these most of these works coincide with the thresholds given by Dodds, Jones & Welch (1998), where in mesotrophic conditions (500-1259 $\mu\text{g L}^{-1}$ of total nitrogen and 25-70 $\mu\text{g L}^{-1}$ of total phosphorus) maximum responses appear, and in eutrophic ones (> 1260 $\mu\text{g L}^{-1}$ of total nitrogen and >71 $\mu\text{g L}^{-1}$ of total phosphorus) the suppression of them. The reason of the decline of responses can be very complex and not straightforward, however Woodward *et al.* (2012) attributes the measured inhibition of leaf breakdown to the decrease of consumers that happens beyond a nutrient level. Additionally, the effects of the dose

depend on the exposure time. For instance, Camargo & Alonso (2006) considered harmful for sensitive aquatic animals in acute toxicity tests (96 h) concentrations of ammonium and nitrate higher than 0.1 mg L^{-1} and 17 mg L^{-1} respectively, whereas in chronic toxicity tests (>30 d) at 0.05 and 1.1 mg L^{-1} .

On the other hand, toxic pollutants reduce the abundance and affect the composition of biofilms (Wilson *et al.*, 2003) and invertebrate communities (Muñoz *et al.*, 2009; Alexander *et al.*, 2013; Clements, Cadmus & Brinkman, 2013), and depress ecosystem functioning (Bundschuh *et al.*, 2009; Moreirinha *et al.*, 2011; Rosi-Marshall *et al.*, 2013). Studies of these effects of these contaminants are mostly from ecotoxicological tests (24 to 96 h) that study the survivor of algae, invertebrate (*i.e.* *Daphnia*) or fish in very high concentrations (Solomon *et al.*, 1996; Cleuvers, 2004; Franz *et al.*, 2008; Morin *et al.*, 2010b). In a longer manipulative experiment (11 d) where a mixture of pharmaceuticals in high concentrations (5 $\mu\text{g L}^{-1}$ in total) was provided, Corcoll *et al.* (2015) measured a decrease in biofilm growth rate, biomass and composition. In some correlative field studies there have been reported negative effects at concentrations that appear in rivers for pharmaceuticals, beta-blockers or pesticides (Lawrence *et al.*, 2005; Muñoz *et al.*, 2009; Ricart *et al.*, 2010; Rosi-Marshall *et al.*, 2013). As far as we know,

no correlative field studies have been published regarding the potential effects of emerging contaminants, mainly because they appear and affect biofilm with other contaminants (*e.g.* Proia *et al.*, 2013).

In the real world, most often assimilable and toxic pollutants appear together. Most published information report the stress effects to overwhelm the subsidy effects (*e.g.* Wagenhoff *et al.*, 2011; Wagenhoff, Townsend & Matthaei, 2012; Aristi *et al.*, 2015). Nevertheless, interaction among pollutants potentially add a further level of complication to the SSH scheme. It has been hypothesized that, because they promote biological activity, moderate nutrient concentrations can help organisms to withstand stress, and thus, reduce their sensitivity to toxic pollutants (Koelmans *et al.*, 2001; Guasch *et al.*, 2004; Morin *et al.*, 2010a). Nevertheless, this hypothesis has been rarely tested, especially regarding stream ecosystem functioning.

We performed a mesocosmos experiment with artificial streams to characterize the interaction between assimilable (*i.e.* nutrients) and toxic (a mixture of emerging pollutants) pollutants on stream biofilms structure and function. We provided nutrients at 3 different concentrations; low concentrations (0.04 mg L⁻¹ of ammonium, 1.70 mg L⁻¹ of nitrate, and 0.04 mg L⁻¹ of phosphorus), medium concentrations (0.20 mg L⁻¹ of ammonium, 5.00 mg L⁻¹ of nitrate, and

0.20 mg L⁻¹ of phosphorus) and high concentrations (1 mg L⁻¹ of ammonium, 25 mg L⁻¹ of nitrate, and 1 mg L⁻¹ of phosphorus). And a mixture of 6 emerging contaminants (ciprofloxacin, diclofenac, erythromycin, methylparaben, sulfamethoxazole and triclosan) was provided with at a final nominal concentration of 4.3 µg L⁻¹. We hypothesized i) that biofilm biomass and functioning would be subsidized by moderate nutrient enrichment, but posteriorly stressed by higher levels of nutrients; ii) that the emerging pollutants would impair biofilm structure and function at any concentration; and iii) that this reduction would be less pronounced at moderate nutrient concentrations.

Methods

Experimental design

The experiment was performed in 18 artificial channels located at the Experimental Streams Facility of the Catalan Institute for Water Research (Girona, Spain). The experimental design consisted of a 3 x 2 factorial structure with 3 nutrient levels [low (L), medium (M) or high (H)] and two levels of emerging pollutants [no emerging (NE) or emerging (E)] (Table 1). Most of the published manipulative works where hump-shape responses have been described (*e.g.* Wagenhoff *et al.*, 2012; 2013) coincide

with the thresholds given by Dodds, Jones and Welch (1998), but when studying ecosystem responses at correlative field studies these concentrations are much higher. It is thus, that nutrient concentrations we provided are much higher to ensure to capture these responses. Each artificial stream was assigned to a different treatment following a randomized complete block design, with three replicates per treatment distributed in 3 separate arrays of 6 artificial streams, with each treatment represented once per array. The concentration of pollutants remained constant during the experiment by renewing a part of the water every 2 h. Biofilm variables were measured one day before and 14, 21 and 28 d after the onset of the treatment.

Experimental conditions

Artificial streams consisted on 2 m long, 10 cm wide Plexiglas channels, with a constant slope and an independent source of water per stream. They operated as flow-through systems with a constant flow of 50 mL s⁻¹, resulting in mean water velocity of 0.58 ± 0.06 cm s⁻¹, a water depth over the plane bed between 2.2 and 2.5 cm. Each channel was filled with approximately 5 L of sand extracted from the nearby headwater Fuirosos Stream (D₅₀ = 0.70 mm), resulting in a sandy streambed 2.5 cm deep. The sediment was sterilized at 120 °C for 2 h with a

Presoclave-II 30L autoclave (JP Selecta S.A., Barcelona, Spain), and evenly distributed in the artificial streams. At complete water saturation, the porosity of the sand yielded a water content of 25% of the wet weight. Daily cycles of photosynthetic active radiation (PAR) were defined as 10 h daylight + 14 h darkness. PAR was held constant at 173.99 ± 33 μE m⁻² s⁻¹ during the daytime, and was recorded every 10 min using 4 quantum sensors located across the whole array of streams (sensor LI-192SA, LiCOR Inc, Lincoln, USA). Water temperature was held constant at 20 °C and recorded every 10 min during the entire experiment by means of a cryo-compact circulator (Julabo CF-31, Seelbach, Germany).

Prior to the experiment, nutrients were held constant at 0.040, 1.7 and 0.040 mg L⁻¹ of phosphate (P-PO₄³⁻), nitrate (N-NO₃⁻) and ammonium (N-NH₄⁺) by means of injection of concentrated solutions (KH₂PO₄, and NH₄Cl, respectively) and using a peristaltic pump (IPC pump, Ismatec, Switzerland). For these conditions nitrate was not added, and rain water provided the system with it. With the experiment implantation, oligotrophic treatments remained at same levels, but to get mesotrophic conditions nutrients were added at 0.2, 5 and 0.2 mg L⁻¹ (P-PO₄³⁻, N-NO₃⁻, and N-NH₄⁺, respectively), and for eutrophic 1, 25 and 1 mg L⁻¹, for which nitrate was supplied adding NaNO₃

solutions with the peristaltic pumps. Emerging contaminant mixture consisted of a mixture of 6 compounds at a final nominal concentration of $4.3 \mu\text{g L}^{-1}$ (more specifically; $0.1 \mu\text{g L}^{-1}$ of Methylparaben, $0.2 \mu\text{g L}^{-1}$ of Triclosan and $1 \mu\text{g L}^{-1}$ of Ciprofloxacin, Diclofenac, Erythromycin and Sulfamethoxazole). All solutions were freshly prepared twice a week and stored at cold temperatures in coolers. Background concentrations were assessed every week from water collected from the end-channel area to control the steadiness of the concentrations of the injected pollutants. Water was filtered through $0.7 \mu\text{m}$ glass fiber filters (Whatman GF/F, Kent, UK) into pre-washed polyethylene containers for nutrients and through $0.45 \mu\text{m}$ (Whatman GD/X) into amber glass bottles for emerging pollutants. The concentration of soluble reactive phosphorus was determined with an Alliance-AMS Smartchem colorimeter (AMS, Frepillon, France). The concentrations of N-NO_3^- and N-NH_4^+ were determined on a Dionex ICS-5000 ion chromatograph (Dionex Corporation, Sunnyvale, USA). The concentration of dissolved organic carbon (DOC) was measured on a Shimadzu TOC-V CSH coupled to a TNM module (Shimadzu Corporation, Kyoto, Japan). Dissolved oxygen (DO), pH and specific conductivity were measured once a week by noon at each artificial stream using

WTW (Weilheim, Germany) hand-held probes.

Biofilm was allowed to grow in all channels for 20 d before the exposure to treatments. It was inoculated twice per week during the colonization period using inocula from epilithic and epipsammic biofilms of the unpolluted Llémèna River (Sant Esteve de Llémèna: UTM X 467232; UTM Y 4657846). Water in the channels was sampled once a week to measure the concentration of nutrients and emerging pollutants. Samples for nutrients were filtered through $0.7 \mu\text{m}$ glass fiber filters (Whatman GF/F, Kent, UK), those for emerging pollutants through $0.45 \mu\text{m}$ (Whatman GD/X), and were analyzed as described before.

Biofilm structure and function

The response of biofilm to different treatments was assessed weekly in terms of biomass [chlorophyll a (Chl-*a*) and ash-free dry mass (AFDM)], basal fluorescence (F_0), autotrophic capacity [effective quantum yield (Y_{eff}), and gross primary production (GPP)] and heterotrophic capacity [activities of 4-MUF-phosphatase (APA) and 4-MUF-B-D-Glucosidase (BG), and community respiration (CR)]. F_0 and Y_{eff} were measured *in situ*, one measurement per artificial stream at each time. The rest (Chl-*a*, AFDM, APA and BG) were carried out at the laboratory immediately

after sample collection (one core sample (*ca.* 1.6 cm³ \approx 1.6 cm²) of the sediment was taken per artificial stream at each time).

To measure Chl-*a*, collected samples were frozen at -18 °C. Samples were later thawed and measured after extraction in 90% acetone for 12 h in the dark at 4 °C (Steinman, Lamberti & Leavitt, 2006). Complete extraction of Chl-*a* was ensured with sonication (30 s, 360 W power, 50/60 Hz frequency, JP Selecta SA, Barcelona, Spain). After filtration (Whatman GF/C) of the extract, the Chl-*a* concentration was determined spectrophotometrically using a Lambda UV/VIS spectrophotometer (U-2000 Spectrophotometer; Hitachi, Tokyo, Japan) and following Jeffrey & Humphrey (1975). AFDM was used as a surrogate of total biomass in the biofilm. For its determination, biofilm samples were dried at 60 °C to constant weight, combusted at 450 °C for 4 h, and reweighed.

F₀ and Y_{eff} were measured *in situ* with a portable pulse amplitude modulate fluorometer (Diving-PAM; WALZ, Effeltrich, Germany) to evaluate the structural and functional changes in the algal component of the biofilm. F₀ was used as an estimation of algal biomass (Schmitt-Jansen & Altenburger, 2008) and Y_{eff} reflects the photosynthetic efficiency of energy conversion at Photosystem II (PS II) reaction centers (Schreiber *et al.*, 2002). They are commonly used to evaluate the physiological state of algae

and their response to different environmental stressors such as toxicants, light stress or desiccation (Sabater *et al.*, 2007; Corcoll *et al.*, 2012; Timoner *et al.*, 2012).

APA and BG activities were determined using substrate analogues of MUF (methylumbelliferyl). Core samples and MUF substrate blank were placed in individual glass vials in 4 mL of filtered stream water (Whatman Nylon Membrane 0.2 μ m) and incubated with 0.120 mL of each substrate (0.3 mmol L⁻¹ to ensure substrate saturation, Romaní & Sabater, 1999). Incubation was done in dark conditions and continuously shaken for 1 h at 20 °C. Two blanks of filtered stream water (Wathman Nylon Membrane 0.2 μ m) were also incubated. After addition of 4 mL of 0.05 M glycine buffer, pH 10.4, samples were frozen at -18 °C. Later, samples and standard calibrating solutions of MUF were thawed and quantified by spectrofluorometry (Romaní & Sabater 1999) (Hitachi Fluorescence Spectrophotometer F-7000).

Net ecosystem metabolism and CR were assessed by means of oxygen variations in cylindrical recirculating chambers (Acuña *et al.*, 2008), which enclosed trays containing 160 cm³ of streambed sediments, one from each artificial stream. The chambers (volume 0.96 L) were made of acrylic glass (PMMA) and operated simultaneously. The incubations for each metabolism rate

lasted for 1 h, and were carried out inside an incubator chamber (Radiber AGP-700-ESP, Barcelona, Spain) at the same temperature as in the artificial streams. Net ecosystem metabolism was measured under a PAR of $168 \pm 2 \mu\text{E m}^{-2} \text{s}^{-1}$, similar to the irradiance received at the artificial streams, and CR was measured in darkness. DO concentration inside the chambers was measured continuously and logged at 15 s intervals with oxygen sensors (PreSens OXY-10mini, Regensburg, Germany). Trays were returned to the corresponding artificial stream after the measurements. Metabolism rates were calculated according to Acuña *et al.* (2008), with GPP estimated as the sum of net ecosystem metabolism and CR.

Data analysis

After treatment implementation, differences between time and treatments were tested with 3-way repeated measurements ANOVA with time, nutrient treatment and emerging pollutant treatment as fixed factors, and arrays (*i.e.* replicates) as random factor ($n = 72$). Interactions were also tested to see if treatments affected biofilm development. Additionally, Post hoc Tukey tests were done for each sampling day to see whether general trends changed or not. Pearson

moment correlation analysis was used with the averaged values of each treatment ($n = 24$) to identify the direction and strength of the relationships between variables. Normality of all variables was checked with the Kolmogorov-Smirnov test, and variables were log-transformed when necessary. All analyses were considered significant at $P < 0.05$, and were performed with the R software (version 3.1.1; R Development Core Team, Vienna, Austria).

Results

Experimental conditions

The achieved ammonium and phosphate concentrations were lower (80 to 90% and 25 to 80% less, respectively) than the nominal concentrations (0.04, 0.2 and 1 mg L^{-1} for L, M and H), whereas the achieved nitrate concentration in L and M was similar to the nominal one (1.7 and 5 mg L^{-1}), but not in H, which was 10% lower (25 mg L^{-1}) (Table 1). The concentrations of emerging contaminants reached in the experiment ranged from 3.4 to 3.7 $\mu\text{g L}^{-1}$ in total (15 to 25% lower than the nominal concentration of 4.3 $\mu\text{g L}^{-1}$) (Table 1).

Environmental conditions showed no statistically significant differences between channels before the onset of the

Table 1. Average (\pm SD) of nutrient and emerging pollutant concentrations in each treatment with emerging pollutants.

	Low	Medium	High
Ammonium (mg N-NH ₄ ⁺ L ⁻¹)	0.004 \pm 0.002	0.041 \pm 0.012	0.184 \pm 0.004
Nitrate (mg N-NO ₃ ⁻ L ⁻¹)	1.685 \pm 0.007	5.570 \pm 0.735	22.955 \pm 0.502
Phosphate (mg P-PO ₄ ³⁺ L ⁻¹)	0.008 \pm 0.001	0.115 \pm 0.030	0.743 \pm 0.004
Erythromycin (μ g L ⁻¹)	0.625 \pm 0.104	0.635 \pm 0.087	0.636 \pm 0.131
Sulfamethoxazole (μ g L ⁻¹)	0.805 \pm 0.141	0.849 \pm 0.125	0.756 \pm 0.193
Ciprofloxacin (μ g L ⁻¹)	1.234 \pm 0.133	1.336 \pm 0.144	1.171 \pm 0.216
Diclofenac (μ g L ⁻¹)	0.812 \pm 0.115	0.834 \pm 0.111	0.754 \pm 0.166
Triclosan (μ g L ⁻¹)	0.003 \pm 0.001	0.004 \pm 0.005	0.003 \pm 0.001
Methylparaben (μ g L ⁻¹)	0.037 \pm 0.032	0.039 \pm 0.034	0.032 \pm 0.034

treatments (Table 2). Afterwards, temperature increased during the entire experiment 0.8 °C in average, but averaged 20 °C in all treatments (Table 2). Similarly, DO was steady throughout the entire experiment and among treatments, with values between 9.99 and 10.41 mg L⁻¹. pH increased slightly during the experiment. Conductivity changed during the experiment due to the nutrient treatments, with values around 218 μ S cm⁻¹ in L nutrient treatments, 250 μ S cm⁻¹

in M and 382 μ S cm⁻¹ in H ones. Interaction of time and nutrient was also significant (Table 2). Conductivity, pH and temperature were positively correlated to nutrients ($R^2 > 0.24$, $P < 0.001$), but DO was not correlated to the other variables.

Biofilm

Before exposure to treatments, biofilm biomass averaged 0.0047 g AFDM cm⁻² and 24.39 μ g Chl-*a* cm⁻², and enzyme

Table 2. Average (\pm SD) of physical and chemical water characteristics of the artificial streams exposed to different treatments before and after the treatment implementation. Results (P values) for the 3-way ANOVA are also shown.

Treatment	Temperature (°C)	Dissolved oxygen (mg L ⁻¹)	pH	Conductivity (μ S cm ⁻¹)
<u>Before treatment implementation</u>				
All artificial streams	19.94 \pm 0.59	9.13 \pm 0.71	8.06 \pm 0.24	187.64 \pm 23.33
<u>After treatment implementation</u>				
NE_L	19.73 \pm 0.12	10.24 \pm 0.53	8.43 \pm 0.32	218.56 \pm 6.54
NE_M	19.73 \pm 0.17	10.19 \pm 0.74	8.69 \pm 0.18	254.67 \pm 7.51
NE_H	19.78 \pm 0.19	10.03 \pm 0.47	8.67 \pm 0.25	386.44 \pm 2.69
E_L	19.92 \pm 0.15	9.99 \pm 0.46	8.69 \pm 0.16	218.44 \pm 9.62
E_M	19.88 \pm 0.19	10.10 \pm 0.57	8.74 \pm 0.31	246.89 \pm 8.85
E_H	19.82 \pm 0.21	10.41 \pm 0.26	9.00 \pm 0.37	378.89 \pm 3.66
3-way ANOVA				
Day	<0.0001	0.173	<0.0001	<0.0001
Nutrients	0.778	0.886	0.198	<0.0001
Emerging	0.289	0.838	0.058	0.292
Day:Nut	0.879	0.812	0.693	<0.0001
Day:Emerg	0.738	0.158	0.372	0.948
Nut:Emerg	0.999	0.550	0.835	0.762
Day:Nut:Emerg	0.940	0.914	0.936	0.888

Table 3. Results (P values) for the 3-way ANOVA for biofilm variables. **AFDM**; Ash free dry mass; **Chl-*a***; Chlorophyll-*a*; **AP**; Alkaline phosphatase; **BG**; Beta glucosidase.

	Biomass		Enzyme activities		Diving-PAM		Metabolism			
	AFDM	Chl- <i>a</i>	AP	BG	F ₀	Y _{eff}	GPP	ER	NEM	EF
Day	<0.0001	0.511	<0.0001	<0.0001	<0.0001	0.487	<0.0001	<0.0001	0.005	<0.0001
Nutrient	0.030	0.067	<0.0001	0.350	<0.0001	0.103	0.001	<0.0001	0.011	<0.0001
Emerging	0.027	0.167	0.017	0.075	0.437	0.576	0.274	0.023	0.604	0.151
Day : Nut	0.990	0.976	<0.0001	0.145	0.006	0.865	0.803	0.198	0.454	0.941
Day : Emerg	0.520	0.142	0.960	0.727	0.117	0.376	0.203	0.874	0.108	0.305
Nut : Emerg	0.407	0.090	0.111	0.055	0.794	0.937	0.274	0.101	0.467	0.193
Day : Nut : Emerg	0.478	0.465	0.261	0.813	0.625	0.814	0.663	0.996	0.526	0.760

activity 427.62 $\mu\text{mol h}^{-1} \text{cm}^{-2}$ for APA and 9.61 $\mu\text{mol h}^{-1} \text{cm}^{-2}$ for BG. After treatment AFDM and BG increased whereas APA decreased (Fig. 1, Table 3). AFDM and

APA were affected both by nutrients and emerging pollutants, but only APA showed a significant difference for the interaction of time and nutrients. Chl-*a* did

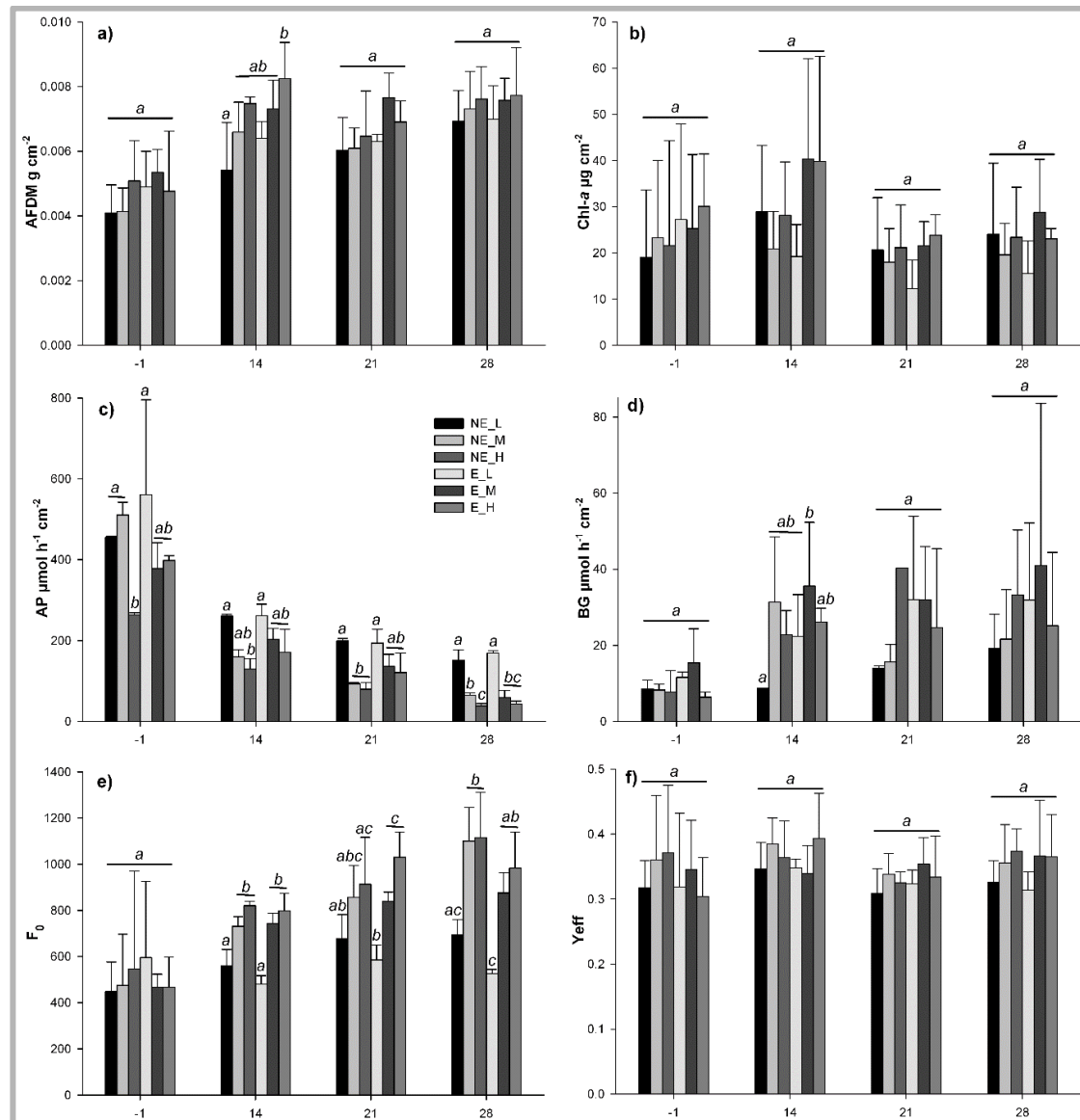


Figure 1. Changes in biofilm measurements, expressed as the means (\pm SD) of the values per treatment and per time of a) AFDM, b) Chl-*a*, c) AP, d) BG, e) F₀, and f) Y_{eff}. Results for Post hoc Tukey test are shown in letters for each day.

implementation (Fig. 2). F_0 increased during the experiment, was affected by nutrients, and their interaction with time was significant. Y_{eff} did not change during the experiment and was not affected by any treatment (Table 3). F_0 and Y_{eff} averaged 499.61 and 0.34, respectively, before treatment the experiment and was not affected by any of the treatments. No significant interactions were found for nutrients and emerging pollutants for these variables. Post hoc Tukey tests showed no significant changes among treatments for AFDM, Chl-*a*, BG and Y_{eff} , but for APA and F_0 nutrient effects were more marked at the end of the experiment. These differences were especially marked for F_0 at the end of the experiment, which showed a linear

increase due to the nutrient treatments, and lower values under emerging pollutants. Most of the biofilm structural measurements, excluding Chl-*a*, were positively correlated between them ($R^2 > 0.17$, $P < 0.04$), negatively with APA ($R^2 > 0.57$, $P < 0.0001$), and positively with conductivity and nutrients ($R^2 > 0.18$, $P < 0.035$). On the other hand, Chl-*a* was positively correlated with DO, ammonium and phosphate ($R^2 > 0.17$, $P < 0.046$).

Before treatment implementation GPP averaged $16.06 \text{ g O}_2 \text{ m}^{-2} \text{ d}^{-1}$, ER $-3.45 \text{ g O}_2 \text{ m}^{-2} \text{ d}^{-1}$, NEP $12.61 \text{ g O}_2 \text{ m}^{-2} \text{ d}^{-1}$ and EF $19.51 \text{ g O}_2 \text{ m}^{-2} \text{ d}^{-1}$ (Fig. 2). After treatment implementation all metabolic metrics changed significantly, increasing until day 21 and decreasing afterwards. Nutrient treatments affected positively all

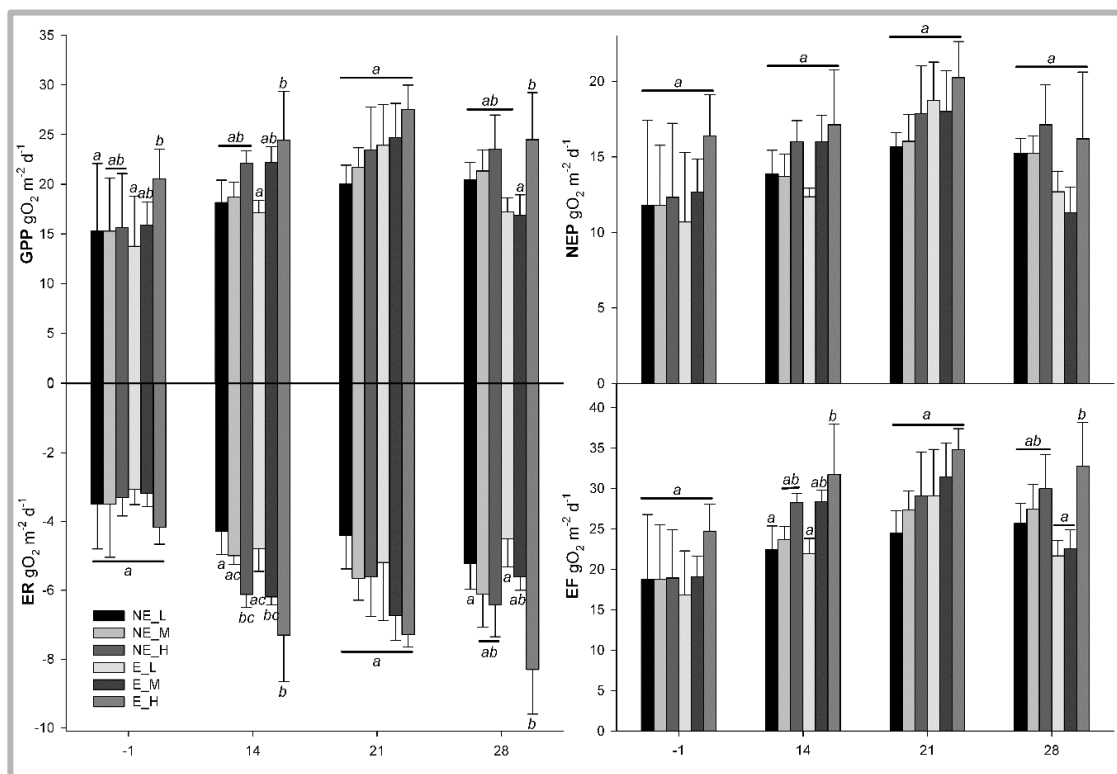


Figure 2. Changes in metabolic rates, expressed as the means (\pm SD) of the values per treatment and per time of GPP (left up), ER (left down), NEP (right up) and EF (right down). Results for Post hoc Tukey test are shown in letters for each day.

metrics, but only ER was affected by emerging pollutants (Table 3). No significant interactions were found for time, nutrients and/or emerging pollutants. Post hoc Tukey test revealed that at the end of the experiment (day 28) emerging pollutants affected negatively GPP and ER in L and M nutrient treatments, but in H treatments these rates were unaltered. Similar results were observed for NEP and EF. On the other hand, differences among nutrient treatments were more accused in E treatments than in NE treatments for ER. NEP, GPP, ER and EF were correlated between them ($R^2 > 0.54$, $P < 0.0001$) and they were positively correlated with conductivity, nutrients ($R^2 > 0.18$, $P < 0.040$), AFDM, BG and F0 ($R^2 > 0.17$, $P < 0.047$), and negatively with APA ($R^2 > 0.23$, $P < 0.016$).

Discussion

Biofilm structure and functioning changed during the experiment, as most variables were linearly affected by the nutrient treatments. Nutrients promote biomass and activity of both primary producers (algae, macrophytes) and microbial heterotrophs (bacteria, fungi), which are able to use dissolved nutrients and organic matter (Stelzer, Heffernan & Likens, 2003), and so responded the AFDM. Besides, AFDM and CR correlation is similar to what we would expect at ecosystem scale for organic matter and CR (Acuña *et al.*,

2004). Besides, the expected coupling for GPP with biomass (Hill, Mulholland & Marzorf, 2001) was found with F₀, which is used as an estimation of algal biomass (Schmitt-Jansen & Altenburger, 2008). APA, contrarily to AFDM, showed high values at the beginning of the experiment, but when structural needs were fulfilled (Schimel, Balsler & Wallenstein, 2007) measured activity reduced, especially in treatments with more nutrients (Proia, Romani & Sabater, 2012). Besides, BG increased during the experiment, mostly due to the higher labile algal material (*e.g.* Romani & Sabater, 2001). Sabater *et al.* (2011) described a similar trend in a manipulated river where APA decreased and BG increased during a nutrient addition experiment. Nevertheless, hump-shape responses could not be measured for any biofilm variable at concentrations that Camargo & Alonso (2006) reported to cause acute and chronic effects on sensitive aquatic biota. We provided nutrients at concentrations that were much higher than those described as mesotrophic and eutrophic (Dodds, Jones & Welch, 1998) which enhanced but also inhibited biofilm and macroinvertebrate responses in manipulative experiments (Wagenhoff *et al.*, 2012; 2013). Besides, mentioned manipulative experiment lasted from 18 to 21 days, a week less than our experiment, and thus we should expect some inhibitory responses. Nevertheless, these works studied changes on the composition of

biofilm and macroinvertebrates communities, rather than the effect on ecosystem functioning. In correlative field studies, where these ecosystem processes have been studied, linear responses have been measured up to 3.8 mg L^{-1} of total nitrogen and 1.8 mg L^{-1} of total phosphorus (Yates *et al.*, 2013; Silva-Junior *et al.*, 2014), and suppression of these responses have been measured beyond these concentrations (Izagirre *et al.*, 2008; Dunck *et al.*, 2015). These concentrations are much higher from the thresholds that most manipulative works have based their hypotheses. These suggest that ecosystem processes need more time to see the effects of nutrients, like the ones we find in real rivers, perhaps because ecosystem processes are integrative processes involving many promoters (Graça, 2001; Hieber & Gessner, 2002; Enquist *et al.*, 2003).

Emerging pollutants, on the other hand, did not affect the biofilm responses from the beginning of the treatment implementation as initially hypothesized, although the effects appeared at the end of the experiment, suggesting a long-term effect. Provided concentration reflected the highest concentrations in rivers, like those found downstream WWTP effluents (Haggard & Bartsch, 2009; Waiser *et al.*, 2010). In acute toxicological studies (24 – 48 h) the effects of these pollutants have been reported at much higher concentrations (Franz *et al.*, 2008; Morin

et al., 2010b; Cleuvers, 2004), but similar ecotoxicological works have also determined that the concentrations at which these contaminants can start affecting these species is very low ($0.15 \mu\text{g L}^{-1}$ of sulfamethoxazole, Kim *et al.*, 2007). The absence of changes during the first weeks in Chl-*a* and Y_{eff} together with an increase in GPP suggested a change on the biofilm composition, probably due to a higher proportion of cyanobacteria with a more efficient use of the light (*e.g.* Sabater *et al.*, 2001). Algal taxa richness loss seemed to be related to emerging contaminants, as it has been reported that some of the provided emerging pollutants can decrease this richness, mostly the one of diatoms (Wilson *et al.*, 2003). Besides, Post-hoc Tukey tests revealed that the effects of emerging pollutants appeared at the end of the experiment (day 28), a time frame proposed by Culp & Baird (2006) to be long enough to see multiple stressors' effects, and reported to be so for pharmaceuticals, beta-blockers or pesticides (Lawrence *et al.*, 2005; Muñoz *et al.*, 2009; Ricart *et al.*, 2010; Rosi-Marshall *et al.*, 2013). As far as we know, apart from acute tests the effects of emerging contaminants have not been studied separately.

Initially hypothesized hump-shape responses for nutrients together with the stress effects of emerging contaminants would have led us to describe all kind of synergistic or antagonistic interactions in

the applied mixtures (Coors & Meester, 2008; Wagenhoff *et al.*, 2013). Stress effects of nutrients, and thus, a synergistic interaction with the effects of emerging contaminants (Wagenhoff *et al.*, 2012), could not be measured in this work. Besides, implemented nutrient treatments led biofilm to respond linearly, been the treatment with more nutrients the one with the highest values for the affected variables. Combining this response with the negative one of emerging contaminants, we could measure a mitigation of the stress effect, especially at high nutrient levels. Similarly to this work, other manipulative experiments have reported some stress mitigation due to the nutrients that overwhelm the stress effects of pesticides (Lozano & Pratt, 1994; Traas *et al.*, 2004). Besides, a correlative field experiments have also reported similar mitigations in biofilms affected by the pesticides in a gradient of vineyard land (Morin *et al.*, 2010a).

Conclusions

Variables responded linearly to nutrients, showing highest responses at high nutrient concentrations. Emerging pollutants, on the other hand, stressed some of the measured variables, but effects were evident only at the end of the experiment indicating mid to long-term effects. Besides, we observed that nutrients alleviated the stress caused by emerging

pollutants, mostly at high nutrient concentrations. Our results highlight the need of more manipulative experiments for a better understanding of the effects of multiple stressors on freshwater ecosystems. Moreover, we identified that the duration of those manipulative experiments is crucial to detect potential harmful effects on the ecosystem, thus we advocate for more low-dose long-term studies.

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CHAPTER 6

General discussion



In this dissertation we explored the effects of the most important impacts caused by global change on the functioning of Mediterranean rivers (*i.e.* flow regulation and chemical pollution). We did so by combining broad geographic surveys of a single process (organic matter breakdown) at multiple sites (chapter 2) with comparisons of multiple processes upstream and downstream from known impacts (reservoirs, chapter 3, and WWTP effluents, chapter 4), and with controlled experiments in artificial streams (chapter 5). Some of the chapters assessed a single impact (regulation or pollution), whereas others incorporated multiple stressors. Altogether, our results provide evidence of the important ecological effects of environmental change on Mediterranean rivers, and suggest important consequences for the society, in terms of ecosystem services. Nevertheless, we must acknowledge that this dissertation is only a step in the understanding of the effects of global change on the functioning of Mediterranean rivers. No doubt, many stressors remain little studied, and even for those we studied, the response might differ depending on specific site characteristics, as well as on the distance to the stressor. Therefore, caution is needed to extrapolate our results to entire river segments downstream of the stressors, to the entire Mediterranean basin, and to rivers from other regions.

As shown in **Chapter 2**, we identified the main factors controlling decomposition of organic matter in Iberian rivers. Results showed large differences in this process, and quantile regressions suggested its maximum rate to be driven by 7 variables. The main factors controlling maximum breakdown rate seem to be temperature and phosphorus concentration, the latter showing a hump-shaped relationship, with low breakdown rates at both low and high phosphorus concentrations, and high rate at medium phosphorus concentration. However, land use, pollution, invertebrate communities and riparian vegetation also drove breakdown. Anyway, it must be stressed that these variables control the peak breakdown rate, but that within any combination of them the breakdown rate can show strong variations, probably caused by other factors. The breakdown of organic matter is inherently a highly variable process (Pérez *et al.*, 2011), and we can only speculate about other factors that might play an additional role, such as water velocity (Ferreira & Graça, 2006) or channel complexity (Elosegi, Díez & Mutz, 2010). It is worth mentioning that breakdown rate was consistently low in the headwater reaches studied, but that variance increased downstream, thus showing how multiple stressors accumulate in lowland areas, which are more affected by human activities (Lobera *et al.*, 2015), and where the breakdown rate can be hard to predict.

Chapter 3 showed how, in Mediterranean rivers, where the flow regime is highly seasonal (Gasith & Resh, 1999), reduction of the hydrologic variability and suppression of extreme flow events by reservoirs promotes the accumulation of organic carbon, and increases the ecosystem metabolism of downstream reaches. These results suggest that flow regulation will reduce the nutrient turnover length (Newbold *et al.*, 2012), thus altering the flux of materials and energy along the river. In a parallel work (von Schiller *et al.*, *in review*), we determined that the discontinuity caused by reservoirs was bigger for nitrogen than for other elements. Although it is beyond the scope of the present dissertation, it would be interesting to know whether this differential dynamics affect nutrient limitation downstream. Another question not addressed is the length of the river stretch where reservoirs affect ecosystem functioning. We can only speculate about this question, and the answer is probably site-dependent, as the distance affected by reservoirs will obviously depend on incoming tributaries. Nevertheless, the effects of regulation will likely be detected far away from reservoirs, as a consequence of the reduction in large floods. Additionally, by blocking sediment transport, reservoirs and other dams have a large impact on channel dynamics, resulting in severe incision and bed armouring that extends for many

kilometres downstream (Ibisate *et al.*, 2013). Both absence of floods and increased stability of the sediments (armouring) promote biofilm growth and metabolism, and thus, our results might be generalised to many Mediterranean rivers. On the other hand, both incision and water abstraction promote channel narrowing, what amounts to ecosystem contraction (Stanley, Fisher & Grimm, 1997). Channel narrowing reduces the active surface of the river, and thus, can counterbalance in part the increased productivity. Clearly, this is a topic worth being investigated if we want to understand the effects of regulation on the global metabolism of rivers, which is necessary, for instance, to comprehend the role of rivers in the global carbon balance or on greenhouse gases emissions by rivers (Cole *et al.*, 2007; Raymond *et al.*, 2013).

Chapter 4 showed the complex effects created by WWTP effluents on river ecosystem functioning. The effluent promoted biological activity, as has been shown elsewhere, (Martí *et al.*, 2004; Gücker, Brauns & Pusch, 2006; Ribot *et al.*, 2012), but not for all organisms. Thus, detailed analyses of the production-irradiance curves also showed some stress on primary producers, whereas this was not the case for heterotrophs. Therefore, the effluent produced a spatial discontinuity in ecosystem functioning along the river, and altered the balance between autotrophic and heterotrophic

processes. Although WWTP themselves are not the source of pollution, but a filter between the source and the receiving freshwater ecosystems, their effluents have still the potential to trigger significant biological responses. Contrasting with chapter 3, in this chapter we focused on a single system, but assessed the effects of the chemical pollution at different distances from the stressor, what allowed identifying the spatial extent of the impact. However, the followed approach did not allow discerning the effects of different types of chemical pollutants, such as nutrients versus pharmaceuticals, whose environmental impacts are still poorly understood (Muñoz *et al.*, 2009).

Chapter 5 aimed to disentangle the effects of two different types of chemical stressors, assimilable and toxic pollutants. This was done combining nutrients and emerging pollutants in a manipulative mesocosmos experiment. We expected toxic effects at the highest nutrients concentrations, as well as synergistic toxic effects with the presence of emerging pollutants. In contrast to those expectations, all experimental nutrient concentrations provoked a subsidy effect, and thus, no synergistic toxic effect was observed. Furthermore, the nutrient subsidy partly counteracted the toxicity of emerging pollutants, which only had an effect in treatments with low to mid nutrient concentrations and after 4 weeks of exposure. Two important conclusions

can be drawn from this experiment: 1) that low concentrations at the long term can exert toxic effects on stream biofilm functioning, and 2) that the subsidy effect by nutrients can partly counteract the toxic effects of emerging pollutants. Contrary to what we observed in chapter 4, the effects of both nutrients and emerging pollutants were similar for autotrophic and heterotrophic processes, thus indicating that further experimental research is needed to properly explain field observations.

The knowledge regarding the combined effects of stressors is increasing, but we are still far from completely understanding the myriads of combinations affecting simultaneously freshwater ecosystems. The effects of stressors will likely affect increasingly longer river sections as humans are keep developing lands (Foley *et al.*, 2005). Therefore, it is urgent to take action to protect and restore Mediterranean rivers, as they are the most vulnerable to environmental damage, and hot-spots of biodiversity (Myers *et al.*, 2000; Smith & Darwall, 2006; Bonada *et al.*, 2007).

1. Breakdown of organic matter showed very large variability across Iberian rivers as a result of multiple stressors. Organic matter breakdown was consistently slow in high elevation rivers with low levels of human impact, whereas variance increased in lowland areas subject to multiple stressors. The main factors controlling maximum breakdown rate seem to be temperature and phosphorus concentration, the latter showing a hump-shaped relationship, with low breakdown rates at both low and high phosphorus concentrations, and high rate at medium phosphorus concentration. Whatever the factors governing spatial variations in organic matter breakdown, tongue depressors offer a simple but powerful method to measure river ecosystem functioning.
2. Flow regulation by reservoirs had strong effects on the ecosystem processes of the studied Mediterranean rivers. Reservoirs reduced hydrological variability and dampened floods. Longer inter-flood periods allowed large downstream increases in benthic organic matter and primary producers, which in turn fostered ecosystem respiration and gross primary production. River reaches downstream reservoirs had a higher capacity to process organic carbon than those upstream reservoirs. Higher processing capacities downstream reservoirs could lead to a shorter organic carbon turnover length, less carbon exported to downstream systems and a higher self-purification capacity.
3. Wastewater treatment plant effluents acted mainly as a subsidy, but there were more limited evidences of them acting as a stressor. Most biofilm variables biofilms showed no change, with the exception of leucine-amino-peptidase, which followed the pollution gradient most likely driven by changes in organic matter availability. The effluent produced mixed effects on ecosystem-scale metabolism. It promoted respiration (subsidy effect), probably as a consequence of enhanced availability of organic matter. On the other hand, photosynthesis-irradiance relationships showed that the effluent partly decoupled primary production from light availability. This together with an increase on non-photochemical dissipation of energy at biofilm scale suggested a stress effect. The complementary response detected at the biofilm and the ecosystem scales stresses the need to study both in order to fully understand the impact of WWTP effluents on river ecosystems.
4. Variables responded linearly to nutrients, showing highest responses at high nutrient concentrations. Emerging pollutants, on the other hand, stressed some of the measured variables, but effects were evident only after 4 weeks of exposure

indicating mid to long-term effects. Besides, we observed that nutrients alleviated the stress caused by emerging pollutants, mostly at high nutrient concentrations. Our results highlight the need of more manipulative experiments for a better understanding of the effects of multiple stressors on freshwater ecosystems. Moreover, we identified that the duration of those manipulative experiments is crucial to detect potential harmful effects on the ecosystem, thus we advocate for more low-dose long-term studies.

CHAPTER 8

References



Assessing the effects of multiple stressors on the functioning of Mediterranean rivers using poplar wood breakdown

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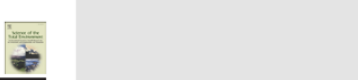
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Mixed effects of effluents from a wastewater treatment plant on river ecosystem metabolism: subsidy or stress?

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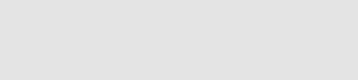
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Flow regulation by dams affects ecosystem metabolism in Mediterranean rivers

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Biofilm responses to flow regulation by dams in Mediterranean rivers

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Impact of water abstraction on storage and breakdown of coarse organic matter in mountain streams

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Geomorphic status of regulated rivers in the Iberian Peninsula

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Supporting information

Table S1. (Chapter 3). Mean and standard error of physicochemical characteristics for each reach with the effect size of the dam and the results of the mixed model comparison (Fixed factors = River, Reach and Season; Random factor = Site).

Variable	Cinca	Montsant	Siurana	All rivers	Variable	Cinca	Montsant	Siurana	All rivers
GD (mm)	C = 109.87 ± 9.00	C = 2007.77 ± 244.28	C = 277.06 ± 88.25	C = 751.63 ± 85.75	Conductivity ($\mu\text{S cm}^{-1}$)	C = 315.73 ± 1.46	C = 539.73 ± 45.63	C = 683.73 ± 7.24	C = 513.07 ± 27.34
	I = 105.58 ± 4.50	I = 3742.54 ± 288.45	I = 673.68 ± 149.38	I = 1527.40 ± 122.86		I = 347.67 ± 11.45	I = 413.77 ± 6.09	I = 614.73 ± 4.98	I = 458.72 ± 17.71
	C > I (-3.9%)	C < I (+86.4%)	=	C < I (+103.2%)		C < I (+10.1%)	C > I (-23.3%)	C > I (-10.1%)	C > I (-11.6%)
	n= 609 $F_{1,603}=22.3$ p<0.0001	n= 539 $F_{1,533}=20.5$ p<0.0001	n= 500 $F_{1,494}=3.2$ p=0.073	n= 1648 $F_{1,1640}=42.9$ p<0.0001		n= 30 $F_{1,22}=9.6$ p=0.005	n= 30 $F_{1,22}=13.5$ p=0.001	n= 30 $F_{1,22}=65.1$ p<0.0001	n= 90 $F_{1,80}=12.0$ p<0.0001
Width (m)	C = 27.38 ± 2.10	C = 3.14 ± 0.63	C = 3.59 ± 0.36	C = 11.93 ± 1.96	DO (mg L^{-1})	C = 10.98 ± 0.39	C = 10.24 ± 0.47	C = 10.33 ± 0.55	C = 10.52 ± 0.27
	I = 8.72 ± 0.75	I = 3.64 ± 0.37	I = 2.4 ± 0.28	I = 5.16 ± 0.53		I = 10.71 ± 0.51	I = 10.68 ± 0.40	I = 10.80 ± 0.34	I = 10.73 ± 0.24
	C > I (-68.2%)	=	C > I (-33.2%)	C > I (-56.7%)		=	C < I (+4.3%)	C < I (+4.5%)	=
	n= 30 $F_{1,22}=132.0$ p<0.0001	n= 30 $F_{1,22}=3.4$ p=0.078	n= 23 $F_{1,15}=5.8$ p=0.030	n= 83 $F_{1,73}=26.8.1$ p<0.0001		n= 30 $F_{1,22}=2.7$ p=0.113	n= 30 $F_{1,22}=18.0$ p<0.0001	n= 30 $F_{1,22}=4.8$ p=0.040	n= 90 $F_{1,80}=1.6$ p=0.207
Depth (m)	C = 0.27 ± 0.02	C = 0.18 ± 0.03	C = 0.13 ± 0.03	C = 0.20 ± 0.02	Alkalinity (mg L^{-1})	C = 143.89 ± 1.00	C = 159.39 ± 6.52	C = 172.56 ± 4.53	C = 158.61 ± 3.44
	I = 0.24 ± 0.04	I = 0.19 ± 0.03	I = 0.16 ± 0.01	I = 0.20 ± 0.02		I = 123.25 ± 4.34	I = 155.99 ± 2.64	I = 166.63 ± 5.58	I = 148.62 ± 4.35
	=	=	=	=		C > I (-14.3%)	=	=	C > I (-6.3%)
	n= 30 $F_{1,22}=2.4$ p=0.140	n= 30 $F_{1,22}=0.02$ p=0.897	n= 23 $F_{1,15}=3.0$ p=0.104	n= 83 $F_{1,73}=0.002$ P=0.096		n= 18 $F_{1,12}=28.8$ p=0.0002	n= 18 $F_{1,12}=0.7$ p=0.414	n= 18 $F_{1,12}=1.3$ p=0.273	n= 54 $F_{1,46}=12.9$ p<0.0001
Discharge ($\text{m}^3 \text{ s}^{-1}$)	C = 1.79 ± 0.16	C = 0.04 ± 0.01	C = 0.02 ± 0.00	C = 0.66 ± 0.14	pH	C = 8.48 ± 0.06	C = 8.18 ± 0.08	C = 8.26 ± 0.01	C = 8.31 ± 0.04
	I = 0.21 ± 0.01	I = 0.09 ± 0.02	I = 0.02 ± 0.00	I = 0.11 ± 0.02		I = 8.39 ± 0.05	I = 8.19 ± 0.04	I = 8.27 ± 0.02	I = 8.28 ± 0.03
	C > I (-88.3%)	C < I (+126.2%)	=	C > I (-83.3%)		C > I (-1.1%)	=	=	=
	n= 30 $F_{1,22}=907.6$ p<0.0001	n= 30 $F_{1,22}=20.2$ p<0.0001	n= 23 $F_{1,15}=0.2$ p=0.668	n= 83 $F_{1,73}=4.6$ p=0.036		n= 30 $F_{1,22}=13.9$ p=0.001	n= 30 $F_{1,22}=0.1$ p=0.784	n= 30 $F_{1,22}=0.2$ p=0.624	n= 90 $F_{1,80}=0.9$ p=0.338

Table S1. (Chapter 3). Continuation

Variable	Cinca	Montsant	Siurana	All rivers	Variable	Cinca	Montsant	Siurana	All rivers
Velocity (m s ⁻¹)	C = 0.26 ± 0.02	C = 0.09 ± 0.02	C = 0.07 ± 0.02	C = 0.15 ± 0.02	Nitrate (mg L ⁻¹)	C = 0.24 ± 0.01	C = 0.94 ± 0.28	C = 0.27 ± 0.03	C = 0.48 ± 0.10
	I = 0.18 ± 0.04	I = 0.14 ± 0.03	I = 0.06 ± 0.01	I = 0.13 ± 0.02		I = 0.29 ± 0.05	I = 0.61 ± 0.05	I = 0.14 ± 0.03	I = 0.35 ± 0.04
	C > I (-31.7%)	=	=	=		=	=	C > I (-46.9%)	=
	n= 30 F _{1,22} =14.1 p=0.001	n= 30 F _{1,22} =2.4 p=0.135	n= 23 F _{1,13} =0.0 p=0.974	n= 83 F _{1,73} =0.2 p=0.693		n= 30 F _{1,22} =0.3 p=0.621	n= 30 F _{1,22} =1.9 p=0.177	n= 30 F _{1,22} =31.2 p<0.0001	n=90 F _{1,80} =0.03 p=0.868
Temperature (°C)	C = 9.38 ± 1.63	C = 10.71 ± 1.43	C = 12.88 ± 2.19	C = 10.99 ± 1.03	Ammonium (mg L ⁻¹)	C = 0.012 ± 0.003	C = 0.011 ± 0.001	C = 0.012 ± 0.001	C = 0.011 ± 0.001
	I = 11.27 ± 2.11	I = 10.24 ± 1.32	I = 10.40 ± 1.05	I = 10.64 ± 0.88		I = 0.016 ± 0.003	I = 0.009 ± 0.001	I = 0.022 ± 0.005	I = 0.016 ± 0.002
	C < I (+20.2%)	=	=	=		=	=	C < I (+93.7%)	=
	n= 30 F _{1,22} =40.7 p<0.0001	n= 30 F _{1,22} =0.6 p=0.439	n= 30 F _{1,22} =0.1 p=0.711	n= 90 F _{1,80} =0.6 p=0.454		n= 30 F _{1,22} =4.0 p=0.059	n= 30 F _{1,22} =1.2 p=0.276	n= 30 F _{1,22} =7.9 p=0.010	n= 90 F _{1,80} =3.5 p=0.066
Light (MJ m ⁻² d ⁻¹)	C = 17.49 ± 3.39	C = 24.80 ± 3.10	C = 22.62 ± 4.28	C = 21.64 ± 2.10	Phosphate (mg L ⁻¹)	C = 0.003 ± 0.0003	C = 0.018 ± 0.0016	C = 0.004 ± 0.0005	C = 0.008 ± 0.0012
	I = 20.04 ± 3.67	I = 10.11 ± 2.82	I = 10.85 ± 3.42	I = 13.67 ± 1.99		I = 0.002 ± 0.0002	I = 0.005 ± 0.0004	I = 0.003 ± 0.0003	I = 0.003 ± 0.0002
	C < I (+14.6%)	C > I (-59.2%)	C > I (-52.1%)	C > I (-36.8%)		C > I (-23.1%)	C > I (-74.2%)	=	C > I (-62.5%)
	n= 30 F _{1,22} =30.7 p<0.0001	n= 30 F _{1,22} =59.2 p<0.0001	n= 30 F _{1,22} =28.7 p<0.0001	n= 90 F _{1,80} =34.5 p<0.0001		n= 30 F _{1,22} =5.9 p=0.024	n= 30 F _{1,22} =174.8 p<0.0001	n= 30 F _{1,22} =0.9 p=0.362	n= 90 F _{1,80} =32.4 p<0.0001