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

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SUMMARY

1. The effluents of wastewater treatment plants (WWTPs) include a complex mixture of nutrients and pollutants. Nutrients can subsidise 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.

2. 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.

3. The photosynthetic capacity and enzymatic activity of biofilms showed no change, with the exception of leucine aminopeptidase, 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.

4. 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 subsidisers.

Keywords: ecosystem functioning, metabolism, photosynthesis versus irradiance curve, pollution, subsidy–stress effect

Introduction

Pollution from point sources such as wastewater treatment plants (WWTPs) 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 2500 WWTPs have been put into operation over the last three decades in Spain (Serrano, 2007). As WWTPs do not remove all contaminants from sewage waters (Rodriguez-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, Petrović & 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 subsidise biological activity (at least up to a threshold beyond which they can suppress it), and toxic contaminants, which are deleterious

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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 fresh waters 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 fresh waters 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 (Cleuvers, 2003). Toxic contaminants reduce the abundance, 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 hypothesised: (i) that WWTP effluents affect autotrophic and heterotrophic metabolism differently; (ii) 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 (iii) 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 *c.* 30 000 population equivalents.

We compared a control reach (CR) upstream from the WWTP effluent with a 4000-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 were obtained from the meteorological station of the Catalan

Meteorological Service (Das, Catalan Meteorological Service, located at *c.* 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, U.S.A.). 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). 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, U.S.A.) 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, UK) and frozen at −20 °C until analysis. Ammonium concentration was analysed by ion chromatography using a DIONEXI C5000 (Dionex Corporation, Sunnyvale, CA, U.S.A.), 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, U.K.) 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 was loaded on the on-line chromatographic system (Thermo Scientific EQuanTM, Franklin, MA, U.S.A.) consisting of two 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). Chromatographic separation was achieved using a Thermo Scientific Hypersil GoldTM (50 × 2.1 mm, 1.9 μm particle size) column. Target compounds were analysed under dual negative/positive electrospray ionisation 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 to 7.86 ng L^{-1} (sulfamethoxazole and venlafaxine, respectively).

Benthic organic matter and biofilm characteristics

Five Surber net (0.09 m^2, 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 cm^2 was scraped with a knife and pooled together to obtain a mixed sampling area of 9–18 cm^2 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 colonised artificial substrata. Unglazed ceramic tiles of 1.25 × 1.25 cm were glued in groups of 110 units onto flat 20 × 20 cm bricks, and 3 flat bricks per segment incubated at a depth of 30 cm in the field during 6 weeks (30 August 2012 to 10 October 2012) to allow for biofilm colonisation. 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) underwa-

ter 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-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride) to INT-formazan (iodonitrotetrazolium formazan) (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 3 mL 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 μg L^{-1} of INT-formazan (Sigma-Aldrich, St Louis, MO).

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 aminopeptidase (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-leucin aminomethylcoumarin (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 carried out 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 three times the v : k ratio and the two-station 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 days (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, OH, U.S.A.) from which 10 days 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 three 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 *c.* 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). 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 by measuring the time between the peaks of the break-through 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 analysed 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 nonlinear regression (STATISTICA, version 8; StatSoft Inc., Tulsa, OK, U.S.A.), the hyperbolic tangent function including or excluding temperature dependence:

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

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^2 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 Kolmogrov–Smirnov test, and variables were log-transformed when necessary. Differences of measured variables among sites were analysed by means of generalised 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 compar-

Table 1 Water physicochemical characteristics for each river segment (mean ± SD)

	CR	IR1	IR2	IR3
Discharge (m³ s⁻¹)	0.29 ± 0.03	0.50 ± 0.17	$0.64 \pm 0.03^*$	$0.83 \pm 0.24^*$
Velocity (m s⁻¹)	0.18 ± 0.06	0.20 ± 0.08	0.38 ± 0.14	0.33 ± 0.08
Depth (m)	0.14 ± 0.02	0.15 ± 0.01	0.19 ± 0.05	$0.23 \pm 0.01^*$
Width (m)	11.90 ± 0.85	10.25 ± 2.47	9.45 ± 1.34	10.70 ± 0.42
GLR (MJ m⁻² day⁻¹)	4.62 ± 0.82	$9.52 \pm 1.69^*$	$11.93 \pm 2.12^*$	$14.34 \pm 2.54^*$
LAI	2.52 ± 0.83	1.76 ± 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 (°C)	13.58 ± 1.41	13.80 ± 1.10	13.49 ± 0.87	13.60 ± 0.86
pH	8.54 ± 0.39	8.63 ± 0.01	8.55 ± 0.12	8.65 ± 0.25
Conductivity (µS cm⁻¹)	180.90 ± 0.85	$225.75 \pm 13.79^*$	$214.5 \pm 2.12^*$	$207.75 \pm 7.42^*$
Ammonium (mg L⁻¹)	0.012 ± 0.001	$1.92 \pm 1.03^*$	$0.90 \pm 0.41^*$	$0.37 \pm 0.33^*$
Phosphate (mg L⁻¹)	0.039 ± 0.001	$0.292 \pm 0.111^*$	$0.200 \pm 0.020^*$	$0.182 \pm 0.004^*$
DOC (mg L⁻¹)	2.54 ± 0.15	$3.67 \pm 0.41^*$	$3.14 \pm 0.34^*$	2.79 ± 0.16
SPOM (mg L⁻¹)	2.90 ± 0.08	$4.48 \pm 0.51^*$	3.04 ± 0.08	3.02 ± 0.34

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.
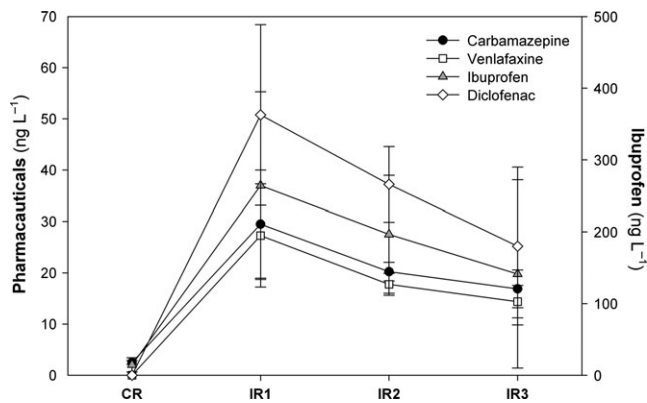*Significant difference ($P < 0.05$) in comparison with CR site.

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

isons 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

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 (0.01–1.9 mg L^{-1}) and phosphate 7.5-fold (0.04–0.3 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 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 analysed 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 in 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.

Dissolved organic carbon 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

Table 2 Benthic organic matter and biofilm characteristics in each river segment (mean $\pm$ SD)

	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 (μg 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 (μg 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

BOM, benthic organic matter; Chl-a, chlorophyll-a; Ymax, maximum photosynthetic capacity; Yeff, effective quantum yield; PQ, photochemical quenching; NPQ, non-photochemical quenching; ETS, electron transport system; AP, alkaline phosphatase; BG, β-glucosidase; LAP: leucine aminopeptidase.
*Significant difference ($P < 0.05$) in comparison with CR site.

decreased linearly with distance from the WWTP ($R^2 > 0.51$, $P < 0.05$), until they approached pre-disturbance values (Table 1).

Benthic organic matter and biofilm characteristics

Benthic organic matter 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 *c.* 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 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

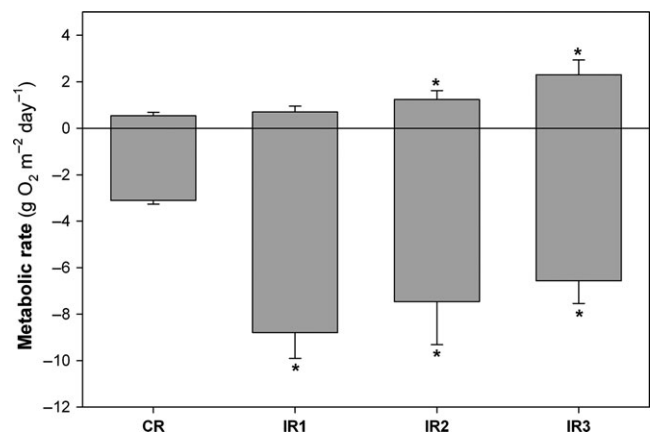Ecosystem metabolism followed contrasting longitudinal patterns. There was an almost threefold increase in ER

Fig. 2 Daily metabolic rates (mean ± 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 with CR site.
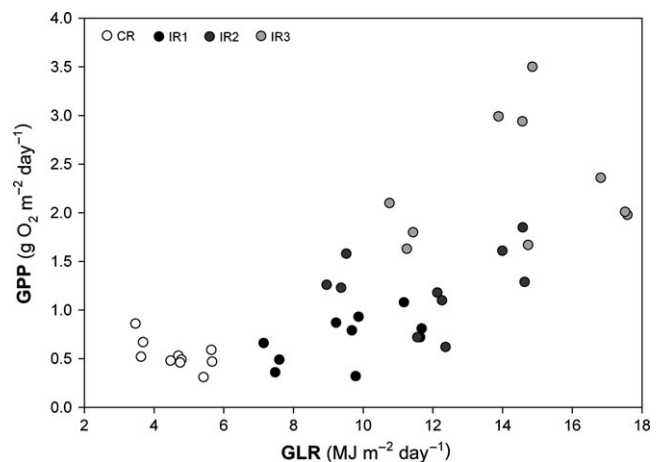

Fig. 3 Daily gross primary production (GPP) in relation to the received total GLR. Values from 10 days are shown for each river segment.

from CR to IR1 (from 3.1 to 8.8 g O$_2$ m^{-2} day^{-1}; Fig. 2, Table 3) and a decrease along the impact reach down to 6.6 g O$_2$ m^{-2} day^{-1} at IR3, a value still two times higher than the control. Overall, ER was significantly higher in the impact reach than in the CR, and the decrease down-

Table 3 Metabolism parameters (mean ± SD) for each river segment

	CR	IR1	IR2	IR3
GPP (g O$_2$ m^{-2} day^{-1})	0.54 ± 0.15	0.70 ± 0.25	1.24 ± 0.38*	2.30 ± 0.64*
ER (g O$_2$ m^{-2} day^{-1})	−3.11 ± 0.16	−8.79 ± 1.11*	−7.46 ± 1.85*	−6.56 ± 0.98*
NEM (g O$_2$ m^{-2} day^{-1})	−2.57 ± 0.23	−8.09 ± 0.87*	−6.22 ± 1.87*	−4.6 ± 1.41*
P/R	0.17 ± 0.05	0.08 ± 0.02*	0.18 ± 0.06	0.36 ± 0.14*

GPP, gross primary production; ER, ecosystem respiration; NEM, net ecosystem metabolism; P/R, production to respiration ratio.
*Significant difference ($P < 0.05$) in comparison with CR site.

stream 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 BOM ($R^2 = 0.91$ $P = 0.043$). GPP averaged 0.5 g O_2 m^{-2} day^{-1} in CR (Table 3), 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 on the light availability ($R^2 = 0.51$ $P < 0.0001$) (Fig. 3). All river segments were heterotrophic, with NEM values averaging -2.6 O_2 m^{-2} day^{-1} in CR, increasing to -8.1 O_2 m^{-2} day^{-1} in IR1 and then decreasing downstream to -4.3 O_2 m^{-2} day^{-1} in IR3. NEM was significantly higher in all impact segments than in CR. The P/R ratio averaged 0.17 in CR, 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.

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.

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
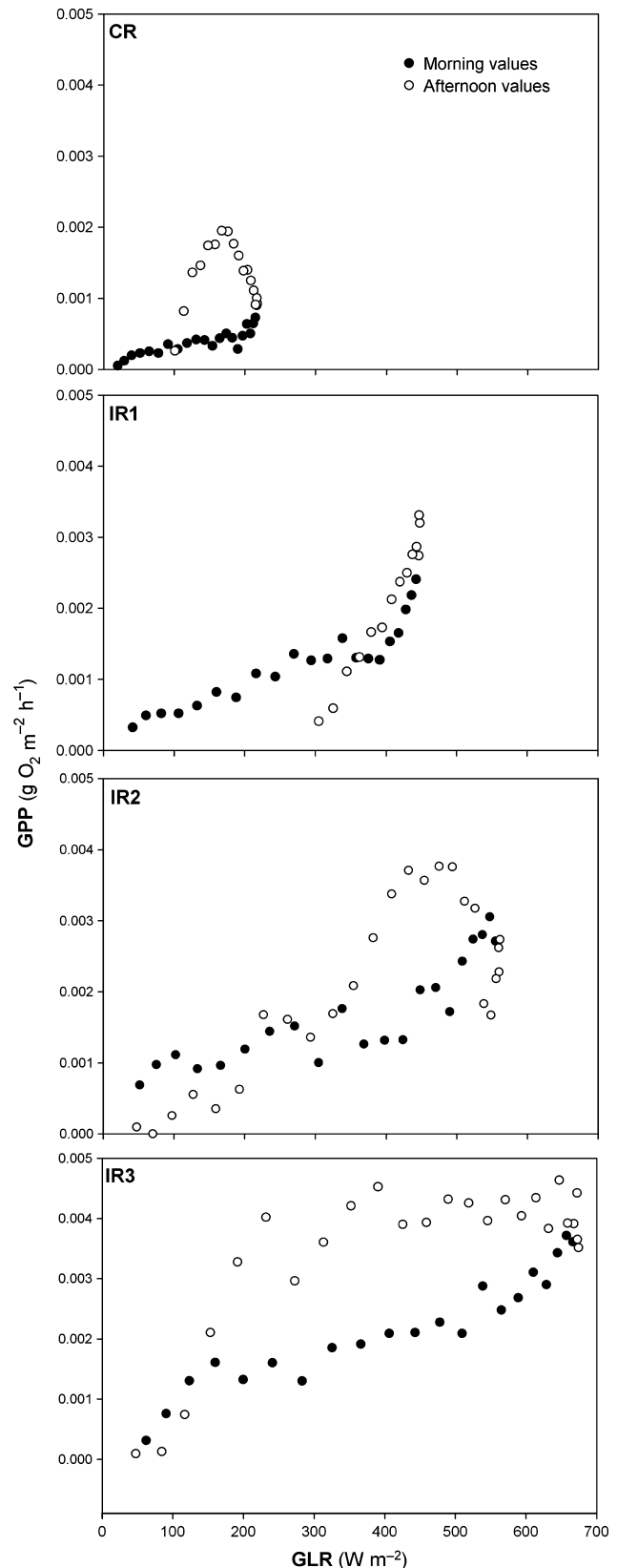

Fig. 4 Gross primary production (GPP) versus GLR for the first day of measurement for each reach. Measurements in the morning are indicated by black circles and those in the afternoon by white circles.

Table 4 Production–Irradiance (P-I) relationships and calculated parameters (mean ± SD) for each river segment

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

*Significant difference ($P < 0.05$) in comparison with CR site.

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 (vonSchiller *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 down-

stream 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 ± 10%, and for diclofenac of 12 ± 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 nonlinear 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 fivefold increase after the WWTP effluent input, followed by a reduction downstream to values three 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 abun-

dance 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 subsidised, following a pattern similar to that of organic matter. Although the low number of river segments analysed 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 (Yates *et al.*, 2013; Silva-Junior *et al.*, 2014), 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 regard 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 subsidised, 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.

Acknowledgments

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