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Testing wastewater treatment plant effluent effects on microbial and detritivore performance: A combined field and laboratory experiment

Libe Solagaistua[a,*], Ioar de Guzmán[a], Miren Barrado[a], Leire Mijangos[b], Nestor Etxebarria[b,c], Gonzalo García-Baquero[a], Aitor Larrañaga[a], Daniel von Schiller[a], Arturo Elosegi[a]

[a] Department of Plant Biology and Ecology, Faculty of Science and Technology, University of the Basque Country (UPV/EHU), 48080 Bilbao, Spain
[b] Department of Analytical Chemistry, Faculty of Science and Technology, University of the Basque Country (UPV/EHU), 48080 Bilbao, Spain
[c] Research Centre for Experimental Marine Biology and Biotechnology (PIE), University of the Basque Country (UPV/EHU), Areatza 48620 Plentzia, Spain

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ABSTRACT

The amount of pollutants and nutrients entering rivers via point sources is increasing along with human population and activity. Although wastewater treatment plants (WWTPs) greatly reduce pollutant loads into the environment, excess nutrient loading is a problem in many streams. Using a Community and Ecosystem Function (CEF) approach, we quantified the effects of WWTP effluent on the performance of microbes and detritivores associated to organic matter decomposition, a key ecosystem process. We measured organic matter breakdown rates, respiration rates and exo-enzymatic activities of aquatic microbes. We also measured food consumption and growth rates and RNA to body-mass ratios (RNA:BM) of a dominant amphipod *Echinogammarus berilloni*. We predicted responses to follow a subsidy-stress pattern and differences between treatments to increase over time. To examine temporal effects of effluent, we performed a laboratory microcosm experiment under a range of effluent concentrations (0, 20, 40, 60, 80 and 100%), taking samples over time (days 8, 15 and 30; 4 and 10 replicates to assess microbe and detritivore performance respectively, per treatment and day). This experiment was combined with a field *in situ* Before-After Control-Impact Paired (BACIP) experiment whereby we added WWTP effluent poured (10 L s^{-1} during 20–40 min every 2 h) into a stream and collected microbial and detritivore samples at days 8 and 15 (5 and 15 replicates to assess the microbe and detritivore performance respectively, per period, reach and sampling day). Responses were clearer in the laboratory experiment, where the effluent caused a general subsidy response. Field measures did not show any significant response, probably because of the high dilution of the effluent in stream water (average of 1.6%). None of the measured variables in any of the experiments followed the predicted subsidy-stress response. Microbial breakdown, respiration rates, exo-enzymatic activities and invertebrate RNA:BM increased with effluent concentrations. Differences in microbial respiration and exo-enzymatic activities among effluent treatments increased with incubation time, whereas microbial breakdown rates and RNA:BM were consistent over time. At the end of the laboratory experiment, microbial respiration rates increased 156% and RN:BM 115% at 100% effluent concentration. Detritivore consumption and growth rates increased asymptotically, and both responses increased with by incubation time. Our results indicate that WWTP effluent stimulates microbial activities and alters detritivore performance, and stream water dilution may mitigate these effects.

1. Introduction

The world population, the per-capita rate of resource use, the industrial production, the proportion of people living in cities and the number of livestock units in large farms, all are rising steadily (Grimm et al., 2008; Steffen et al., 2007). With them, the amount of pollutants and nutrients entering rivers via point source are also increasing. Nowadays, over 80% of worldwide wastewater is released directly to the environment without an adequate treatment (UNESCO, 2017). In order to reduce this impact, most countries are investing in wastewater treatment plants (WWTPs). For instance, more than 2500 WWTPs have been put into operation over the last three decades only in Spain (Serrano, 2007). WWTPs have been built to mainly reduce loads of nutrients and organic matter, but they also function to some extent as a filter for other pollutants. However, they are still a major point-source pollution in many river ecosystems (Carey and Migliaccio, 2009; Munz

* Corresponding author.
E-mail address: libe.solagaistua@ehu.eus (L. Solagaistua).

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et al., 2017), as their effluents consist of complex mixtures of nutrients, organic matter, metals, and other pollutants, including regulated and emerging pollutants (Gros et al., 2007; Petrovic et al., 2002). Some of these compounds (e.g. pesticides) are toxic and reduce biological activity, whereas others (e.g. nutrients), can subsidize biological activity (Martí et al., 2001; Ribot et al., 2012) up to a concentration threshold, beyond which they also become stressful, a pattern that results in the so-called 'subsidy-stress response' (Odum et al., 1979).Many WWTP effluents are discharged into streams, where allochthonous organic matter such as leaf litter is a key resource for aquatic food webs (Petersen and Cummins, 1974; Tank et al., 2010). Litter decomposition is a complex process in which microbial decomposers and macroinvertebrate detritivores play a leading role (Hieber and Gessner, 2002). It is highly sensitive to changes in environmental conditions, such as nutrient availability (Ferreira et al., 2006; Niyogi et al., 2003; Woodward et al., 2012) and the presence of toxic compounds (Lecerf et al., 2006). WWTP-derived nutrients may subsidize microbial activity (e.g. respiration and exo-enzymatic activity) and thus promote litter decomposition (Ferreira et al., 2006; Niyogi et al., 2003). However, excessive concentration of nutrients and other pollutants can be toxic for microbes and macroinvertebrates (Baldy et al., 2007; Camargo and Alonso, 2006; Duarte et al., 2009). Moreover, macroinvertebrate detritivores depend on microbes to condition plant litter and increase its palatability (Bärlocher and Kendrick, 1975; Graca et al., 2001). Thus, direct and indirect effects can alter consumption rates, body condition and death rates of macroinvertebrate consumers (Bundschuh et al., 2013, 2011a). Moreover, if the species sensitive to WWTP effluents are key consumers, such as detritivores that process extraordinary amounts of leaf litter, this pollution might eventually affect rates of ecological processes and the entire food web (Bundschuh et al., 2011b).

Ecotoxicological assays have evolved from those focused on survival facing acute toxicity to assays recording sublethal endpoints, including mobility, carcinogenic effects, hormonal disruption and histopathological, cytological or molecular-level stress biomarkers (Gorokhova et al., 2010; Löf et al., 2016; Wigh et al., 2017). The results of these assays are essential to rank pollutants according to their environmental risk. Nevertheless, individual stress signals do not always translate into responses at the levels of the community or ecosystem function (Lukančič et al., 2010; Souza et al., 2010; Trapp et al., 2015). Moreover, results in laboratory experiments cannot be directly transferred to consequences in the field, where biotic interactions and environmental complexity can modulate the responses. Community-Ecosystem Functioning assays (hereafter CEF) are those assays that construct simplified ecosystems in the laboratory where biotic communities (microbes and macroinvertebrates and their interactions) and ecosystem functions are tested for environmental conditions or pollutants. An increasing number of studies show that CEF can respond in contrasting ways to different pollutants and their interactions (Rasmussen et al., 2012; Zubrod et al., 2015). However, as many factors differ among CEF studies, it is difficult to draw general conclusions or to rank the different toxicants in terms of toxicity as with simpler toxicological studies. One of the varying factors among the studies is the taxa tested. Using standard model taxa as in classic toxicology (e.g. *Gammarus* or *Daphnia*) helps homogenizing studies, but these taxa might not occur in the target ecosystems. Another challenge of CEF studies is the lack of a standardized experiment duration. Temporal consistency of the responses has seldom been studied in CEF assays, despite being a stepping stone for these approaches to become more prevalent.

Here, following a CEF approach, we assessed the effect of the concentration of a WWTP effluent on organic matter decomposition and performance of microbes (decomposition, respiration and exo-enzymatic activity) and macroinvertebrate detritivores (food consumption, growth and RNA to body mass ratio). We combined a highly controlled and standardized laboratory experiment with a field bioassay of the effects of WWTP effluents in a stream. The former was used to build an effluent concentration-dependent response model, whereas the later tested the validity of this model in natural ecosystems. We repeated samplings over time in both experiments to assess the consistency of the diagnoses. Our main predictions were: i) microbial and detritivore performance variables will present a subsidy-stress type response along the effluent concentration gradient; ii) differences along the effluent concentration gradient will increase with time, and iii) field measurements will follow patterns observed in the laboratory experiment.

2. Materials & methods

2.1. WWTP characteristics

The selected WWTP (Apraitz) is located in Elgoibar, N Iberian Peninsula (43°13′41.1″N 2°23′56.3″W). The facility treats the sewage water of approximately 90,000 population equivalents, mostly urban, but also including industrial sources. An average daily amount of 29.90 m^3 of wastewater is subjected to primary and secondary treatment (http://acciona-agua.com/). An additional tertiary treatment is also carried out, where phosphorus is chemically precipitated via $FeCl_3$ addition. The WWTP is based on sequencing batch reactors in which the sewage water is mixed in large tanks with activated sludge, and alternatively subject to anaerobic and aerobic conditions to reduce organic matter. This process results in pulsed releases of effluent during ca. 20–40 min every 2 h. The effluent is released into the Deba River (av. discharge, 11.4 $m^3 s^{-1}$) right beside the junction with the Apraitz Stream, a small tributary with average discharge of 0.118 $m^3 s^{-1}$, and 2.2 m of channel width that flows next to the WWTP and that was used for the field experiment.

2.2. Organic matter and experimental taxa

We used leaf litter of black alder (*Alnus glutinosa* (L.) Gaertner) as the model organic material in the experiment because it is one of the most common riparian tree species in Europe, it has closely related species that are common throughout the Holarctic region and it has been widely used in decomposition studies in streams (Boyero et al., 2011; Woodward et al., 2012)(Boyero et al., 2011; Woodward et al., 2012). Freshly fallen alder leaves were collected in September 2016 and disks (14 mm in diameter) were extracted, air-dried and stored in Petri dishes in a dark dry place until further use. The microbial assemblage used in the experiment was obtained by incubating the organic material on an experimental solution (see below). As the test detritivore we chose an amphipod crustacean; this taxonomic group shows a relative tolerance to pollution and is often key for the detritic pathway of many freshwater ecosystems (Besse et al., 2013; Woodward et al., 2008). The amphipod we selected, *Echinogammarus berilloni* (Catta) is one of the most abundant macroinvertebrate species in the streams of the region (Larrañaga et al., 2009a,b) and has demonstrated to be sensitive to food quality (Larrañaga et al., 2009a,b,2014). Individuals were kick-sampled (500 µm pore size) in the Apraitz stream, in a reach upstream from the WWTP, where levels of pollution are low but nevertheless some pollutants such as caffeine are detected, what suggests that sampled individuals might be pre-adapted to some degree of pollution. The animals so captured were enclosed individually and carried to the laboratory in stream water. There, we selected individuals with a first thoracic segment length between 0.45 and 0.95 mm (6.40 and 13.62 mm total body length), excluding breeding females, which show a clearly differentiated biochemical composition (Larrañaga et al., 2009a,b).

2.3. Laboratory experiment

For the laboratory experiment, we collected an integrated effluent sample between April 24 and 27, 2017. Each day, 10 L of effluent was collected continuously through the day, filtered (0.1 mm mesh size) into a large container, and then, frozen (-20 °C) in smaller bottles (see

below). Although freezing affects DOC concentration (Fellman et al., 2008), because we wanted to change water periodically, we thought it was better to freeze the effluent than to use a different effluent every week or preserving unfrozen the water, as it would change the chemical properties even more (Fedorova et al., 2014; Morosini et al., 2017). The experiment lasted 30 d and water was renewed every week to prevent changing chemical conditions. For the experiment, we prepared a set of microcosms (test tubes 2 cm diameter and 20 cm long, filled with 40 mL of solution, enough to allow free movement of the detritivore) with a range of concentrations (0, 20, 40, 60, 80 and 100%) by diluting the effluent in filtered (0.1 mm mesh) stream water from the reach the detritivores were collected from.

Alder disks were conditioned in the laboratory to reduce the amount of leachates and to encourage microbial colonization. Conditioning was performed by incubating five alder disks in each microcosm for 15 d at 15 °C, 12:12 light photoperiod and constant aeration. After conditioning (stream water was the only source of microbial propagules), a set of 96 microcosms was kept in incubation for an additional 30 d to measure microbial breakdown. The rest of the microcosms were used for invertebrate performance by adding one individual of *E. berilloni* per microcosm. Microcosms were sampled just after the conditioning for initial values (day 0) and later at days 8, 15 and 30. Replication for each sampling and effluent concentration was of 4 in the microbial incubation (4 replicates × 6 concentrations × 4 sampling times = 96 microcosms in total) and of 10 for the detritivore incubation (10 replicates × 6 concentrations × 3 sampling times = 180 microcosms in total).

2.4. Microbial performance

The disks incorporated into each microcosm were weighed before conditioning. Initial dry mass of the disks was estimated with a correction factor (air dried-to-leached oven dried) obtained from a set of 4 tubes with 5 disks at each effluent concentration that were subject to the same initial conditioning of 15 d. At each sampling time, 3 out of 5 disks were retrieved from the microcosms, oven dried (70 °C; 72 h) and ashed (500 °C; 4 h) to obtain the ash free dry mass (AFDM). The remaining 2 disks were used to measure microbial respiration with a RC650 Respirometer coupled to a SI929-6 Channel oxygen meter (Strathkelvin Instruments, Scotland). The respirometer cells were filled with 3 mL of M9 medium (adapted from Sambrook et al., (2001); supplementary material C) that was oxygen-saturated. The disks were kept in the respirometer for 40 min with constant agitation, and the last 20 min were used to measure the rate of oxygen depletion in each cell. Additional chambers with oxygen-saturated M9 medium and without disks were used as a control. Oxygen consumption was estimated by the subtraction of the slope in the control cells (mean: -1.33; SE: ± 1.07 μg $O_2 \cdot h^{-1}$) to the slope of each respirometer cell and corrected for the remaining AFDM of the disks (μg $O_2 \cdot$ mg $AFDM^{-1} \cdot h^{-1}$). The disks used for respirometry were ground and homogenized with a IKA Ultra-Turrax T25 Basic grinder (Saufen, Germany) in 20 mL of M9 medium for exo-enzymatic activity measurements. Microbial exo-enzymes contributing to the degradation of cellulose and hemicellulose (β-glucosidase, BG) and to the acquisition of organic phosphorus (alkaline phosphatase, AP) were assessed following Saiya-Cork et al. (2002). Potential activity of both enzymes was estimated fluorometrically (360 nm excitation, 450 nm emission, 37 °C) using the substrate 4-Methylumbelliferyl β-D-glucopyranoside for BG and 4-Methylumbelliferyl phosphate for AP. Assays were carried out in 96-well microplates and fluorescence was determined with a Tecan GENios microplate reader (Cavro Scientific Instruments, Salzburg, Austria). The required controls and blanks were used to determine autoflorescence and quenching, and the results were expressed as μmol · mg $AFDM^{-1} \cdot h^{-1}$.

2.5. Detritivore performance

Consumption rates were calculated from the difference between estimated initial dry mass and final weighted dry mass of the 5 alder disks in microcosms with detritivores. *E. berilloni* individuals were photographed at the beginning and at the end of the experiments with a binocular microscope (Leica M165FC, Wetzlar, Germany). From these photographs, the dorsal length of the first thoracic segment (DL) was measured using the "Leica Application suite V4″ program (LAS V4.1). Initial and final total body lengths (BL) were calculated using Eq. (1) (Flores et al., 2014). Instantaneous growth rate (IGR) for each individual was calculated using Eq. (2) (Flores et al., 2014) where t is time, BL_t is the body length at time t and BL_0 is the initial body length. Finally, by means of the Eq. (3) the body mass (BM) was calculated at the beginning and at the end of the experiment (Flores et al., 2014), and the geometric mean of the body mass was used as the descriptor for each detritivore mass throughout the experiment. The equation fits for the detritivores of the present study it was derived from the same species collected in nearby streams.

$$BL = 14.458 \cdot DL - 0.110; \text{ (mm)} \quad (1)$$

$$IGR = (\ln(BL_t) - \ln(BL_0)) / t; \text{ (mm} \cdot d^{-1}) \quad (2)$$

$$BM = 0.8213BL - 4.3025; \text{ (mg)} \quad (3)$$

For each individual, the concentration of RNA was quantified fluorometrically on microplates, to assess individual metabolic status. The measurements were performed using RiboGreen to quantify RNA of detritivores after the extraction with N-laurylsarcosine and followed by RNase digestion as described by Gorokhova and Kyle (2002). Fluorescence was measured in a Tecan GENios microplate reader (Cavro Scientific Instruments, Sunnyvale), filters: 485 nm for excitation and 520 nm for emission and black solid flat-bottom microplates. The plate was scanned with 0.2 s · $well^{-1}$ measurement time, with 10 measurements per well at constant temperature (37 °C).

2.6. Field experiment

To test how well laboratory experiments fit responses in the field, we performed an *in situ* bioassay experiment using a comparable experimental design and measuring the same set of variables. This experiment was conducted from February 28 to May 30, 2017. We experimentally diverted part of the WWTP effluent, thus polluting the lowermost 150 m of the Apraitz Stream before it joins the Deba River. The experiment followed a Before-After Control-Impact Paired (BACIP) design (Downes et al., 2002), which allows detecting the effect of the tested impact while controlling for the effect that temporal and spatial changes could have on the response. Two 100-m long reaches were defined: a Control reach (C) just upstream from the effluent addition point and an Impact reach (I) below that point. At both reaches, the variables were measured Before (B) and After (A) the start of the addition of the effluent to the Impact reach. The WWTP released a mean discharge of 10 L · s^{-1} of effluent into the Impact reach for around 20–40 min every 2 h. The effluent was diluted to 1.6% on average along the duration of the experiment (stream discharge measured every 5 min by a level-logger ((Solinst Edge 3001; Solinst Canada Ltd., Georgetown)). Fine-mesh bags (20 x 10 cm, 0.5 mm pore size) filled with two alder disks each (24 mm diameter) were conditioned in stream water in the laboratory at 15 °C (mean temperature in the field: 12.1 °C) for 15 d. After the conditioning, 20 bags were used to measure microbial performance (5 replicates per sampling day and reach, which were distributed along each reach). Another 60 bags with alder disks and one *E. berilloni* individual in each were incubated to assess detritivore performance (15 replicates per sampling day and reach, distributed along each reach). In each sampling period (Before/After), reach (Control/Impact) and sampling day (Day8/Day15) 20 bags (5 for microbial performance and 15 for detritivore performance) were retrieved (in total 160 bags). The addition started on May 3, with the Before incubation spanning from March 14 to 30 and the After incubation from

May 16 to 30.

2.7. Chemical analysis

Effluent conductivity and pH data are continuously measured in the WWTP. During the field experiment, stream water physicochemical characteristics (temperature, dissolved oxygen, pH, oxidation reduction potential (ORP), conductivity and total dissolved solids (TDS)) were measured continuously during 1.5 h (30 m downstream from the effluent input; June 12, 2017) with a multiparametric probe (EXO 2, YSI, USA). Water physicochemical characteristics were also measured in the Control and Impact reaches during the experiment.

Nitrogen and phosphorus concentrations were analyzed in the integrated sample used in the laboratory experiment and in the samples collected in the field (from the lower end of the Control and Impact reaches and directly from the effluent). Samples were filtered through pre-combusted glass-fiber filters (Whatman International, 0.7 μm) and stored at -20 °C until analysis. The concentration of soluble reactive phosphorus (SRP) (molybdate method (Murphy and Riley, 1962)) and ammonium (salicylate method (Reardon et al., 1966)) were determined colorimetrically on a UV-1800 UV–vis Spectrophotometer (Shimadzu, Shimadzu Corporation, Kyoto, Japan). The concentration of nitrate and nitrite were determined with capillary ion electrophoresis (Agilent CE) (Environmental Protection Agency, 2007). Dissolved inorganic nitrogen (DIN) was calculated as the sum of nitrate, nitrite and ammonium.

Additionally, 41 priority and emerging organic compounds (supplementary material A) were analyzed following Mijangos et al. (2018a,b), including herbicides, hormones, life style products (personal care products, stimulants and artificial sweeteners), industrial chemicals (corrosion inhibitors and fluorinated compounds), and pharmaceuticals (antibiotics, tricyclic antidepressants, antihypertensives, anti-inflammatories, β-blocker cardiovascular drugs, lipid-regulating and anticonvulsants), which cover a wide variety of emerging contaminants typically found in WWTP effluents. The selection of the target pollutants was carried out taking into account their presence and relevance in the environment (Brack et al., 2017; Busch et al., 2016; Tousova et al., 2017). Compound families, names, CAS numbers, molecular formulas and other relevant physicochemical properties for all the target compounds are summarized in supplementary material (A). These analysis were performed on the integrated effluent sample used in the laboratory experiment and in the case of field experiment, spot samples (May15, 2017) were taken from the Control, Impact and WWTP effluent simultaneously every 5 min during 1 h. In both cases (laboratory and field experiment) water samples were kept in the fridge at 4 °C before analysis, which was performed within 24 h according to a previously validated SPE procedure (Mijangos et al., 2018a). Additionally, in the case of the field sample, time weight average concentrations (C_{TWA}) of the stream (Control and Impact) were calculated from May15 to June 12, 2017 by means of passive samplers. At both reaches, a canister containing two polar organic chemical integrative samplers (POCIS) was deployed at ~50-100 cm below the surface. POCIS were prepared according to the procedure described by Mijangos et al. (2018b) (sorbent material mixture of 100 mg mixed-mode anion exchange (Strata X-AW) and 100 mg HLB (Plexa) and using a highly porous (30 μm pore size) Nylon membrane sampler). POCIS were transported at -4 °C to the lab and stored at −20 °C before being processed as described in supplementary material (D).

Emerging organic compound analysis was carried out using a HPLC-QqQ (Agilent 1260 series LC coupled to an Agilent 6430 triple quadrupole) equipped with electro spray ionization (ESI) source (Agilent Technologies) according to a previously optimized method (Mijangos et al., 2018a). The separation of the target analytes was accomplished at a flow of 0.3 mL·min^{-1} using a Kinetex F5 100 Å core-shell (2.1 mm × 100 mm, 2.6 μm) column coupled to a Kinetex F5 pre-column (2.1 mm × 4.6 mm, 2.6 μm). The column temperature and the injection volume were set to 35 °C and 5 μL, respectively. Under optimized conditions, a binary mixture consisting of a mobile phase A of water: MeOH (95:5) and mobile phase B of MeOH: water (95: 5), both containing 0.1% of formic acid was used for gradient separation of the target analytes. The gradient profile started with 30% B which was increased to 50% in 4 min and maintained for 12 min. Then it was increased to 90% B where it was maintained constant for 10 min. Initial gradient conditions (30% B) were then achieved in 6 min, where it was finally held for another 10 min (post-run step). ESI was carried out using a nitrogen flow rate of 12 L·min^{-1}, a capillary voltage of 3500 V, a nebulizer pressure of 45 psi, and a source temperature of 350 °C. Both, negative and positive voltages, according to the target analytes, were simultaneously applied in a single injection. Quantification was performed in the selected reaction monitoring (SRM) acquisition mode. Fragmentor voltage and collision energy values for each target analyte and the determined apparent recoveries and method limits of quantification (MQLs) are included in supplementary material (D). Instrumental operations, data acquisition and peak integration were performed with the Mass Hunter Workstation Software (Qualitative Analysis, Version B.06.00, Agilent Technologies).

2.8. Data analysis

Consumption of organic matter was calculated by subtracting final AFDM to the initial AFDM values. Initial dry mass was corrected for leaching, and microbial decomposition was removed from the total consumption in test tubes with *E. berilloni* to estimate the consumption by the detritivore. Thus, microbial decomposition was expressed as the depleted AFDM per sampling day. For *E. berilloni* consumption, depleted AFDM was corrected by the dry body mass of the individual per day (mg AFDM · mg BM^{-1} · d^{-1}). The detritivore death rate was calculated per treatment at each sampling day and corrected with the incubation time. Respiration and exo-enzymatic activities were corrected by the remaining AFDM of alder disk at the sampling day. Although RNA:DNA ratio is widely used in the literature (Gorokhova, 2003; Vrede et al., 2002), total nucleic acids and DNA concentrations are determined sequentially from the same sample and RNA concentrations extrapolated subtracting both values. The numerator is thus dependent on the denominator, magnifying error and thus potential variability in the index (Suthers et al., 1996). Therefore, individual RNA concentrations were corrected with the BM of the individuals to obtain the RNA:BM ratio. For the laboratory experiment, we fitted Gaussian models (Madsen and Thyregod, 2010; Zuur and Ieno, 2010) as our data accommodated satisfactorily to their requirements. We used these kinds of models to test for the effect of treatment and time on the response variables: microbial decomposition, respiration and exo-enzymatic activity, and detritivore death rate, consumption rate, growth rate and total concentration of nucleic acid. For parameter estimation, restricted maximum log-likelihood (Pinheiro and Bates, 2000) was used, via the lme() function of the package nlme (Pinheiro and Bates, 2016). The fixed structure of the model included treatment (fitted as a continuous explanatory variable, 0–100% effluent concentration), time (fitted as a discrete explanatory variable, with three levels, Day 8, Day 15 and Day 30) and the interaction between both. The quadratic term of Treatment was also included in the model to test the fit to a subsidy-stress response pattern. A variance structure was also added to the model to deal with observed heterogeneity, allowing different variances per stratum (varIdent(form = ~1|Day)). *In situ* bioassay data were analyzed via linear models, where period (BA) and reach (CI) were treated as fixed factors, and the spatial variation was fitted as a random factor nested in reaches. The interaction between period and reach was also fitted. The models were fitted using the lm() function of the nlme package (Pinheiro and Bates, 2017). Data from days 8 and 15 were analyzed separately, so that the responses for each day were assessed separately. In all cases significance was accepted when $p < 0.05$. All statistical analyses were conducted using R statistical software (version 3.1.2, R Core Team, 2017).

Table 1
Basic water chemical characteristics of the WWTP effluent used during the laboratory and field experiment and the Control reach of Apraitz stream during 2016-2017. Mean values and standard errors are shown. Letters display statistical differences.

Variables	WWTP effluent laboratory experiment	WWTP effluent field experiment	Stream water
Temperature (ºC)	15_a	20.02 ± 1.13 (n = 4)$_b$	14.79 ± 1.07 (n = 8)$_a$
Conductivity (µs · cm^{-1})	649 ± 18 (n = 3)$_a$	765 ± 25 (n = 8)$_b$	351 ± 60 (n = 8)$_c$
DO (%)	100_a	7.75 ± 3.87 (n = 4)$_b$	102.5 ± 1.01(n = 8)$_a$
DIN (mg · L^{-1})	8.03 (n = 1)$_a$	1.05 ± 0.07 (n = 9)$_b$	
SRP (mg · L^{-1})	0.77 (n = 1)$_a$	10.59 ± 2.11 (n = 8)$_a$	0.02 ± 0.01 (n = 9)$_b$
		1.65 ± 0.25 (n = 2)$_a$	

3. Results

3.1. Laboratory experiment

3.1.1. Effluent characteristics

The WWTP effluent had substantially higher conductivity, DIN and SRP concentrations than the stream water (Table 1). Twenty five out of the 41 emerging contaminants analyzed in the integrated effluent sample were above quantification limits, the highest values corresponding to valsartan (26,870 ng L^{-1}) and caffeine (14,555 ng L^{-1}). Other compounds, such as acesulfame (2610 ng L^{-1}), sucralose (1865 ng L^{-1}), irbesartan (3665 ng L^{-1}), eprosartan (2899 ng L^{-1}) and telmisartan (1637 ng L^{-1}), also exhibited high concentrations (Table 2). In the Control reach, only caffeine was detected by active sampling and 13 analytes with the POCIS (Table 2).

3.1.2. Microbial performance

Overall, the effect size of the response was higher for microbes than for the detritivore. The effluent promoted microbial decomposition of organic matter (Treatment, $p = 0.039$, Table 3, Fig. 1), which in the most concentrated treatment increased by 120% for day 30. Neither time nor the time:treatment interaction were significant, but variance decreased from the first sampling to the last sampling. Microbial respiration increased with effluent concentration (Treatment, $p < 0.030$, Table 3, Fig. 1) and time of exposure (Day, $p < 0.001$), with highest values at 100% effluent concentration and day 30. The interaction between effluent concentration and time of exposure was also significant (Treatment:Day, $p = 0.006$), as the positive relationship was only observed after 15 and 30 d of incubation. Exo-enzymatic activities were also promoted by the effluent concentration (Table 3, Fig. 1). The response of both exo-enzymatic activities to the effluent concentration depended on the exposure time (Day, $p < 0.001$). For AP, the relationship with the effluent concentration only became significant after day 30, for BG was significant after day 15. The quadratic term, which characterizes the hump-shaped curve, was only significant for AP (Treatment^2, $p = 0.026$). The maximum rate of AP activity was predicted to be at 29.4, 42.5, 67.0 and 390.7% of effluent concentration, at 0, 8, 15 and 30 days of exposure. For both exo-enzymes, the interaction between effluent concentration and incubation time was significant (Treatment:Day, $p < 0.001$).

3.1.3. Detritivore performance

In total, 51 out of 180 individuals died during the experiment, but the effluent did not affect detritivore death rate (Treatment, $p = 0.464$, Table 3, Fig. 1). The effluent concentration affected the food consumption rate of the detritivore (Treatment, $p = 0.036$, Table 3, Fig. 1). The response followed a hump-shape pattern (Treatment^2, $p = 0.037$) across the concentration range, with the highest consumption rates (0.510 mg AFDM · mg BM^{-1} · d^{-1}) at day 8 and 40% concentration. Food consumption decreased gradually over the experiment (Day, $p < 0.001$). At 100% effluent concentration it decreased by half (from 0.262 to 0.130 mg AFDM · mg BM^{-1} · d^{-1}) from day 8 to day 30. Growth rate of the detritivore was overall not affected by treatment (Treatment, $p = 0.591$, Table 3, Fig. 1), although the significant quadratic term (Treatment^2, $p < 0.041$) showed a significant subsidy response at medium concentrations. Despite reduced food consumption over time, growth rate increased with time (Day, $p < 0.008$). The RNA:BM ratio (as well as RNA concentration, not shown) increased with the effluent concentration (Treatment, $p < 0.001$, Table 3, Fig. 1), but not with time. The overall effect size of the response was lower for the detritivore than for microbes.

3.2. Field experiment

3.2.1. Water quality

In the Before period, temperature, conductivity, dissolved oxygen, DIN and SRP (Table 1), did not differ between Control and Impact reaches. However, within 5 min of effluent discharge, dissolved oxygen, pH and ORP fell, whereas temperature, conductivity and TDS increased (Fig. 2). Dissolved oxygen did not reach hypoxic conditions (i.e. it decreased from approx. 100% to 80%) and pH remained neutral (i.e. it decreased from approx. 7.9 to 7.0). Water characteristics returned to initial values after approx. 20 min, when the effluent discharge stopped. This intermittent pattern was repeated every 2 h during the whole study period. The concentration of emerging contaminants measured with the grab sample also varied during one pulse (before effluent input, during input and after input; supplementary material B). Prior to each effluent discharge, caffeine was the only analyte detected measuring spot samples analysis in both Control (20 ng L^{-1}) and Impact (39 ng L^{-1}) reaches. 20 min after the pouring started, 8 more emergent pollutants were detected in the Impact reach (supplementary material B). After pouring, most compounds returned to not detected below quantification levels (supplementary material B). Moreover, by using POCIS, a technique which allows to detected lower concentrations in comparison with the conventional grab samples approach (Miège et al., 2015), time average concentrations (28 days) in the range of 0.3–128 ng L^{-1} and 0.6–40 ng L^{-1} were obtained in the Control and Impact reach, respectively. In the Control reach, the highest concentration corresponded to caffeine (128 ng L^{-1}), whereas the rest of compounds did not exceed 11 ng L^{-1}. In any cases, these values are lower in comparison with previous reported aquatic media affected by wastewater inputs (Guibal et al., 2018; Mijangos et al., 2018b). In the Impact reach, OBT (40 ng L^{-1}), Irbesartan (37 ng L^{-1}) and caffeine (34 ng L^{-1}) were the most concentrated compounds (Table 2).

3.2.2. Microbial performance

Microbial decomposition of alder leaf disks was not affected by the effluent (BA:CI, Day 8; $p = 0.146$, Day 15; $p = 0.572$, Table 4, Fig. 3). Neither sampling period nor reach affected microbial decomposition. Microbial respiration only showed differences between the Before and After period at day 8 (BA, $p = 0.031$, Table 4, Fig. 3). However, the effect of the effluent input on microbial respiration was not significant (BA:CI, Day 8; $p = 0.198$, Day 15; $p = 0.811$). Both measured exo-enzymatic activities differed between periods at both sampling days, with higher activities in the Before period (Table 4). However, the effluent did not affect any of the exo-enzymatic activities.

3.2.3. Detritivore performance

Food consumption of detritivores enclosed in bags was not affected by the effluent (BA:CI, p = 0.841, Table 4, Fig. 3), but there were differences between incubation period and reach by day 15 (BA, $p = 0.035$; CI, $p = 0.028$). No effect of effluent addition, period or sampling

Table 2
Mean concentrations ($ng \cdot L^{-1}$) of the target analytes determined by means of active sampling for the laboratory experiment (integrated effluent sample) and the field experiment (Control and Impact reach during input and WWTP effluent discharge) as well as the time average concentrations (POCIS, $ng \cdot L^{-1}$). Mean values and standard errors are shown.

	Laboratory experiment	Field experiment				
	Integrated sample	Spot sample			POCIS	
	WWTP effluent	Control reach	WWTP effluent	Impact reach	Control reach	Impact reach
Acesulfame	2610 ± 160	n.d.	n.d.	n.d.	n.d.	6 ± 2
Acetaminophen	n.d.	< mql	< mql	< mql	n.d.	19 ± 3.5
Amitriptyline	52 ± 2	n.d.	72 ± 2	26 ± 3	0.9 ± 0.1	6 ± 2
Atrazine	< mql	n.d.	< mql	< mql	0.1	11 ± 2
Bezafibrate	< mql	n.d.	n.d.	n.d.	n.d.	0.6 ± 0.1
Butylparaben	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Caffeine	14,555 ± 373	20 ± 6	76 ± 5	46 ± 6	128 ± 10	34 ± 6
Carbamazepine	104 ± 2	n.d.	122 ± 6	42 ± 3	0.31 ± 0.04	9 ± 2
Ciprofloxacin	100 ± 2	< mql	98 ± 8	44 ± 3	–	–
Clofibric acid	n.d.	n.d.	n.d.	n.d.	–	–
Clomipramine	8.1 ± 0.2	n.d.	12 ± 1	< mql	–	–
Diclofenac	843 ± 3	n.d.	472 ± 61	102 ± 11	–	–
Diuron	325 ± 12	n.d.	267 ± 13	99 ± 12	0.67 ± 0.02	20 ± 5
Eprosartan	2899 ± 63	n.d.	691 ± 65	174 ± 43	–	–
Genistein	n.d.	n.d.	n.d.	n.d.	–	–
Genistin	n.d.	n.d.	n.d.	n.d.	–	–
Glycitin	n.d.	n.d.	n.d.	n.d.	–	–
Imipramine	n.d.	n.d.	n.d.	n.d.	–	–
Irbesartan	3665 ± 33	< mql	3639 ± 71	1161 ± 110	0.5 ± 0.1	37 ± 7
Isoproturon	< mql	n.d.	< mql	< mql	–	–
Ketoprofen	135 ± 3	n.d.	126 ± 10	46 ± 6	0.29 ± 0.03	4 ± 1
Losartan	37.3 ± 0.6	n.d.	14 ± 1	< mql	–	–
Metylparaben	n.d.	n.d.	n.d.	n.d.	–	–
Norfloxacin	34 ± 3	n.d.	34 ± 2	< mql	–	–
Nortriptyline	10.5 ± 0.6	n.d.	13 ± 1	< mql	–	–
Obt	367 ± 15	n.d.	415 ± 16	151 ± 21	10.6 ± 0.8	40 ± 9
Pfbs	260.4 ± 0.8	n.d.	147 ± 16	52 ± 11	0.8 ± 0.1	2.5 ± 0.8
Pfoa	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Pfos	19 ± 3	n.d.	10 ± 1	< mql	0.8 ± 0.2	4 ± 2
Pfosa	n.d.	n.d.	n.d.	n.d.	–	–
Phenytoin	162 ± 24	n.d.	1882 ± 95	549 ± 40	0.3 ± 0.1	12 ± 4
Progesterone	n.d.	n.d.	n.d.	n.d.	0.4 ± 0.1	5 ± 1
Propranolol	34 ± 2	n.d.	35 ± 3	14 ± 1	–	–
Simazine	n.d.	n.d.	n.d.	n.d.	–	–
Sucralose	1855 ± 132	< mql	1977 ± 155	< mql	–	–
Sulfadiazine	5.0 ± 0.3	n.d.	n.d.	n.d.	–	–
Sulfamethoxazole	22 ± 2	n.d.	67 ± 5	37 ± 5	–	–
Telmisartan	1637 ± 111	n.d.	1771 ± 252	341 ± 70	0.38 ± 0.09	4.6 ± 0.9
Testosterone	n.d.	n.d.	n.d.	n.d.	–	–
Trimethoprim	56.7 ± 0.3	n.d.	44 ± 3	25 ± 2	–	–
Valsartan	26,870 ± 190	n.d.	403 ± 71	< mql	–	–

Table 3
Statistical results for the laboratory experiment.

Microbial performance

Source of variation	Decomposition, mg AFDM $\cdot d^{-1}$			Respiration, µg $O_2 \cdot$ mg AFDM$^{-1} \cdot h^{-1}$			AP, µmol$\cdot h^{-1} \cdot$ mg AFDM^{-1}			BG, µmol$\cdot h^{-1} \cdot$ mg AFDM^{-1}		
	DF	F-value	p-value	DF	F-value	p-value	DF	F-value	p-value	DF	F-value	p-value
Treatment	1	4.43	0.039	1	4.86	0.030	1	4.07	0.047	1	0.97	0.328
Day	2	1.17	0.316	3	17.74	< 0.001	3	52.07	< 0.001	3	61.08	< 0.001
Treatment^2	1	1.59	0.211	1	1.42	0.236	1	5.16	0.026	1	2.57	0.113
Treatment:Day	2	0.36	0.699	3	4.48	0.006	3	36.00	< 0.001	3	16.99	< 0.001

Detritivore performance

Source of variation	Consumption, AFDM $\cdot$ mg BM $^{-1} \cdot d^{-1}$			Instantaneous growth rate, mm $\cdot d^{-1}$			RNA:BM, µg $\cdot$ mg BM^{-1}			Death %		
	DF	F-value	p-value	DF	F-value	p-value	DF	F-value	p-value	DF	F-value	p-value
Treatment	1	4.49	0.036	1	0.29	0.591	1	12.11	< 0.001	1	0.58	0.464
Day	2	31.06	< 0.001	2	5.09	0.008	2	0.67	0.512	2	0.98	0.405
Treatment^2	1	4.44	0.037	1	4.29	0.041	1	0.01	0.934	1	0.39	0.545
Treatment:Day	2	3.59	0.060	2	1.55	0.217	2	1.06	0.350	2	0.60	0.577

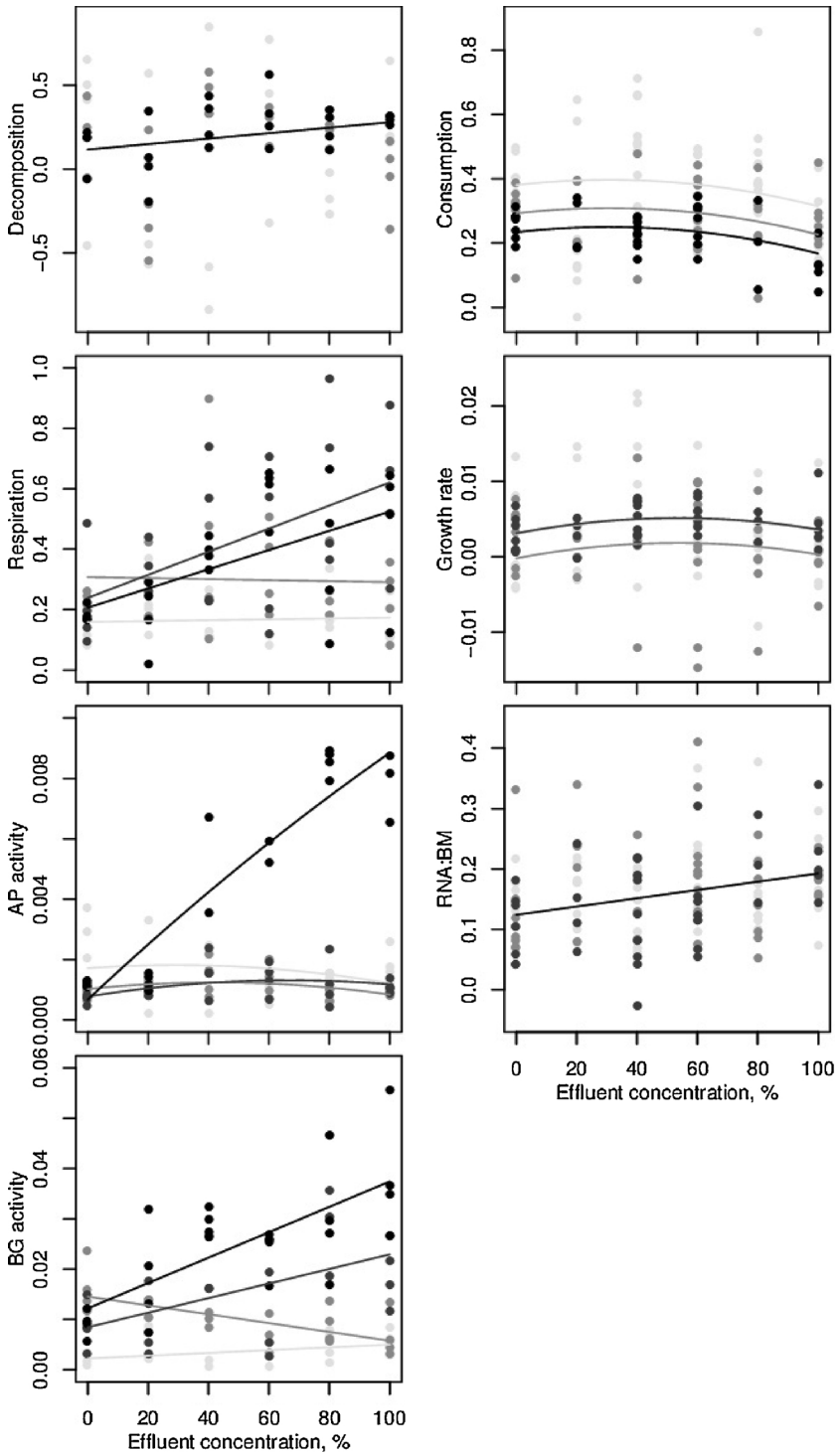

Fig. 1. Laboratory experiment results. Left, microbial performance: ecomposition (mg AFDM · d^{-1}), respiration (μg O_2 · mg $AFDM^{-1}$ · h^{-1}), AP and BG exo-enzymatic activities (μmol · mg $AFDM^{-1}$ · h^{-1}). Right, detritivore performance: food consumption (mg AFDM · mg BM^{-1} · d^{-1}), growth rate (mm · d^{-1}) and RNA:BM ratio (μg RNA · mg BM^{-1}). Lighter to darker symbols represent time from 0 (or 8 if no Day-0 measure available) to 30 d of incubation. Regression lines are drawn with the significant coefficients from model and a single line is represented if incubation time was not significant.

day was detected for growth rate (Table 4). However, the RNA:BM ratio was reduced because of the effluent on both sampling days (BA:CI, Day 8; $p = 0.020$, Day 15; $p = 0.026$, Table 4, Fig. 3). This ratio decreased 30.9% from Control to Impact reach at day 8 and 25.3% at day 15 (Fig. 3).

4. Discussion

WWTP effluents consist on complex mixtures of compounds, some of which, such as nutrients, are expected subsidize biological activity, whereas others, such as pesticides or many emerging contaminants, are expected to stress biological activity. Therefore, the overall effect of WWTP effluents can depend on their exact composition and final concentration, as well as on the characteristics of receiving water bodies.

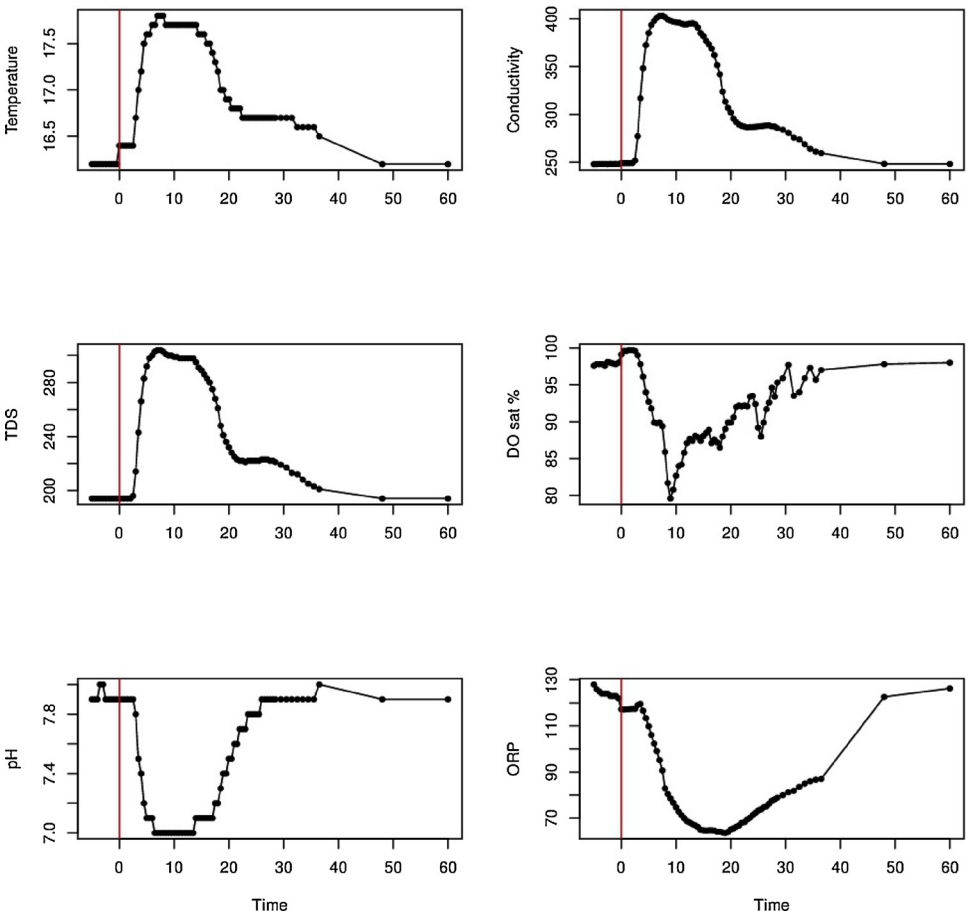

Fig. 2. Physicochemical characteristics in the Impact reach recorded every 5 min during one cycle of the effluent discharge. Temperature (ºC), conductivity (μs · cm^{-1}), total dissolved solid (TDS, mg · L^{-1}), dissolved oxygen saturation (DO sat %), pH and oxidation reduction potential (ORP, mV). The effluent discharge starts at time 0 and lasted 20 min.

Many studies have shown that in nutrient-limited streams, nutrient addition promotes microbial decomposition (Ferreira et al., 2006; Niyogi et al., 2003; Suberkropp and Chauvet, 1995). In our case, microbial decomposition was subsidized significantly along the effluent concentration gradient in the laboratory experiment, suggesting a positive nutrient effect. A similar pattern was observed by Biasi et al. (2017), who found that increased nutrient concentrations in laboratory microcosms stimulated microbial activity (e.g. respiration), which was

Table 4
Statistical results for the field experiment. BA: Before-After; CI: Control-Impact.

Microbial performance

Source of variation	Decomposition, mg AFDM · d^{-1}			Respiration, μg O_2 · mg $AFDM^{-1}$ · h^{-1}			AP, μmol · h^{-1} · AFDM mg^{-1}			BG, μmol · h^{-1} · AFDM mg^{-1}		
	DF	F-value	P-value	DF	F-value	P-value	DF	F-value	P-value	DF	F-value	P-value
Day 8												
BA	1	2.82	0.115	1	5.71	0.031	1	5.45	0.035	1	47.93	< 0.001
CI	1	1.33	0.268	1	3.33	0.089	1	0.19	0.668	1	0.14	0.711
BA:CI	1	2.37	0.146	1	1.82	0.198	1	0.65	0.432	1	0.01	0.942
Day 15												
BA	1	2.27	0.154	1	2.51	0.135	1	35.69	< 0.001	1	11.41	0.004
CI	1	0.41	0.531	1	0.06	0.817	1	0.29	0.597	1	3.37	0.088
BA:CI	1	0.33	0.572	1	0.06	0.811	1	2.74	0.120	1	0.76	0.397

Detritivore performance

Source of variation	Consumption, AFDM · mg BM^{-1} · d^{-1}			Instantaneous growth rate, mm · d^{-1}			RNA:BM, μg · mg BM^{-1}		
	DF	F-value	P-value	DF	F-value	P-value	DF	F-value	P-value
Day 8									
BA	1	0.01	0.911	1	0.38	0.539	1	9.80	**0.003**
CI	1	2.75	0.105	1	0.42	0.520	1	1.64	0.207
BA:CI	1	1.45	0.236	1	1.82	0.184	1	5.90	**0.02**
Day 15									
BA	1	4.87	**0.035**	1	0.98	0.330	1	0.29	0.593
CI	1	5.32	**0.028**	1	0.05	0.819	1	0.01	0.927
BA:CI	1	0.04	0.841	1	0.28	0.598	1	5.51	**0.026**

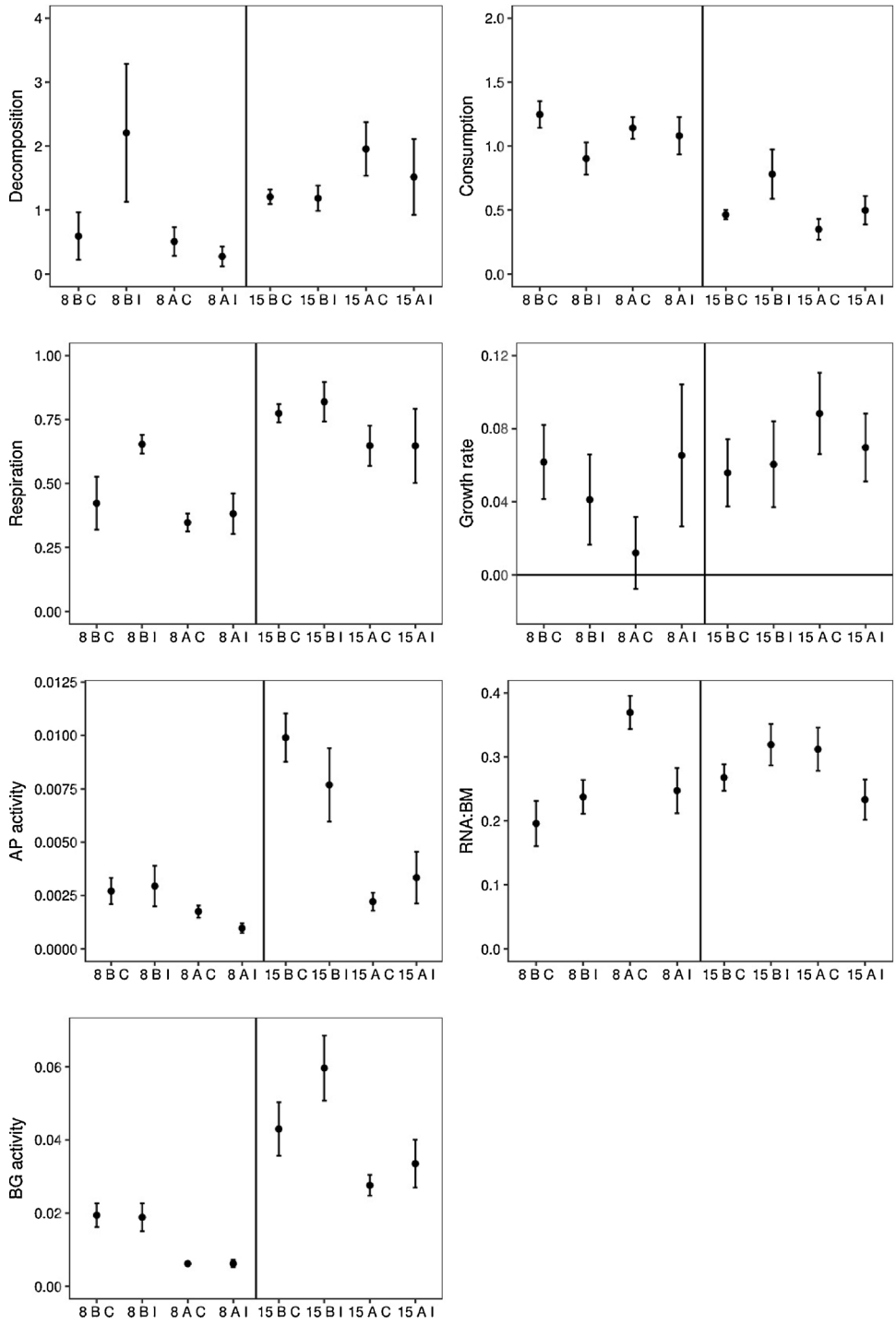

Fig. 3. Field experiment results. Left, microbial performance: decomposition (mg AFDM · d^{-1}), respiration (µg O_2 · mg $AFDM^{-1}$ · h^{-1}), AP and BG exo-enzymatic activities (µmol · mg $AFDM^{-1}$ · h^{-1}). Right detritivore performance: food consumption (mg AFDM · mg BM^{-1} · d^{-1}), growth rate (mm · d^{-1}) and RNA:BM ratio (RNA µg · BM mg^{-1}). Results for Before-After (BA) periods and Control-Impact (CI) reaches at sampling days 8 and 15. Mean values and standard error are represented.

translated into accelerated organic matter decomposition. Similarly, the observed increase in exo-enzymatic activities could be explained by the nutrient enrichment due to the effluent addition (Carreiro et al., 2000). Together with nutrients, the effluent used in the experiment comprised an array of emerging contaminants (i.e. trimethoprim, diclofenac, sulfamethoxazole, sulfadiazine) in the same range as those detected in surveys of WWTP effluents across Europe (Beckers et al., 2018; Loos et al., 2013). In fact, Loos et al. (2013) monitored 90 European WWTPs and found the sartan family to be one of the most relevant emerging contaminant groups with a median concentration of 480 ng L^{-1}, 368 ng L^{-1} and 227 ng L^{-1} for irbesartan, telmisartan and eprosartan, respectively. The integrated effluent used in the laboratory experiment exceeded these concentrations up to 4 (telmisartan) or 12 (eprosartan) fold depending on the compound. A variety of the emerging contaminants, and even nutrients above a certain concentration, can have adverse effects on the biota. Leaf decomposing microbial communities are affected by WWTP effluent inputs (e.g. Feckler et al., 2018; Gardeström et al., 2016), although we cannot test this point as we did not analyze microbial communities. Regardless of the changes in community composition, in the present study there were no negative

effects at the functional level. In this line, Hughes et al. (2016) did not observe any effect on microbial breakdown or detritivore consumption of leaf litter when they were exposed to a mixture of pharmaceuticals. The exact concentration of a cocktail of pollutants at which a certain response variable switches from a subsidy to stress response pattern depends on the species/community (King and Richardson, 2007), and can be especially difficult to predict for complex mixtures of substances as in the case of WWTP effluents (Culp et al., 2000; Jackson et al., 2016; Paine et al., 1998).

Regarding detritivores, in our laboratory experiment, the observed hump-shape response of consumption and growth rate (Fig. 1) did not reach to stress levels as anticipated by our first prediction as none of the effluent concentrations reduced the response below the control treatment.. A similar pattern was observed by Woodward et al. (2012), who described an increase in invertebrate-mediated breakdown in European rivers until 18 $\mu g \cdot L^{-1}$ SRP and 1000 $\mu g \cdot L^{-1}$ DIN, followed by a reduction, which did not reach stress values (i.e. values below control). Similar hump-shape responses for detritivore consumption rate were reported by Dunck et al. (2015) and Pereira et al. (2016). Our highest consumption rates were observed at 40% effluent concentration. Even if the overall effect size (i.e. differences with the control) was small for consumption, the quadratic term of the treatment, which describes the hump-shape response, was statistically significant. *E. berilloni* is a key consumer that has been described in mildly polluted sites (Pérez et al., 2013), and thus, was likely quite resistant to the treatment, especially when, as was our case, the individuals were not collected in a pristine site, which probably pre-adapted them to certain levels of contamination. Moreover, high nutrient concentrations in the field can result in eutrophication and hypoxia, which have direct negative effects on biota. However, the constant aeration in the laboratory experiment avoided hypoxic conditions in the microcosms, thereby increasing the capacity of the animals to resist toxicity.

RNA concentration reflects the rate of protein synthesis, including those produced in detoxification processes (Elser et al., 2000). In our laboratory experiment, RNA concentrations steadily increased along the effluent gradient (Fig. 1), which might suggest that the effluent was activating the defense systems and repair processes and, with that, protein turnover (Maltby, 1999). For instance, sucralose has been shown to induce neurological and oxidative mechanisms with important consequences on crustacean behavior and physiology (Wiklund et al., 2014). Similarly, propranolol can have negative effects on *Gammarus spp*. physiology (Oskarsson et al., 2012). The increase of RNA concentration observed in our study may have avoided negative implications in the remaining endpoints. Growth rate responded with a significant hump shape (Fig. 1). It has been described that *E. berilloni* individuals can invest more or less in longitudinal growth depending on the environmental conditions (Basset and Glazier, 1995; Glazier, 2000; Larrañaga et al., 2014). We did not study the length-mass relationship of our individuals and growth was derived from body length changes. Thus, we cannot discard that individuals may have switched from longitudinal growth at intermediate concentrations of the effluent to a higher investment in becoming fatter and accumulating more reserves at high effluent concentration. Actually, Larrañaga et al. (2014) noted that mass body condition of *E. berilloni* tended to maximize at higher nutrient concentrations than longitudinal growth in a laboratory experiment.

In general, the response was clearer in the laboratory experiment than in the field experiment. The only variable that responded significantly to the effluent input in the field experiment, the RNA:BM ratio (Table 4), did it in the opposite direction compared to the laboratory response. This contrasting pattern might be caused by biofilm accrual on top of the bags in the Impact reach (L. Solagaistua, personal observation), which might have reduced the oxygenation of the bags. However, being RNA:BM the only variable that responded negatively, extremely deleterious conditions in these bags are discarded. The generally weaker response in the field experiment was probably caused by the dilution of the effluent in stream water (1.6% on average along the duration of the field experiment). Even at minimum discharge conditions for the After period in the Impact reach, the effluent concentration never rose beyond 64%, which would not cause strong responses given the short duration of those peak concentrations. The WWTP effluent was released in pulses, with 20–40 minutes of pouring followed by 100 min of non-pouring. In line with this, Nyman et al. (2013) showed that exposure to low and constant insecticide (imidacloprid) concentration reduced feeding and lipid content of *Gammarus pulex*, whereas the same concentration added in repeated pulses did not affect those variables. Similarly, Alexander et al. (2007) observed that the invertebrates *Epeorus longimanus* (insect, Ephemeroptera) and *Lumbriculus variegatus* (annelid, Lumbriculida) could recover from a 1-d exposure to imidacloprid in 4 d. The time required to recover in this study was 4 times longer than the exposure. In our case, the relative recovery time between effluent pulses was 5 times longer than the exposure, and thus, although the effluent could have generated some effects during peak effluent concentrations, the recovery time seems to be enough to avoid changes in the measured variables. Nevertheless, both works cited above assessed the effect of a unique chemical while the effluents used in the present study consist of a mixture of compounds, making direct comparisons complicated.

CEF assays, like the one performed here, have not reached maturity compared to more standardized toxicological assays. Among other methodological differences, the incubation time differs greatly between studies (Arroita et al., 2016; Englert et al., 2013). Our results show incubation time to be a significant factor in the laboratory experiment, as “Day” significantly affected 6 out of 8 measured variables (Table 3). We found that consumption and growth rate of detritivores were significantly affected by incubation time. Both consumption and growth are commonly measured response variables (e.g. Danger et al., 2012; Mas-Martí et al., 2015), whereas incubation time ranges from a week to up to 4 months. There are some generalities that have been pointed out regarding the effect of incubation time on the performance of detritivores. Hessen et al. (2013), for instance, noted that reduced feeding rate along time in microcosm experiments might be caused by decreased energy requirements in laboratory conditions. In our case, we cannot rule out the possibility that reduced consumption was a consequence of decreased food quality over time due to the accumulation of toxicants in the microbial layer. Additionally, in 3 out of 8 variables, incubation time significantly interacted with the treatment (Table 3). In those cases, longer incubations showed clearer differences between treatments, as expected from Haber´s rule. These results mean that the conclusions drawn from the assay depend on the incubation time, which has very relevant implications. Therefore, time-dependent standardized protocols should be applied to make studies comparable.

When the tested conditions kill the study organisms, this would clearly affect ecosystem function (e.g. consumption). CEF assays enable primarily focusing on sub-lethal effects (e.g. changes in appetite or body condition), that are inherently linked to predicting mid-to-long term effects on processes. From our results, it seems logical then to favor 30 d as preferred incubations for future experiments, as the differences among effluent dilutions were clearer. However, other variables (microbial decomposition and consumer RNA:BM ratio) did not present significant interaction with time, which would make them especially suitable for bio-assessment, as they would yield higher comparability between studies. Besides, longer incubations (months or even years) of macroinvertebrates would include sensitive life stages, and consequently, show impacts that might be more relevant for macroinvertebrate communities in the long-term (Hoguet and Key, 2008). For instance, emerging contaminants released from the WWTP could have affected the reproduction capacity of the detritivore, as pointed out by Wigh et al., (2017), who found that the toxicity of several effluents reduced the fecundity and fertility of *Gammarus fossarum*. Indeed, Englert et al., (2013) found WWTP effluents to affect macroinvertebrate community structure. As amphipod detritivores are very

important for organic matter processing and secondary production (Piscart et al., 2009; Woodward et al., 2008), a reduction in fecundity would probably have important consequences for ecosystem function.

CEF assays, as the one described in the present paper, mimic simplified ecosystems as biotic communities (microbes and macroinvertebrates) and interactions between them are included. Measuring the response at different trophic levels increases the ecological relevance of the assay, yielding a better understanding of how ecosystems respond to particular stressors. Moreover, our findings demonstrate that responses are modulated through time and can produce very different acute or chronic effects on ecosystems. However, there is a lack of standardized protocols for CEF assays, which complicates comparisons between studies as well as drawing general conclusions. Therefore, while the effects of new pollutants are measured for different taxa, our efforts should also focus on developing common procedures (e.g. incubation time, measured variables) to have more comprehensive CEF assays in the future.

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