



## Research article

## Treated and highly diluted, but wastewater still impacts diversity and energy fluxes of freshwater food webs



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## ABSTRACT

Wastewater treatment plants (WWTPs) have greatly improved water quality globally. However, treated effluents still contain a complex cocktail of pollutants whose environmental effects might go unnoticed, masked by additional stressors in the receiving waters or by spatiotemporal variability. We conducted a BACI (Before-After/Control-Impact) ecosystem manipulation experiment, where we diverted part of the effluent of a large tertiary WWTP into a small, unpolluted stream to assess the effects of a well-treated and highly diluted effluent on riverine diversity and food web dynamics. We sampled basal food resources, benthic invertebrates and fish to search for changes on the structure and energy transfer of the food web with the effluent. Although effluent toxicity was low, it reduced diversity, increased primary production and herbivory, and reduced energy fluxes associated to terrestrial inputs. Altogether, the effluent decreased total energy fluxes in stream food webs, showing that treated wastewater can lead to important ecosystem-level changes, affecting the structure and functioning of stream communities even at high dilution rates. The present study shows that current procedures to treat wastewater can still affect freshwater ecosystems and highlights the need for further efforts to treat polluted waters to conserve aquatic food webs.

## 1. Introduction

The implementation of wastewater treatment plants (WWTP) led by environmental regulations such as European Water Framework Directive (2000/60/EC) or the North American Clean Water Act has turned into significant improvements in water quality by reducing the amount of contaminants reaching aquatic ecosystems (Brion et al., 2015; Brauns et al., 2022). However, treated sewage still consists of a complex mixture of pollutants, nutrients, and pathogens (Pascual-Benito et al., 2020; Weitere et al., 2021). Some of these compounds are always toxic

(Vasilachi et al., 2021; Kurwadkar et al., 2022) and can have detrimental effects on biota, whereas others, like nutrients, can also subsidize biological activity but become hazardous above certain concentrations (Wang et al., 2019). The composition of the pollutants in effluents vary depending on the design and performance of WWTPs, which can include primary (physical) and secondary (biological) treatments, or even a more advanced tertiary treatment to reduce nutrients, organic matter and metals (Roccaro, 2018). In Europe for instance, 69% of the population is connected to a WWTP performing some kind of tertiary treatment, and another 13% to a WWTP with a secondary treatment (EEA,

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2021). These WWTPs have significantly contributed to the recovery of freshwater diversity in European rivers, but worryingly, this recovery rate has stopped during the last two decades (Haase et al. Accepted), suggesting the need for further improvements in the treatment of sewage. In addition to their composition, the effects of effluents on rivers strongly depend on dilution capacity (Büttner et al., 2022), which is globally being reduced due to climate change and human activities (Shi et al., 2019). Operating flaws or low discharges in receiving water bodies are not the only challenges that water depuration suffers from, potentially explaining the observed pause in the improvement of water quality. The effects of well-treated (i.e. tertiary treated) and highly diluted effluents, although weaker, could be significant, since even low pressures can result in clear effects on ecological responses if they are maintained in time (Hillebrand et al., 2020). These more subtle effects could remain unnoticed by common monitoring surveys, such as the ones comparing upstream and downstream reaches from WWTPs (Comber et al., 2022), as receiving water masses are often subject to other co-occurring stressors or because of difficulties to isolate the effects of interest from confounding factors inherent to spatiotemporal variability. This difficulty of properly estimating effects of WWTPs calls for carefully designed experimental studies, such as BACI (Before-After/Control-Impact) experiments that control spatiotemporal variations (Downes et al., 2002).

Treated wastewater effluents can trigger bottom-up trophic cascades by stimulating algal growth (Pereda et al., 2020), and affect the composition and structure of food webs by altering density and diversity of freshwater communities (González et al., 2023). Thus, as biodiversity has been positively linked to ecosystem functioning (Loreau et al., 2021) and ecological services (Isbell et al., 2011), effluents can ultimately influence ecosystem functions (Brauns et al., 2022). In this regard, food web studies provide a quantitative framework that combine community ecology with ecosystem ecology, merging both, species-centred approaches and energy flux-centred approaches (Thompson et al., 2012). In this line, a useful way to infer how food webs respond to stressors is to analyse the distribution of individual body sizes (i.e. community size-spectra), which is a reinterpretation of the trophic pyramids (Trebilco et al., 2013) and informs about how efficiently energy is transferred along the food web or the ecological pyramid (White et al., 2007; Collyer et al., 2023), and therefore about the capacity of the community to sustain species from higher trophic levels. This approach has been proposed as a tool for ecological assessment (Petchev and Belgrano, 2010) but has not been used to determine the effects of WWTP impacts. In this context, WWTP effluents are expected to have complex and entangled effects of community size-spectra. The nutrient input from WWTP effluents for instance, can increase trophic transfer efficiency by reducing the stoichiometric gap between consumers and resources (Mulder and Elser, 2009; Ott et al., 2014) increasing the slope of the community size spectra. Besides, toxic compounds can decrease the abundance of a specific size class (Baho et al., 2019) or increase the abundance of tolerant taxa (Peralta-Maraver et al., 2019). In addition, changes in composition, diversity and structure of communities can lead to changes in the amount of energy fluxes transferred along food webs. These alterations can also provide information about modifications in ecosystem functions such as herbivory, detritivory of carnivory (Barnes et al., 2018).

In the present study, we aim at addressing the effects of well treated and highly diluted effluent on communities and ecosystem functionalities. For that, a whole-ecosystem manipulation experiment following a BACI design was performed, where part of the effluent of a WWTP was diverted to a nearby, unpolluted stream to assess the response of the ecosystem to the effluent at different ecological scales. More precisely, we quantified (i) the response of biodiversity using classical metrics, (ii) community structure through the use of size spectra and (iii) ecosystem functions through the calculation of energy fluxes in food webs. Previous laboratory and field studies showed little toxicity of the effluent for invertebrates and microbes (Solagaistua et al., 2018; González et al.,

2023). Still, we expected the most sensitive taxa to decrease in abundance, inducing detrimental effects on diversity at different scales. We also predicted the effluent addition to promote energy flux through the green food web (i.e. biofilm-based), increasing herbivory and the dependency of the food web on biofilm. We finally predicted that enhanced nutrient inputs would promote the efficiency of the energy transfer along the food web, leading to shallower slopes of the size spectra.

## 2. Materials and methods

### 2.1. Study site and experimental design

The experiment was conducted in the Apraitz Stream (N-Iberian Peninsula, 43°13'41.1"N 2°23'56.3"W), a small unpolluted stream with a mean discharge of 0.12 m<sup>3</sup> s<sup>-1</sup> draining a 7 km<sup>2</sup> catchment over sandstone and shale. In the lowermost 300 m, its riverbed is dominated by bedrock and cobbles. The young riparian forest surrounding it is mainly composed of black alder (*Alnus glutinosa* (L.) Gaertn.), hazel (*Corylus avellana* L.) and ash (*Fraxinus excelsior* L.). This reach runs next to the WWTP of Apraitz which releases the treated sewage into the Deba River (mean ± SE discharge during our study 10.9 ± 0.7 m<sup>3</sup> s<sup>-1</sup>, <http://gipuzkoa.eus/>) through a regularly pulsed discharge (20–40 min every 2 h). The WWTP receives the sewage of approximately 90,000 population equivalents from urban and industrial areas, treating on average 29,904 m<sup>3</sup> of wastewater per day in sequential biological reactors (<https://www.accion-agua.com>). After mixing the sewage with activated sludge and subjecting it to aerobic and anaerobic processes to reduce the load of organic matter and nitrogen, it receives tertiary treatment (precipitation with ferric sulphate) to reduce phosphorus concentrations.

The experiment followed a BACI design (Fig. S1), which allows controlling both temporal and spatial variability to isolate the effect of interest (Downes et al., 2002). We diverted part of the WWTP effluent into the lowermost 150 m of the Apraitz Stream to get a final dilution rate similar to that of the effluent discharged into the Deba River (0.2–4% and 0.1–9% of range of effluent concentration, respectively; Pereda et al., 2020). We studied two 100-m long reaches: a Control reach, upstream from the effluent addition point and an Impact reach just below. Reaches were separated by a 20 m-long buffer reach that was composed by a man-made concrete flat surface that was able to reduce water depth to less than 5 cm and that ended in an overhung waterfall of about 50 cm making upstream migration of fish virtually impossible during low flow periods. Both reaches formed a continuum only on the strongest spates. For this study, one sampling was conducted at each reach before (September 2016) and after (October 2017) the start of the effluent addition (May 2017), both low-flow periods, entailing a low dilution of the effluent during the after period. The effluent release changed significantly water physico-chemical characteristics at the impact reach (Pereda et al., 2020): dissolved oxygen saturation and pH decreased by 10% but temperature remained unaffected. Nutrient concentrations significantly increased, especially ammonium and soluble reactive phosphorous (5.2 and 2.4 times respectively). However, other nutrients such as dissolved organic carbon or sulphate were not altered (Pereda et al., 2020). Yet, physico-chemical characteristics in the impact reach during the addition of the effluent were within the very good status category described in Mendiguren et al. (2021).

### 2.2. Sampling and sample processing

#### 2.2.1. Biofilm

Before the start of the experiment, we deployed artificial substrata (granite paving stones of 20 × 10 × 8 cm) along each reach (covering less than 0.5% of the streambed) to allow for biofilm colonization. According to Pereda et al. (2020), biofilm was formed by a similar amount of photoautotrophic and heterotrophic microorganisms, with a tendency to net autotrophy. In each sampling period and reach, we scrapped the whole surface of five randomly chosen paving stones and processed

them to obtain ash-free dry mass per surface unit ( $\text{g m}^{-2}$ ) (complete methodology in [Pereda et al., 2020](#)).

### 2.2.2. Benthic organic matter and macroinvertebrates

We collected nine benthic Surber samples (surface of  $0.09 \text{ m}^2$ , mesh of  $0.5 \text{ mm}$ ) randomly along each reach of the Apraitz Stream in each sampling period and reach. From each sample, the organic matter retained on an  $8\text{-mm}$  sieve was gathered, leaves were separated from the rest of the coarse detritus and both categories were separately processed to obtain their ash-free dry mass in each sample (see [Pereda et al., 2020](#)). Since consumption of wood and other recalcitrant materials by macroinvertebrates is negligible compared to leaves ([Diez et al., 2002](#)) we only retained leaves (hereafter coarse detritus). Before getting the ash-free dry mass of coarse detritus, a subsample was kept for the assessment of carbon and nitrogen concentrations. From these Surber samples, macroinvertebrates collected in a  $0.5\text{-mm}$  sieve were preserved in 96% ethanol. In the laboratory they were sorted, identified to the lowest possible taxonomic level following [Tachet et al., \(2010\)](#) (mostly to genus-level, except for some Diptera identified to subfamily level and Annelida to subclass level) and counted to obtain population densities. In addition, we measured the body length of up to 30 randomly selected individuals of each taxon in every sample (except for oligochaetes, planarians and leeches, which were not measured) with a binocular microscope (Leica M165FC, Wetzlar, Germany) equipped with a Leica DFC310FX camera using "Leica Application suite V4" software program. Total body length was considered as the distance from the anterior part of the head to the posterior part of the last abdominal segment excluding antennae and tails ([Meyer, 1989](#)). For gastropod molluscs, we measured the maximum length of the shell ([Meyer, 1989](#)), and for crustaceans of the genus *Echinogammarus*, the dorsal length of the first abdominal segment was measured to posteriorly obtain body length ([Flores et al., 2014](#)). Individual body mass (mg dry mass) was derived using published length-mass relationships ([Meyer, 1989](#); [Stoffels et al., 2003](#); [Larrañaga et al., 2009](#)).

### 2.2.3. Fish

We conducted fish samplings along the  $100\text{-m}$  long reaches by depletion electrofishing with a backpack-electrofishing unit with variable output current (MARTIN PESCADOR III, Alborlan S.L.). Stop-nets were set at the upstream and downstream ends of the reaches and up to three runs were made. All fish were anaesthetized with MS-222, identified, counted and weighed (to the nearest g). The study was approved by the Ethics Committee for Research Involving Animals at the University of the Basque Country (M20/2016/177) and was conducted in conformance with all applicable laws. We converted wet mass into ash-free dry mass through conversion factors published in [www.fishbase.se](http://www.fishbase.se).

### 2.2.4. Quality of basal food resources

Six samples of biofilm were collected in each reach by scraping the entire surface of randomly chosen cobbles and collecting the slurry in filtered river water ( $0.7 \mu\text{m}$  pore size, Whatman GF/F). Six fine detritus samples were randomly collected per reach and period using a sediment corer (surface  $81.7 \text{ cm}^2$ ). Biofilm and fine detritus samples were frozen ( $-20 \text{ }^\circ\text{C}$ ) and freeze-dried (VirTis Benchtop 2 K) (from 12 to 72 h depending on their water content). Each subsample of coarse detritus was oven dried ( $72 \text{ h}$ ,  $70 \text{ }^\circ\text{C}$ ). Freeze-dried and oven-dried material was grounded (Vibration mill MM301, Fisher Bioblock Scientific) and weighed (approximately  $10 \text{ mg}$  for fine detritus and  $2 \text{ mg}$  for the other basal resources) into tin capsules (Lüdiwiss Sn 98,  $5 \times 8 \text{ mm}$ ) for carbon and nitrogen analysis. Carbon and nitrogen analyses for biofilm and fine detritus were performed at the Helmholtz-Zentrum für Umweltforschung (Magdeburg, Germany) by a flash combustion on a Flash 2000 elemental analyzer (Thermo Fisher Scientific, Bremen, Germany) and for coarse detritus at the Stable Isotope Facility of the University of California – Davis on a PDZ Europa ANCA-GSL elemental analyzer

(Sercon Ltd., Cheshire, UK).

## 2.3. Data treatment

### 2.3.1. Invertebrate diversity

We assessed invertebrate taxa diversity at different scales through Hill numbers (i.e. number equivalent,  ${}^qD$  ([Jost, 2006](#))) with the entropart package for R ([Marcon and Hérault, 2015](#)). We used Hill numbers of order 0 ( ${}^0D$ , species richness, which is insensitive to the abundance of individuals of each taxon, highlighting the response of rare taxa), 1 ( ${}^1D$ , the exponential of Shannon's entropy, which weighs each taxon according to its log-transformed abundance), and 2 ( ${}^2D$ , inverse of Simpson concentration, which weighs each taxon according to its abundance, highlighting the response of dominant taxa) ([Jost, 2006](#)). We computed alpha-diversity per sample in each period and reach for the three Hill number orders and beta-diversity among samples (i.e. pairwise dissimilarity ([Socolar et al., 2016](#))) within each period and reach for orders 0 and 1 of diversity measures. Beta-diversity ( $D_\beta$ ) for the diversity orders ( $q$ ) 0 and 1 was transformed from beta entropy ( $H_\beta$ ) as described in [Marcon and Hérault \(2015\)](#):

$$qD_\beta = e^{\frac{q H_\beta}{1 - (q-1)H_\beta}} \quad (1)$$

### 2.3.2. Food webs, energy flux and dependency on basal resources

Following the number of replicates of Surber samples per period and reach we constructed 9 local food webs. Information about organic matter and invertebrates was obtained from the Surber samples. In the case of biofilm, we used the average biomass per paving-stone surface in each period and reach to estimate total biomass in each food web. Fine detritus was a scarce basal resource with a heterogeneous distribution along the reach and was not quantified during the sampling campaign, so it was equalled to the mean biofilm biomass values recorded. In the case of fish, assuming a homogeneous distribution of fish along the reach, we estimated the total biomass related to each Surber sample ( $0.09 \text{ m}^2$ ). For every invertebrate taxon in each sample, we calculated mean body mass from the individual masses measured and estimated the total biomass per node. We also estimated total metabolic rate for each invertebrate and fish nodes based on individual metabolic rate, calculated for each individual using an allometric equation derived from [Brown et al. \(2004\)](#):

$$X = \exp((a \cdot \ln(BM) + x_0) + E / kT) \quad (2)$$

where  $X$  is the metabolic rate (in watts, W),  $a$  is the allometric exponent ( $0.71$ ),  $BM$  is the body mass (g),  $E$  is the activation energy ( $0.63 \text{ eV}$ ),  $k$  is the Boltzmann's constant ( $8.62 \cdot 10^{-5} \text{ eV K}^{-1}$ ),  $T$  is the temperature (K) and  $x_0$  is a normalization factor ( $17.17$  for invertebrates and  $18.47$  for fish). All these parameters were extracted from [Brown et al. \(2004\)](#). We gathered mean daily temperature of the 190 days before the sampling date in each reach.

To estimate energy fluxes between nodes of local food webs, we used an adapted food-web energetics approach ([Barnes et al., 2018](#); [Gauzens et al., 2019](#)) by means of the "fluxweb" package ([Gauzens et al., 2019](#)). This approach uses allometric scaling laws to quantify metabolic rates ([Brown et al., 2004](#)). The model assumes a steady-state system, where the energetic losses of nodes in each food web, estimated by metabolic rate of consumer  $j$  ( $X_j$ ) and predation on consumer  $j$  by higher trophic levels ( $k$ ), need to be balanced by the energetic gains defined through resource consumption and assimilation ([Barnes et al., 2018](#)). The flux of energy  $F_{ij}$  from resource  $i$  to consumer  $j$  was calculated as:

$$\sum_i \mathcal{E}_{ij} F_{ij} = X_j + \sum_k W_{jk} F_k \quad (3)$$

where  $\mathcal{E}_{ij}$  is the efficiency in which consumer  $j$  assimilates the energy consumed from resource  $i$ . Energy fluxes to each consumer are defined as  $F_{ij} = W_{ij} F_j$ , where  $F_j$  is the sum of all the ingoing fluxes to consumer  $j$

and  $W_{ij}$  is the proportion of  $F_j$  obtained from resource/prey  $i$ , after scaling consumer preferences  $w_{ij}$  to the biomass ( $B$ ) of the different resources/preys as:

$$W_{ij} = \frac{w_{ij} B_i}{\sum_k w_{kj} B_k} \quad (4)$$

For that, an adjacency matrix with trophic links among all taxa present in our study and feeding preferences for each food resource was created based on the literature (Tachet et al., 2010; Gray et al., 2015) and our own gut content findings (see provided dataset in de Guzman et al., 2023). Based on feeding preferences three trophic groups were considered: primary consumers (feeding on basal food resources), omnivores (feeding on basal food resources and preys), carnivores (feeding on preys). For carnivore taxa we assumed that preferences were equally distributed amongst prey species. For omnivore invertebrates and primary consumers  $w$  values were given following preferences in Tachet et al. (2010), where traits related to consumed food are quantified using affinity scores between 0 and 5. For omnivores, affinity scores related to predation were equally distributed amongst prey species. For cannibalistic species, we set the preference for cannibalism to 0.01 in the adjacency matrix to minimize the amount of energy a consumer could ingest from its own biomass pool. Assimilation efficiencies ( $\mathcal{E}$ ) for the consumption of food resources were calculated deriving a formula from Lang et al. (2017):

$$\mathcal{E} = \frac{e^{\mathcal{E}' e^{E(T-T_0)/kTT_0}}}{1 + e^{\mathcal{E}' e^{E(T-T_0)/kTT_0}}} \quad (5)$$

where  $\mathcal{E}'$  is normalization constant for assimilation efficiency ( $-1.670$  for detritivory,  $0.179$  for herbivory and  $2.260$  for carnivory),  $E$  is the activation energy ( $0.164$  eV),  $k$  is the Boltzmann's constant and  $T$  is temperature (K) and  $T_0$  temperature normalized to  $20$  °C. Parameters were extracted from Lang et al. (2017).

We calculated whole-food web energy flux as the sum of energy fluxes within each local food web (each Surber sample). To calculate the consumption on biofilm, detritus and preys, we summed all the outgoing energy fluxes from each food resource. Thus, we quantified three consumption pathways: herbivory (consumption of biofilm), detritivory (consumption of coarse and fine detritus) and carnivory (consumption of animals) in the entire food web and within each trophic group (primary consumers, omnivores and carnivores) of each local food web. In addition, we calculated the dependency of each trophic group on basal food resources (biofilm and detritus) with the "NetIndices" package (Soetaert and Kones, 2014).

### 2.3.3. Size spectra

We constructed size spectra for the entire community (including both invertebrate and fish assemblages), and for primary consumers, omnivores and carnivores, separately. We used body mass of the measured (invertebrates) and weighed (fish) organisms. Since the log-transformed length values followed a normal distribution, we obtained body mass of the remaining non-measured invertebrates by means of the "truncnorm" package (Mersmann et al., 2018), based on the mean, standard deviation, minimum and maximum values of each taxon in each sample. We used animals with body mass higher than  $0.1$  mg to construct size spectra, since organisms with lower weights are undersampled as they are mostly washed through the  $0.5$  mm mesh aperture sieves (Gruenert et al., 2007). We divided the total range of body mass ( $\log_{10}$  body mass) values into 8 logarithmic bins of the same width and regressed density of organisms ( $\log_{10}$  density) against the centre of the bin (White et al., 2007).

### 2.3.4. Statistical analyses

We conducted linear models with the factors period (Before and After), reach (Control and Impact) and their interaction (to assess the effect of the effluent) as sources of variation (as well as the covariate

body mass for size spectra comparisons). This kind of BACI models do sometimes pose some challenges when graphically interpreting the results, as the stressor can dissipate instead of amplify initial differences between control and impact sites (see Fig. S2 in Supporting Information for an illustrative explanation). Coefficients for the interaction term point the direction of the effect of interest. Some variables were log-transformed to fulfil the requirements for linear models. We conducted all the analyses using R software, ver. 3.6.0. (R Core Team, 2019). For details regarding the code and collected data, see de Guzman et al. (2023).

## 3. Results

### 3.1. Stock and quality of basal resources

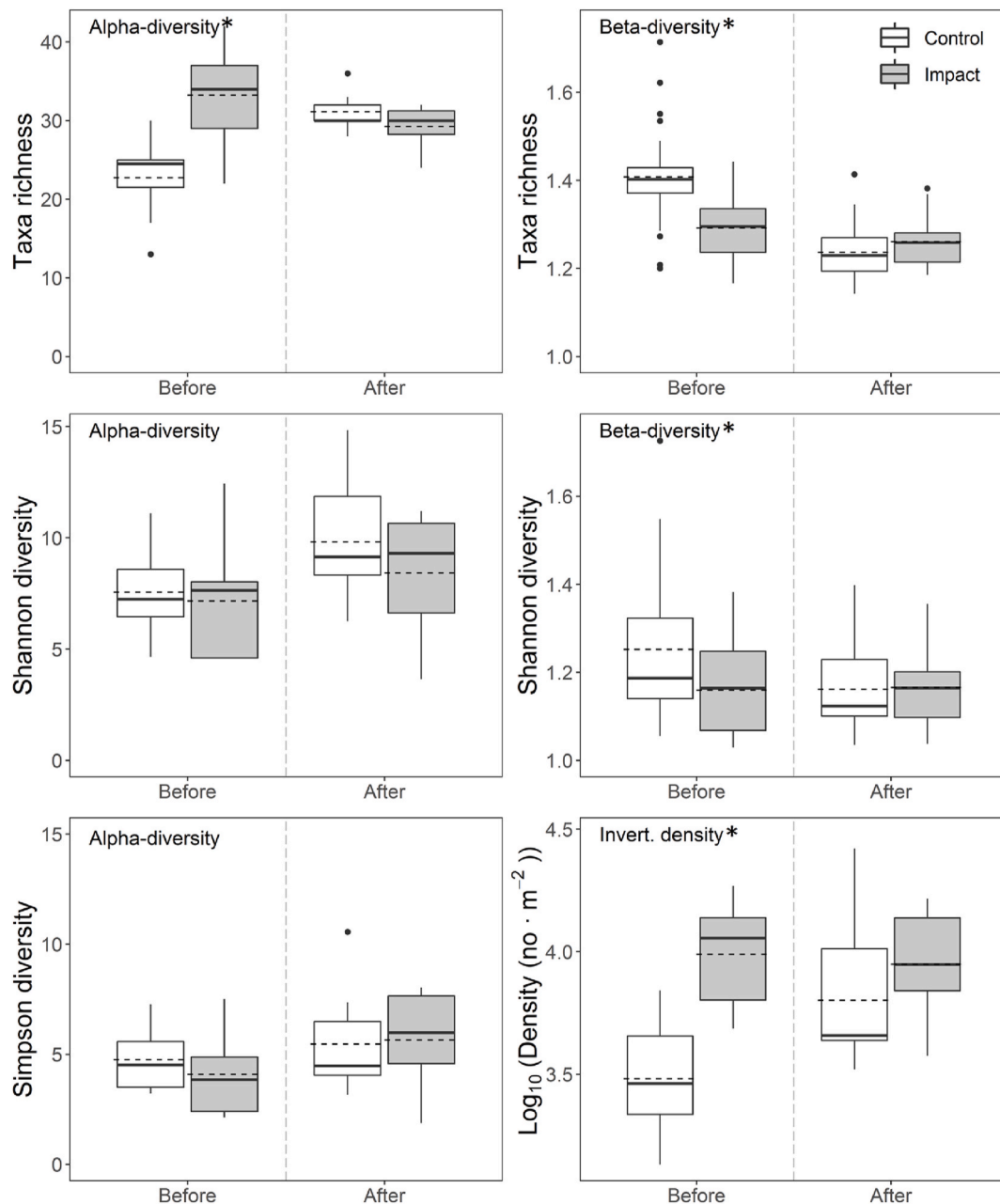
Coarse detritus stock tended to be higher in the impact reach ( $F_{1,31} = 3.90$ ,  $p = 0.057$ , Fig. S3, Table S1). Biofilm stock differed between periods ( $F_{1,16} = 5.56$ ,  $p = 0.031$ , Fig. S3), and exhibited a marginally significant increase as a consequence of the addition of the effluent ( $F_{1,16} = 3.84$ ,  $p = 0.068$ , Fig. S3, Table S1). In addition, carbon-to-nitrogen ratios (Table S2) significantly differed among basal food resources ( $F_{2,75} = 240.8$ ,  $p < 0.001$ ), with biofilm showing the lowest and coarse detritus the highest ratios, but the addition of the effluent affected none (Table S1).

### 3.2. Invertebrate and fish diversity and abundance

Alpha-diversity of macroinvertebrate taxa richness significantly differed between reaches (Fig. 1, Table 1) and with the addition of the effluent decreased in the impact reach respecting the control reach ( $F_{1,30} = 16.07$ ,  $p < 0.001$ , Fig. 1, Table 1). However, the effluent did not significantly affect Shannon and Simpson's diversity (Fig. 1, Table 1). Macroinvertebrate beta-diversity for taxa richness was initially higher in the control reach, but the difference disappeared with the addition of the effluent ( $F_{1,124} = 27.89$ ,  $p < 0.001$ , Fig. 1, Table 1). We observed a similar pattern for Shannon at beta-diversity scale: effluent addition led to less homogeneous communities ( $F_{1,124} = 5.73$ ,  $p = 0.018$ , Fig. 1, Table 1). Moreover, the effluent had a negative net impact on invertebrate density, as the initial differences found between the two experimental reaches decreased ( $F_{1,30} = 4.76$ ,  $p = 0.037$ , Fig. 1, Table 1). Regarding fish assemblages, four species were present during the study, and in both periods, more individuals and higher biomass were found in the impact reach, but overall, individuals at the control reach were bigger (Table S3).

### 3.3. Energy fluxes and dependency on basal food resources

Mean body mass, total biomass and metabolic rate of the three trophic groups did not change with the addition of the effluent (Fig. S4, Table S1). However, energy fluxes of the entire community differed between periods and reaches (Fig. 2, Table 1), and the addition of the effluent caused a negative net effect on them ( $F_{1,30} = 6.62$ ,  $p = 0.015$ , Fig. 2, Table 1). Moreover, some fluxes were also sensitive to the effluent. Herbivory increased with the addition of the effluent ( $F_{1,30} = 7.75$ ,  $p = 0.010$ , Fig. 2, Table 1), and in the after period differences in detritivory between reaches decreased ( $F_{1,30} = 10.23$ ,  $p = 0.003$ , Fig. 2, Table 1). However, although carnivory significantly varied between periods and reaches, it was unaffected by the addition of the effluent (Fig. 2, Table 1). Omnivores were responsible for the increase in herbivory during the addition of the effluent, as energy fluxes significantly increased within this trophic group ( $F_{1,30} = 5.47$ ,  $p = 0.026$ , Fig. S5, Table S1). On the other hand, the decreasing differences in detritivory between reaches after the addition of the effluent, were driven by the decrease in detritivory among primary consumers ( $F_{1,30} = 58.62$ ,  $p < 0.001$ , Fig. S5, Table S1). Carnivory was unaffected in any trophic group with the addition of the effluent (Fig. S5, Table S1). Overall, all trophic



**Fig. 1.** Diversity and density of macroinvertebrate assemblage: alpha- and beta-diversity for taxa richness and for Shannon diversity, alpha-diversity for Simpson diversity and density. The box plots show the median, the interquartile range and the tails of the distribution. Dots represent outliers and dashed lines mean values. Asterisks indicate the effect of the effluent (Period:Reach interaction) was significant.

groups depended on detritus more than on biofilm (Fig. S6). However, dependency of carnivores on biofilm significantly increased with the addition of the effluent ( $F_{1,30} = 7.66$ ,  $p = 0.010$ , Fig. S6; Table 1), while dependency on detritus decreased ( $F_{1,30} = 7.99$ ,  $p = 0.010$ , Fig. S6; Table 1), with no other group showing a variation on their dependency on biofilm or detritus.

### 3.4. Size spectra

The slope of the size spectrum of the entire community was unaffected by the addition of the effluent (Fig. S7, Table 2: Body mass:BA:CI, Fig. S8), as well as the slopes of the three trophic groups of consumers: primary consumers, omnivores and carnivores (Fig. S7, Table 2).

Nevertheless, the addition of the effluent significantly reduced the intercept of the primary consumers (Table 2: BA:CI; coefficient AI: 0.636).

## 4. Discussion

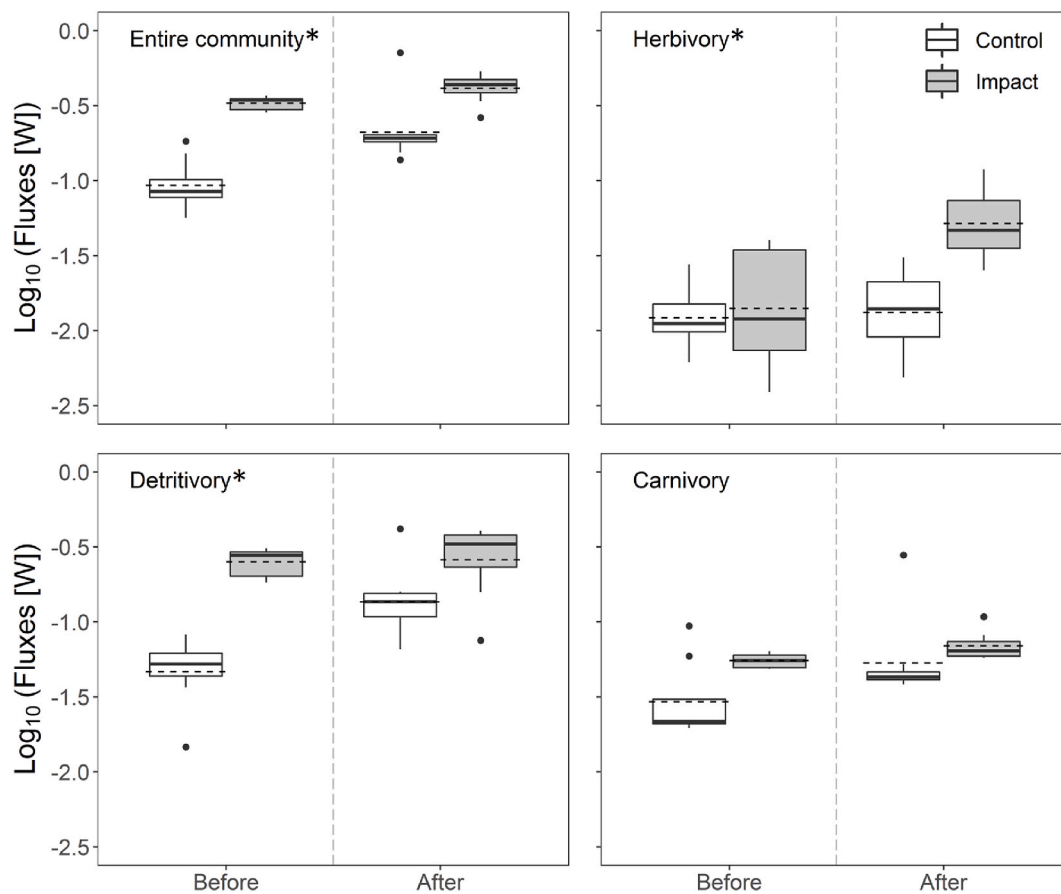
Effects of treated wastewater effluents depend on effluent composition, dilution and the characteristics of the receiving ecosystem. Our whole-ecosystem manipulation experiment shows that even a well-treated and highly diluted (mean dilution 3%, range 0.2–4%) effluent alters diversity and energy pathways on food webs.

**Table 1**

Linear model results comparing invertebrate density and diversity, energy fluxes throughout food webs and dependencies of trophic groups on basal resources between control (C) and impact (I) reaches before (B) and after (A) the start of effluent addition to assess its effects (BA:CI).

		BA			CI			BA:CI		
		F	p	Coeff.	F	p	Coeff.	F	p	Coeff.
<b>Invertebrate community descriptors</b>										
Taxa richness	Alpha	1.6	0.216		7.83	0.009	10.47	16.07	<0.001	-12.33
	Beta	52.21	<0.001	-0.17	11.76	0.001	-0.12	27.89	<0.001	0.14
Shannon diversity	Alpha	4.31	0.046	2.25	1.06	0.311		0.32	0.574	
	Beta	3.35	0.069		4.81	0.03	-0.09	5.73	0.018	0.1
Simpson diversity	Alpha	2.76	0.107		0.12	0.734		0.38	0.542	
Invertebrate density*		2.11	0.156		15.65	<0.001	0.51	0.51	0.037	-0.36
<b>Energy fluxes</b>										
Entire community*		16.46	<0.001	0.35	71.6	<0.001	0.55	6.62	0.015	-0.26
Herbivory*		8.66	0.006	0.04	11.62	0.002	0.06	7.65	0.010	0.53
Detritivory*		8.83	0.006	0.47	50.86	<0.001	0.73	10.23	0.003	-0.45
Carnivory*		6.2	0.019	0.26	8.44	0.007	0.28	1.45	0.238	
<b>Dependencies on resources</b>										
Primary consumers	Biofilm	0.29	0.591		0.36	0.555		2.36	0.135	
	Detritus	0.29	0.591		0.36	0.555		2.36	0.135	
Omnivores	Biofilm	0.45	0.507		0.23	0.633		3.2	0.084	
	Detritus	0.45	0.507		0.23	0.633		3.2	0.084	
Carnivores	Biofilm	0.32	0.579		0.61	0.440		7.66	0.01	0.21
	Detritus	0.49	0.490		0.34	0.566		7.99	0.01	-0.23

Footnote: Asterisks indicate log<sub>10</sub> transformed variables. Coefficients are shown for significant responses ( $p < 0.05$ ) and consider B and C as reference in all cases. In dependencies on basal resources  $F$  and  $p$  values are identical within each trophic group because the sum of dependencies on biofilm and detritus equals 1.



**Fig. 2.** Energy fluxes of stream food webs and functions: fluxes throughout the entire food web and herbivory, detritivory and carnivory related fluxes. The box plots show the median, the interquartile range and the tails of the distribution. Dots represent outliers and dashed lines mean values. Asterisks indicate the effect of the effluent (Period:Reach interaction) was significant.

**Table 2**

Linear model results comparing size spectra between control (C) and impact (I) reaches before (B) and after (A) the start of effluent addition to assess its effects (BA:CI) and considering body mass.

	Entire community			Primary consumers			Omnivores			Carnivores		
	F	p	Coeff.	F	p	Coeff.	F	p	Coeff.	F	p	Coeff.
Body mass	304.20	<0.001	-0.95	164.55	<0.001	-0.78	245.34	<0.001	-0.79	66.70	<0.001	-0.28
BA	6.03	0.022	0.58	11.58	0.002	0.54	13.04	0.001	0.19	13.34	0.001	0.45
CI	0.02	0.888		0.13	0.722		21.15	<0.001	0.39	0.00	0.954	
Body mass:BA	0.16	0.695		6.00	0.022	0.13	0.86	0.364		0.00	0.968	
Body mass:CI	0.66	0.423		1.71	0.203		3.99	0.057	-0.27	0.87	0.359	
BA:CI	0.89	0.356		10.48	0.004	-0.64	1.54	0.227		0.91	0.350	
Body mass:BA:CI	0.04	0.844		2.07	0.163		0.13	0.720		0.10	0.757	

Footnote: Coefficients are shown for significant responses ( $p < 0.05$ ) and consider B and C as reference in all cases.

#### 4.1. The effluent promoted biofilm biomass

Although the effect of the effluent addition on biofilm biomass was marginally significant for the sampling campaigns in which food web energy fluxes and size spectra were quantified, Pereda et al. (2020) reported a highly significant increase when considering samples collected every second month during the year the effluent was poured in the same experimental setup. Biofilm growth is known to increase under enhanced nutrient concentration (Dodds and Smith, 2016) and under sewage inputs (Pereda et al., 2021). Many studies have also reported that increased concentrations of dissolved nutrients promote litter quality (Cross et al., 2003) and decomposition (Ferreira et al., 2014). Pereda et al. (2020) also observed an increase in detritus decomposition in the same experimental setup, although we did not observe changes in the quality of detritus in the present study. Surprisingly, in presence of effluent, we did not observe alterations in the carbon-to-nitrogen ratio of biofilm either, which contrasted with other studies addressing effects of nutrient enrichment even downstream from effluent discharge points (Scott et al., 2008).

#### 4.2. Effects on diversity were scale-dependent

The effluent reduced invertebrate richness and density, but Shannon and Simpson at alpha-diversity scale were unaffected. Studies addressing effects of treated wastewater effluents have reported increased invertebrate abundance and decreased diversity as a consequence of the loss of the most sensitive taxa and the stimulation of the resistant ones (Mor et al., 2019; Hamdhani et al., 2020; Weitere et al., 2021), indicating that these effluents act as environmental filters that select pollution tolerant taxa. In this regard, González et al. (2023) also observed a sensitivity dependant response of the abundance, i.e. the most sensitive species reduced their abundance and the most tolerant ones increased it. In the current study, the highly diluted effluent did still negatively impact taxa richness at alpha-diversity scale. Contrarily, we observed that the effluent significantly increased beta-diversity, indicating increased spatial turnover across local communities. Mor et al. (2019) also reported an increase in beta-diversity between samples obtained in reaches downstream from WWTP effluents and attributed it to increases in local habitat heterogeneity, to opportunistic responses of biota to increased productivity or to basal environmental differences between sites. In our study, we could associate the increase in beta-diversity to the stronger role of stochastic assembly events caused by the increase in productivity (Chase, 2010), which promotes the opportunistic responses of some species (Langenheder et al., 2012), and to the uneven elimination of some sensitive species to pollutants (Trubina and Vorobeichik, 2012). Treated wastewater effluents have been reported to reduce the abundance and biomass of fish (Weitere et al., 2021) as well as fish diversity (Hamdhani et al., 2020). In contrast, in our study the effluent had no clear effects on fish diversity and densities, likely due to the high dilution rate. Overall, more and smaller individuals were found in the impact reach, but the effluent did not affect abundance nor body mass.

#### 4.3. Herbivory was stimulated, but total energy flux reduced

We expected increased biofilm to promote the energy flux along the green food web, as it is of higher nutritional quality than detritus (Cross et al., 2005) and can sustain a high production of primary consumers (McCutchan and Lewis, 2002). Several studies reported that nutrient addition increased secondary production as it increased the quality of basal resources (Demi et al., 2018; Eckert et al., 2020). We observed no increment in mean body mass, total biomass or estimated metabolic rate for any trophic group. However, we observed a net decrease in the total energy flux of the food web and a change in the amount of energy provided to consumers through different pathways, with increased herbivory and reduced detritivory. A similar shift from brown to green food-web pathways was reported through the increase of herbivore abundance in a nutrient enrichment experiment where detritus was also the most abundant basal resource (Bumpers et al., 2017). In our experiment the energy fluxes towards omnivores and primary consumers shifted, although none of these groups increased in biomass or abundance. Omnivores, able to consume detritus, biofilm and preys, increased the consumption of high-quality biofilm, whereas primary consumers reduced the consumption of low-quality detritus. Carnivory did not increase, but carnivores became more dependent on biofilm through the higher consumption of herbivores, which shows that the effects of the effluent reached the top of the food web.

#### 4.4. Community size spectra were unaffected

Water nutrient enrichment can increase the quality (i.e. reduce Carbon:nutrient ratio) of autotrophs and detritus (Evans-White and Halvorson, 2017) narrowing the stoichiometric gap between basal resources and primary consumers and increasing the efficiency of trophic transfer (Mulder and Elser, 2009). We did not see a shift in carbon-to-nitrogen ratio of basal resources, although we observed an increase of the relative abundance of biofilm over detritus, which enhanced the total availability of nutrients for consumers. Therefore, we expected a shallower slope in the body mass-abundance size spectra (Mulder and Elser, 2009; Ott et al., 2014), as nutrient subsidy favours organisms with higher per capita metabolic requirements (Cross et al., 2003) and increases the capacity of the system to support larger individuals (Juvigny-Khenafou et al., 2021). However, the effluent did not change the slope of size spectrum. This finding could suggest that the nutrient enrichment was not high enough to trigger a change in energy transfer efficiency. Additionally, toxicity of the effluent might not have been strong enough to modify the slope of the size spectra either, following other studies reporting size specific responses to contaminants (Gergs et al., 2015; Taddei et al., 2021). The only sign of the effect of the toxicity of the effluent on size spectra was the lower intercept for primary consumers, which is in line of the subtle effects of treated and diluted effluent on ecophysiology of consumers (Solagaistua et al., 2018) and ecosystem functioning (Pereda et al., 2020).

## 5. Conclusion

Our whole-ecosystem manipulative experiment shows that even well treated and highly diluted WWTP effluents can still modify the structure and functioning of stream communities. The reduction of alpha- and the increase of beta-diversity show that the effluent reduced the number of species, but increased the dissimilarity among the different patches of the riverbed. The overall size structure of the community did not change, energy fluxes through herbivory increased and those through detritivory decreased, but a negative effect on the total flux along the entire community was triggered. Our experiment underlines that current procedures to treat contaminated waters might not be enough to preserve natural properties of food webs and that further efforts might be needed when dealing with sensitive or highly valuable aquatic ecosystems.

## Author statement

AE, AL, DvS and JMG designed the study. IdG, AE, AL, DvS, JMG, LEP, AA and NO contributed to collecting the data. IdG and LEP conducted laboratory work. IdG, AL, JMM, BG and UB discussed and analysed data. IdG, AL and JMM drafted the manuscript and all the authors provided feedback, contributed to the writing and gave final approval for publication.

## Open research statement

The original data that supports the findings of this study and the code are openly available in Figshare (<https://doi.org/10.6084/m9.figshare.21185224.v1>) and GitHub.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

The original data that supports the findings of this study and the code are openly available in Figshare (<https://doi.org/10.6084/m9.figshare.21185224.v1>) and GitHub

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.jenvman.2023.118510>.

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