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Tertiary wastewater treatment combined with high dilution rates fails to eliminate impacts on receiving stream invertebrate assemblages

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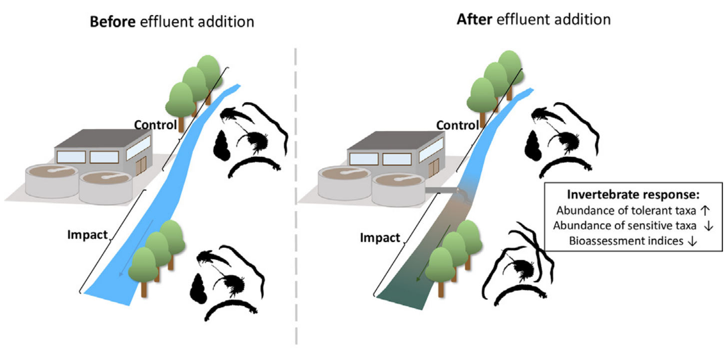
HIGHLIGHTS

- This field experiment tests the effects of a tertiary wastewater treatment plant.
- The plant altered taxonomic composition of the stream invertebrate assemblages.
- Sensitivity to environmental degradation explained change in taxa abundances.
- Current wastewater treatment does not avoid impacts on receiving streams.

GRAPHICAL ABSTRACT



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ABSTRACT

The amount of wastewater processed in treatment plants is increasing following more strict environmental regulations. Treatment facilities are implementing upgrades to abate the concentrations of nutrients and contaminants and, thus, reduce their effects on receiving systems. Although many studies characterized the chemical composition and ecotoxicological effects of treated wastewater, its environmental effects are still poorly known, as receiving water bodies are often subjected to other stressors. We performed a field manipulative experiment to measure the response of invertebrate assemblages to one year of tertiary-treated wastewater discharges. We poured treated wastewater from an urban wastewater treatment plant into the lower-most 100-m of a previously unpolluted stream (3.6 % daily flow on average) while using another upstream reach as control. The positive correlation between effect sizes of abundance changes and IBMWP scores suggested assemblage modifications were following taxa tolerance to ecological impairment. The treatment increased the temporal variability of $SPEAR_{organic}$, EPT relative abundance, and invertebrate functional redundancy. Our results show that even in this best-case scenario of tertiary-treated and highly diluted wastewater, the abundance of the most sensitive taxa in the aquatic assemblages is reduced. Further improvements in wastewater treatments seem necessary to ensure these effluents do not modify receiving water ecosystems.

1. Introduction

Global human population is expected to add over 2 billion people between 2019 and 2050 (United Nations, Department of Economic and Social Affairs, Population Division, 2019). This growth, combined with increasing per capita resource consumption, is accelerating waste production (Wen et al., 2017). Because urban populations are growing even faster than the global population, total production of urban wastewater is expected to increase even more sharply, booming from the current 380 km^3/year to 574 km^3/year in 2050 (Qadir et al., 2020).

In the past, urban wastewater was released untreated into nearby ecosystems, polluting them with a mixture of water, salts, metals, organic matter, and nutrients, as well as pathogens. The Industrial Revolution added

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pesticides and emergent pollutants such as pharmaceuticals and their metabolites, personal care products, and industrial chemicals, to wastewaters which increased the impact on receiving ecosystems (Petrie et al., 2015). Today, although half of global wastewater is still released untreated (Jones et al., 2021), environmental regulations such as European Water Framework Directive (2000/60/EC), North American Clean Water Act or Water Law of the People's Republic of China, are encouraging the implementation of wastewater treatment plants (WWTPs), especially in developed countries (Wen et al., 2017), as a mean to achieve some of the Sustainable Development Goals, such as no. 6, “Clean water and sanitation”, and no. 11, “Sustainable cities and communities” (United Nations, 2015). Therefore, an increasingly large fraction of aquatic ecosystems in the world receive wastewater treated in WWTPs.

WWTPs differ greatly in design and performance: the common WWTP includes primary (physical) and secondary (biological) treatments, whereas more advanced plants also include tertiary treatments aiming at further improving water quality, for instance, by reducing nutrient, organics and metal concentrations (Roccaro, 2018). However, wastewater treated in tertiary WWTPs still holds many biologically active substances (Rout et al., 2021), which may affect the receiving aquatic systems even at low concentrations (Kienle et al., 2019). Testing the environmental effects of these effluents in the real world is often difficult because they can be blurred by other impacts such as diffuse pollution or hydromorphological alterations, which also affect the receiving ecosystems (Burdon et al., 2016).

We experimentally assessed the ecological effects of treated wastewater on a previously unpolluted stream in the best-case scenario, that is, by releasing wastewater processed in a modern WWTP with advanced tertiary treatment at a high dilution rate, with treated wastewater accounting for only 3.6 % of mean streamflow. We are aware of only two field assessments of WWTP impacts on invertebrate assemblages in reaches that received WWTP effluents at smaller or similar concentrations than here,1 % in both the Saale River (Spänhoff et al., 2007) and the Demntzer Mill Brook (Gücker et al., 2006), and 4.7 % in the 2007 sampling campaign of the Vistre River (Arce et al., 2014). To our knowledge, in all the other field studies dealing with WWTP effects on invertebrate assemblages (Gücker et al., 2011; Prat et al., 2013; Arce et al., 2014; Poulton et al., 2015; Burdon et al., 2016; Johnson et al., 2019), wastewater was discharged at greater concentrations than here (average: 81.8 % of mean flow, range: 15–274 %).

As treated effluents in most modern WWTPs, the wastewater used in our experiment contained nutrients and emerging pollutants (e. g., dissolved inorganic nitrogen concentration 10.59 mg/L, Valsartan concentration of 26.9 μg/L, Solagaistua et al., 2018). One year of experimental addition of treated wastewater resulted in increased biofilm biomass (2.1 ×), chlorophyll-a (2.3 ×) and activity (phosphatase: 2.2 ×, glucosidase: 4.1 ×), and litter decomposition (1.4 ×), but no changes in biofilm gross primary production or community respiration (Pereda et al., 2020). Those responses of biofilm and ecosystem processes to treated wastewater addition were linked to of nutrient enrichment (Pereda et al., 2020).

Here, we quantify the effects of the treated wastewater on invertebrate assemblages, a group of organisms that play a key role in river ecosystems and show large variation in their sensitivities to anthropogenic stressors (Wallace and Webster, 1996; Sumudumali and Jayawardana, 2021). As Juvigny-Khenafou et al. (2021) recommended, we combine taxonomical and functional diversity assessments to get a better understanding of multiple-stressor effects. Therefore, we measured functional diversity using the biological traits maximum body size, respiration, diet, and number of generations per year (Table 1).

Although the treated effluent accounted for <5 % of mean streamflow, we expected discharge of treated wastewater to affect invertebrate taxonomical and functional diversity and bioassessment metrics, as a consequence of differences in their ability to take advantage of nutrient enrichment and/or their sensitivity to pollutants (Dolédec et al., 2006; Hamdhani et al., 2020). Specifically, we firstly expected the abundance of pollution-resistant taxa to increase as they would not be filtered out by the conditions created by our experiment, but they would instead benefit from nutrient enrichment (Pereda et al., 2020). Secondly, we anticipated treated wastewater to reduce the abundance of sensitive taxa and, hence, also bioassessment indices and taxonomic diversity. Functional diversity would also decrease as wastewater filters out some trait combinations. Finally, because of temporal variation in the dilution of the treated wastewater discharged to the stream, we anticipated the experiment to increase seasonal variability in invertebrate assemblages.

Table 1
Biological traits used to measure functional diversity and rationale to expect changes caused by treated wastewater discharges.

Biological trait	Rationale	Sources
Maximum body size	Increased nutrient inputs may benefit bigger invertebrates because their higher energy demands. Moreover, their smaller surface/volume ratio decreases their exposure to pollutants.	Brown et al. (2004); Wiberg-Larsen et al. (2016)
Respiration mode	Air-breathers do not experience oxygen limitation associated to eutrophication. Furthermore, they are less exposed to pollutants than invertebrates obtaining oxygen from the water.	Arce et al. (2014); Collins and Fahrig (2020)
Diet	Greater biofilm biomass found in the experiment can benefit microphyte-eating invertebrates. Nutrient enrichment may also benefit coarse detritus-eating fauna because of increased detritus food value.	Greenwood et al. (2007)
Number of generations per year	Producing more than one generation per year increases population resilience.	Liess and Beketov (2011)

2. Methods

2.1. Study site

This research was performed in the lowermost section of the Apraitz Stream (43°13′45″ N, 2°23′52″ W), a perennial tributary of the Deba River, which flows into the Bay of Biscay, southwestern Europe. It drains a 7-km^2 catchment of sandstone and shale covered mainly by pastures and conifer plantations, and with only 16 very sparsely distributed residences. It is close to the northern coast of Iberian Peninsula, at 80 m of altitude in Gipuzkoa, Basque Country, a region with temperate, humid climate. Mean annual temperature is 12.3 °C and mean annual precipitation 1565 mm (http://agroclimap.aemet.es). The flow regime of the Apraitz Stream includes frequent spates at any time of the year, with lowest flows in summer (Fig. 1). The studied section has steep banks covered by a narrow, dense, and deciduous riparian forest dominated by alder, *Alnus glutinosa* (L.) Gaertn., hazel, *Corylus avellana* L. and ash, *Fraxinus excelsior* L. The stream channel is narrow, with a succession of riffles and pools, and the bed is dominated by bedrock and cobbles.

Near the confluence of the Apraitz Stream with the Deba River, the Apraitz WWTP performs a tertiary treatment of urban-industrial wastewater equivalent to over 90,000 inhabitants. It is a biological sequential reactor that releases treated water in 20–40 min pulses every 2 h to the Deba River near the confluence with the Apraitz. The characteristics of the Apraitz WWTP and its outflow were fully described in Pereda et al. (2020).

2.2. Experimental design

We performed an experiment following the before-after-control-impact series (BACI) design (Downes et al., 2002). We diverted part of the WWTP treated wastewater to Apraitz Stream by means of a pipe, in such a way that the diverted effluent had a dilution rate in the receiving stream similar to that of the full effluent in the Deba River. We characterized the invertebrate assemblages in two 100 m-long reaches, a control reach upstream from the wastewater addition site and an impact reach downstream from it, six times during the year before starting discharges from the WWTP and six more during the following year.

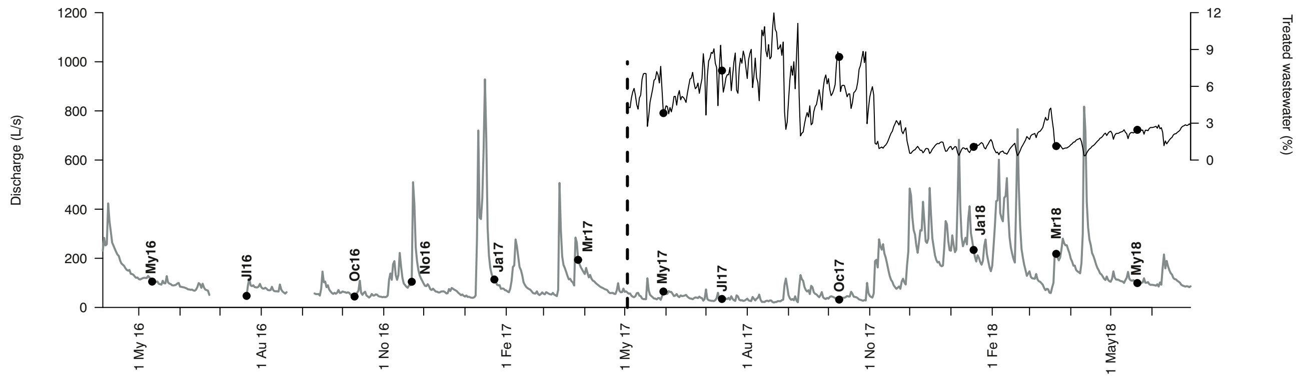

Fig. 1. Discharge of the Apraitz Stream during the study period (grey line) and percent contribution of treated wastewater to discharge in the impact reach (black). Circles mark when the benthos samples were taken. The vertical dashed line marks the start of treated water addition.

2.3. Treated wastewater volume and characteristics

From 3 May 2017 to 30 June 2018, 10 L/s of treated wastewater was released for 20–40 min every 2 h to the impact reach, accounting for 0.3–12 % (mean 3.6 %) of the Apraitz Stream daily water flow. Treated wastewater had 3 × conductivity, 0.5 × oxygen saturation, and 9.3 × total dissolved nitrogen, and 55 × soluble reactive phosphorus concentration of the stream water. It also contained some emerging pollutants, especially renin-angiotensine II receptor blockers, caffeine, and sugar substitutes (Pereda et al., 2020). Four of the 41 organic compounds that we analyzed in the treated water discharged to the stream were herbicides, but only diuron was detected and it had mean concentration in stream water during treated wastewater releases of 0.099 μg/L (Solagaistua et al., 2018). During the releases of treated wastewater to the stream, Pereda et al. (2020) found that conductivity and the concentrations of NH_4^+, dissolved inorganic nitrogen and soluble reactive phosphorus were higher in the impact reach (mean, 427 μS/cm, 0.2 mg N/L, 1.9 mg N/L, and 0.2 mg P/L, respectively) than at the control reach (mean, 289 μS/cm, 0.01 mg N/L, 0.7 mg N/L, and 0.02 mg P/L, respectively), whereas dissolved oxygen saturation and pH decreased (mean at the impact reach: 92 % and 7.1; mean at the control reach: 100 % and 7.7). During no-release periods, mean NH_4^+ concentration at the impact reach remained 4 times higher than the values found at the control reach (mean values 0.036 and 0.009 mg N/L, respectively), but concentration of other chemicals fell to basal levels. Water temperature at the impact reach was not affected by the treated wastewater.

2.4. Invertebrate assemblage characterization

We took nine benthos samples per reach and sampling occasion with a Surber net (0.09 m^2, 500 μm) and stored them in ethanol before sorting, identifying, and counting the fauna. We identified the animals to genus level, except Diptera which we identified to family, and Hydracarina and Oligochaeta, which were left as such. One benthos sample taken in the impact reach in October 2017 was lost. Another sample taken in the control reach in October 2016 with only 11 animals and three taxa was repeatedly identified as an outlier in data analyses. Therefore, we excluded it from all the analyses and figures.

For each sample, we calculated invertebrate and EPT (Ephemeroptera, Plecoptera and Trichoptera) abundance, and used the vegan package (Oksanen et al., 2020) to calculate ^qD Hill numbers for q = 0, 1, and 2 (Jost, 2006) as measures of taxonomical and EPT richness (^0D, i.e., number of taxa present) and diversity (^1D and ^2D, being ^2D more sensitive to the relative abundances of taxa than ^1D, Jost, 2006). To calculate taxonomical richness at reach scale, we grouped all samples taken at the same sampling occasion and reach to obtain 24 measures (12 sampling occasions x 2 reaches).

We determined for each benthos sample the IBMWP index (Alba-Tercedor et al., 2004), a modification of the Biological Monitoring Working Party to Iberian rivers. It detects anthropic impacts by summing a score (1–10, quantifying sensitivity to organic pollution) per each family found. It is therefore a qualitative index that produces higher values (i.e., better ecological status) for assemblages composed by more invertebrate families with higher scores irrespective of their abundances. Although the scores depend mainly on sensitivity to organic pollution, values of the IBMWP index are also negatively correlated with other stressors such as nutrients, acidification, and organic toxicants (Couto-Mendoza et al., 2015). We also calculated SPEAR$_{organic}$ (Beketov and Liess, 2008), which addresses the sensitivity of invertebrate assemblages specifically to organic chemicals. SPEAR$_{organic}$ classifies taxa as vulnerable to organic toxicants when their physiological sensitivity to them is greater than −0.36, where 0 is the sensitivity shown by *Daphnia magna* (von der Ohe and Liess, 2004). To compute the value of the index we use the following equation:

$$\mathrm{SPEAR}_{\mathrm{organic}} = \frac{\sum_{i=1}^{S} \log(4x_i + 1) \times y_i}{\sum_{i=1}^{S} \log(4x_i + 1)}$$

where S is taxon richness, and x_i is the abundance of taxon “i”, y_i was set to 1 or to 0 accordingly only to taxon “i” sensitivity to organic toxicants retrieved from the database available with the SPEAR$_{pesticide}$ (Version 2021.02) software https://www.systemecology.de/indicate/). We did not use information on generation time, dependence on refuge areas, and exposure potential during spring-summer of the studied taxa because these characteristics will hardly increase invertebrate resistance or resilience in our experiment, in which the treated wastewater is discharged 12 times a day in a 100 m-long reach of a small stream. SPEAR$_{organic}$ increases with the proportion of invertebrates sensitive to organic pollutants.

We evaluated functional redundancy (Pillar et al., 2013), and three components of functional diversity, i.e., functional richness, evenness, and divergence (Villéger et al., 2008) in the functional space defined by the biological traits of the groups maximum size, respiration, diet, and number of generations per year, as classified in the Tachet et al. (2010) database. Functional redundancy measures diversity of species bearing similar biological traits, and it may improve resilience of ecosystem processes (Pillar et al., 2013). Functional diversity describes the distribution of the species and the abundance of a community in the niche space defined by the traits and informs on ecosystem processes as resource use (Mason et al., 2005). We computed functional redundancy as the difference between the taxonomical Gini-Simpson index and the Rao index computed using the four trait groups (Pillar et al., 2013; Juvigny-Khenafou et al., 2021). We calculated functional richness as the volume of the functional space occupied by the assemblages, functional evenness as the regularity of the abundance in that functional space, and functional divergence as taxa deviance from the centroid of the functional space (Villéger et al., 2008). These four functional indices were calculated using the FD package (Laliberté et al., 2014) in R.4.0.4 (R Core Team, 2021). We used the Tachet et al. (2010) database to determine the affinity of each taxon for each trait.

2.5. Effect sizes and coefficients of variation

We calculated the effect sizes of the treated wastewater addition on the estimated parameters using the mean values at each period x reach combination as:

$$\frac{Impact_{after}/Control_{after}}{Impact_{before}/Control_{before}}$$

If treated wastewater discharge increases a given metric, the effect size will take a value >1 that will inform on the magnitude of such increase. In the same way, if the experiment reduces the value of any metric, the effect size will be smaller than 1 and its value will inform on the magnitude of such reduction. To calculate the confidence intervals of the effect sizes, we bootstrapped 10,000 times each set of period x reach combinations obtaining a population of 10,000 replicates of effect size and extracted from it the 2.5 and 97.5 percentiles. We assessed the relationships between the effect sizes of the experiment on abundances of taxa with total numbers >1000 individuals, and (a) their IBMWP scores and (b) sensitivity to organic toxicants constructing general linear models (GLMs).

To compare stability before and after the start of treated wastewater inputs, we calculated coefficients of variation of all the above parameters in these two periods. Then, we computed the effects sizes and confidence intervals for the coefficients of variation as shown above.

2.6. Statistical modeling of response variables

We used generalized linear mixed models (GLMMs), constructed with the package glmmTMB (Brooks et al., 2017) to assess the effects of the treated wastewater addition on total invertebrate, EPT, and dominant taxa (those accounting for >5000 animals) abundances. We also used GLMMs to analyse the effects on taxonomic diversity, IBMWP index, $SPEAR_{organic}$, EPT proportion and functional richness, evenness, divergence, and redundancy. These GLMMs included period (before and after intervention), reach (control and impact) and their interaction as fixed factors, and both sampling occasion and sampling occasion x reach as random factors to account for no independence of data taken at the same reach and occasion. Using GLMMs in these contexts avoids problems with the estimation and interpretation of parameters associated to the Stewart-Oaten et al. (1986) method (McDonald et al., 2000). Because we did not find that our intervention altered trends in the response variables, we used the BACI contrast, that is, the period x reach interaction, as an indication of the effect of the treated wastewater inputs. In the GLMMs we used gaussian distributions for taxonomic diversity, $SPEAR_{organic}$, functional richness, evenness, and divergence. We used negative binomial distributions for IBMWP index, total invertebrate, EPT and dominant taxa abundances, beta distributions for EPT proportion and functional redundancy, and generalized Poisson distributions for taxonomic and EPT richness. We also plotted the scaled residuals of the GLMs and the GLMMs to compare them with their predicted values to assess if the models were reliable using the package DHARMa (Hartig, 2021).

As we tested effects of treated wastewater on 23 variables, we used False Discovery Rates (Benjamini and Hochberg, 1995) to correct *p*-values. Therefore, p-values larger than 0.015 (see Appendix A) were considered too big to truly indicate an effect.

Composition of the invertebrate assemblages was represented using nonmetric multidimensional scaling (NMDS). To construct the plots, we calculated a Bray-Curtis dissimilarity matrix between the invertebrate abundances found at each sample. We decided to perform a two-dimensional NMDS ordination after examining a scree plot of stress against a number of dimensions, and a Shephard plot of distance between points in the biplot against their dissimilarity. We tested changes in the taxonomical composition with a Permanova, constraining the 9999 permutations within samples taken at each sampling occasion. NMDS and Permanova were performed using the package vegan 2.5–6 (Oksanen et al., 2020).

3. Results

3.1. Nutrient flow

Treated wastewater supplied 2.73 mg/s of soluble reactive phosphorus to the stream water which equals 101.6 % of soluble reactive phosphorus influx from the control reach at the same period (2.68 mg/s). The experiment also provided 20.3 mg/s of dissolved inorganic nitrogen, and 25.3 mg/s of total dissolved nitrogen to the impact reach, accounting for respectively, 21.6 and 17.1 % the inflows from the control reach (93.9 mg/s of dissolved inorganic nitrogen, and 147.5 mg/s of total dissolved nitrogen).

3.2. Invertebrate abundance

In the 215 benthos samples we found 147,855 animals belonging to 101 taxa. Five dominant invertebrate taxa, Chironomidae, Oligochaeta, *Echinogammarus*, *Potamopyrgus* and *Baetis*, together accounted for 73 % of the total abundance (Table 2), whereas the 17 taxa with total abundances >1000 animals were 93 % of all the fauna found.

Inputs of treated wastewater increased the abundance of one dominant taxon, Oligochaeta (effect size of 3.39, Table 2), immediately after the start of the treated wastewater inputs (Fig. 2). In contrast, *Potamopyrgus* and *Baetis* abundance dropped, with effect sizes of 0.24 and 0.36, respectively (Table 2, Fig. 2). However, treated wastewater did not alter the abundance of the two other dominant taxa, Chironomidae and *Echinogammarus* (Table 2, Fig. 2).

3.3. Composition and diversity of the assemblages

The input of treated wastewater impacted the taxonomic composition of the assemblages ($p < 0.001$ for the BACI contrast, Permanova). When we represented it with a two-dimensional NMDS (stress = 0.156, Fig. 3), we found ample variation during the study period. In most sampling

Table 2
Responses of invertebrate abundances to the input of treated wastewater. BACI coefficients consider B and C as references. Effect sizes with their confidence intervals (CI), results of the mixed models concerning the BACI interactions of the GLMMS, and relative abundances of the dominant taxa are also shown. SE = standard error, z = z statistic, p = *p*-value. Those *p*-values smaller than 0.015 (the limit for maintaining 5 % probability of rejecting null hypotheses in this paper using False Discovery Rate, see methods) are shown in bold.

	Effect size (CI)	BACI coefficient	SE	z	p	Relative abundances (%)
Total abundance	1.32 (0.87–1.89)	0.113	0.269	0.418	0.676	
Chironomidae	1.77 (0.85–3.32)	0.566	0.273	2.072	0.038	43.2
Potamopyrgus	0.24 (0.11–0.47)	−0.851	0.216	−3.933	<0.001	11.7
Echinogammarus	0.78 (0.45–1.34)	−0.201	0.225	−0.892	0.372	8.8
Oligochaeta	3.39 (1.57–7.39)	1.065	0.305	3.485	<0.001	5.6
Baetis	0.36 (0.23–0.58)	−1.149	0.276	−4.170	<0.001	3.8

occasions after treated wastewater addition, differences in assemblage composition between the impact and the control reach were greater than those observed before it (Fig. 3), but this was not observed in the samples taken in January and March 2018. Addition of treated wastewater did not change taxonomical richness and diversity at the sample scale (Table 3, Fig. 4). Taxonomical richness also remained unaltered by treated wastewater at the reach scale (effect size of 0.99); mean values before adding treated wastewater were 48.8 at the control and 48.3 at the impact reach, and 53.3 and 52.3 after it. In the same way, functional richness, evenness, divergence and redundancy were not impacted by the experiment (Table 3, Fig. 4).

3.4. Pollution indices and sensitive taxa

The treated wastewater discharges severely reduced both absolute and relative abundance of EPT (effect sizes of 0.58 and 0.71, respectively, Table 4, Fig. 5). However, the experiment did not reduce EPT richness and diversity, IBMWP index or $SPEAR_{organic}$ (Table 4, Fig. 5). Effect sizes for the density of the 17 most abundant taxa were negatively related to their IBMWP scores (Fig. 6, $p = 0.002$, GLM) and explained 47 % of its deviance. Most of these effect sizes were smaller than 1, meaning reduced invertebrate numbers after treated wastewater addition, but Oligochaeta and Chironomidae, which were the most resistant taxa to pollution (lowest IBMWP scores), showed effect sizes over 1.5 (Fig. 6). Contrarily, no relationship was found between effect sizes of the experiment and taxa sensitivity to organic toxicants (Fig. 6, $p = 0.907$, GLM).

3.5. Stability

In four out of 20 variables, the inputs of treated wastewater altered the coefficient of variation (i.e., stability). Treated wastewater increased the coefficient of variation for $SPEAR_{organic}$ (effect size of 1.95), the relative abundance of EPT (2.01), and the functional redundancy (2.26, Fig. 7). The coefficient of variation only decreased for *Potamopyrgus* abundance (effect size of 0.64, Fig. 7).

4. Discussion

4.1. Changes on invertebrate abundance

As in most other works on the effects of treated wastewater effluents on stream invertebrate assemblages (see Hamdhani et al., 2020 for a review), we found a marked increase in the abundance of Oligochaeta, a

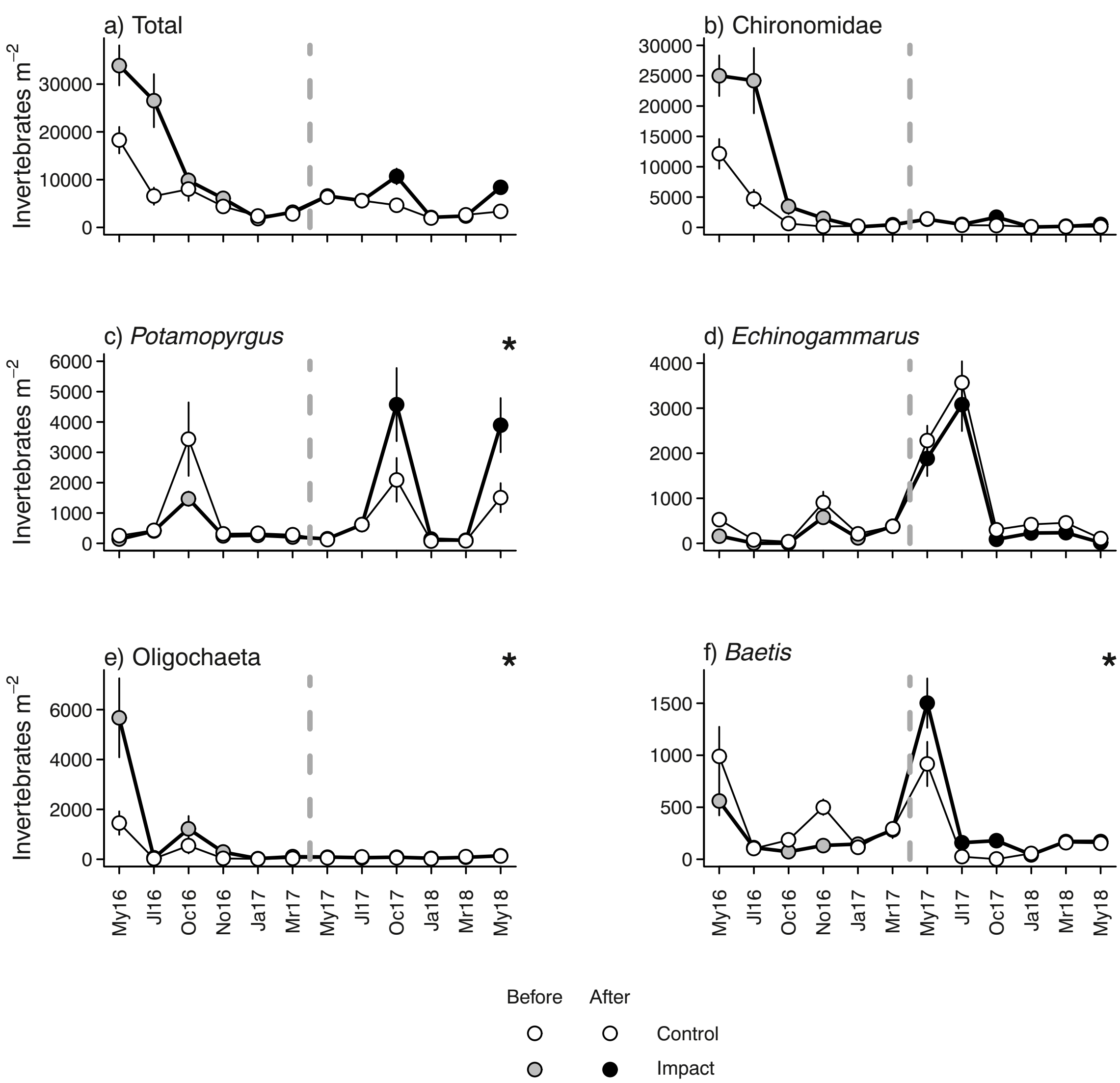

Fig. 2. Mean (±SE) density at the control and impact reach before and after discharging treated wastewater of a) total invertebrates and the five dominant taxa: b) Chironomidae, c) *Potamopyrgus*, d) *Echinogammarus*, e) Oligochaeta, and f) *Baetis*. The vertical dashed line divides the before and after periods. Asterisks indicate effect of treated wastewater on invertebrate density.

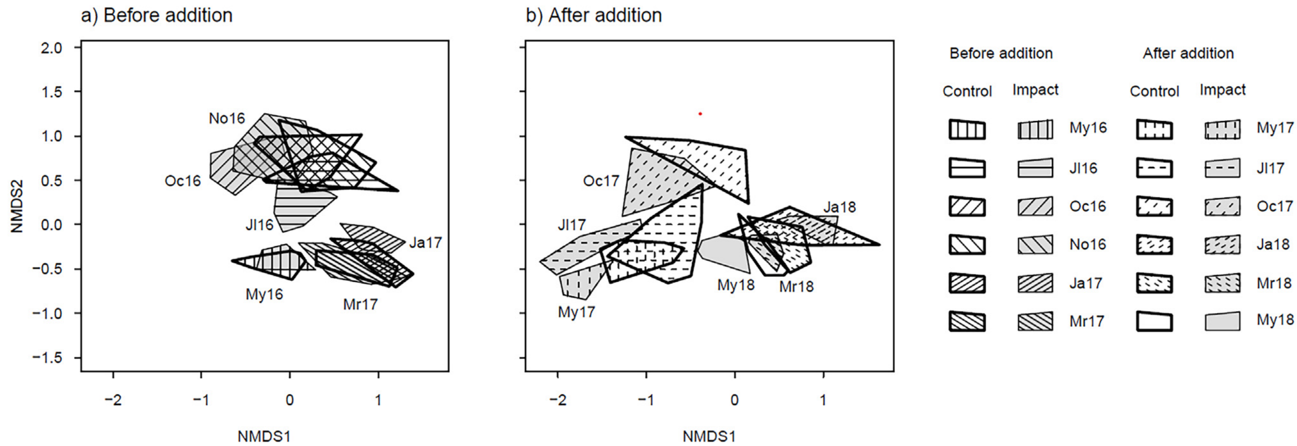

Fig. 3. Two-dimensional nonmetric multidimensional scaling (NMDS) plots representing the invertebrate assemblages a) before, and b) after wastewater addition. Each polygon includes all the samples taken at each reach and sampling occasion.

group highly tolerant to organic toxicants (sensitivity of −1.1), and to general environmental degradation (IBMWP score of 1). Since resistance to organic toxicants was not related to changes in abundance of the 17 main taxa, our results suggest toxicity was not the main driver of changes observed here, probably because of the low concentration of wastewater in the receiving stream. IBMWP scores of individual taxa were however a good predictor of abundance changes of these main taxa, as the density of the 15 main taxa with IBMWP scores greater than three was reduced by treated wastewater. Because IBMWP scores respond to general stream impairment (Couto-Mendoza et al., 2015), these results are compatible with treated wastewater generating multiple causes of stress as increased nutrient concentrations, pulses of higher temperature and conductivity but lower concentration of dissolved oxygen, and, partly, greater organic toxicant concentrations (Lemm et al., 2021). Therefore, wastewater input to an unpolluted stream favoured tolerant invertebrates and reduced the abundance of sensitive invertebrates.

Inputs of treated wastewater did not alter total invertebrate abundance, as the increase of Oligochaeta was compensated by the decrease of other dominant taxa such as *Potamopyrgus* and *Baetis*. This result is contrary to most previous works on the effects of WWTPs (Hamdhani et al., 2020), but other authors have also reported unchanged invertebrate abundance (Mor et al., 2022), and even reduced abundance (Aristone et al., 2022) below tertiary treated WWTP effluents. The effect of treated wastewater discharges depends on their concentration in the receiving system. Nevertheless, in our experiment, these discharges increased the availability of nitrogen by >17 % and doubled that of soluble reactive phosphorus, enough to modify ecosystem process rates in these systems (Pereda et al., 2020). The fact that this increase in nutrient availability did not affect total invertebrate abundance shows that other components of treated wastewater counterbalanced the effects of nutrients.

4.2. Changes in bioassessment indices

Treated wastewater caused rapid and substantial declines in EPT abundance that are in accordance with the high sensitivity of these insects to a great variety of environmental impacts (Valente-Neto et al., 2018). However, consistent with the lack of relationship between sensitivity to organic compounds and effect size of the experiment on each taxon abundance, the experiment did not reduce $SPEAR_{organic}$. Therefore, the increase in the relative abundance of invertebrates tolerant to organic toxicants is not a universal feature of streams receiving tertiary treated wastewater. On the other hand, the IBMWP index at the impact reach was not affected by the treated wastewater input, and kept above 127 throughout the study, indicating good ecological status. This lack of response of the IBMWP index contrasts with the good performance of IBMWP scores predicting abundance changes of the main taxa. Therefore, the stability of the IBMWP index is likely due to it being calculated using only the presence, not the abundance, of invertebrate families, which allows drifting animals from the control reach to prompt a high IBMWP index. Conversely, the transitory presence of those sensitive animals in low abundances will have much lower effect on EPT abundances.

4.3. Consequences on invertebrate diversity and functioning

Our experiment did not reduce any measure of taxonomic diversity at the sample or reach scales, although it did modify the taxonomic composition of invertebrate assemblages. Such modification was observed at the three first and the last sampling occasions with treated wastewater. In January and March 2018, the taxonomic composition of the assemblages at the impact reach overlapped very much with those found at the control, suggesting that higher dilution reduced the impact of treated wastewater. In May 2018, after one month of lower stream flow and dilution, assemblages at the impact reach showed again different composition to that in the control

Table 3
Responses of taxonomical and functional diversity of the invertebrate assemblages to the input of treated wastewater. BACI coefficients consider B and C as references. Effect sizes with their confidence intervals (CI), and results of the mixed models concerning the BACI interactions of the GLMMS are also shown. SE = standard error, z = z statistic, p = *p*-value.

	Effect size	BACI coefficient	SE	z	p
Taxonomical richness	0.89 (0.80–0.98)	−0.115	0.064	−1.807	0.071
Taxonomical ^{1}D	0.86 (0.76–1.01)	−1.391	0.845	−1.647	0.100
Taxonomical ^{2}D	0.91 (0.80–1.10)	−0.605	0.659	−0.918	0.359
Functional richness	0.51 (0.20–1.28)	−1.435	0.962	−1.491	0.136
Functional evenness	0.98 (0.92–1.05)	−0.007	0.017	−0.425	0.671
Functional divergence	0.96 (0.89–1.03)	−0.016	0.025	−0.623	0.533
Functional redundancy	0.83 (0.75–0.92)	−0.466	0.198	−2.357	0.018

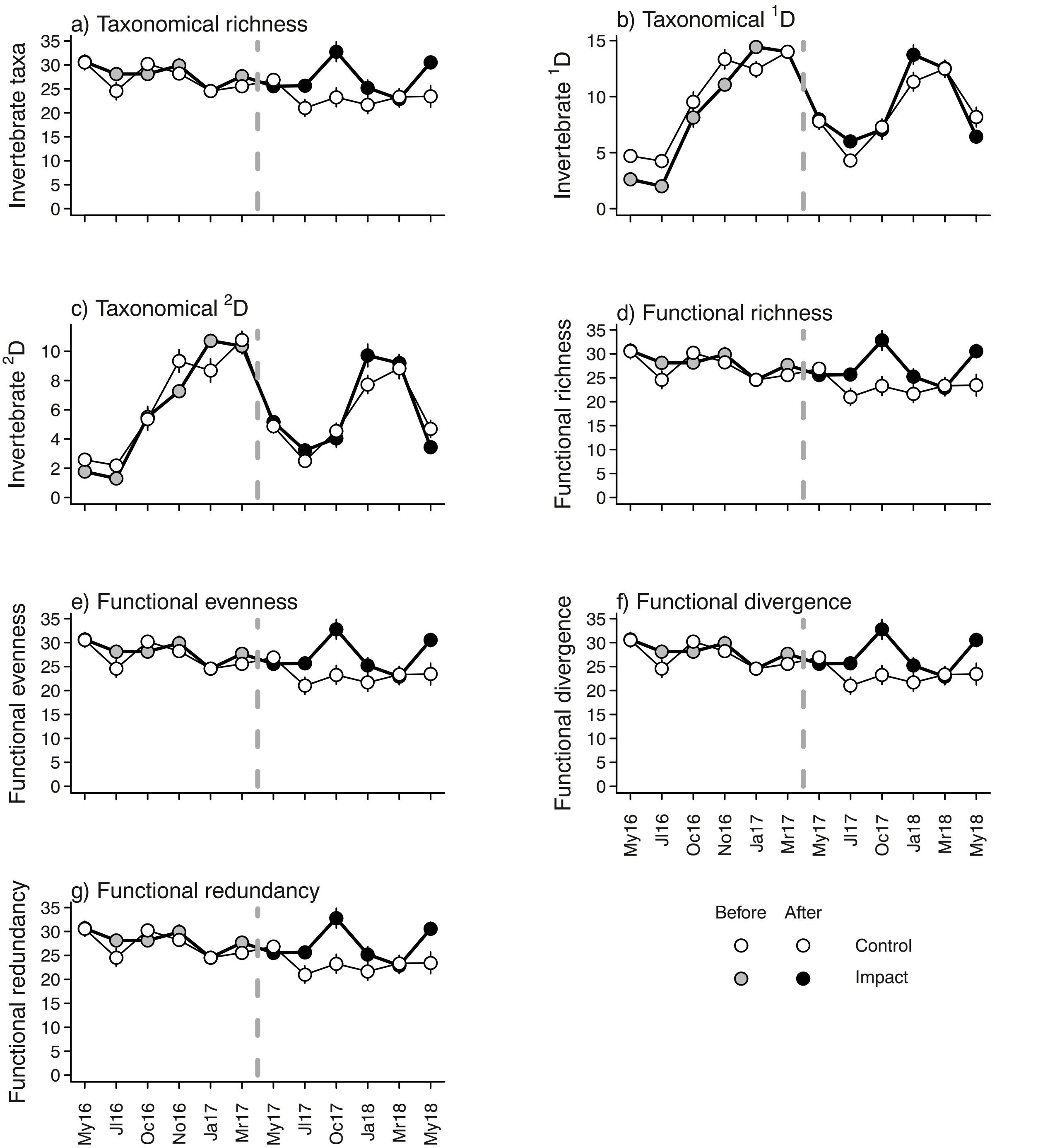

Fig. 4. Mean (±SE) values at the control and impact reach before and after discharging treated wastewater of a) taxonomical richness b) taxonomical ^{1}D, c) taxonomical ^{2}D, d) functional richness, e) functional evenness, f) functional divergence, and g) functional redundancy. ^{1}D and ^{2}D are respectively Hill numbers for q = 1 and q = 2.

reach. These patterns suggest again that flow conditions of the receiving stream modulate the effects of treated wastewater discharge on invertebrate assemblages (Mor et al., 2022) and that low flow conditions may act as bottlenecks impairing the assemblages.

Functional richness, evenness, and divergence of these new assemblages were similar to those found in the control reach, i.e., the extension of functional space used by the assemblages, and the distribution of invertebrates inside this functional space were not altered by the experiment and showed no signs of reduced stability and resilience as judged by functional redundancy (Biggs et al., 2020). We cannot rule out, however, changes in energy flows because, as found by Mor et al. (2022), small shifts in taxonomic composition associated to WWTP effluents can modify secondary production of these assemblages.

4.4. Changes in temporal variation of assemblages

Treated wastewater inputs increased temporal variation of functional redundancy and two additional metrics, $SPEAR_{organic}$ and % EPT, both directly related to the ecological status of the stream. This increased variation was expected because of the 35-fold changes in dilution rate of treated wastewater as a consequence of changes in stream discharge. However, our hypothesis was only partially fulfilled because

Table 4
Responses of pollution indices and Ephemeroptera, Plecoptera and Trichoptera (EPT) metrics to the input of treated wastewater. BACI coefficients consider B and C as references. Effect sizes with their confidence intervals (CI), and results of the mixed models concerning the BACI interactions of the GLMMS are also shown. SE = standard error, z = z statistic, p = p-value. Those p-values smaller than 0.015 (the limit for maintaining 5 % probability of rejecting null hypotheses in this paper using False Discovery Rate, see methods) are shown in bold.

	Effect size (CI)	BACI coefficient	SE	z	p
IBMWP	0.91 (0.81–1.01)	−0.087	0.074	−1.167	0.243
$SPEAR_{organic}$	0.94 (0.88–0.99)	−0.039	0.019	−2.021	0.043
EPT abundance	0.58 (0.41–0.82)	−0.640	0.211	−3.035	0.002
EPT richness	0.85 (0.75–0.95)	−0.165	0.080	−2.059	0.040
EPT ^1D	0.85 (0.75–0.96)	−1.056	0.561	−1.883	0.060
EPT ^2D	0.84 (0.73–0.97)	−0.839	0.417	−2.014	0.044
% EPT	0.71 (0.59–0.87)	−0.552	0.153	−3.601	<0.001

stability of other analyzed invertebrate metrics was not modified by treated wastewater inputs (16 out of 20), and stability of *Potamopyrgus* abundance was increased by the experiment.

5. Conclusions

The experimental addition of well-treated and highly diluted wastewater from an urban WWTP with tertiary treatment had subtle but potentially important consequences for the invertebrate assemblages, and thus, ecosystem functioning in the receiving stream. The alterations found might have gone unnoticed if we had not measured invertebrate abundances using a very controlled experimental design (BACI) and a high replication, per sampling (9 samples per sampling occasion) and

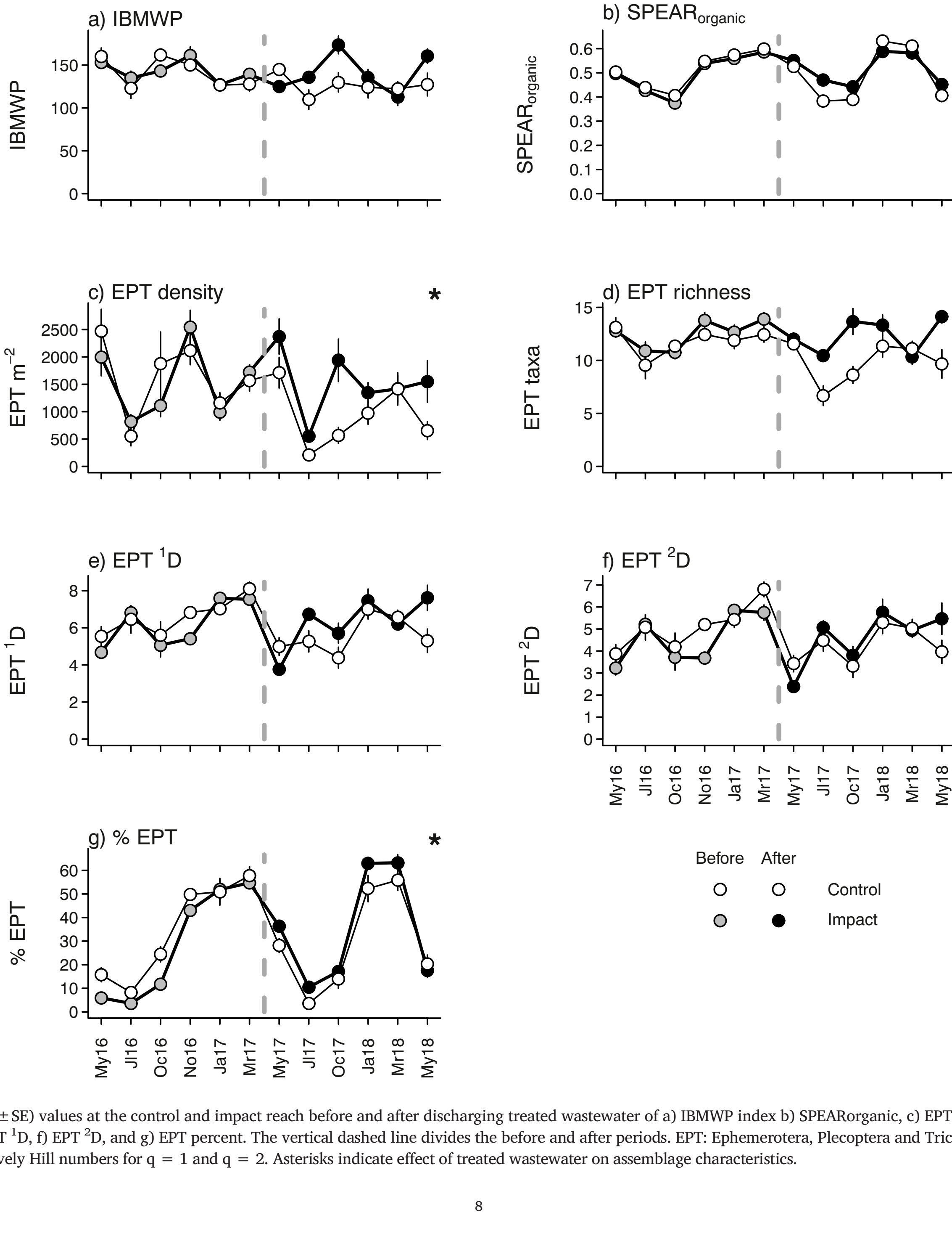

Fig. 5. Mean (±SE) values at the control and impact reach before and after discharging treated wastewater of a) IBMWP index b) SPEARorganic, c) EPT density, d) EPT richness, e) EPT ^1D, f) EPT ^2D, and g) EPT percent. The vertical dashed line divides the before and after periods. EPT: Ephemerotera, Plecoptera and Trichoptera. ^1D and ^2D are respectively Hill numbers for q = 1 and q = 2. Asterisks indicate effect of treated wastewater on assemblage characteristics.

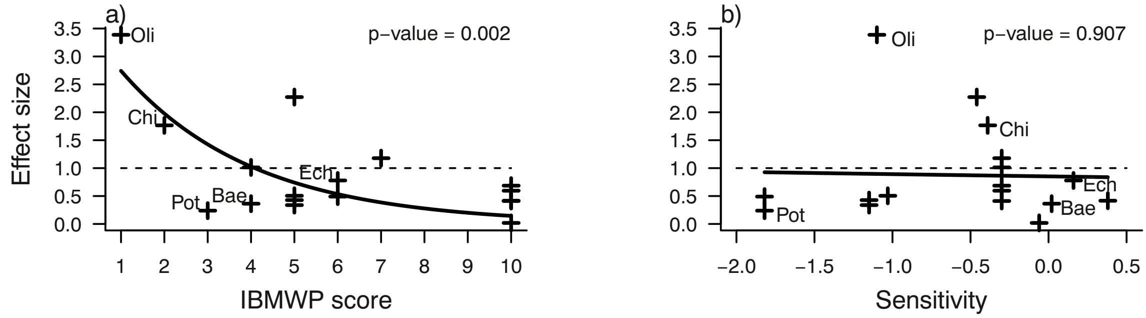

Fig. 6. Effect size of treated wastewater addition on abundant taxa density plotted against a) IBMWP score and b) sensitivity to organic pollutants. Positions of the five dominant taxa are shown. Bae: *Baetis,* Chi: Chironomidae, Ech: *Echinogammarus,* Oli: Oligochaeta, and Pot: *Potamopyrgus*. *P*-values of the models relating these variables are shown.

per period (6 samplings in each). We show that in this kind of experiments, regularly used bioassessment metrics, as the IBMWP index, that rely in presence of taxa irrespective their abundance can fail to detect impairments, whereas EPT metrics can be more sensitive. IBMWP scores can be however useful to anticipate the changes of abundance of individual taxa.

The effects of treated wastewater discharges are predicted to be stronger in high quality ecosystems with diverse communities, where sensitive taxa are more abundant. Therefore, increased efforts are needed to further reduce the water quality alterations associated to discharges from WWTPs, either by improving water treatment efficiency, reducing the use of contaminants difficult to eliminate in WWTPs or diverting the treated effluent to less sensitive water bodies.

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CRediT authorship contribution statement

J.M. González: Writing – original draft, Writing – review & editing, Data curation, Methodology. **I. de Guzmán:** Writing – review & editing, Data curation, Methodology. **A. Elosegi:** Conceptualization, Writing – review & editing, Project administration, Resources. **A. Larrañaga:** Writing – review & editing, Data curation, Supervision.

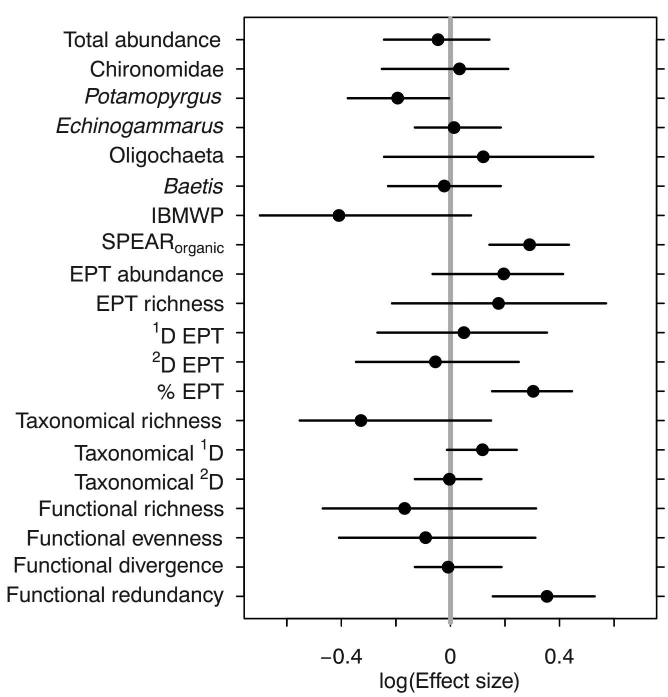

Fig. 7. Effect size of treated wastewater addition on coefficients of variation of invertebrate abundances (circles) and their 95 % confidence intervals (lines). The vertical dashed line marks effect size = 1.

Data availability

Invertebrate data used in this paper are publicly available at González, José M.; de Guzmán, Ioar; Elosegi, Arturo; Larrañaga, Aitor (2022), “Apraitz fauna”, Mendeley Data, V1, doi: 10.17632/gbjppjcznk.1

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.

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