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From biomarkers to community composition: Negative effects of UV/chlorine-treated reclaimed urban wastewater on freshwater biota

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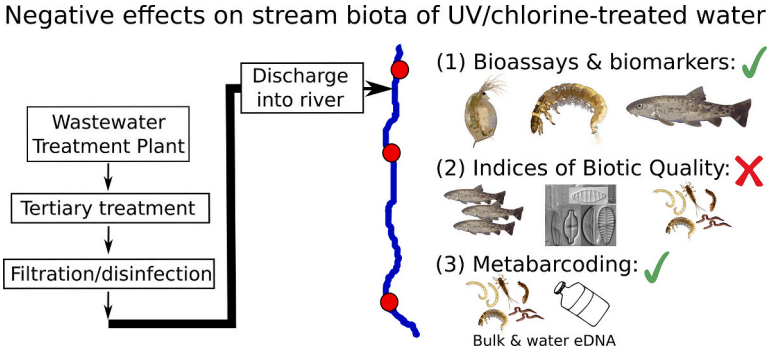
HIGHLIGHTS

- UV/chlorine-treated water caused changes in feeding rates of *Daphnia magna*.
- *Hydropsyche exocellata* changed oxidative status when exposed to UV/chlorine water.
- Biotic indices did not discriminate between UV and UV/chlorine water treatments.
- Metabarcoding captured changes in composition of sensitive species across treatments.
- UV-treated is less harmful for freshwater biota than UV/chlorine-treated water.

GRAPHICAL ABSTRACT



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ABSTRACT

The use of urban wastewater reclaimed water has recently increased across the globe to restore stream environmental flows and mitigate the effects of water scarcity. Reclaimed water is disinfected using different treatments, but their effects into the receiving rivers are little studied. Physiological bioassays and biomarkers can detect sub-lethal effects on target species, but do not provide information on changes in community structure. In contrast, official monitoring programs use community structure information but often at coarse taxonomic resolution level that may fail to detect species level impacts. Here, we combined commonly used biomonitoring approaches from organism physiology to community species composition to scan a broad range of effects of disinfection of reclaimed water by UV-light only and both UV/chlorine on the biota. We (1) performed bioassays in one laboratory species (water flea *Daphnia magna*) and measured biomarkers in two wild species (caddisfly *Hydropsyche exocellata* and the barbel *Luciobarbus graellsii*), (2) calculated standard indices of biotic

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quality (IBQ) for diatoms, benthic macroinvertebrates, and fishes, and (3) analysed community species composition of eukaryotes determined by Cytochrome Oxidase C subunit I (*cox1*) metabarcoding. Only the UV/chlorine treatment caused significant changes in feeding rates of *D. magna* and reduced antioxidant defenses, increased anaerobic metabolism and altered the levels of lipid peroxidiation in *H. exocellata*. However, inputs of reclaimed water were significantly associated with a greater proportion of circulating neutrophils and LG-PAS cells in *L. graellsii*. Despite IBQ did not discriminate between the two water treatments, metabarcoding data detected community composition changes upon exposure to UV/chlorine reclaimed water. Overall, despite the effects of UV/chlorine-treated water were transient, our study suggests that UV-light treated is less harmful for freshwater biota than UV/chlorine-treated reclaimed water, but those effects depend of the organizational level.

1. Introduction

Owing to the ongoing water scarcity crisis, water reclamation and reuse are important water resources for water managers in Mediterranean and Middle East countries. The use of reclaimed water requires additional water treatments so that effluents from conventional wastewater-treatment plants (WWTPs) must meet more restrictive requirements of water quality (Deng et al., 2019). Common uses of reclaimed wastewater include agricultural irrigation, industrial processes, environmental restoration, groundwater replenishment or even drinking water (Levine et al., 2010). Depending on the use of reclaimed water, effluents of conventional WWTPs are treated using different technologies, including chlorine, ozone and ultraviolet (UV) light to remove pathogens (e.g. bacteria and their spores, viruses, protozoa and their cysts, worms), which are of major concern for authorities (Wang et al., 2012; Chen et al., 2022). However, chlorine addition generates toxic compounds including trihalomethanes or haloacetic acid, both of which can have cytotoxic, mutagenic or ecotoxic effects (Petala et al., 2009; Srivastav et al., 2020). Moreover, chlorine treatments do not fully inactivate all pathogens and additional treatments such as UV do not disinfect water completely because some adenovirus and rotaviruses bypass the treatment (Srivastav et al., 2020; Chen et al., 2022). Therefore, authorities do combine different technologies to ensure water disinfection (Wang et al., 2012; Chen et al., 2022; Ye et al., 2022).

To determine the potential risks into ecosystems and the aquatic biota of different disinfecting technologies to get reclaimed water, the use of bioassays and biomarkers is advisable to detect sub-lethal effects at the individual level. The bioassay involves an experimentally controlled "ex situ" exposure of organisms to a toxicant and the assessment of their behavioural, reproduction, or survival responses, whereas biomarkers are the physiological responses measured within the organism. Biomarkers can be measured in organisms exposed to a bioassay or directly in organisms captured in the field without controlled exposure to toxicants. For the later, the normal range of variation for many physiological parameters is unknown, and consequently, the pathological condition is established based on a comparison of effects between 'exposed' and 'non-exposed' individuals (Colin et al., 2016a, 2016b). An example of well-established biomarkers is the oxidative status such as antioxidant ones (e.g. superoxide dismutase, catalase, glutathione peroxidase, reduced glutathione) because pollutants often increase the production of Reactive Oxygen Species (ROS) within organisms and hence alter antioxidant responses (Barata et al., 2007; Damásio et al., 2008; Faria et al., 2009; Prat et al., 2013). However, when the capacity of an organism to deal with ROS is overcome, then oxidative cell damages appear, such as lipid peroxidation, changes in the proportion of circulating white and red blood cells, and erythrocytic nuclear abnormalities (ENAs), and finally the organism can die (Field et al., 1943; Damásio et al., 2008; Faria et al., 2009; Maceda-Veiga et al., 2015; Farag and Alagawany, 2018). When the pollutant is expected to have sub-lethal or lethal effects on the target species, it would be advisable to combine the use of bioassays and biomarkers with other bioindicators either at the individual level, such as body-condition indices based on mass-length relationships, or at the community level using indices of biotic quality (IBQ) (Peig and Green, 2009; Prat et al., 2013; Maceda-Veiga et al., 2014; Colin et al., 2016a).

Biomonitoring programs under the European Water Framework Directive (WFD, Directive 2000/60/EC of the European Parliament, https://eur-lex.europa.eu/eli/dir/2000/60/oj) use IBQ to assign a value of biological quality to a water body. IBQ are calculated based on the taxonomic identity of a particular community such as diatoms, macrophytes, benthic macroinvertebrates, or fishes. Commonly, a significant decrease in their relative abundance or when a taxon disappears, the value of IBQ changes, but IBQs are unable to detect sub-lethal effects as biomarkers do. Given each particular taxa differs in their response to different stressors, various groups of sentinels are combined in IBQs to effectively detect ecological impacts of sewage inputs. For instance, the community structure of diatoms is particularly sensitive to organic pollution, whereas that of fishes is sensitive to the presence of introduced species and hydromorphological alterations (Colin et al., 2016a, 2016b). For macroinvertebrates, the major caveat of using them is that several impacts cannot be detected at the taxonomic family or genus levels that are commonly used by the time and cost needed for sorting specimens and their challenging identification.

New approaches that use species-level taxonomic information based on DNA sequencing were proposed for a fast and replicable methodology to calculate IBQ (Baird and Hajibabaei, 2012; Pawlowski et al., 2018). For metazoans, individuals can be assigned to a species using a fragment of the mtDNA gene Cytochrome Oxidase C subunit I (*cox1* or COI), referred to as the DNA barcode (Hebert et al., 2003). The recent optimization of high-throughput sequencing protocols had greatly reduced the cost of sequencing DNA barcodes of the whole community, the so-called metabarcoding (Taberlet et al., 2012). Once DNA of the whole community is extracted, there is an amplification of DNA barcodes, the pooling of samples for massive sequencing, and the bioinformatic work to assign individual DNA barcodes to their respective samples (Hajibabaei et al., 2011). The two most popular metabarcoding techniques used to determine biological quality in rivers are tissue homogenization of all organisms captured in a community (bulk sample) and the remains of fragments of DNA released by organisms into river water or sediment together with faeces, mucus, skin cells, organelles, gametes or even extracellular DNA (eDNA, environmental DNA) (Creer et al., 2016). These two metabarcoding techniques are expected to be complementary because bulk samples target local communities whereas eDNA can also capture the presence of other organisms at a sub-catchment scale (Bista et al., 2017; Deiner et al., 2017; Deiner and Altermatt, 2014; Macher et al., 2018). However, these two techniques have the limitation that DNA barcode reference libraries for assigning taxonomic species are still far from complete (Múrria et al., 2020).

Here, we study the effects of reclaimed water from an urban WWTP on the receiving biota, and compared the use of two different disinfection processes: UV light only or a combined treatment of UV light and chlorine (thereafter UV-light versus UV/chlorine treatments) (see details in Mujeriego et al., 2008). Given the many physiological and ecological pathways by which water treatments may affect the receiving biota (Colin et al., 2016a, 2016b), we assessed the effects of the two disinfection processes by means of an interdisciplinary perspective in taxa (diatoms, benthic macroinvertebrates, and fishes) and across multiple study methods: (1) bioassays to monitor sub-lethal effects on the

water flea *Daphnia magna* (Crustacea, Branchipoda); (2) biomarkers to detect sub-lethal effects on two feral taxa, namely the caddisfly *Hydropsyche exocellata* (Insecta, Trichoptera) and the barbel *Luciobarbus graellsii* (Actinopterygii, Cyprinidae); (3) the commonly used IBQ for diatoms, benthic macroinvertebrates and fishes to elucidate if IBQ can capture the effect of reclaimed water on the ecosystem; and (4) species level data from bulk samples and water eDNA metabarcoding for macroinvertebrates and fishes to determining effects downstream of reclaimed water discharge. We expect that UV/chlorine treatment would negatively affect taxa located downstream of reclaimed water input and that UV-light treatment should have a negligible effect because UV-light disinfection does not release any toxic compound into waters (Noga, 2000). Moreover, we also expect a short-term effect of the UV/chlorine treatment water, which should be detected only at the physiological level by sub-lethal effects, whereas a long-term persistent effect can be detected at the community level, which is not expected here. However, if impacts are severe, biota responses should manifest at all the levels of biological organization studied from biomarkers to changes in community composition.

2. Methods

2.1. Study design and sample collection

An initiative from the Catalan Water Agency consisted of pumping reclaimed water 16.6 km upstream the reclamation facility and releasing it into the river to keep environmental flows along 8.5 km of the Llobregat River, before part of the water flow is diverted to generate drinking water (Munné et al., 2023). This lower part of the Llobregat River has already severe ecological impacts and poor biological status by industrial and urban activities and the entire studied reach can be considered homogeneous in both biotic and abiotic conditions (Prat and Rieradevall, 2006). In order to determine the effect of the reclaimed water on the freshwater biota over time, we sampled chronologically three sites between July and November 2019 (Fig. 1): R0 was located upstream the discharge site where individuals were 'non-exposed' to reclaimed water, and therefore it is considered as the control site; R1 was located at 500 m downstream of the discharge site where individuals were highly 'exposed' to reclaimed water; and R2 was located at 3 km downstream where individuals were less 'exposed' to reclaimed water than at R1. To simplify terminology, each sampling is thereafter called "treatment" following this scheme: on 15th July (T1), WWTP effluent was treated with UV-light, whereas on 25th July (T2) the WWTP effluent was treated with UV/chlorine. Sampling on 7th November (T3) was used to study whether the effects of reclaimed water input persist over time and also to test if R0, R1 and R2 were comparable. We acknowledge that T3 sampling may integrate seasonal effects and carry-over effects (i.e. persistence of the toxicants) from T1 and T2. During T1 and T2 river flow was set as low as possible around 1–2 m^3/s and reclaimed water was released at circa 1.6 m^3/s to reach a dilution rate close to 1:1 or 1:3. Chlorine was added at 13 mg Cl_2/L (see more details in Munné et al., 2023).

To test the effects of different treatments occurring within the WWTP and inside the water reclamation facility, we had four additional sampling points for metabarcoding after tertiary treatment during T2 (W1), following filtration and disinfection during T2 (W2) and at the pipe during discharge to the river during T1 and T2 (Dis.T1, Dis.T2) (Fig. 1). Also, water collected at site Dis.T2 was used for "ex situ" bioassays of *D. magna*.

Prior to sampling organisms, water temperature, electrical conductivity, total dissolved solids and pH were measured in each site using a multi-parametric digital probe YSI® Pro Plus. Ten individuals of the species *H. exocellata* were collected and frozen in N_2 one by one in the field for biomarker analysis. Finally, five liters of water were collected for "ex situ" *D. magna* bioassays at each site and other five liters of reclaimed water were directly collected from the discharge pipe (Dis). Diatoms, fishes, and macroinvertebrates were sampled following international standardized procedures to calculate IBQ (see below).

2.2. Daphnia magna feeding and survival bioassay

We followed previous procedures by Rivetti et al. (2015) with minor modifications. Once in the laboratory, and for each water sample, 20 four-day-old juveniles of *D. magna* were inoculated in 1 L and left exposed to water in an orbital wheel set to 1 rpm for 24 h at 20 °C. Two replicates were performed. In parallel, a laboratory control was prepared with the same number of individuals exposed also to 1 L of clean water (ASTM hard water standard). At the end of the incubation, the survival rate was estimated, and alive organisms were used to determine feeding rate effects. To do so, five individuals pre-exposed to Llobregat water were cultured in 50 mL of clean laboratory water medium (ASTM hard water) with 5 × 105 cells/mL of *Chlorella vulgaris* for 4 h under darkness. Between 5 and 7 replicates were made for each sample, and blanks with no animals were also included. Feeding rates were determined as the decrease in chlorophyll-a absorbance (λ = 665 nm), measured in a Cecil-CE 9200 spectrophotometer (Cambridge, UK), between blanks and the samples with individuals. Final feeding results were reported as % relative to laboratory controls.

2.3. Hydropsyche exocellata biomarkers

Different biomarkers related to oxidative stress were measured in ten individuals per site of *H. exocellata*: lipid peroxidation, reduced glutathione-GSH, enzymatic activities of super-oxide dismutase-SOD, catalase-CAT, glutathione S transferase-GST conjugation and anaerobic metabolism/stress-lactate de-hydrogenase-LDH (Colin et al., 2016a; Faria et al., 2009). Briefly, lipid peroxidation was measured colorimetrically by means of quantifying the number of equivalents of malonyl

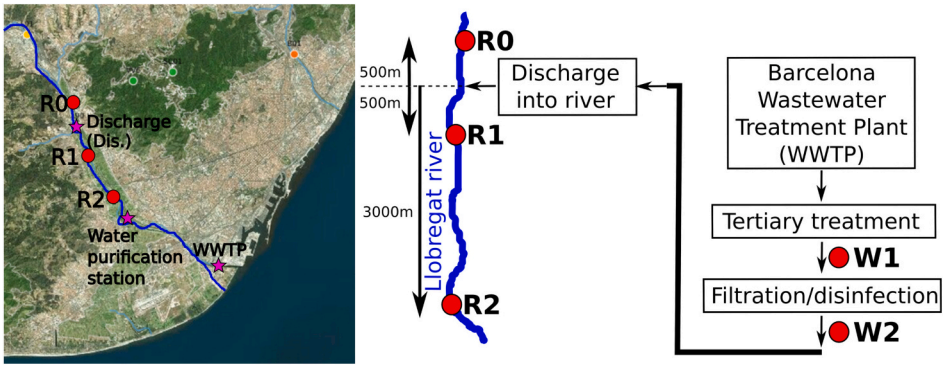

Fig. 1. Location of the wastewater-treatment plant (WWTP), the discharge site (Dis.), and the water-purification station. Experimental design consisted of two sampling points inside the WWTP (W1 and W2), one sampling site (R0) upstream of the discharge site and two sites downstream the Llobregat river (R1 and R2).

dialdehyde, which is a by-product of lipid oxidation induced by ROS. The rest of the biomarkers were determined using a spectrophotometer Cecil-CE 9200 (Cambridge, UK) and fluorimetric methods at 20 ± 0.5 °C with a multiple detection microplate reader (BioTek ®, Vermont, USA).

2.4. Luciobarbus graellsii biomarkers

Fish were captured at all mesohabitats (rapids, tables and pools) with a portable electrofishing equipment following well-established international procedures (CEN 14011:2003; Maceda-Veiga et al., 2014). During electrofishing, fish were kept alive in buckets provided with air pumps and at the end of the survey, all fishes were counted and identified to the species level for calculating IBQ (see below). The focal specimens for the biomarker study were juveniles of *L. graellsii* because this species was captured in enough numbers in all samplings. Juveniles of *L. graellsii* may be advantageous for biomonitoring because they are likely to have more site-fidelity than adults, which conduct upstream reproductive migrations (Colin et al., 2016a). Ten juveniles per site were collected and the rest of fishes were released at the site of capture.

For fish biomarkers, we followed the guidelines of the animal welfare committee at the University of Barcelona (Num. 87/15). Barbels were anaesthetized with MS-222 (2 %), measured (furcal length, ±1 mm), and weighted (wet mass, ± 0.01 g). The entire external surface of measured fish was inspected for gross signs of disease. Blood was obtained via caudal venipuncture using an insulin syringe rinsed in lithium heparin. A blood smear was then prepared for each fish by stained with Diff-Quick® and mounted with DPX®. Following the methods outlined in Maceda-Veiga et al. (2017), differential white blood cell count was calculated based on the inspection of 100 white blood cells and the relative abundance of ENAs was calculated based on the inspection of 1000 red blood cells. Finally, we calculated the Scaled Mass Index (SMI) using mass-length regressions of fish individuals following the methods outlined in Maceda-Veiga et al. (2014). The SMI detects any physiological impact in animals that affects individuals' weight or shape, and so, it can be used as a general biomarker of a fish health status (Colin et al., 2016a, 2016b).

2.5. Calculation of IBQ based on diatoms, macroinvertebrates and fishes

For diatoms and macroinvertebrates, sampling, preparation and counting for calculating IBQs followed well-established international methodologies (Barber and Haworth, 1981; Hofmann et al., 2011; Colin et al., 2016b; Prat et al., 2013). Diatoms were brushed from the top of ten stones collected at each site. Aquatic macroinvertebrates were captured along 100 m of river in all possible habitats during 10 min using a 20 × 20 cm 250 μm-mesh D-net sampling. Those samples were preserved in the field in 96 % alcohol and frozen in the laboratory at −20 °C until identification. To determine the biological quality of each sampling site, the most common metrics used in the Iberian Peninsula were applied for diatoms (IPS, CEMAGREF, 1982), macroinvertebrates (IBMWP index, Alba-Tercedor et al., 2004) and fishes (e.g. IBICAT index 2010 and IBICAT2b, Sostoa et al., 2010).

2.6. DNA extraction, PCR amplification and library preparation

For the bulk sample technique, the first 2500 macroinvertebrates were sorted from each sampling site under a stereoscope. To validate the molecular results, and despite it is not required for calculating IBQ, all individuals were identified at the lowest taxonomic level as possible, usually genus, using Tachet et al. (2010), but with some Diptera identified to family and annelids and acari to phylum and subclass levels, respectively. Following identification, all individuals from the same site were pooled and stored overnight in an oven for alcohol evaporation before the DNA extraction. Macroinvertebrates were then homogenized with liquid nitrogen and 0.3 g subsequently transferred to extract DNA using the PowerSoil kit (Qiagen) in a laminar flow cabinet sterilised with UV light between samples.

For the eDNA technique, 2 L of water were collected in four sterile bottles of 0.5 L and were frozen at −20 °C for extracting eDNA as soon as possible. At least two of the four samples of 500 mL of water were filtered through a 0.22 μm Sterivex filters (Merck) to retain as much as the DNA dissolved in the water. The final filtered volume varied in function of water turbidity. Each eDNA filter was stored at −20 °C in sterile plastic bags until DNA extraction. DNA retained in the filters was extracted in the cabinet sterilised with UV light using PowerWater kit (Qiagen).

The universal Leray-XT primer set (Wangensteen et al., 2018) was used for amplifying a 313 bp fragment of the mitochondrial marker *cox1*. The Leray-XT marker was optimized for a nearly full amplification for almost all main eukaryotic lineages covering all freshwater macroinvertebrate lineages. For each sample, forward and reverse primers included an 8-base specific tag (identical on both sides) for further sample identification. A variable number of N from two to four were also attached also to the forward and reverse primers to improve sequence diversity for Illumina processing. Two replicates of PCR were run per each DNA extraction, and three negative controls (PCR mixture without DNA template) and one negative control of each DNA extraction method (distilled water was filtered for eDNA) were performed following standard conditions for *cox1* amplifications (Wangensteen et al., 2018). None of the negative PCR controls yielded bands on agarose gels.

PCR products were pooled, purified, and concentrated using MinElute PCR purification kit (Qiagen). A Qubit fluorometer was used to check DNA concentrations. A single Illumina library was built using the Nextflex PCR-free library preparation kit (Perkin-Elmer), and paired-end sequenced on an Illumina MiSeq V3 run (2 × 250 bp).

2.7. Bioinformatic analyses

Most steps of the bioinformatic analyses were based on the OBITools package (Boyer et al. 2016) and followed similar pipelines used in Antich et al. (2021a). The *Illuminapairedend* program was used to align paired-end reads and only those with >40 alignment quality score were kept. Reads were demultiplexed using *ngsfilter* and only reads with the same primer tags at both extremes were kept.

Only reads between 300 and 319 bp and with no other than A, C, T or G nucleotides were retained using *obigrep*, and *obiuniq* was further used to dereplicate sequences. Chimeric amplicons were removed using the *Uchime-denovo* algorithm from VSEARCH v2.7 (Rognes et al., 2016). Sequences were then clustered into molecular operational taxonomic units (MOTUs) with SWARM (Mahé et al., 2022) using d = 13 (Antich et al., 2021b). MOTUs with less than five reads were removed to ensure quality of data. Finally, MOTUs were assigned to species names, or higher taxonomic level if a species name was not reported, using *ecotag* and a custom database containing sequences from the EMBL nucleotide database, Barcode of Life Database (BOLD) and the Iberian DNA barcode reference library (Múrria et al., 2020). Poorly assigned sequences were further improved by querying the BOLD database, and only MOTUs with an identity match >85 % in BOLD were kept. All sequences were filtered by taxonomy, and only Metazoa were retained for further analyses. All analyses of the sequenced data were performed using the fourth square root-transformed values of the relative frequencies of read numbers of MOTUs in each sample.

2.8. Statistical analyses

We performed two-way ANOVA followed by Tukey's post-hoc multiple comparison test (Zar, 1996) to explore the effects of sampling site (R0, R1 and R2) and treatment (T1, T2 and T3) on the biomarkers of *H. exocellata* and *L. graellsii*. The same statistical technique was used for the *D. magna* bioassay, which included water samples from inside the WWTP and the discharge pipe of reclaimed water (sampling site: R0, R1, R2, Dis.1, Dis.2). Residuals of ANOVA were inspected for normality and

variance homoscedasticity and transformed when required (e.g. log for discrete variables). All this analysis were run in R (R Core Team, 2023).

The similarity of the community composition of macroinvertebrates was explored using non-metric multidimensional Scaling analysis (nMDS) after building the Bray-Curtis dissimilarity matrices. To visualize species names on the nMDS axes, we only plotted a list the 10 most characteristic species per site and treatment based on a high probability of being preferentially recorded using the indval function of the R package labdsv (Roberts, 2023). Statistical differences in the community similarity was tested between the two type of metabarcoding techniques by a permutational multivariate analysis of variance (PERMANOVA), which tests the null hypothesis that the centroids and dispersion of the groups are equivalent for all groups (Anderson, 2001). Because each metabarcoding technique captured significantly a different community composition, statistical differences in the community similarity were tested separately by metabarcoding technique among sampling sites, treatments and their additive effect using PERMANOVA.

3. Results

The study design reflected the intended gradient in water quality before and after the discharge of reclaimed water (Table 1). In particular, values of conductivity and TDS were higher upstream (R0) than downstream the discharge site of reclaimed water (R1 and R2). Differences in conductivity values among sampling sites were particularly marked during UV/chlorine disinfection (T2) than with UV-light treatment (T1) and three months after the discharge of reclaimed water (T3). As expected, values of water temperature were higher at T1 and T2 samplings in July than at T3 in November (Table 1).

3.1. Bioassays of D. magna and biomarkers of H. exocellata and L. graellsii

For *D. magna*, the bioassays showed significant differences in feeding rates ($p < 0.05$; $F_{9,\ 52} = 4.6$) (Fig. 2). Interestingly, feeding rates of *D. magna* exposed to water just released from the UV/chlorine treatment (Dis.) were similar to those in individuals exposed to water from R1 and R2 (Fig. 2). However, there was greater mortality with discharged water (Dis.). Three months later during T3, effects on *D. magna* were still detectable only at site R1, whereas R0 and R2 showed similar values (Fig. 2).

Almost all biomarkers measured in *H. exocellata* showed significant effects among treatments (Fig. 3, Table 2). Changes attributable to the UV/chlorine treatment (T2) were detected for CAT as this enzymatic activity was significantly lower in individuals exposed to the discharge (R1) compared to 'non-exposed' individuals in R0 during T2, and the lowest values were found in individuals captured in T3 at the three sites. LPO levels also reached the lowest levels in the study at R1 in T2, while the highest values were at R0 in T3. The lowest levels of GSH and GST activities were also in individuals from R1 in T2. A potential biomarker more affected by the sampling season than by input of reclaimed water was SOD activity, which showed remarkable high levels at R2 but only in T3, whereas similar values were found across sites within each treatment. Likewise, LDH activities were higher in T2 but without clear differences among sites.

For *L. graellsii*, blood biomarkers performed better in detecting differences among treatments than the SMI body condition index (Table 3). *Luciobarbus graellsii* from the 'exposed' site R1 significantly had a greater percentage of circulating white blood cells (neutrophils and LG-PAS + cells) compared to *L. graellsii* from 'non-exposed' R0, especially in T3 (Fig. 4). *Luciobarbus graellsii* from sites R1 and R2 also significantly differed in the percentage of red blood cells with nuclear abnormalities (ENAs) but patterns of ENAs were not significantly related to any of the reclaimed water treatments. As for the other taxa, the UV-light treated water was not significantly related to any remarkable effect in biomarker responses of *L. graellsii* (Figs. 2–4).

Table 1
Date and hour of sampling, treatment, site and values of water temperature, conductivity, total dissolved solids and pH recorded.

Sampling	Date	Site	Hour	Temp (°C)	Cond (µS/cm)	TDS	pH
Treatment 1	15/07/19	R0	10:15 AM	24.1	1301	858	8.33
		R1	12:30 PM	26	1466	936	8.42
		R2	04:00 PM	27.1	1573	975	8.61
Treatment 2	25/07/19	R0	10:30 AM	25.9	1643	1059.6	8.33
		R1	12:30 PM	27.4	1790	1118	8.29
		R2	03:00 PM	29.3	1855	1118	8.36
Treatment 3	11/07/19	R0	10:00 AM	13.1	1377	1157	7.88
		R1	11:15 AM	13.7	1439	1196	8.01
		R2	01:40 PM	14	1904	1566.5	8.07

3.2. Indices of biotic quality (IBQ): diatoms, macroinvertebrates, and fishes

Metrics for diatoms (Annex 1), macroinvertebrates (Annex 2) and fishes reported the bad–poor ecological status of the entire study area across treatments and sites (Table 4). Therefore, effects of the treatments were not detectable in values of IBQs. Across sites, only the diatom-based index IPS tended to have greater scores towards the most downstream site of the study area (R2).

3.3. Changes in species composition of macroinvertebrates

Results of metabarcoding indicated that the study area had a total taxonomic richness of 150 Metazoa MOTUs (Annex 3 and Fig. 6). The bulk samples contributed the most to the total amount of reads (3,085,926 reads), whereas 74,992 reads corresponded to water eDNA. Both bulk samples and eDNA captured 48 common MOTUs, but the bulk samples captured more unique MOTUs (75) than water eDNA (27). Also the number of MOTUs per site was higher for bulk sample than water eDNA (Fig. 5). PERMANOVA test showed significant differences in taxonomic composition at species level between bulk samples and eDNA ($F = 13.197$, $p < 0.001$). For comparison with IBQ results, the majority of benthic macroinvertebrate families used to estimate IBQ were captured also for metabarcoding techniques, especially in the bulk samples (Fig. 6). Notably, metabarcoding provided a species level resolution, which is important for diverse groups such as Baetidae (4 species), Chironomidae (19 species) and Oligochaeta (41 species). For fishes, eDNA detected all the fish species and resolved uncertainties in taxonomy of juveniles (e.g. *Gobio* species). Moreover, eDNA detected several species of birds and reptiles such as *Gallinula chloropus*, *Larus marinus* and *Trachemys scripta*.

Concerning the effects of effects of disinfection of reclaimed water, species richness along sites within and across treatments was similar for each technique, except for bulk sample during treatment 1 (UV-light treated water), which diversity decreased downstream (Fig. 5). The community composition for bulk sample and eDNA on nMDS axes showed spatial segregation between the 'non-exposed' site R0 (see blue dots in Fig. 7) and the two sites located downstream the discharge site of reclaimed water (R1 and R2), except for T3. Despite the dissimilarity of species composition across sites recorded by bulk samples, the PERMANOVA test did not change significantly across sites ($F = 0.9$, $p = 0.61$), but it varied significantly among treatments ($F = 1.58$ $p = 0.046$). Also the additive effects of the two factors was only significant for the

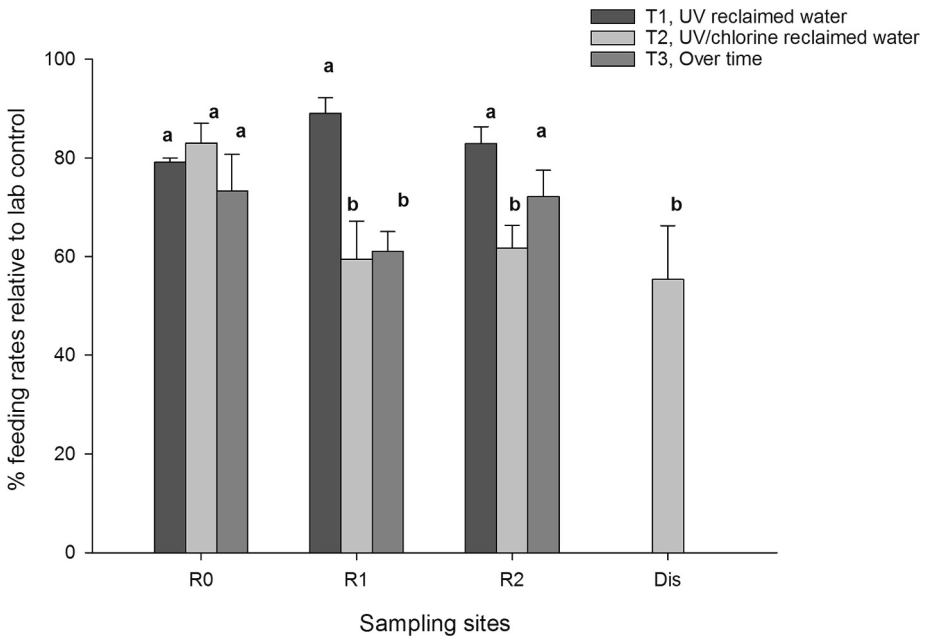

Fig. 2. Changes in feeding rates of *Daphnia magna* individuals (mean ± SE, n = 5–7) exposed to river water sampled before (R0, 'non-exposed'), at the outflow (Dis) and downstream (R1 and R2, 'exposed') the discharge site of the wastewater-treatment plant in relation to laboratory control in three periods (T1: UV only, T2: UV/Chlorine and T3: over time). Different letters show differences between treatments based on ANOVA outputs followed by Tukey's multiple comparisons.

effect of site on treatment ($F = 1.63$ $p = 0.03$), which indicates that the site composition was different across treatments. Similarly, for eDNA, taxonomic composition significantly varied among treatments ($F = 1.66$, $p = 0.042$), but it did not change significantly across sites ($F = 1.45$, $p = 0.12$). However, the additive effects of site on treatment ($F = 0.75$, $p = 0.69$) and treatment on sites ($F = 0.46$, $p = 0.95$) were non-significant.

Besides assessing the effects of reclaimed water on the taxonomic composition of the river communities, eDNA was used for determining the community composition in two sites inside the water treatment plants (W1 and W2) and the reclaimed water just when discharged in the river (Dis.T1, Dis.T2). In this regard, eDNA data showed similar richness compared with samples from the river (Fig. 5), but different taxa composition and high dissimilarity from samples collected in the river (i.e., R0, R1 and R2) (Figs. 6, 7). Within the water treatment plant, community composition following the tertiary treatment (W1) was similar to water discharged in the river (Dis.T1, Dis.T2), whereas reclaimed water disinfected by UV/chlorine showed the most different composition with any specific taxa (W2) (Fig. 7).

4. Discussion

Our interdisciplinary study in taxa and methodology (bioassays, biomarkers, IBQ and metabarcoding) showed that reclaimed water disinfected by UV/chlorine caused more physiological alterations and greater changes in the community structure of the Llobregat River when compared to the UV-light treatment. Effects of the input from the WWTP were evident in *D. magna*, *H. exocellata* and *L. graellsii*, which have different trophic ecology (Colin et al., 2016a). Potential general multi-trophic impacts in the biological community due to UV/chlorine were suggested by metabarcoding data because the UV/chlorine treatment was significantly related to changes in the total community composition of eukaryotes. Interestingly, this result refused the expectation of a short-term transitory impact of the UV/chlorine treatment given that the treatment caused a severe structural change in the community. In contrast, no major changes were observed in IBQs of diatoms, macro-invertebrates and fishes, all of which are widely used indices in river monitoring (Water Framework Directive 2000/60/EC).

The feeding rates of *D. magna* were sensitive to the discharge of UV/chlorine-treated reclaimed water and its effects were still visible even three months later. This is in line with prior work showing that *D. magna* is a suitable taxon for "ex situ" bioassays to detect the impacts of multiple chemicals present in rivers (McWilliam et al., 2002; Barata et al., 2008; Damásio et al., 2008). Further support for the use of the *D. magna* bioassay is that changes in biomarkers of oxidative stress status in wild individuals of *H. exocellata* were also related to the input of reclaimed UV/chlorine-treated water in the river. Compared to 'non-exposed' individuals of *H. exocellata*, the 'exposed' ones showed low antioxidant defense activities (inhibition of CAT activity), increased anaerobic energy metabolism (increased LDH activity) and altered levels of peroxidized lipids (lower levels of LPO). All three responses provide evidence for tissue damage induced by oxidative stress (Barata et al., 2005a, 2005b; Faria et al., 2009, 2010). *Hydropsyche exocellata* metabolism probably increased energy demand at first instance to deal with the effects of UV/chloride water but its capacity to deal with ROS produced was overcome and then ROS inhibited CAT activity and altered lipid peroxidation (Diamantino et al., 2001; Dotan et al., 2004; Colin et al., 2016a). This sequence illustrates that, in contrast to river monitoring programs based on values of IBQ alone, the use of biomarkers can reveal sub-lethal effects of using reclaimed water in organisms, and may identify the potential mechanisms by which reclaimed water may be affecting the health status of freshwater biota.

Our findings also supported previous data showing that fishes 'exposed' to WWTPs often exhibit increased percentages of circulating neutrophils, which are general indicators of stress in vertebrates (Davis et al., 2008; Maceda-Veiga et al., 2010, 2013). Additionally, we found a clear positive association between reclaimed water input and the percentage of LG-PAS cells, which still have an unclear biological function but some studies suggest it is similar to that of neutrophils (Maceda-Veiga et al., 2015). However, the type of water treatment was not significantly related to the percentages of neutrophils and LG-PAS cells. Furthermore, there was no clear association of body-condition index SMI values with UV/chlorine or UV-light treated water, which indicates that neither of the two treatments caused additional stress in *L. graellsii*. Overall, UV/chlorine treatment probably had mild effects in these fish individuals or had affected tissues other than those examined for this

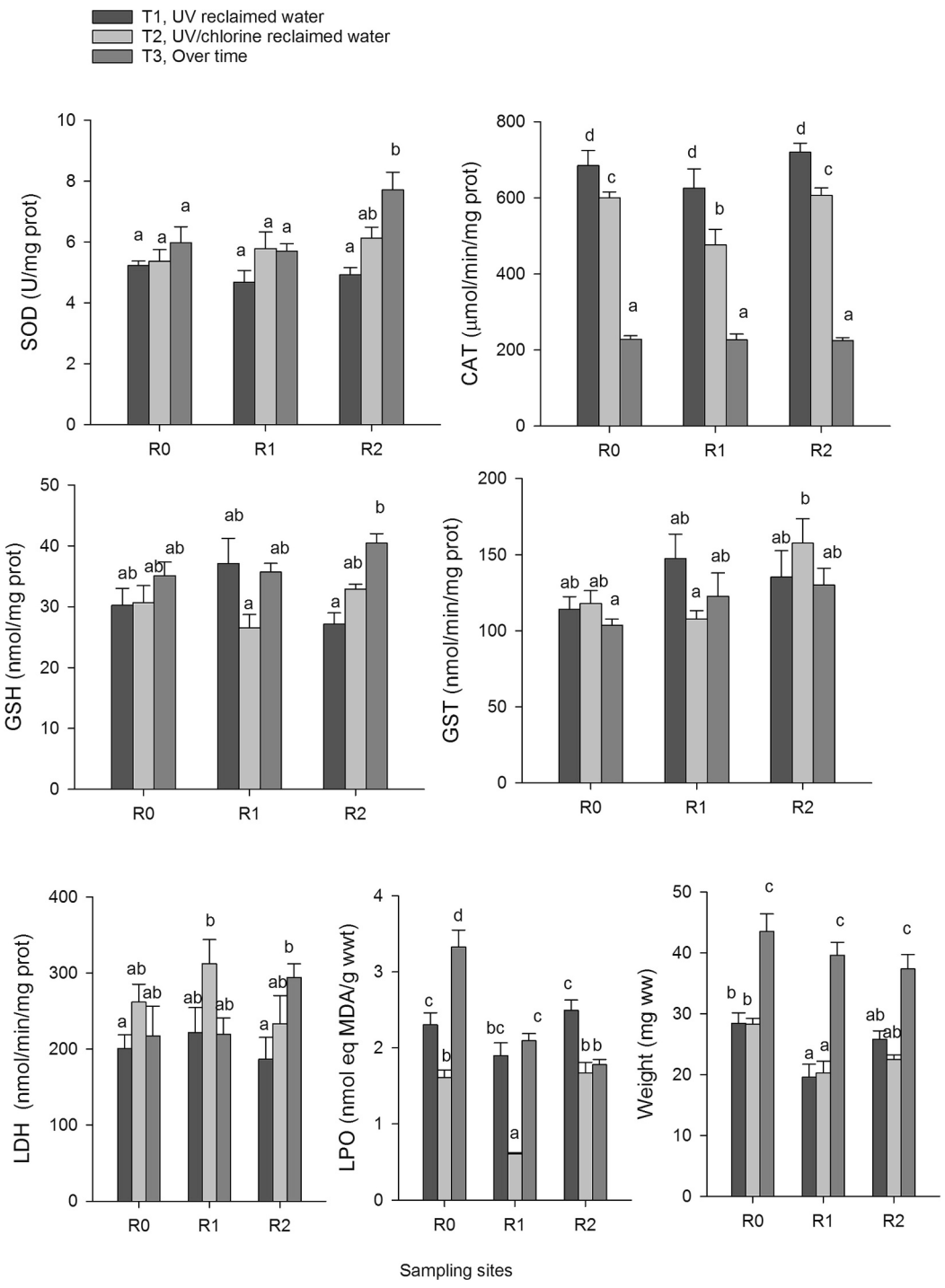

Fig. 3. Changes in biomarkers of oxidative stress in *Hydropsyche exocellata* (mean ± SE, n = 7–10) collected in the river before (R0, 'non-exposed') and downstream (R1 and R2, 'exposed') the discharge site of the wastewater-treatment plant in three periods (T1: UV only, T2: UV/Chlorine and T3: over time). Different letters show differences among samples based on ANOVA analyses followed by Tukey's multiple comparisons.

study (e.g. gills). However, the absence of overt signs of disease (e.g. ulcers, fin erosions, skeletal deformities), and the lack of clear association of erythrocytic nuclear abnormalities with the UV/chlorine or UV-light treated water, suggests a lack of serious health issues in *L. graellsii* attributable to the type of reclaimed water (Maceda-Veiga et al., 2015), at least along this severely human altered river reach (Prat and Rieradevall, 2006; Munné et al., 2023).

Besides identifying physiological effects of UV/chlorine treatment on *D. magna* and *H. exocellata*, our study also reported that the entire community structure of eukaryotes was significantly altered by reclaimed water treatments. Metabarcoding data showed major changes in species composition associated to disinfection treatment, which were not discernible using IBQs of diatoms, macroinvertebrates and fishes. The lack of signal for IBQ was probably because the studied community has a long history of altered hydromorphology and severe water degradation (Prat and Rieradevall, 2006; Munné et al., 2023), and therefore sensitive taxa were already extirpated. For instance, the fish community was dominated by introduced species (*Silurus glanis*, *Cyprinus carpio*, *Lepomis gibbosus*), which generally have a wider tolerance to changes in water quality than have Llobregat native species (e.g. *Barbus*

Table 2
Comparison of biomarker responses of *Hydropsyche exocellata* collected from sites R0, R1 and R2 during the three treatments of reclaimed water. SOD, super-oxide dismutase; CAT, catalase; GSH, reduced glutathione; GST, glutathione S transferase; LDH, metabolism/stress-lactate de-hydrogenase; LPO, Lipid peroxidation. df, degrees of freedom; F, Fisher's coefficient. In bold p-values < 0.05.

	Treatment			Site			Interaction treatment × site		
	df	F	p-Value	df	F	p-Value	df	F	p-Value
SOD	2.56	10.4	**<0.001**	2.56	4	**0.02**	4.56	2.5	0.056
CAT	2.58	167	**<0.001**	2.58	4.9	**0.01**	4.58	1.5	0.217
GSH	2.56	7	**<0.001**	2.56	0.3	0.73	4.56	3.4	**0.016**
GST	2.52	0.9	0.4	2.52	4.4	**0.02**	4.52	1.5	0.205
LDH	2.57	4.2	**0.02**	2.57	0.6	0.56	4.57	2.1	0.094
LPO	2.58	55.4	**<0.001**	2.58	31.6	**<0.001**	4.58	14.2	**<0.001**
Fresh weight	2.58	10.5	**<0.001**	2.58	68	**<0.001**	4.58	1.2	0.304

Table 3
Statistical comparison of fish body condition measurements of *Luciobarbus graellsii* among sampling sites (R0, R1 and R2) during the three treatments of reclaimed water. The body condition measures were the Scaled Mass Index (SMI) based on mass-length relationships and proportions of neutrophils, Large Granulocytic – PAS + cells (LG-PAS) and the Erythrocytic Nuclear Abnormalities (ENAs). In bold p-values < 0.05.

	Treatment		Site		Site × treatment	
	Wald test	p-Value	Wald test	p-Value	Wald test	p-Value
SMI	2.47	0.29	0.08	0.95	1.65	0.79
Neutrophils	7.11	**0.03**	0.79	0.67	1.96	0.74
LG-PAS	10.19	**0.01**	13.41	**<0.001**	19.71	**<0.001**
ENAs	18.04	**<0.001**	0.88	0.64	10.05	**0.03**

haasi) (Maceda-Veiga and De Sostoa, 2011). For macroinvertebrates, IBQ were calculated at the family level and sampled communities had mostly tolerant taxa adapted to water polluted conditions (e.g. Baetidae, Caenidae, Chironomidae, Oligochaeta, (Alba-Tercedor et al., 2004), making difficult to detect the additional effect of the reclaimed water effluent (Prat et al., 2013), which was detected at the species level. Hence, our findings indicate that future studies improving IBQ calculated based on species level identification obtained using metabarcoding data (Pawlowski et al., 2018; Cordier et al., 2019, 2021), could increase the diagnostic power of macroinvertebrate communities once the tolerance ranges of these 'new' species will be established.

Concerning the two techniques of metabarcoding used to detect effects of the different disinfection treatments, communities assigned by these two techniques distinguished the communities impacted by UV-light and UV/chlorine treated water, which were in turn different from communities recorded at the 'non-exposed' site located upstream from the discharge. However, it is relevant to emphasize that differences in community composition captured by bulk sample during discharge of UV/chlorine and UV-light treated water from the community found three months after the treatments (T3) (Fig. 7), indicates that macroinvertebrates communities have changed by the effects of water condition generated by the discharge of reclaimed water, but also by seasonality. For water eDNA samples, communities assigned along the river were clearly different of the communities found at the reclaimed water (i.e., W1, W2 and Dis.). Along the river, the communities found by eDNA at the 'exposed' site R1 were subtly closer of those found in reclaimed water, but in general a small part of the communities in the reclaimed water were also captured in the river. Surprisingly, and despite some overlap in community composition found for eDNA, community composition was different among sites, which is unexpected because the high proximity of sites (~2.5 km). In general, water eDNA captures cells and free DNA from upstream communities at a sub-catchment scale and previous results showed high similarity of communities along the river course (Deiner et al., 2016; Deiner and Altermatt, 2014; Macher et al., 2018). Given most of organisms sequenced by water eDNA are planktonic small, floating organisms that inhabit the water column, our findings suggest that these plankton communities are sensitive to water conditions induced by the discharge of reclaimed water. As a result, our findings suggest communities captured by eDNA are quickly affected and the effects of the reclaimed water downstream are higher than the effects of the homogenization expected by water transportation.

5. Conclusions

Our study reveals the ecological impact of UV/chlorine treated reclaimed water compared to an UV-light treated water in a 3 km stream reach of the Llobregat river, and identifies the potential physiological mechanisms by which UV/chlorine-treated water may be affecting aquatic taxa. We found support for the UV/chlorine treatment reducing feeding intake in *D. magna* and causing oxidative stress in *H. exocellata* but no support for the UV/chlorine treatment causing DNA damage or affecting the immune status or the whole-body condition of *L. graellsii*. Besides effects at the individual scale, our study also showed impacts at the whole-community structure of eukaryotes as quantified by metabarcoding. Changes in species community composition performed better as indicators of impact than conventional IBQs of diatoms, macroinvertebrates and fishes. Based on our findings, the development of new IBQs metrics based on community composition captured by the bulk samples and eDNA metabarcoding are warrant, since they could provide a more accurate diagnosis of human pressures. Our results provide additional empirical data to support the utility of eDNA tools to provide environmental friendly metrics for assessing water quality, which can be especially relevant in these situations when morphological-based biological indexes at the family level are inappropriate because tolerant taxa are abundant and biological responses are at the species taxonomic level. Overall, despite the effects of UV/chlorine-treated reclaimed water on the ecosystem were transitory, we recommend the use UV-light disinfected reclaimed water as a lesser aggressive alternative source of water during episodes of water scarcity.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.169561.

CRediT authorship contribution statement

Cesc Múrria: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. **Alberto Maceda-Veiga:** Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Carlos Barata:** Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Joan Gomà:** Data curation, Formal analysis, Methodology, Writing – review & editing. **Melissa Faria:** Data curation, Formal analysis, Methodology. **Adrià Antich:** Data curation, Formal analysis, Methodology, Writing – review & editing, Software. **Miquel A. Arnedo:** Conceptualization, Funding acquisition, Methodology, Writing – review & editing. **Núria Bonada:** Conceptualization, Funding acquisition, Project

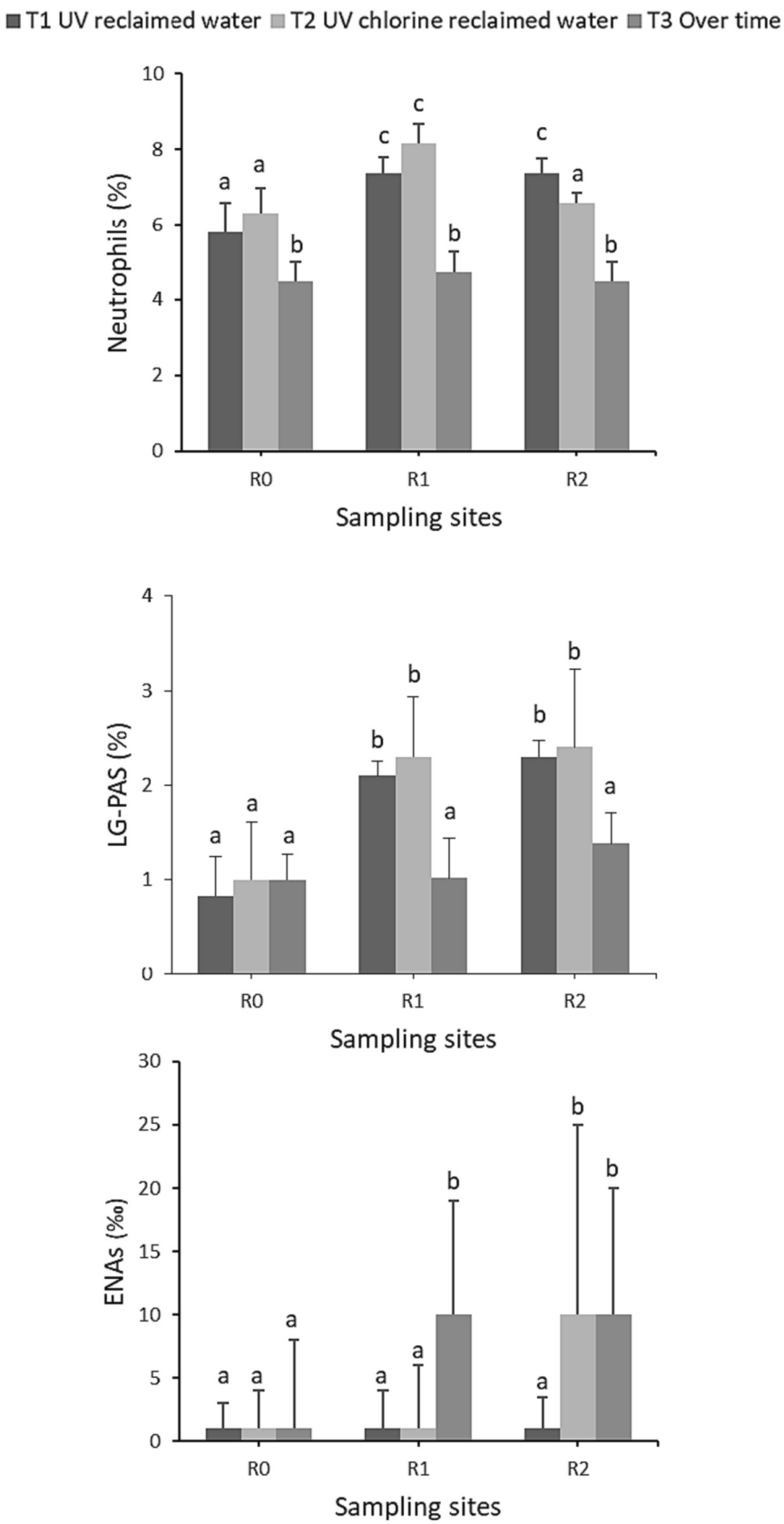

Fig. 4. Changes (mean ± SD) in biomarkers of immune status and overall stress status (white blood cells: % Neutrophils and % Large Granulocytic – PAS + cells) and genotoxic damage (Erythrocytic Nuclear Abnormalities: ENAs %0) in the cyprinid fish *Luciobarbus graellsii* collected in the river before (R0, 'non-exposed') and downstream (R1 and R2, 'exposed') the discharge site of the wastewater treatment plant in three periods (T1: UV only, T2: UV/Chlorine and T3: over time). Letters grouped samples at $p > 0.05$.

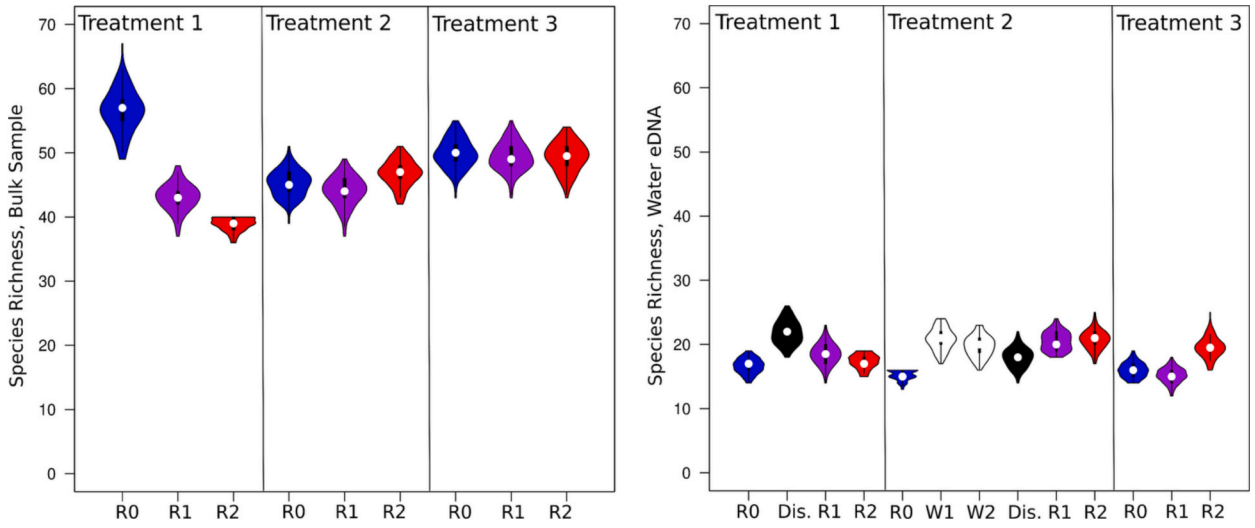

Fig. 5. Mean of species richness (number of taxa) of macroinvertebrates per each replicate per site and sampling for the two molecular methods. In blue, upstream the discharge of the regenerated water (R0, 'non-exposed'); in purple, just after the discharge (R1,'exposed'); in red, downstream (R2, 'exposed').

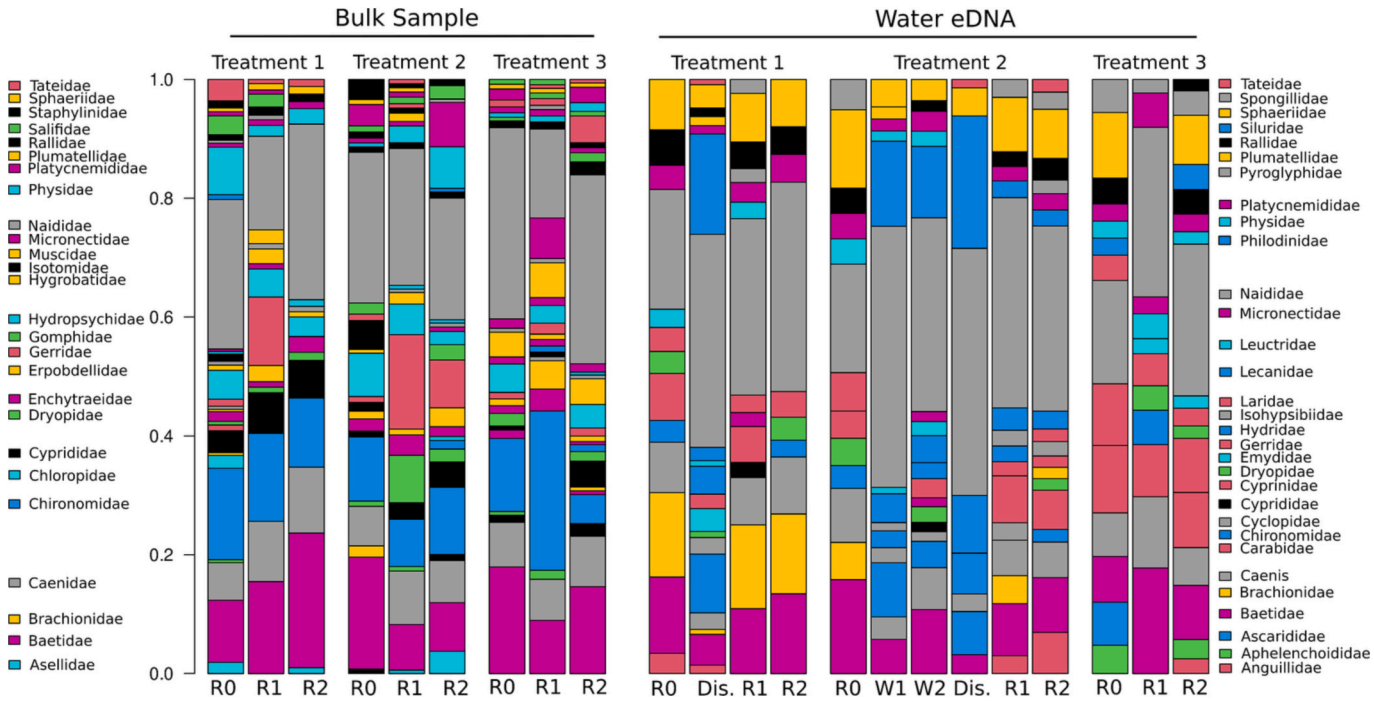

Fig. 6. Relative abundance of families recorded in each site and sampling. The most abundant families are indicated ordered alphabetically separated for bulk sample and water eDNA.

Table 4
Values of richness, Shannon diversity and indices of biotic quality for macroinvertebrates, diatoms and fishes.

	Treatment 1			Treatment 2			Treatment 3		
	R0	R1	R2	R0	R1	R2	R0	R1	R2
Richness (macroinvertebrates)	13	18	13	15	15	15	16	14	16
Shannon diversity (macroinvertebrates)	1.28	1.3	1.24	1.41	0.84	1.26	1.65	1.64	1.63
IBMWP (macroinvertebrates)	39	53	37	48	49	54	48	48	47
Richness (diatoms)	44	42	37	45	42	32	44	51	40
Shannon diversity (diatoms)	2.85	2.86	1.73	2.07	2.28	1.65	1.68	3.08	0.97
IPS (diatoms)	5.4	5.5	7.4	6.6	10	7.7	8.1	5.7	9.6
IBICAT 2010 (fish)	2	3	2.5	2	2	3	2	2	2
IBICAT 2b (fish)	2	2	2	2	2	2	2	2	2

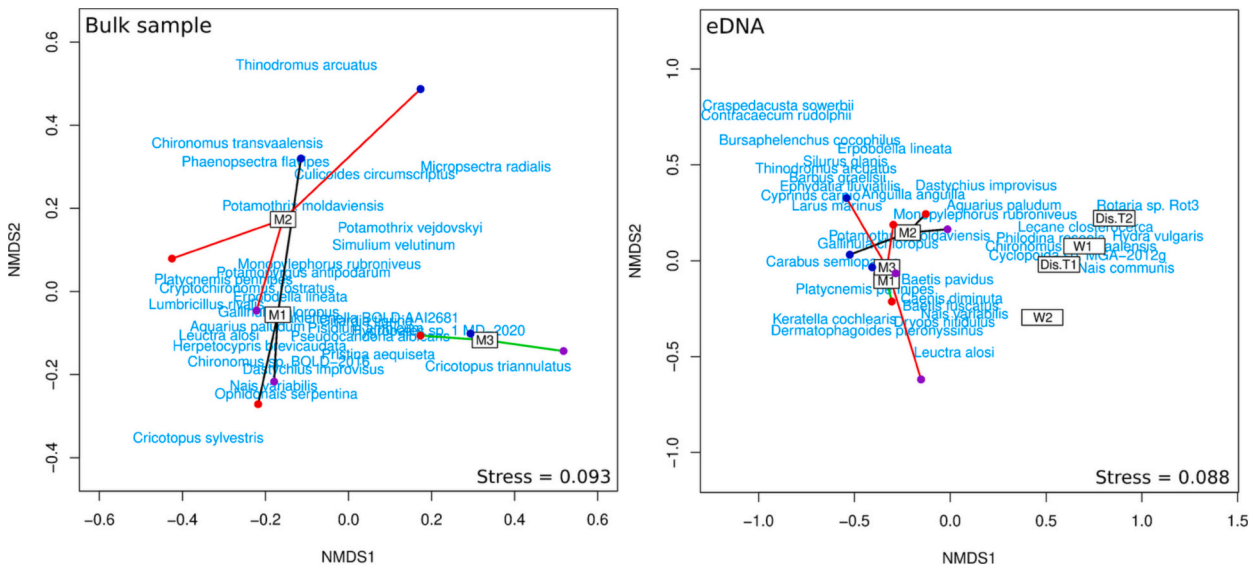

Fig. 7. Results of Multidimensional scaling (MDS) analysis showing the level of composition similarity across sites and treatments for macroinvertebrates bulk sample and water eDNA metabarcoding. In blue, upstream the discharge of the regenerated water (R0, 'non-exposed'); in purple, just after the discharge (R1, 'exposed'); in red, downstream (R2, 'exposed'). All three sampling are indicated as T1, T2 and T3.

administration, Writing – review & editing. **Narcís Prat:** Conceptualization, Data curation, Funding acquisition, Project administration, Writing – review & editing.

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

Data used is available in Annexes 1-3

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