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Diatom responses to sewage inputs and hydrological alteration in Mediterranean streams☆

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ABSTRACT

We analyzed the conjoint effects of sewage inputs and hydrological alteration on the occurrence of teratological forms and on the assemblage composition of stream benthic diatoms. The study was performed in 11 Mediterranean streams which received treated or untreated urban sewage (Impact sites, I), whose composition and morphological anomalies were compared to upstream unaffected (Control, C) sites. The impact sites had high concentrations of ammonium, phosphorus, and pharmaceutical compounds (antibiotics, analgesics, and anti-inflammatories), particularly in those receiving untreated sewage. Impact sites had a higher proportion of teratological forms as well as a prevalence of diatom taxa tolerant to pollution. The differences in the diatom assemblage composition between the paired C and I sites were the largest in the impacted sites that received untreated sewage inputs as well as in the systems with lower dilution capacity. In these sites, the diatom assemblage was composed by a few pollution-tolerant species. Mediterranean river systems facing hydrological stress are highly sensitive to chemical contamination, leading to the homogenization of their diatom assemblages.

1. Introduction

Algal assemblages in rivers are potentially affected by multiple stressors, including chemical contamination, irradiance excess and high water temperatures, as well as hydrological alterations. Mediterranean streams are naturally characterized by periods of hydrological stability, only interrupted by autumnal floods and intermittency during the summer period (Lake, 2003; Sabater and Tockner, 2010). Most climate change scenarios predict low-flow waters to become more pronounced in the next century in the Mediterranean region (Giorgi and Lionello, 2008). Further, rising human exploitation of water resources directly affects river ecosystems (Alcamo et al., 2007). As a result, streams and rivers show temporal and spatial increases of hydrological stability (i.e. more extended and frequent basal flow periods) and even non-natural loss of flowing water or streambed desiccation.

Non-natural conditions of low flow and hydrological stability in human-impacted Mediterranean rivers may be associated to higher concentrations of nutrients, organic matter (Almeida et al., 2014) and organic micropollutants (Sabater et al., 2016). Wastewater discharges of urban origin reach freshwater systems either via wastewater treatment plants (WWTP) or through direct sewage inputs. Wastewaters carry pharmaceutically active compounds (PhACs) together with other micro-contaminants, and organic matter (Gros et al., 2007; Muñoz et al., 2009). These may reach potentially hazardous concentrations (Gros et al., 2007) when entering watercourses with reduced dilution capacity.

The co-occurring chemical stressors and hydrological alterations in Mediterranean river systems produce a conjoint pressure on biological assemblages. Amongst them, the diatoms are suitable ecological indicators of the effects of these stressors, given their high sensitivity to chemical and physical conditions (Pan et al., 1999). Diatoms are siliceous algae which make up the largest fraction of algal assemblages colonizing the streambed. Diatoms may be affected morphologically by different sources of stress, producing teratologies (Cantonati et al., 2014), and their assemblages respond to the stressors by shifting on their composition and relative abundances (Tornés et al., 2007; Sabater et al., 2016). The diatom taxa show specific tolerances to stressors, some (e.g.

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Simonsenia delognei) being able to face harsh conditions during desiccation (Souffreau et al., 2013; Novais et al., 2014; Falasco et al., 2016) and to acclimate to unfavorable hydric conditions (Souffreau et al., 2013), while many others have defined sensitivities to chemical contamination due to nutrients, organic matter and organic micropollutants (e.g. *Nitzschia palea*, *Navicula gregaria*; Tornés et al., 2007; Sabater et al., 2016).

While the separate effects of hydrological and chemical stress on diatom assemblages have been well studied (e.g. Muñoz et al., 2009; Barthès et al., 2014; Teittinen et al., 2015; Piano et al., 2017), their response to the two conjoint stressors is still uncertain (but see Corcoll et al., 2015; Ponsatí et al., 2016). We here analyzed the combined effects of sewage inputs and hydrological alteration on stream benthic diatoms and non-siliceous algae in small and medium-sized Mediterranean rivers. We selected 11 streams receiving treated or untreated sewage discharges from urban point sources. The streams differed on the received chemical impact as well as on their dilution capacity.

We assumed that the potential chemical impact on the diatom assemblages would be a function of the dilution capacity of the receiving system as well as of the type of sewage entering each of the systems. We performed this analysis by comparing the occurrence of teratologic forms and the compositional change of diatom assemblages in the impact sites with respect to their respective upstream (control) sites. We hypothesized (i) that chemical pollution would drive the changes in morphology and taxonomic composition to the downstream sites, (ii) that the lower the dilution capacity of the receiving system, the higher the changes in diatom composition, and (iii) that the most affected impact sites will converge in the composition of their diatom assemblages by favoring the dominance of the pollution-tolerant taxa.

2. Material and methods

2.1. Study sites

The study was conducted in 11 Mediterranean streams tributaries from the lower part of the Ebro River (NE Iberian Peninsula) (Fig. 1). The streams were comparable in terms of geology (mostly calcareous), and ranged between orders 2 to 4. All the sites shared a similar light regime, all being of small to medium order with sparse canopy cover. Most of the rivers were poorly urbanized, mostly having forested and agricultural land uses in their basins. All of them receive strong pressure for the use of their water resources, in the forms of direct water abstraction or groundwater exploitation. We selected two sites (i.e. Control-upstream and Impact-downstream) in each of these systems. The Control (C) sites did not receive direct inputs from human activities in its vicinity, but were submitted to hydrological alteration because of the intensive use of water resources. The Impact (I) sites were immediately downstream to wastewater discharges from urban sources (small cities ranging 540–7000 inhabitants), and were submitted to analogous hydrological alterations than those affecting the C site. The distance between the C and I sites ranged from a few hundred meters in the smaller systems to a few kilometers in the larger systems. The sites were selected not to have tributaries or dams entering or intercepting the stream in between the C and I sites. Some of the sites received treated effluents from WWTP while others directly received untreated urban sewage (Table 1). In the smaller rivers (having the lowest water flow) the sewage outflow represented a moderate increase to the basal water flow but in most of the sites the change was inappreciable (Table 1).

2.2. Physical and chemical measurements

We conducted sampling surveys during early summer (June) of 2015 and spring (April) of 2016. These two sampling periods respectively covered lower-water flow (summer) and higher-water flow (spring) conditions. Water depth, velocity, and instant discharge were measured at each sampling campaign with an acoustic Doppler velocity meter (ADV; Flow Tracker, SonTek Handheld-AD®, P-4077). Water pH, dissolved oxygen, and electrical conductivity were measured *in situ* using hand-held probes at each sampling campaign (WTW, Weilheim, Germany). One water sample for nutrient analyses (nitrate (NO_3^-, μg N·L^{-1}), nitrite (NO_2^-, μg N·L^{-1}), ammonium (NH_4^+, μg N·L^{-1}) and phosphate (PO_4^{3}, μg P·L^{-1})), and dissolved organic carbon (DOC, mg·L^{-1}) were collected at each site, filtered in 0.7 μm GF/F filters (Whatman Int. Ltd., Maidstone, UK) and kept at −20 °C until analysis. Phosphate concentration was determined colorimetrically using a spectrophotometer (Alliance-AMS Smartchem 140, AMS, Frepillon, France), after Murphy and Riley, (Murphy and Riley, 1962). Nitrite, nitrate and ammonium concentrations were determined on a Dionex ICS-5000 ion chromatograph (Dionex Co., Sunnyvale, USA; Hach, 2002). DOC concentrations were determined on a Shimadzu TOC-V CSH coupled to a TNM-1 module (Shimadzu Co., Kyoto, Japan). DOC results were only available for the second sampling campaign, and therefore were not used in the statistical analyses (see below).

Pharmaceutical products were assumed to be the dominant microcontaminants given the urban sources of wastewaters. Their continuous discharge into the aquatic environment makes the PhACs pseudo-persistent contaminants, potentially able to cause adverse effects on living organisms and the environment (Daughton and Ternes, 1999). The PhACs analysis in water samples was conducted following the method developed by Gros et al. (2012). Briefly, the analyses were carried out with an off-line solid phase extraction (SPE) followed by ultra-high-performance liquid chromatography coupled to triple quadrupole linear ion trap tandem mass spectrometry (UHPLC-QqLIT-MS^2). Chromatographic separations were carried out with a Waters Acquity Ultra-Performance™ liquid chromatography system, coupled to a 5500 QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) with a turbo Ion Spray source. Quantification was carried out by isotope dilution. Finally, all data were acquired and processed using Analyst 1.5.1 software, while quantification was carried out by isotope dilution. Detailed information regarding chemicals and reagents used, as well as method performance parameters of target compounds including limits of detections (LODs), limits of quantifications (LOQs) and recovery rates are described in detail in Mandaric et al. (2018).

2.3. Algal (diatom) sampling and analysis

The algal collection was performed simultaneously to the physical and chemical measurements, in June 2015 and April 2016. For the algal collection, at least five stones were randomly collected from the stream bottom in riffle sections of the C and I sites. The substrata were scraped with a knife and a hard bristled toothbrush to fully detach the algal assemblage to a final area of ~50 cm^2. Samples were preserved in 4% formaldehyde until analysis. Each algal sample was partitioned in the laboratory for the taxonomic analysis of diatoms, and for the determination of non-diatom algae and cyanobacteria.

The non-diatom algal fraction was inspected under light microscopy (Nikon Eclipse 80i, Tokyo, Japan) at a magnification of 400×, after performing 50 random microscope fields per aliquot. Taxonomic determination was performed at the genus level and estimated after semi-quantitative analysis based on cell numbers

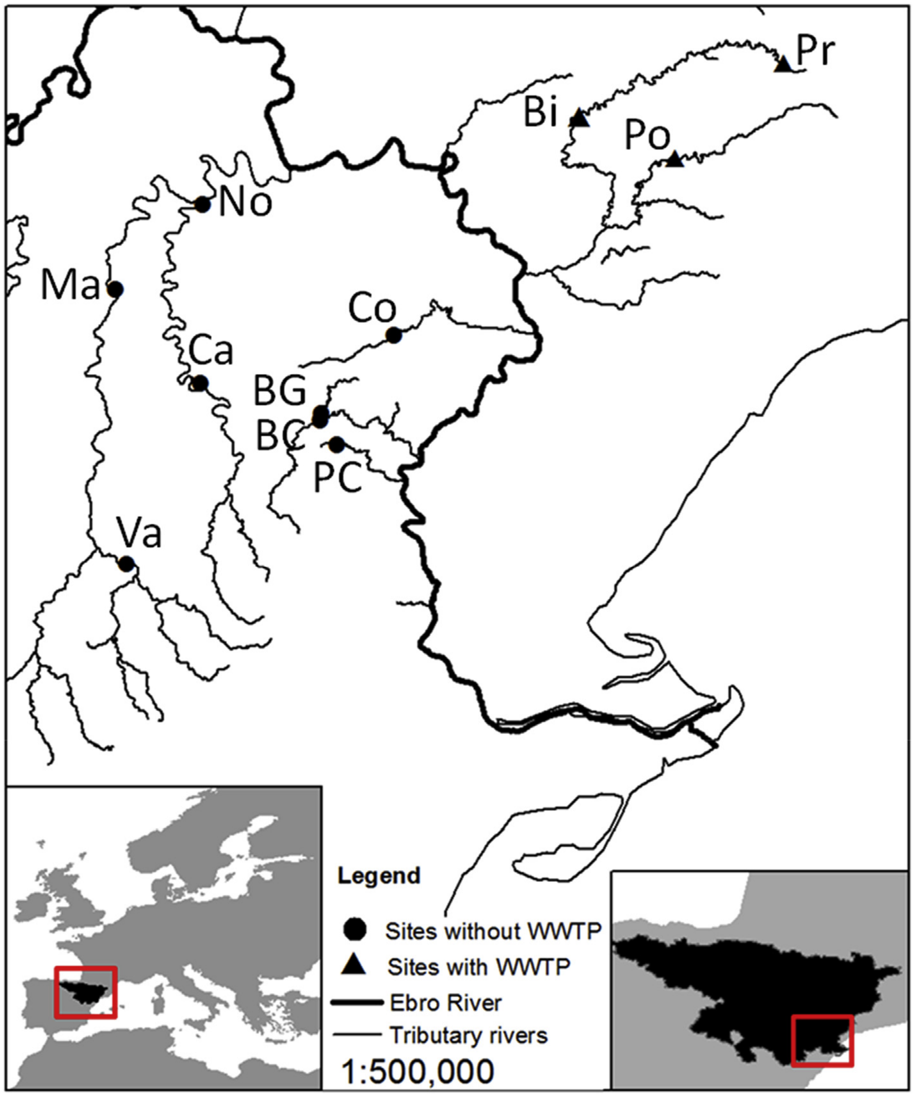

Fig. 1. Map of the sampling locations. Prades (Pr), Bisbal F. (Bi), Poboleda (Po), Maella (Ma), Vallderoures (Va), Nonasp (No), Caseres (Ca), Bot Gandesa (BG), Bot Canaleta (BC), Corbera d'Ebre (Co), Prat de Compte (PC).

and ranked on the following scale: 0.1 (presence), 1 ($\leq$5%), 2 (>5 to $\leq$25%), 3 (>25 to $\leq$50%), 4 (>50 to $\leq$75%) and 5 (>75%). Reference books used for the determination of non-diatom algae and cyanobacteria were those of John et al. (2011) and Wehr and Sheath (2003).

The diatom analysis was performed after cleaning diatom frustules from organic matter. This cleaning was performed in boiling hydrogen peroxide, and cleaned frustules were mounted on permanent slides using Naphrax (r.i. 1.74; Brunel Microscopes Ltd., Chippenham, Wiltshire, UK). Up to 400 valves were counted on each slide by performing random transects under light microscopy (Nikon Eclipse 80i, Tokyo, Japan) using Nomarski differential interference contrast optics at a magnification of 1000×. Diatom taxa were identified according to reference floras (Krammer and Lange-Bertalot (1991a,b), Krammer and Lange-Bertalot (1991a,b, 1997a,b), Hofmann et al., 2011), and complemented through the monographs series of "Diatoms of Europe" and "Bibliotheca Diatomologica". We inspected the samples for the presence of teratological forms for each diatom taxa. Teratological forms are non-adaptive phenotypic abnormalities usually involving anomalous valve outlines or modification in their striation patterns (Falasco et al., 2009a). The teratological forms were recorded and quantified in both C and I sites, and summarized in a single value when different types of deformities occurred for a given taxon. For all types of teratologies we considered from slight to marked teratologies (Fig. S5) following Cantonati et al. (2014) and Lavoie et al. (2017).

2.4. Data analysis

The chemical variability at the sites (C and I) was explored by means of a Principal Components Analysis (PCA). PCA allows summarizing the variables data provided the Principal Components can be identified as descriptors of a given gradient. Candidate physical and chemical variables were nutrient concentrations, conductivity, dissolved oxygen and PhACs concentrations (Tables 1 and S1). Pearson's correlation was performed between the candidate variables, and those strongly correlated to each other (correlation coefficient was >0.8) were unselected to avoid multicollinearity. This resulted in the final selection of phosphate, ammonium, nitrate, conductivity (EC), dissolved oxygen (DO), analgesics/anti-inflammatories and antibiotics. Concentrations of

Table 1
Major physical and chemical variables at sampling locations. C control sites, I impact sites. Numbers in sample labels represent sampling (1, June 2015; 2, April 2016). The annual discharge mean and standard deviation (SD) for each site is provided besides the corresponding discharge of each period. Codes with asterisk (*) correspond to those locations with WWTP upstream of impact sites, while those not showing it received direct (untreated) sewage in the impact sites.

Location	Site	Code	$N\text{-}NH_4$	$N\text{-}NO_3$	$P\text{-}PO_4$	DOC	EC	DO	T	pH	Width	Discharge	Annual discharge (m3/s)		
			(mg/L)	(mg/L)	(mg/L)	(mg/L)	(µs/cm)	(mg/L)	(°C)		(m)	(m^3/s)	Average		SD
Prades	C	Pr1	0.017	1.741	0.012	–	598	7.3	–	7.4	2	0.002	0.004	±	0.003
Prades	I	Pr1*	6.638	1.193	0.825	–	639	3.5	–	7.6	2	0.01	0.018	±	0.012
Bisbal de Falset	C	Bi1	0.008	0.347	0.005	–	546	5.7	22.7	7.5	2	0.007	0.227	±	0.382
Bisbal de Falset	I	Bi1*	0.028	0.269	0.019	–	546	5.5	21.1	7.8	3	0.005	0.216	±	0.369
Poboleda	C	Po1	0.022	0.012	0.016	–	1068	4.3	24	7.9	0.1	0.001	0.032	±	0.048
Poboleda	I	Po1*	3.002	0.39	0.632	–	1122	2.3	22.2	7.4	1	0.001	0.011	±	0.007
Maella	C	Ma1	0.001	7.433	0.002	–	887	11.5	27	8.1	6	0.009	0.329	±	0.6
Maella	I	Ma1	2.637	1.048	0.203	–	1148	9.9	25.8	7.9	4	0.018	0.319	±	0.577
Vallderoures	C	Va1	0.001	2.622	0.003	–	472	8.7	23.3	8.3	10	0.276	0.477	±	0.338
Vallderoures	I	Va1	0.274	2.963	0.025	–	509	7.4	24.2	8.2	12	0.236	0.53	±	0.384
Nonasp	C	No1	0.003	3.498	0.004	–	1031	8.6	25.3	8.1	4	0.036	0.253	±	0.389
Nonasp	I	No1	0.001	3.243	0.017	–	1048	5.6	25.8	7.6	2	0.042	0.317	±	0.503
Caseres	C	Ca1	0.001	8.026	0.002	–	748	6.9	23.7	7.7	7	0.182	0.339	±	0.39
Caseres	I	Ca1	0.001	7.548	0.004	–	750	6.9	23.3	8	14	0.161	0.225	±	0.252
Bot Gandesa	C	BG1	0.006	0.389	0.002	–	2180	8.1	18.7	7.9	2	0.007	0.023	±	0.038
Bot Gandesa	I	BG1	0.931	0.747	0.088	–	2190	7.2	19.6	7.9	2	0.007	0.029	±	0.03
Bot Canaleta	C	BC1	0.001	1.236	0.002	–	1056	7.7	21.4	8	7	0.029	0.068	±	0.07
Bot Canaleta	I	BC1	0.001	1.147	0.002	–	1170	7.3	21.7	7.9	1	0.049	0.077	±	0.072
Corbera d'Ebre	C	Co1	0.013	9.756	0.427	–	2170	7.1	21.2	8.1	2	0.012	0.013	±	0.006
Corbera d'Ebre	I	Co1	1.149	8.84	0.538	–	2130	5.8	21.3	8.1	1	0.02	0.023	±	0.01
Prades	C	Pr2	0.012	2.945	0.011	1.4	605	9.6	14.9	7.9	2	0.006	0.004	±	0.003
Prades	I	Pr2*	2.692	3.198	0.394	6.8	585	7.4	11.1	7.6	3	0.027	0.018	±	0.012
Bisbal de Falset	C	Bi2	0.001	0.112	0.002	3.8	481	10.4	15	7.8	4	0.082	0.227	±	0.382
Bisbal de Falset	I	Bi2*	0.009	0.153	0.008	3.7	481	9.7	14.2	7.8	2	0.068	0.216	±	0.369
Poboleda	C	Po2	0.001	0.018	0.002	1.6	624	10	14.7	7.9	6	0.104	0.032	±	0.048
Poboleda	I	Po2*	0.001	0.044	0.011	1.7	621	10.3	13.2	7.9	2	0.016	0.011	±	0.007
Maella	C	Ma2	0.001	2.991	0.002	1.3	641	10.5	15.2	8.2	9	0.074	0.329	±	0.6
Maella	I	Ma2	0.102	1.507	0.002	3.3	754	4.7	15	7.6	6	0.057	0.319	±	0.577
Vallderoures	C	Va2	0.001	0.489	0.002	0.8	460	12.4	13.6	8.4	7	0.425	0.477	±	0.338
Vallderoures	I	Va2	0.403	0.744	0.023	1.3	482	9.4	13.6	8.1	6	0.448	0.53	±	0.384
Nonasp	C	No2	0.001	1.073	0.002	2.2	944	9.5	18.2	7.9	3	0.133	0.253	±	0.389
Nonasp	I	No2	0.047	1.106	0.014	1.2	862	6	16.9	7.7	3	0.133	0.317	±	0.503
Caseres	C	Ca2	0.001	1.806	0.003	–	620	10.3	15	8.2	16	0.157	0.339	±	0.39
Caseres	I	Ca2	0.009	1.819	0.002	1.8	619	9.9	14.3	8.1	12	0.144	0.225	±	0.252
Bot Gandesa	C	BG2	0.001	0.514	0.002	4	2400	11.8	14.6	8	2	0.001	0.023	±	0.038
Bot Gandesa	I	BG2	0.136	0.532	0.009	10.5	2440	8.9	14.6	8	1	0.016	0.029	±	0.03
Bot Canaleta	C	BC2	0.001	1.147	0.002	3.6	827	8.5	14.2	7.8	4	0.065	0.068	±	0.07
Bot Canaleta	I	BC2	2.688	1.143	0.083	1.7	925	6.8	14.5	7.8	1	0.066	0.077	±	0.072
Corbera d'Ebre	C	Co2	0.019	4.868	0.704	5.5	2560	12.1	12.6	8	4	0.019	0.013	±	0.006
Corbera d'Ebre	I	Co2	2.929	7.573	0.962	11.7	2700	8.2	12.6	8	2	0.035	0.023	±	0.01
Prat de Comte	C	PC2	0.007	3.85	0.005	1.5	1784	9.9	13.3	8	3	0.008	0.009	±	0.011
Prat de Comte	I	PC2	4.362	1.705	0.43	18.9	1764	0	14.7	7.1	2	0.029	0.02	±	0.013

PhACs below method detection or quantification limits were replaced by a value equal to one-half of the method detection or quantification limit. Before performing the PCA the variables were normalized by subtracting the mean and dividing it by the standard deviation.

The ordination of the diatom assemblage composition was performed using the complete diatom taxa list for all the sites, so forth avoiding arbitrary decisions on removal of rare taxa; down-weighting the contributions of rare taxa is an inherent property of the computation of Bray-Curtis similarities (Capone and Kushlan, 1991; Hansen and Ramm, 1994). We analyzed the spatial and temporal patterns of the diatom assemblages' structure by means of Principal Coordinates Analysis (PCO). PCO can be based on any (symmetric) resemblance matrix like non-metrical multi-dimensional scaling and it is a projection of the points onto axes that minimise residual variation in the space of the resemblance measure chosen (Anderson et al., 2008). The PCO was performed on Bray–Curtis similarities of the diatom assemblages in both the C and I sites. Vector overlay was added on the PCO using Spearman correlations to visualize relationships between taxa and ordination axes. Spearman correlations highlight the overall increasing or decreasing relationships of individual taxa across the plot, instead of Pearson correlations that specifically highlight linear relationships. Tolerance to pollution and desiccation of the different diatom taxa were defined using Van Dam et al. (1994).

The diatom assemblages (Bray-Curtis) similarities (derived from the diatom PCO) and the chemical (Euclidean) distances (derived from the chemical variables included in the PCA) were related to each other. The relationship between the chemical and diatom assemblages in the C and I sites for each location was used to define the degree of change in diatom assemblages composition against the degree of change in the chemical variability. AIC (Akaike Information Index) and RSE (Residual Standard Error) were used to select the best fitted regression curves of the diatoms against the chemical characteristics.

The significance of the occurrence frequency of teratological forms in the I sites was tested using a PERMANOVA. The PERMANOVA was based on the Bray–Curtis similarity scores. The PERMANOVA operates on a resemblance matrix and it is similar to traditional parametric MANOVA (Anderson, 2001). Since C and I pair sites were not independent, we considered in the design the factors *location* and *site (control or impact)* nested in *location* as random factors.

The different PCA, PCO, and PERMANOVA (999 permutations) were performed with PRIMER-E 6 v.6.1.11 and PERMANOVA + v.1.0.1 (PRIMER-E Ltd., Plymouth, UK). Analyses were carried out with fourth-root transformed diatom data further converted into a resemblance matrix using Bray–Curtis similarity. Environmental data (except those expressed as ranked variables) were logarithmically transformed (x + 1) before analyses to reduce skewed distributions. Pearson correlations were calculated using the software package IBM SPSS version 21 (IBM Corporation, Armonk, NY, USA). Regressions were conducted using R version 3.4.2 (R Development Core Team, 2013) applying the package *nlme*.

3. Results

3.1. Physical and chemical characteristics

The sites ranged from 0.1 to 16 m width and between 1 and 400 L/s water flow (Table 1). Discharge was lower during summer 2015 (below annual mean discharge) and higher in spring 2016 (higher than the annual mean for each river). Most of the sites showed high water conductivity (EC), particularly Bot Gandesa, Bot Canaleta, Corbera d'Ebre and Prat de Compte. Oxygen concentrations in the C sites averaged 9.1 ± 2.1 mg/L, and nutrient concentrations in these sites were moderate, particularly regarding ammonium (average 5 ± 6 μg/L) and reactive phosphorus (average 4 ± 4 μg/L), but higher in nitrate (average 2.6 ± 1.6 mg/L). As an exception, the C site of Corbera had high P concentrations in the two sampling occasions (427–704 μg/L). DOC concentrations in the second sampling campaign were low to moderate (0.81–4 mg/L) in the C sites, except in Corbera which also had high DOC concentrations (5.51 mg/L). The I sites had lower oxygen concentration (6.8 ± 2.6 mg/L), and much higher nutrient concentrations (1330 ± 1830 μg/L ammonium, 204 ± 301 μg/L reactive phosphorus, 2.2 ± 2.6 mg/L nitrate). The DOC in the I sites (second sampling campaign) ranged from moderate (Poboleda, 1.67 mg/L) to very high concentrations (10.4 mg/L Bot Gandesa and 11.7 mg/L Corbera d'Ebre).

A total of 76 PhACs, belonging to 15 therapeutic groups (Table S1), were detected in the water samples. The highest concentrations of PhACs were of analgesics and anti-inflammatories, antihypertensives, lipid regulators and cholesterol lowering statin drugs, diuretics, psychiatric drugs and antibiotics. Concentrations of all PhACs moved from 6.53 ± 7.92 ng/L in the C sites to up to 151.54 ± 45.98 ng/L for analgesics and anti-inflammatories on treated-sewage WWTP effluents. PhACs in I sites receiving untreated wastewater discharges reached maximum average concentrations of 1.92 ± 1.38 μg/L for analgesics and anti-inflammatories, and 0.14 ± 0.1 μg/L for antihypertensives (Table S1).

The PCA performed with the selected chemical variables (Fig. 2) explained 45.6% of the total variation in its first component. This axis separated C sites (located on the right part) from I sites (expanded from the center towards the left of the graph). Phosphate, ammonium, analgesics/anti-inflammatories and antibiotics were the variables that contributed most to this axis. Some C sites (Corbera d'Ebre) were located closer to the impact sites because of their higher concentrations of phosphates and nitrates. The second axis (17.7% of the total variation) accounted for the relevance of nitrate, EC and DO, the sampling locations of Corbera d'Ebre, Maella, Prat de Compte and Caseres having the highest values, and opposite to the locations of Bisbal de Falset, Prades and Poboleda.

3.2. Non-diatom algal and cyanobacterial composition

Up to 37 non-diatom taxa were determined in the studied streams (Table S2). While most of them occurred both in C and I sites, a few were exclusive of ones or the others. *Euglena* sp. and *Phacus* sp. were only recorded in I sites. The cyanobacteria *Rhabdoderma* sp., *Rhabdogloea* sp. and *Rivularia* sp. were present in C sites, while the rodophytes *Bangia* sp., *Audouinella* sp. and *Batrachospermum* sp. were recorded in the less illuminated sites of the C sites. Coccoid and filamentous cyanobacteria (*Oscillatoria* sp., *Chroococcus* sp., *Merismopedia* sp., *Lyngbya* sp., *Gloeocapsa* sp.) were moderately abundant in C sites, and some of them dominated in the I sites. The zygnematales *Mougeotia* sp., *Zygnema* sp. and *Spirogyra* sp. preferentially occurred in the C sites of the wider river systems (Vallderoures, Caseres, Nonasp), but also occurred in the I sites. The filamentous green algae *Stigeoclonium* and *Ulothrix* occurred mostly in the I sites. The proteobacteria *Sphaerotilus natans* was present in some C sites but produced large masses in several I sites.

3.3. Diatom assemblage composition

Diatoms were the most abundant (60–100%) component of the algal assemblage in all the studied locations and sites. A total of 231 diatom taxa were recorded (Table S3). The dominant taxa in the C sites were *Achnanthidium minutissimum*, *Achnanthidium lineare* and *Encyonopsis minuta*, while *Amphora pediculus*, *Navicula veneta* and *Achnanthidium minutissimum* were the most abundant taxa and occurred in 80% of the I sites.

Teratological forms occurred in both C and I sites but were more frequent in the I sites (Fig. 3; PERMANOVA; pseudo-$F_{11,20} = 1.723$, $P = 0.049$). Five types of Falasco et al. (2009a) teratologies were found in the studied streams (Fig. 4). There was a clear dominance of those affecting valve outline, followed by changes in striation pattern, raphe canal modifications and valves with more than one type of teratology (mixed type). The taxa *Sellaphora nigri*, *Achnanthidium minutissimum*, *Craticula subminuscula*, *Nitzschia frustulum* and *Planothidium frequentissimum* accounted for most of the occurring teratological forms. The presence of the teratological forms could not be attributed to any particular location (PERMANOVA; pseudo-$F_{10,20} = 0.798$, $P = 0.701$).

The PCO which analyzed the ordination of the diatom assemblages separated those in the C and I sites in the first axis (24.9% of total variation; Fig. 5 and Table S4). *Encyonopsis* ssp., *Achnanthidium* spp., *Gomphonema lateripunctatum* and *Sellaphora stroemii* were separated from *Mayamaea atomus* var. *permitis*, *Planothidium frequentissimum*, *Sellaphora seminulum*, *Craticula subminuscula*, *Nitzschia inconspicua* and *Gomphonema parvulum* according to their Spearman correlations with the PCO axes. The former were characteristic of the C sites, while the latter were dominant in the I sites. In some of the C sites (Corbera, Poboleda) pollution tolerant diatom taxa were quite abundant, and they were arranged close to their respective I sites (Fig. 5).

The second axis of the PCO (15.1% of the total variance) did not provide any biologically meaningful ordination of the diatom taxa. However, the third axis (8.6% of total variation) separated the taxa for their hydrological preferences. Taxa regularly recorded in temporary streams (Van Dam et al., 1994) (i.e. *Amphora indistincta*, *Amphora ovalis*, *Nitzschia frustulum*, *Simonsenia delognei* and *Navicula tripunctata*) were arranged on the upper part of the axis, while *Cymbella excisa*, *Nitzschia fonticola*, *Cocconeis pediculus*, *Fistulifera saprophila* and *Navicula reichardtiana* were arranged on the lower side of the axis and they are taxa mainly occurring in permanent watercourses (Van Dam et al., 1994). The samples from the two sampling campaigns were grouped together, indicating a small effect of the different hydrology of the two periods.

The difference in diatom assemblage composition in the I with respect to the C sites was calculated using the respective pairwise similarities accounted for the Bray-Curtis similarity matrix used in

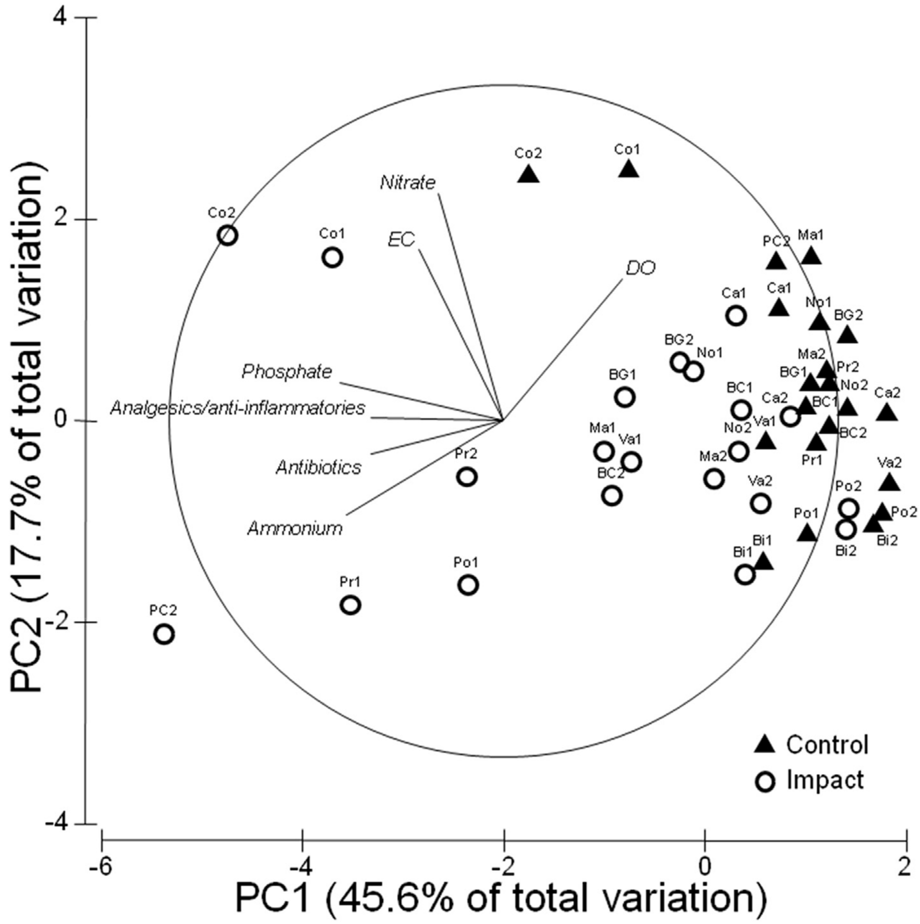

Fig. 2. Principal components analysis (PCA) based on selected chemical variables of all studied streams (including both control and impact sites). The vector length and direction reflects the importance of each variable's contribution to each of the two axes. The circle is a unit circle, whose relative size and centre is arbitrary with respect to the underlying plot. Each vector begins at the centre of the circle and ends at the coordinates (x,y) consisting of the correlations between that variable and each of the PCO axis respectively. Codes correspond to those of Fig. 1 and Table 1. Numbers in sample labels represent sampling (1, June 2015; 2, April 2016).

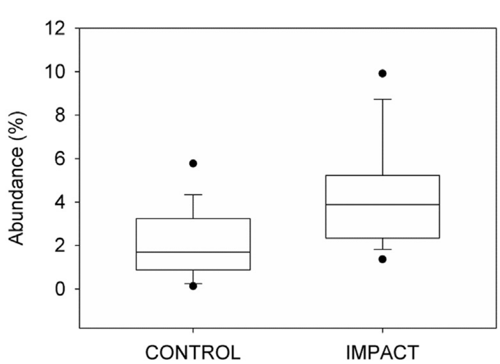

Fig. 3. Percentage of teratological forms in control and impact sites. Boxes represent the median, and 25th and 75th quartiles. 5th and 95th percentiles are also shown with dots.

the PCO (Fig. 6). The I sites receiving treated effluents (i.e. Bisbal de Falset, Poboleda and Prades) had higher similarity to their respective C sites in the two sampling campaigns. The I sites receiving direct sewage inputs but having higher discharges (Vallderoures and Caseres) also presented high similarity to their C sites. The site with the most polluted C samples (Corbera) also showed high similarities to their I samples. The locations with the lowest similarity (and those with the highest impact on their diatom assemblages) were Maella, Bot Gandesa, Nonasp, and Bot Canaleta.

4. Discussion

Overall, the studied set of Mediterranean streams received strong hydrological pressures as well as growing chemical contamination. The impact sites received high concentrations of dissolved organic carbon, ammonium, phosphorus, and pharmaceutical compounds (antibiotics, analgesics, and anti-inflammatories), particularly in those receiving untreated sewage. Concentrations of ammonium and total phosphorus were particularly high in the impact sites (maximum values of 6.64 mg/L and 1.1 mg/L respectively). Pharmaceutical compounds reached maximum concentrations ranging between 2.9 ng/L to 17.5 μg/L (Mandaric et al., 2018). These concentrations were within the range encountered in urban effluents (Gros et al., 2012). The effects of the hydrological and chemical stressors potentially affected the diatom cells as well as their assemblage composition, favoring the contribution of pollution-tolerant taxa.

The diatom taxa were distributed according to their preferences for resistance to desiccation and river size. This was indicated by the third axis of the multivariate ordination analysis (PCO), where taxa occurring in lower discharge (and smaller) sites were categorized as common inhabitants on wet and moist locations (Van Dam et al., 1994), though not as strictly terrestrial taxa. Amongst

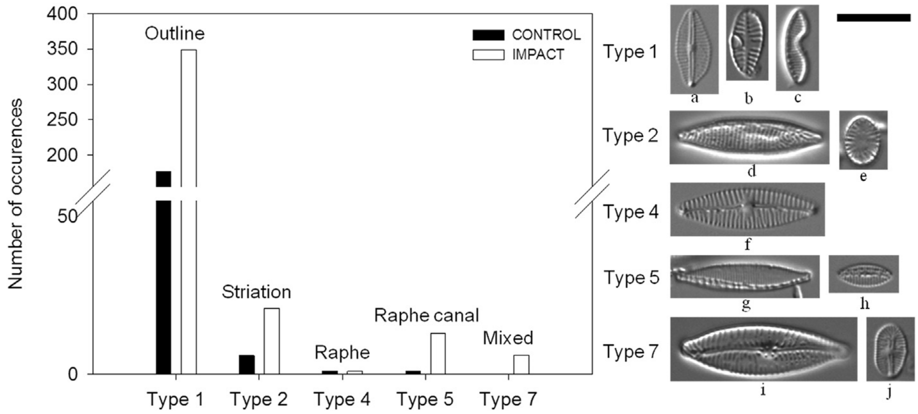

Fig. 4. Left, type of teratologies and their occurrence in C and I sites. Right, examples of the different types of teratologies observed on: (a) *Craticula subminuscula*, (b) *Planothidium frequentissimum*, (c) *Nitzschia frustulum*, (d) *Nitzschia amphibia*, (e) *Planothidium frequentissimum*, (f) *Navicula veneta*, (g) *Nitzschia fonticola*, (h) *Nitzschia inconspicua*, (i) *Gomphonema parvulum*, (j) *Sellaphora nigri*. Scale bar = 10 µm.

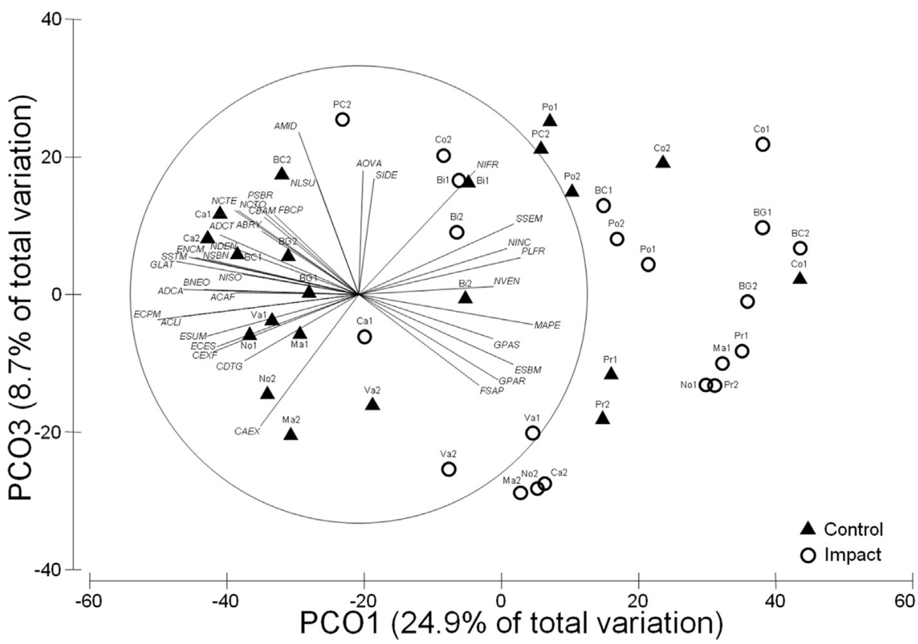

Fig. 5. Principal coordinates analysis (PCO) using diatom assemblages' composition of both control and impact sites, including vector overlay. To improve graphic display, overlay was restricted to include only variables with a vector length greater than 0.5 (See Table S3 for all Spearman correlations). The circle is a unit circle, whose relative size and centre is arbitrary with respect to the underlying plot. Each vector begins at the centre of the circle and ends at the coordinates (x,y) consisting of the correlations between that variable and each of the PCO axis respectively. Site codes correspond to those of Fig. 1 and Table 1. Numbers in sample labels represent sampling (1, June 2015; 2, April 2016). Taxa codes are given in Tables S2 and S3.

these, *Simonsenia delognei* is frequent in exposed habitats (Witkowski et al., 2014), *Amphora indistincta* has been observed in spring-fed streams in Majorca (Delgado et al., 2013) and Sardinia (Lai et al., 2016), and *Amphora ovalis* is common in slow flowing rivers (Levkov, 2009) as well as in spring-fed streams in Majorca (Delgado et al., 2013). On the contrary, the taxa occurring in sites with higher discharges (and larger stream width) are not common in exposed habitats (Van Dam et al., 1994). *Cymbella excisa* and *Nitzschia fonticola* have been indicated as abundant in normal-flow and less common under low-flow conditions (Isheva and Ivanov, 2016). *Navicula reichardtiana* is common in large rivers (Gomà et al., 2005) and in moderately hydrological stable habitats (Tornés and Ruhí, 2013). Other taxa are opportunistic, such as *Fistulifera saprophila*, which is able to thrive under highly to moderately stable hydrological conditions as well as in intermittent sites (Tornés and Ruhí, 2013). This diatom produces mucous films in large quantities (Lange-Bertalot, 2001) and this allows it to adapt to fast current conditions (Wendker, 1992).

Pollution caused, mostly in the small taxa of the genera *Sellaphora, Craticula*, *Achnanthidium*, *Nitzschia* and *Planothidium*, a higher frequency of deformities in their valves. Teratological diatom forms occur because of physical or chemical environmental stress (Lavoie et al., 2017). Unstable environmental conditions, such as wide changes in temperature, light irradiance, or moisture, favor the occurrence of teratological forms (Falasco et al., 2009b). The hydrological severity in our systems could account for valve deformities in some of the C sites, but chemical contamination made the difference between C and I sites and likely drove the higher proportion of deformities. Teratologic diatoms have been observed under different forms of pollution, such as heavy metal

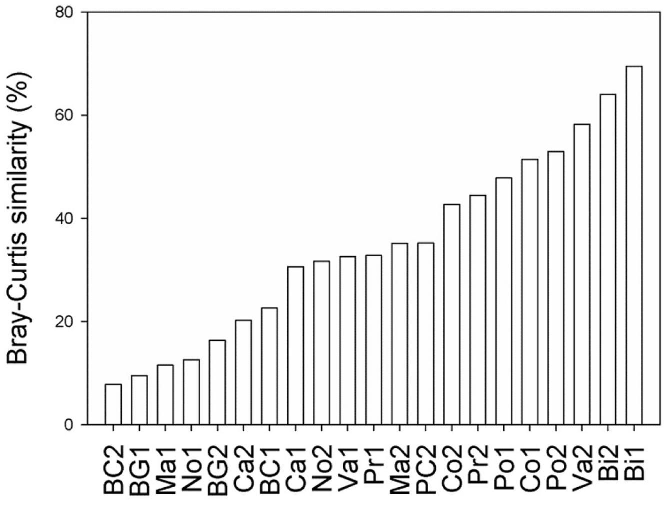

Fig. 6. Bray-Curtis similarity (%) between control and impact sites for each location based on their diatom assemblages. Sites are arranged from left to right in increasing order of similarity. Codes correspond to those of Fig. 1 and Table 1.

contamination (Morin et al., 2008). Even though a clear interpretation of the mechanisms and ecological implications of diatom valve deformities is still unclear (Lavoie et al., 2017), our observations suggest that urban pollution (high abundance of nutrients, organic matter, and PhACs) may reinforce the malformations already occurring under hydrological stress.

The multivariate analysis of the chemical variables indicated the relevance of ammonium, phosphate, antibiotics and analgesics/anti-inflammatories over the other chemical stressors. This chemical contamination likely caused the differences in the diatom assemblage composition between the paired C and I sites, since other environmental factors did not differ between impact and control sites. The differences between the paired C-I sites were the largest in those that received untreated sewage inputs. In those sites a few diatom taxa tolerant to organic and chemical pollution (Tornés et al., 2007) accounted for most of assemblage. Fig. 7 shows the relationship that can be built between the similarity of the diatom assemblages and their corresponding chemical change in the paired C-I sites. This relationship follows a negative power curve, where the higher similarity occurs in the sites showing the lowest chemical difference between C and I, including some C sites affected by upstream chemical pollution (which resembled in their composition to those of their I sites). The power expression also suggests a fast decrease in similarity between the paired C and I sites with higher chemical pollution affecting the impact. In fact, the diatom assemblage in the most polluted sites was largely similar irrespectively of their upstream composition, composed by a few pollution-tolerant species. Goldenberg Vilar et al. (2014) showed that eutrophication favored the decrease in species turnover and the increased homogenization of community composition. In our most polluted systems, the assembled chemical contamination of nutrients, organic matter and microcontaminants (PhACs) was driving the homogenization of the diatom assemblages.

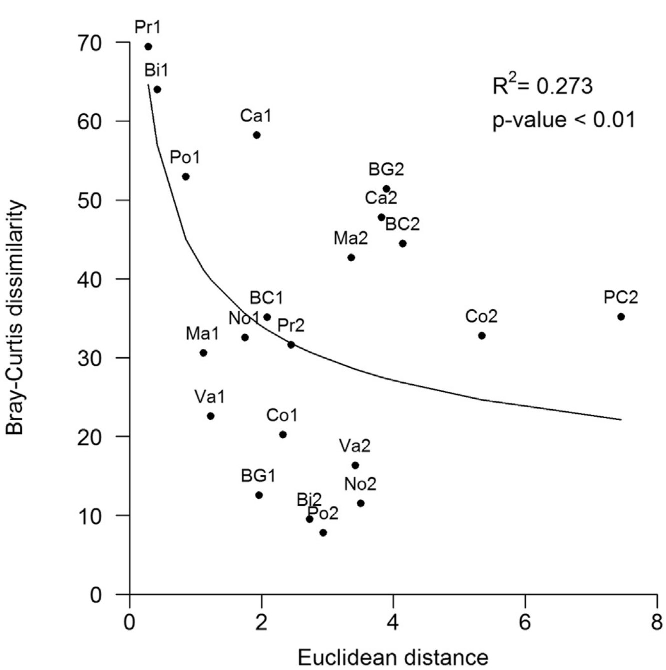

Fig. 7. Relationship between diatom assemblages' (Bray-Curtis) similarities and chemical (Euclidean) distances calculated between control and impact sites for each location. The best fit, the R^2 and the probability of each expression are also indicated.

The dilution capacities of the receiving watercourses (Petrovic et al., 2011) as well as the quality of the sewage entering the systems were determinants of the degree of effect in the diatom assemblage composition. A low dilution capacity not only favors higher contaminant concentrations, but also affects the architecture and function of benthic biofilms (Ponsatí et al., 2016) because of its associated hydrological stability. In our studied set of streams the change in diatom composition between the C and I sites was less pronounced when the latter received treated effluents, and more accentuated when the effluents were untreated (Fig. 8). The potential relation to dilution is suggested by the slightly higher effect (lower similarity) in systems with lower water flow (i.e. lower dilution capacity) (Fig. 8). In the particular set of streams included in this study, where most of them were submitted to water resources exploitation, the extent of the difference between smaller and larger systems is however very limited. We suspect that this difference would be accentuated in rivers not submitted to such strong hydrological pressure than those in our study area.

Our results show that the confluence of chemical pollution (organic matter, nutrients and PhACs) and hydrological alteration enhances the effects of the former on the diatom assemblages. A study performed in temporary streams in Greece showed the prevailing effect of pollution over water stress on diatom assemblage composition (Karaouzas et al., 2018). We also observed that the effects of the two stressors were not as much apparent in non-diatom taxa and cyanobacteria than in diatoms, but some gross effects indeed occurred. Cyanobacteria were the second most abundant taxonomic group in the C sites probably because of their ability to adapt to hydrological alteration using their thick mucilage layer. However, green algae were more common in the I sites, where they showed higher resistance to chemical contamination. An analogous response in the response of algal groups has been observed in a separate experiment under simulated conditions of hydrological stress and pharmaceutical contamination (Serra-Compte et al., 2018).

The effects we described on the structure, composition, and diatom deformitiess, as well as the accompanying results observed in the other algal groups, stress the importance of chemical pollution and altered discharge patterns on the ecological status of Mediterranean streams. The low water flow of Mediterranean streams enhances the potential ecological risk of chemical pollution. Our study revealed that fluvial systems facing hydrological stress, in the way currently occurring in Mediterranean streams, are highly sensitive to chemical contamination and may experience

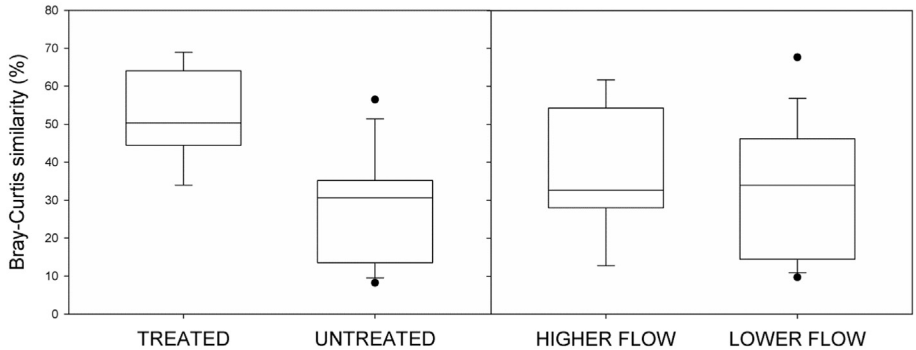

Fig. 8. Bray-Curtis similarity (%) based on diatom assemblages' composition between control and impact sites receiving either treated or untreated wastewater discharges (left), and where the receiving river had higher (>0.05 m^3/s) or lower water flow (<0.05 m^3/s) (right). Boxes represent the median, and 25th and 75th quartiles. 5th and 95th percentiles are also shown with dots.

homogenization in their biological communities. The high susceptibility of these systems to the environmental impact of organic matter, micropollutants and nutrients stresses the relevance of preventing direct sewage inputs to rivers (Muñoz et al., 2009). Particular attention should be placed on adapting management decisions and existing metrics to co-occurring hydrological alteration and pollution (Karaouzas et al., 2018). Focusing on the combined hydrological alteration and chemical contamination is essential to predict potential ecological problems under future climate change scenarios, as well as an essential step to improve the conservation of these fluvial systems.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2018.03.037.

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