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## Impact of wastewater treatment plant discharges on macroalgae and macrofauna assemblages of the intertidal rocky shore in the southeastern Bay of Biscay

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### Abstract :

Rocky intertidal habitats are particularly vulnerable to anthropogenic pressures especially in areas with high urban concentrations such as southeastern Bay of Biscay. This research aims to establish an assessment of the potential impact of sewage discharges on intertidal rocky benthic assemblages on macroalgae and on macrofauna as required by the European Directives (Water Framework Directive - WFD and Marine Strategy Framework Directive -MSFD). The assemblages were sampled at five locations according to a control-impact design. A moderate detectable effect of discharges was highlighted on the assemblage structure by means of multivariate analyses but this was less evident using other biological and ecological metrics. Results would also suggest that benthic macroalgae constitute for the study area the best relevant biotic component to assess the effect of this pressure on the intertidal rocky platform habitats. Changes in the relative abundance of *Ceramium* spp., *Corallina* spp. and *Halopteris scoparia* were mainly responsible of the dissimilarities found. Finally, a pseudo-ecological quality ratio, based on the current WFD metrics, was also calculated for each site within locations (i.e. each distance from the outfall) to assess its sensitivity to this type of pressure. Results were conformed with those of the WFD monitoring because un- or less-impacted sites were ranked as “Good” contrary to the others ranked as “Moderate”. Thus, this work provides additional information for the MSFD and bridges deficiencies emphasized by Directives on the response of biological indicators to various pressures and the biocenosis of southeastern Bay of Biscay.

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## Highlights

► Detectable effects of discharges were highlighted on assemblage structure. ► Macroalgae constituted a relevant biotic component to study impact of WWTP discharges. ► 24 contributors responsible for differences (impacted vs. control) were identified. ► The pseudo-EQR ratio was sensitive to the WWTP pressure.

**Keywords** : bioindicators, impacts, pollution, sewage, Marine Strategy Framework Directive, benthic communities

48 **Introduction**

49 Rocky shore habitats constitute one of the most common environments in coastal areas (Coutinho et  
50 al., 2016). The intertidal zone is a very important part of the coastal ecosystem providing many  
51 services in terms of primary productivity, fisheries and tourism (Seitz et al., 2013). These areas are  
52 governed by particular environmental factors (e.g., hydrodynamics, tides, salinity and temperature  
53 gradients) (Ghilardi et al., 2008) but these coastal habitats are very vulnerable to anthropogenic  
54 pressures (e.g. waste waters, urban runoff, spilled chemicals, overexploitation, invasive species  
55 introduction, habitat fragmentation and destruction) (Becherucci et al., 2016; Crain et al., 2008).

56 Among those pressures, sewage discharges are responsible for nutrient enrichment, turbidity,  
57 increased sedimentation, decreased salinity (Azzurro et al., 2010; Terlizzi et al., 2005) and  
58 contamination (by heavy metals, priority and emerging contaminants) (Costanzo et al., 2001;  
59 Millennium Ecosystem Assessment -MEA, 2005). In this regard, the European Urban Waste Water  
60 Treatment Directive (91/271/EEC) was adopted to protect the water environment from harmful  
61 effects of wastewater discharges (urban and industrial). It constitutes a prerequisite for the  
62 achievement of the objectives within the Water Framework Directive (WFD; 2000/60/EC; EC, 2000)  
63 which aims to attain "good ecological status" of all water bodies by 2020. This obliges politicians to  
64 make additional efforts to increase connections between a given population and wastewater systems  
65 and to improve the running of sewage treatment plants. Monitoring networks also have to be  
66 implemented by scientists and environmental managers to understand benthic communities'  
67 response and to distinguish changes caused by anthropogenic impacts from natural variability  
68 (Veríssimo et al., 2013). Indeed, sewage discharges constitute an important stressor for marine  
69 communities in many intertidal systems around the world (Arévalo et al., 2007; Becherucci et al.,  
70 2016; Borowitzka, 1972; Littler and Murray, 1975; Liu et al., 2007; O'Connor, 2013; Vinagre et al.,  
71 2016a). Depending on their type, source and level, they may have direct or indirect effects on the  
72 environment (Borja et al., 2011). Some studies highlight negative effects such as the alteration of  
73 benthic composition and abundance patterns (Guidetti et al., 2003; Terlizzi et al., 2005, 2002). The

74 consequences may be diverse: a biotic homogenization (Amaral et al., 2018) with a simplification of  
75 community structure through a decrease in macroalgae species richness and abundance (Borowitzka,  
76 1972; Díez et al., 1999; Littler and Murray, 1975), a decrease of pollution-sensitive species (Schnerer  
77 et al., 2013), an increase of pollution-tolerant opportunistic species abundance due to their high  
78 reproductive capacity (Amaral et al., 2018) and a shift from algal-dominated assemblages to  
79 invertebrate-dominated assemblages (Díez et al., 2012). Contaminants released into the  
80 environment may also thereafter be accumulated in biological tissues or cause harmful effects such  
81 as endocrine disruption, behavioral changes, energy metabolism disturbances and genetic responses  
82 (Macdonald et al., 2003). However, other studies did not find an effect of this stressor on species  
83 richness of rocky shores (Archambault et al., 2001; O'Connor, 2013).

84 Over the last decades, large investigations and survey methods have been developed to study  
85 benthic communities of intertidal rocky shores (e.g. Huguenin et al., 2018; Le Hir and Hily, 2005;  
86 Vinagre et al., 2016b, 2016a; Wells et al., 2007; Zhao et al., 2016) in different contexts such as global  
87 climate change prospects (Barange, 2003; Thompson et al., 2002) or ecological status assessment of  
88 water bodies (e.g., WFD) (Borja et al., 2013; Guinda et al., 2014). In addition, the study of  
89 environmental pollution through biotic diversity analyses has become of major importance because  
90 it gives precise information of the deleterious effects of contaminants (Borja et al., 2011). In this  
91 context, and as described by Echavarri-Erasun et al. (2007), effects of sewage discharges have  
92 already been studied on different environmental compartments (e.g. sediments, water body, trophic  
93 web, benthic and pelagic communities). Benthic communities are often used to assess marine  
94 pollutions because they reflect both previous and present conditions to which communities have  
95 been exposed (Reish, 1987).

96 Macroalgae constitute the primary food chain producers and the dominant group on rocky shore  
97 bottoms (Amaral et al., 2018). Because of their sedentary nature and the sensitivity of their  
98 components, they are known to be an accurate bioindicator (e.g., biochemical and physiological) of  
99 environmental changes (e.g. water quality of coastal waters for the WFD (Ar Gall et al., 2016; Borja et

100 al., 2013; Gorostiaga and Diez, 1996). Their assessment is fundamental because their modification  
101 can also alter the trophic structures of other communities (e.g. grazers, carnivorous, scavengers)  
102 (Airoldi et al., 2008; Scherner et al., 2013; Schramm, 1999; Viaroli et al., 2008). Macrofauna has also  
103 to be considered, as it is requested by the Marine Strategy Framework Directive (MSFD; 2008/56/CE;  
104 EC, 2008). The use of mobile macrofauna as an indicator constitutes a “*snapshot in space and time*”  
105 because their community structure respond with short-term variability to environmental changes  
106 (Casamajor (de) and Lalanne, 2016; Davidson et al., 2004; Mieszkowska, 2015; Takada, 1999).  
107 Moreover, sessile species or slightly mobile species cannot redistribute themselves when faced with  
108 disturbances. They are then highly sensitive and constitute the first biological compartment impacted  
109 by environmental stressors (Maughan, 2001; Mieszkowska, 2015; Murray et al., 2006; Roberts et al.,  
110 1998). So, dispersion patterns of sessile macrofauna constitute more precise descriptors of  
111 population dynamics (e.g. recruitment and mortality), community structure, individual performance  
112 (e.g. physiology, morphology and behavior changes) in response to environmental changes  
113 (Mieszkowska, 2015). However, most studies are focused either on the survey of macroalgae or  
114 macrofauna assemblages independently (Anderlini and Wear, 1992; Cabral-Oliveira et al., 2014; Díez  
115 et al., 1999; Souza et al., 2013) and rarely together (Bishop et al., 2002; Echavarri-Erasun et al., 2007;  
116 Littler and Murray, 1975; López Gappa and Tablado, 1990; O’Connor, 2013; Terlizzi et al., 2002;  
117 Vinagre et al., 2016a).

118 The Basque coast (“Bay of Biscay” subregion) displays a set of environmental specificities: mesotidal  
119 conditions, with a magnitude between 1.85 and 3.85 m (Augris et al., 2009), energetic waves (Abadie  
120 et al., 2005), freshwater inputs caused by rainfall and a dense river system (Winckel et al., 2004), N-  
121 NW dominant winds, a specific coast orientation and geomorphology (cliffs, rocky platforms, boulder  
122 fields and semi-enclosed bays with sandy beaches) (Borja and Collins, 2004). In the western Basque  
123 coast (Spanish side), around 90% of the shore is constituted by rocky substrata (Borja and Collins,  
124 2004) whereas in the eastern (French side) it is only 30% (Chust et al., 2009). All those parameters  
125 make this region a heritage area (Augris et al., 2009; Casamajor (de) and Lalanne, 2016) and justify

126 the presence of specific communities in these remarkable habitats (Borja et al., 2004). Thus, rocky  
127 platforms constitute a habitat of European Community importance (High energy littoral rock; EUNIS  
128 A1-1). But, over the last decades, the French Basque coast has been subjected to urban sprawl and  
129 massive summer overcrowding (Le Treut, 2013) which explains the large number of WWTP  
130 (Wastewater Treatment Plant) outfalls along the coast.

131 Studies are scarce and local and are carried out only on the Spanish coastal area on macroalgae (Díez  
132 et al., 2013) and macrofauna (Bustamante et al., 2012) independently. This study therefore aims to  
133 offer a broader and integrated view on the potential impact of these discharges on intertidal rocky  
134 benthic assemblages (macroalgae and macrofauna) in the southeastern Bay of Biscay by comparing  
135 control and impacted locations and sites within locations (i.e. different distances from the outfalls).  
136 The general hypothesis is that if WWTP treatments are efficient, structural parameters of  
137 communities and results based on the WFD monitoring between impacted and control locations  
138 should be similar. This work also provides a framework for future monitoring allowing an assessment  
139 of benthic communities' changes related to WWTP mitigation measures. The interest in studying  
140 both benthic fauna and flora is also discussed in this context.

## 141 **1. Methodology**

### 142 **1.1 Choice of the sampling design**

143 To evaluate the impact of WWTP discharges, a control-impact design was chosen due to the absence  
144 of previous data of benthic assemblages in the impacted locations (before-after design) and models  
145 based on data characterizing the study area under reference conditions. This design is widely used to  
146 study an impact, a perturbation or a stressor on the environment (Murray et al., 2006) and allows  
147 temporal variation to be integrated. Impacted locations (with direct discharges from WWTP) and  
148 control locations (natural conditions) were thus chosen. Control locations were selected by expert  
149 judgment that is to say with similar features to WWTP locations: wave exposure (N-NW) and slight to  
150 moderate slope (<30°).

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## 153 1.2 Study area and sampling locations

154 The study was conducted in the southeastern Bay of Biscay. The field sampling campaign took place  
 155 on intertidal rocky platforms in French and Spanish coastal areas of the Basque coast. The sampling  
 156 was carried out during spring tide periods and in a relatively short period, from March 2<sup>nd</sup> to July 27<sup>th</sup>  
 157 2017 (the same as used within the WFD). A total of five locations were selected (Fig. 1). Three  
 158 locations were potentially impacted by Wastewater Treatment Plants (WWTP): 'WWTP 1' and  
 159 'WWTP 2' in France and 'WWTP 3' in Spain. General information of each WWTP were summarized in  
 160 (Table 1). Two locations were considered as control: 'Control 1' in France and 'Control 2' in Spain.

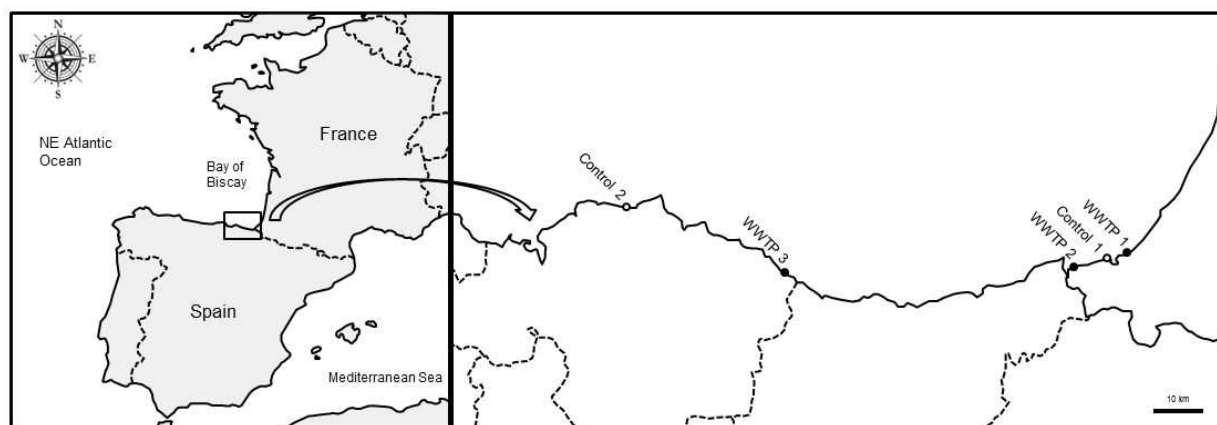
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166 *Fig. 1: Study area and locations: 'Control 1' (France) and 'Control 2' (Spain) (white points) and*  
 167 *'WWTP 1' (France), 'WWTP 2' (France) and 'WWTP 3' (Spain) constitute the impacted locations (black*  
 168 *points).*

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170 *Table 1: General WWTP features*

	<b>WWTP 1</b>	<b>WWTP 2</b>	<b>WWTP 3</b>
Location	France	France	Spain
Population equivalent (PE)	78 217	45 000	27 500
Nominal flow (m <sup>3</sup> /day)	10 450	7 350	5 930
Outfall location	Intertidal zone	Intertidal zone	Intertidal zone

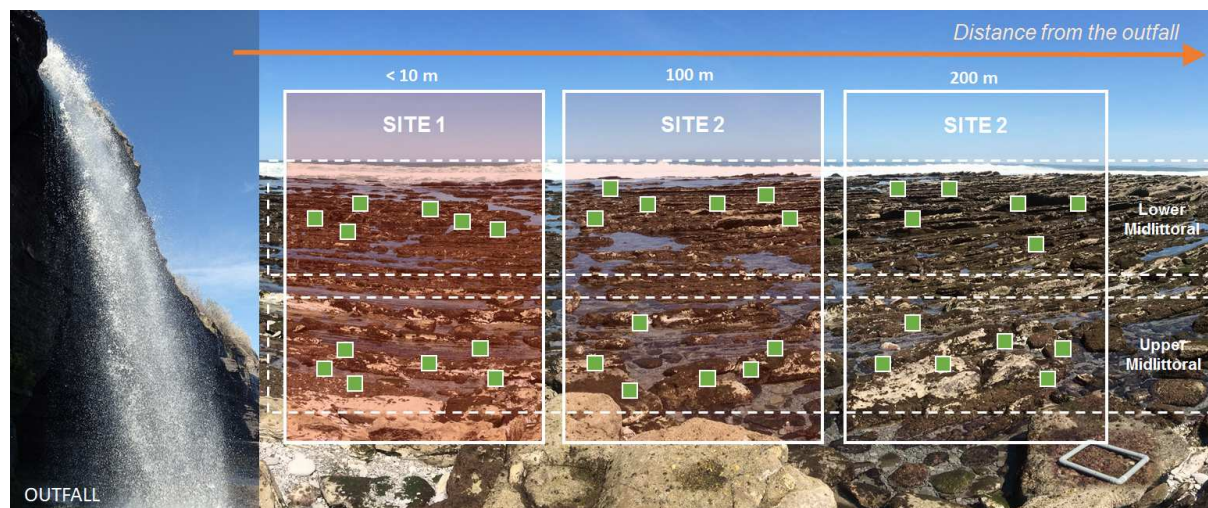
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## 172 1.3 Field data collection strategy

173 Each location was 200 m long and was represented by three sites in order to explore the spatial  
174 variability in a lower spatial scale. The selection of sites was done by means of a random stratified  
175 sampling design (i.e. sites were placed 100 m from each other and within them, the sampling was  
176 done randomly). Within impacted locations, sites corresponded to three distances from the outfall  
177 and were positioned on one side of the outfall, the first one being to a maximum of 10 m from the  
178 discharge. Sites within controls were established along the location maintaining the mentioned  
179 distances (Fig. 2). Within each site, two midlittoral zones were separately sampled: upper and lower  
180 midlittoral zones, characterized by algal-dominated communities described in the WFD "*Corallina*  
181 *spp.* & *Caulacanthus ustulatus*" and "*Halopteris scoparia* & *Gelidium spp.*" (Ar Gall et al., 2016). In  
182 each site, a set of six randomly selected surfaces (33 x 33 cm quadrats) were positioned on  
183 comparable substrata (stable substrate and continuous bedrock) avoiding special microhabitats  
184 (crevices and pools) and separated by at least 1 m. The random sampling design ensures  
185 independence of errors and allows samples to be considered as replicates (Murray et al., 2006). In  
186 each quadrat, the percentage cover of macroalgae and sessile macrofauna (e.g. hexacorallia,  
187 mussels, barnacles, ascidiacea, etc.) was visually estimated and the abundance of mobile or slightly  
188 mobile macrofauna (e.g. gasteropods, crustaceans or limpets) was counted. This size quadrat is the  
189 same as those used for the WFD sampling and allows for direct comparison with others studies (Ar  
190 Gall et al., 2016; Casamajor (de) et al., 2016; Huguenin et al., 2018). Most organisms were identified  
191 *in situ* at species level to limit the sampling impact. When identification was impossible in the field  
192 (especially for small species), specimens were taken to the laboratory for further identification by  
193 taxonomic specialists. Due to the complex taxonomy of certain taxa, some organisms were identified  
194 at genus level (Huguenin et al., 2018).

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Fig. 2: Schematic layout of the sites in each location and midlittoral zone.

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#### 1.4 Statistical analyses

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The variation on the species composition and abundance (community structure) was studied by means of PERMANOVA analysis (Permutational multivariate analysis of variance using distance matrices; with 999 permutations) with pairwise post hoc tests (Anderson, 2001) using the standardized data set with an *a priori* chosen significant level of  $\alpha = 0.05$ . For each midlittoral zone, statistical analyses were carried out separately for both areas (French and Spanish Basque coast), as a consequence of differences in the geomorphology (abrasion platforms vs. sloped platforms, respectively) and hydrodynamics (higher in the Spanish side). 'WWTP' within the French area were also studied separately as they have different features (Table 1). Therefore, each of the three 'WWTP' was compared with their corresponding 'Control'. Two factors were considered: (i) location (fixed, 2 levels) and (ii) sites (fixed and nested in location, 3 levels, representing increasing distances from the outfall in the case of impacted locations) with 6 random replicate samples. To avoid problems with unidentified species, analyses were conducted on aggregated data containing mixed taxonomic levels (species, genus, family, class). Data were standardized (e.g. each counting value, for

213 one taxon, was divided by the maximum reached by this taxon in order to avoid differences in  
214 sampling units (percentage vs. abundance).

215 In order to explore the structure of benthic assemblages among locations (impacted and control) and  
216 within each location (different sites), a non-metric multi-dimensional scaling (nMDS) (after a distance  
217 matrix calculation) and a cluster analysis, based on Bray-Curtis dissimilarity, were conducted. These  
218 tools are useful in benthic marine community studies as they define the relative (dis)similarity  
219 between samples in multidimensional space (in two or more dimensional plots according to a  
220 number of reduced dimensions  $k$  defined by a degree of stress). nMDS does not use the absolute  
221 abundances of species in communities, but rather their rank distances (Clarke and Warwick, 2001;  
222 Murray et al., 2006). To identify the important contributors to differences among assemblages, the  
223 SIMilarity PERcentage (SIMPER) analysis was used (Oksanen et al., 2013). It enables the identification  
224 of taxa which contribute (according to their abundance) to the dissimilarity between locations and  
225 sites within each midlittoral zone.

226 Apart from the community structure, the mean abundance and the total taxonomic richness were  
227 calculated for each ecological group (macroalgae, mobile and sessile macrofauna). The mean  
228 taxonomic richness (MTR) was also calculated for each sample for macrofauna, for macroalgae and  
229 for characteristic and opportunistic taxa, in order to calculate the characteristic/opportunistic MTR  
230 ratio. For the MTR of macrofauna, species were assigned to one of five Ecological Groups (EG I-V)  
231 according to their responses to natural and man-induced changes in water quality: the higher the  
232 group, the higher the tolerance to pollution (Borja et al., 2000). The classification of macroalgae into  
233 characteristic or opportunistic algae was done according to Ar Gall et al. (2016) for French locations  
234 and Juanes et al. (2008) for Spanish ones. The spatial variability of the mean taxonomic richness was  
235 studied by means of PERMANOVA analysis (Permutation analysis of variance; with 999 permutations)  
236 with pairwise post hoc tests (Anderson, 2001) using raw data considering the two factors and the  
237 design mentioned above.

238 The graphs and statistical analyses were undertaken using Excel v7<sup>®</sup> and R<sup>®</sup> software.

## 239 1.5 Ecological quality

240 The quality index, achieved using the “intertidal macroalgae” WFD protocol (Casamajor (de) et al.,  
 241 2016), was calculated for each location to assess its sensitivity to the pressure (Table 2). The WFD  
 242 protocol was based on the Spanish CFR index (Guinda et al., 2008) and it was firstly adapted to  
 243 Brittany by Ar Gall and Le Duff (2007). Then, it was adapted to the Basque coast by Casamajor (de) et  
 244 al.(2010) due to a greater number of warm water species, the absence of large fucoids and a lower  
 245 number of algal belt on the Basque coast. It constitutes a simplified version of the CCO index (Cover  
 246 Characteristic - Opportunistic species; Ar Gall et al., 2016). The final rating of the index used in this  
 247 study (on 1 point) was based on the sum of three subindices: (i) the global cover of macroalgae  
 248 communities [C] (rated on 0.40 points), (ii) the number of characteristic species [N] (rated on 0.30  
 249 points) and (iii) the cover of opportunistic species [O] (rated on 0.30 points) (Casamajor (de) et al.,  
 250 2016). This quality index was also calculated for each distance from the outfall of impacted location.  
 251 It was called “pseudo-index” because it was calculated on only 12 quadrats (6 per midlittoral zone)  
 252 randomly sampled during the campaign, as opposed to 18 (9 per midlittoral zone) in the WFD  
 253 protocol.

254 *Table 2: Ecological quality according to the CFR index.*

<b>Score</b>	<b>Ecological quality</b>
0.80 - 1	<i>Very good</i>
0.60 - 0.79	<i>Good</i>
0.40 - 0.59	<i>Moderate</i>
0.20 - 0.39	<i>Poor</i>
0 - 0.19	<i>Bad</i>

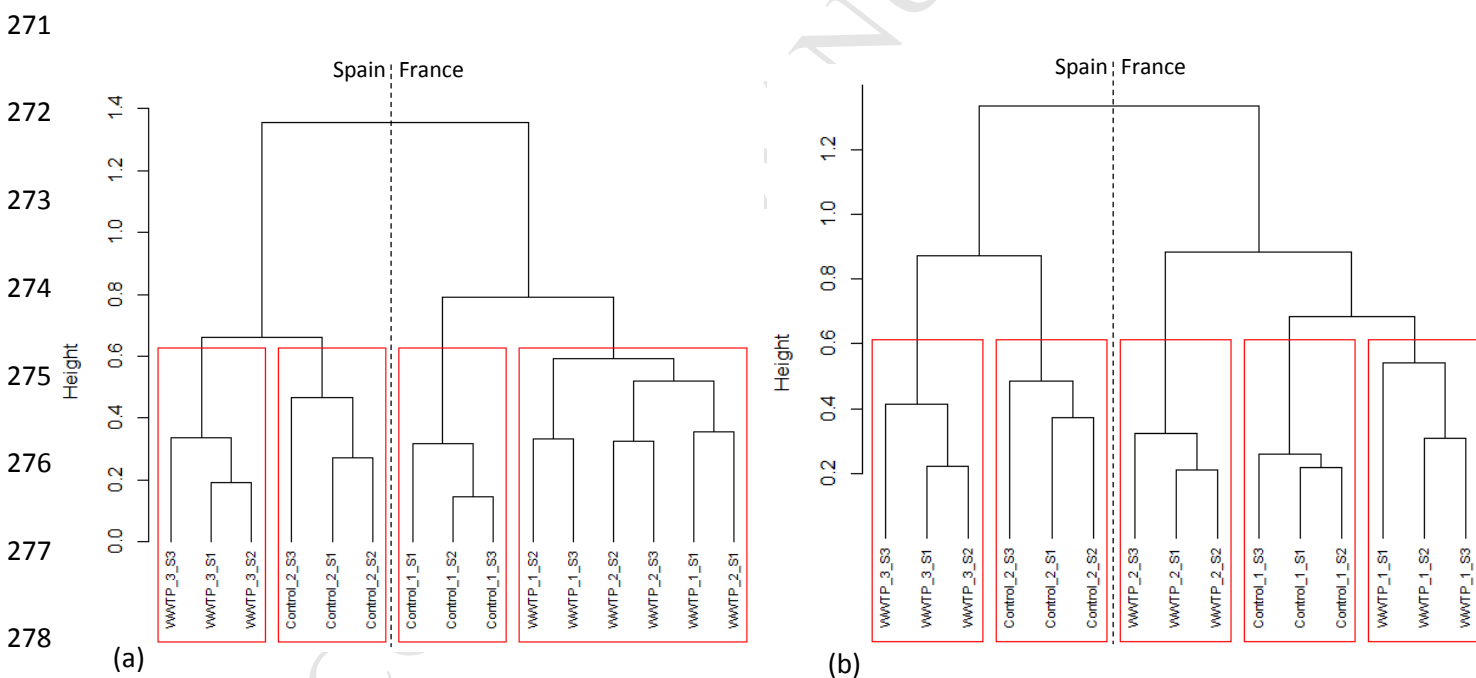
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256 **2. Results**

## 257 2.1 Effects of WWTP discharges on the structure of intertidal rocky benthic assemblages

258 Taking into account the whole study area, benthic assemblages differed in relation to coastal stretch  
 259 (French or Spanish) and locations (WWTP and control) for both midlittoral zones (Fig. 3;  
 260 Supplementary materials 1).

261 The analyses showed significant differences between each WWTP and their respective control  
 262 (PERMANOVA,  $p < 0.05$ ; Table 3). Furthermore, the analyses also detected significant variability at a  
 263 lower scale (sites) in all three cases and for both midlittoral zones (Table 3). Post hoc pairwise  
 264 comparisons revealed significant differences between almost of all the distances from the outfall  
 265 within the French impacted locations ('WWTP 1' and 'WWTP 2') in both midlittoral zones (Table 4). In  
 266 contrast, no such obvious differences were found in the control location ('Control 1') (only  
 267 differences between sites at the upper level). In the Spanish area the variability among sites was  
 268 slightly higher (at both midlittoral zones) in the control ('Control 2') than the impacted one ('WWTP  
 269 3') (Table 4). Differences at the site level were also supported by nMDS and dendrograms which also  
 270 showed clear distinctions between them (Supplementary materials 2; Fig. 3).



279 *Fig. 3: Cluster analysis dendrograms computed on benthic taxon assemblages (macroalgae and*  
 280 *macrofauna) in the upper midlittoral zone (Corallina spp. belt) (a) and the lower midlittoral zone*  
 281 *(Halopteris scoparia belt) (b) of French and Spanish impacted ('WWTP 1', 'WWTP 2', 'WWTP 3') and*  
 282 *control locations ('Control 1', 'Control 2').*

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286 *Table 3: Summary of PERMANOVA results testing for effects of presence of sewage discharges on*287 *benthic assemblages between impacted and control locations ('WWTP 1'/'Control 1' (a), 'WWTP*288 *2'/'Control 1' (b), 'WWTP 3'/'Control 2' (c) in both midlittoral zones.*

(a) 'WWTP 1'/'Control 1'	Df	MeanSqs	F.Model	R2	Pr(>F)	Significance
Upper midlittoral zone						
Locations	1	2.93519	27.8767	0.19025	1.00E-04	***
Locations/Sites	4	0.59622	5.6626	0.15458	1.00E-04	***
Residuals	96	0.10529	0.65517			
Lower midlittoral zone						
Locations	1	2.3794	13.3604	0.11347	1.00E-04	***
Locations/Sites	4	0.50673	2.8453	0.09666	1.00E-04	***
Residuals	93	0.17809	0.78986			
(b) 'WWTP 2'/'Control 1'	Df	MeanSqs	F.Model	R2	Pr(>F)	Significance
Upper midlittoral zone						
Locations	1	4.1775	34.2	0.19055	1.00E-04	***
Locations/Sites	4	0.7719	6.319	0.14084	1.00E-04	***
Residuals	120	0.1221	0.66861			
Lower midlittoral zone						
Locations	1	4.6721	23.5977	0.15924	1.00E-04	***
Locations/Sites	4	0.3758	1.8983	0.05124	0.0011	**
Residuals	117	0.198	0.78952			
(c) 'WWTP 3'/'Control 2'	Df	MeanSqs	F.Model	R2	Pr(>F)	Significance
Upper midlittoral zone						
Locations	1	1.05628	8.1187	0.12313	1.00E-04	***
Locations/Sites	4	0.31941	2.4551	0.14893	2.00E-04	***
Residuals	48	0.1301	0.72795			
Lower midlittoral zone						
Locations	1	2.41452	17.8223	0.22095	1.00E-04	***
Locations/Sites	4	0.50256	3.7096	0.18396	1.00E-04	***
Residuals	48	0.13548	0.59509			

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297 *Table 4: Summary of pairwise post hoc results testing for effects of presence of sewage discharges on*  
 298 *benthic assemblages between sites within each location ('WWTP 1' (a), 'WWTP 2' (b), 'WWTP 3' (c),*  
 299 *'Control 1' (d), 'Control 2' (e)) in both midlittoral zones.*

300	(a)	Upper midlittoral zone		WWTP 1		Site 2	Site 3	Site 2	Site 3
		WWTP 1	Site 2	Site 1	Site 2				
301	(c)	Upper midlittoral zone		WWTP 3		Site 2	Site 3	Site 1	Site 2
		WWTP 3	Site 2	Site 1	Site 2				
302	(d)	Upper midlittoral zone		Control 1		Site 2	Site 3	Site 1	Site 2
		Control 1	Site 2	Site 1	Site 2				
303	(e)	Upper midlittoral zone		Control 2		Site 2	Site 3	Site 1	Site 2
		Control 2	Site 2	Site 1	Site 2				
304	(b)	Lower midlittoral zone		WWTP 1		Site 2	Site 3	Site 1	Site 2
		WWTP 1	Site 2	Site 1	Site 2				
305	(b)	Lower midlittoral zone		WWTP 2		Site 2	Site 3	Site 1	Site 2
		WWTP 2	Site 2	Site 1	Site 2				
306	(c)	Lower midlittoral zone		WWTP 3		Site 2	Site 3	Site 1	Site 2
		WWTP 3	Site 2	Site 1	Site 2				
307	(d)	Lower midlittoral zone		Control 1		Site 2	Site 3	Site 1	Site 2
		Control 1	Site 2	Site 1	Site 2				
308	(e)	Lower midlittoral zone		Control 2		Site 2	Site 3	Site 1	Site 2
		Control 2	Site 2	Site 1	Site 2				

## 2.2 Identification of contributors to differences in assemblages structure

304 SIMPER analyses identified, per each midlittoral zone, taxa responsible for differences between  
 305 impacted and control locations and between sites within each location. Very few macrofauna  
 306 species/taxa appeared in the analyses, consequently only macroalgae taxa identified as significant  
 307 contributors were listed in Supplementary materials 3.

308 In both areas and midlittoral zones, the global dissimilarity between impacted and control locations  
 309 varied from 42% to 71.14% (Table 5). The highest dissimilarity was obtained between 'WWTP 2' vs.  
 310 'Control 1' with 53.81% in the upper zone and 71.14% in the lower zone. The lowest occurred  
 311 between 'WWTP 3' vs. 'Control 2' with 42% and 52.54%, respectively. At a lower scale, the highest  
 312 global dissimilarity always appeared between S1 vs. S3 (the furthest sites) within impacted locations.  
 313 Within control locations, a global dissimilarity between sites was also observed but higher values  
 314 were either between S1 vs. S2 or between S2 vs. S3.

315 Table 5: Summary of global dissimilarities between 2 groups from SIMPER analyses (i.e. between  
 316 impacted and control locations and between sites within each location) for both midlittoral zones  
 317 (upper and lower).

		Global dissimilarity (%)	
		Upper midlittoral zone	Lower midlittoral zone
<b>WWTP 1' vs. 'Control 1'</b>		<b>51.12</b>	<b>63.58</b>
<b>WWTP 2' vs. 'Control 1'</b>		<b>53.81</b>	<b>71.14</b>
'WWTP 1'	S1 vs. S2	43.62	52.00
	S2 vs. S3	39.45	41.41
	S1 vs. S3	52.74	56.96
'WWTP 2'	S1 vs. S2	49.57	56.88
	S2 vs. S3	49.17	55.08
	S1 vs. S3	52.93	57.36
'Control 1'	S1 vs. S2	42.41	56.27
	S2 vs. S3	40.95	61.04
	S1 vs. S3	41.89	57.44
<b>WWTP 3' vs. 'Control 2'</b>		<b>42.00</b>	<b>52.54</b>
'WWTP 3'	S1 vs. S2	31.16	33.44
	S2 vs. S3	33.08	50.08
	S1 vs. S3	36.56	51.28
'Control 2'	S1 vs. S2	32.65	30.99
	S2 vs. S3	40.26	46.28
	S1 vs. S3	37.47	45.78

318

319 In the French area, among species/taxa identified in the upper midlittoral as significant contributors  
 320 to the dissimilarity between impacted and control locations, only *Ceramium* spp. had a contribution  
 321 higher than 10%, being more abundant in 'WWTP 1' than in 'Control 1' (2.07 vs. 0.38)  
 322 (Supplementary materials 3). Furthermore, species such as *Laurencia obtusa* and *Osmundea*  
 323 *pinnatifida* were more abundant in 'Control 1' than in 'WWTP 1' and 'WWTP 2', despite their  
 324 contribution was lower than 10%. Actually, *Osmundea pinnatifida* was not present in 'WWTP 1'. In  
 325 the lower midlittoral zone, the significant contributors (>10%) to the dissimilarity between impacted  
 326 and control locations were *Halopteris scoparia* and *Ceramium* spp. (Supplementary materials 3). The  
 327 former was more abundant in 'Control 1' than in 'WWTP1' (2.62 vs. 1.07) and 'WWTP 2' (2.62 vs.  
 328 0.15), whereas the latter showed higher values in 'WWTP 1' comparing to 'Control 1' (2.40 vs. 0.22).  
 329 With a contribution below 10%, it should be highlighted the absent and the lower abundance of  
 330 *Cystoseira tamariscifolia* in WWTP 1 and WWTP 2 (average abundance of 0.20), respectively,  
 331 comparing to Control 1 (average abundance of 0.78). In the Spanish area, only *Corallina* spp. showed



332 a contribution higher than 10% in the upper midlittoral zone (Supplementary materials 3). The  
333 abundance of this species was higher in 'WWTP 3' than in 'Control 2' (4.56 vs. 3.06). Furthermore,  
334 with a contribution below 10%, *Halopteris scoparia* showed slightly higher abundance in 'Control 2'  
335 comparing to 'WWTP 3' (0.50 vs. 0.03). Regarding the lower midlittoral zone, *Chondria coerulescens*  
336 was the species that contributed most (9.63%) to the dissimilarity between 'Control 2' and 'WWTP 3',  
337 being more abundant in the former location (1.44 vs. 0.03).

338 Focusing on the upper midlittoral zone and within French 'WWTP' locations, *Caulacanthus ustulatus*  
339 was a significant contributor (>10%) in 'WWTP1' (Supplementary materials 3). The abundance of this  
340 species was higher in Site 1 (close to the outfall), decreasing towards Site 3. Within 'WWTP 2',  
341 *Ceramium* spp., *Corallina* spp. and *Laurencia obtusa* showed contributions higher than 10%  
342 (Supplementary materials 3). The former two species showed higher abundances in Site 1, whereas  
343 the latter, absent in Site 1, increased from Site 2 to Site 3 (0.39 to 1.67). Within the French control  
344 location ('Control 1'), four significant contributors were detected: *Halopteris scoparia*, *Caulacanthus*  
345 *ustulatus*, *Enteromorpha* spp. and *Codium adharens* (Supplementary materials 3). It is remarkable the  
346 higher abundance of *Halopteris scoparia* in Site 3 (1.33) comparing to Site 1 (absent), two distant  
347 sites according to the cluster (Fig. 3). It is also noticeable the higher abundance of *Caulacanthus*  
348 *ustulatus* and the lower abundance of *Enteromorpha* spp. in Site 2 comparing to Site 1.

349 Regarding the lower midlittoral zone, four species had a contribution higher than 10% within 'WWTP  
350 1' (Supplementary materials 3). Among them, it should be highlighted the increasing abundance of  
351 *Halopeteris scoparia* from Site 1 to Site 3 (away from the outfall). By contrast, *Ceramium* spp showed  
352 higher abundance (3.08) in Site 1. Within 'WWTP 2', there was no contributor higher than 10%, but  
353 the abundances of *Cystoseira tamariscifolia* and *Halopteris scoparia* were higher in Site 3 (0.56 and  
354 0.28, respectively) (Supplementary materials 3). However, it should be noticed that *Cystoseira*  
355 *tamariscifolia* was also present, with a very low abundance, in Site 1 (the closest from the outfall),  
356 whereas it was not detected in Site 2. Within the French control ('Control 1') and for the same tidal  
357 level no significant contributor was detected. Looking at sites comparisons, the abundance of

358 *Enteromorpha* spp. decreased from Site 1 to Site 3 (from 0.38 to 0.05), whereas *Gelidium* spp.  
359 showed higher abundance in Site 3 comparing to Site 2 (Supplementary materials 3).

360 Regarding the upper midlittoral zone within Spanish 'WWTP 3', *Lithophyllum incrustans* was pointed  
361 as a significant contributor with lower abundance in Site 3 comparing to Site 1 (1.25 vs. 2.17)  
362 (Supplementary materials 3). By contrast, in the Spanish control location ('Control 2') three species  
363 with contributions higher than 10% were detected: *Chondria coerulescens*, *Corallina* spp. and  
364 *Halopteris scoparia* (Supplementary materials 3). The former two showed lower abundances in Site 3  
365 comparing to Site 2, whereas the values of *Halopteris scoparia* were higher in Site 3 than in Site 1. In  
366 relation to the lower midlittoral zone within 'WWTP3', three significant contributors were recorded.  
367 Whilst *Codium adhaerens* increased from Site 1 to site 3, *Ceramium* spp. and *Corallina* spp. were  
368 higher in Site 1 (Supplementary materials 3). By contrast, within 'Control 2', four species had  
369 contribution higher than 10%. *Halopteris scoparia* showed the higher abundance in Site 2 (1.83),  
370 *Corallina* spp. decreased from Site 1 to Site 3 and *Cladostephus spongiosus* and *Codium adhaerens*  
371 were more abundant in Site 3 (Supplementary materials 3).

### 372 2.3 Effects of WWTP discharges on the diversity of intertidal rocky benthic assemblages

373 88 species/taxa were identified during the field campaigns: 59 macroalgae (38 Rhodophyta, 12  
374 Ochrophyta and 9 Chlorophyta), 7 sessile and 22 mobile macrofauna (Table 6).

375

376 *Table 6: List of species/taxa identified into quadrats in control locations ('Control 1' and 'Control 2')*  
377 *and in impacted locations ('WWTP 1', 'WWTP 2' and 'WWTP 3'). Mean abundances (ind./0.1m<sup>2</sup>) and*  
378 *total mean taxonomic richness for each location are shown. Macroalgae were classed into taxonomic*  
379 *groups (red, brown and green) and functional groups (characteristic, opportunistic) according to Ar*  
380 *Gall et al. (2016) for French locations and Juanes et al. (2008) for Spanish ones. Macroalgae were*  
381 *assigned to one of two Ecological Status Groups (ESG) according to morphological and functional*  
382 *characteristics (Ar Gall et al., 2016; Gaspar et al., 2012; Neto et al., 2012; Orfanidis et al., 2011, 2001;*

383 *Vinagre et al., 2016a*). ESG I corresponded to late successional or perennial to annual taxa and ESG II  
384 to opportunistic or annual taxa. Macrofauna species were assigned to one of five Ecological Groups  
385 (EG I–V) according to their responses to natural and man-induced changes in water quality: the  
386 higher the group, the higher the tolerance to pollution (*Borja et al., 2000*). Significance codes: M:  
387 Macroalgae; ESG: Ecological Status Groups for macroalgae species; EG: Ecological groups for  
388 macrofauna; L = Lower midlittoral zone; U = Upper midlittoral zone. Sampling fluctuations were  
389 described by their standard deviation (SD).





416 *Fig. 4: Mean taxonomic richness of macrofauna in the upper (a, c) and lower midlittoral zones (b, d)*  
417 *for each impacted and control locations and site (i.e. each distance) within locations. Macrofauna*  
418 *species/taxa were*  
419 *classified into ecological groups (EG1 in white with black points, EG2 hatched, EG3 in black and others*  
420 *in grey).*

421  
422 In relation to macroalgae MTR, no difference was found between impacted and control in both  
423 countries except between 'WWTP 3' and 'Control 2' in the upper zone (Fig. 5; Supplementary  
424 materials 6). Within impacted locations, the only significant difference between sites occurred within  
425 'WWTP 1' in the lower zone (Site 1 < Site 2 < Site 3) (PERMANOVA; p-value < 0.05). There were also  
426 significant differences within control locations in the lower zone (i.e. between Site 1 and 3 in 'Control  
427 1' and between Site 2 and 3 in 'Control 2') (Supplementary materials 6).

428 Focusing on the ratio characteristic/opportunistic MTR, there were significant differences between  
429 impacted and control locations in both countries and midlittoral zones (except in the upper zone  
430 between 'WWTP 3' and 'Control 2') (PERMANOVA; p-value < 0.05; Supplementary materials 7). The  
431 ratio was always lower in impacted locations than in control with higher opportunistic MTR in  
432 impacted locations (except between 'WWTP 2' and 'Control 1' in the lower zone). This was not so  
433 obvious regarding the characteristic MTR. Indeed, it was higher in the control only between 'Control  
434 1' and 'WWTP 1' in both midlittoral zones. At the site scale within impacted locations, there were  
435 only significant differences within 'WWTP 2' (i.e. between Site 1 and 3 in both zones and between  
436 Site 1 and 2 in the upper zone). In all three cases, the ratio was always lower in Site 1. The  
437 characteristic MTR was higher in furthest sites from the outfall (Sites 2 and 3) contrary to the  
438 opportunistic MTR which was higher in Site 1 in the upper zone. Within control locations, only  
439 'Control 1' in the upper zone presented significant differences between sites (i.e. Site 1 significantly  
440 differed from Site 2 and Site 3).

441

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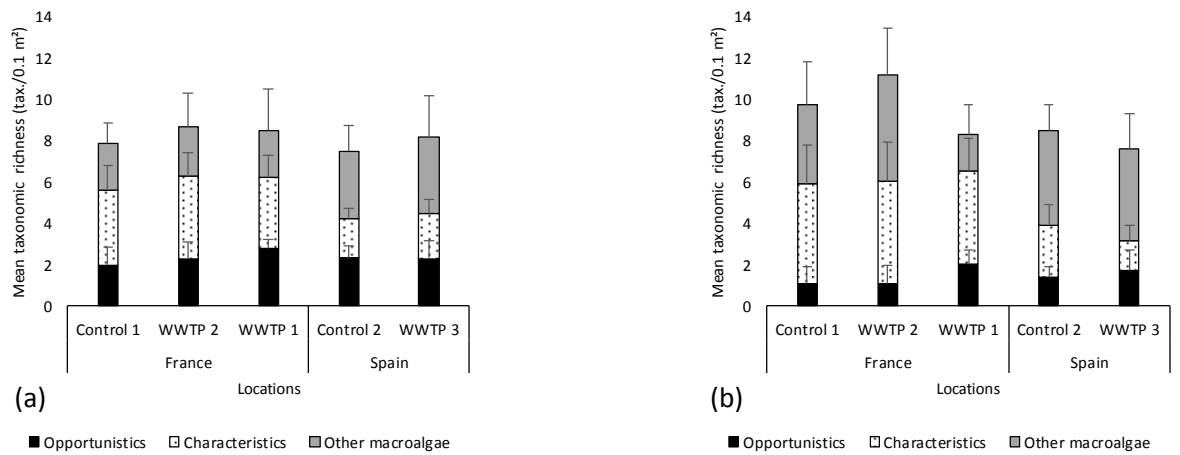
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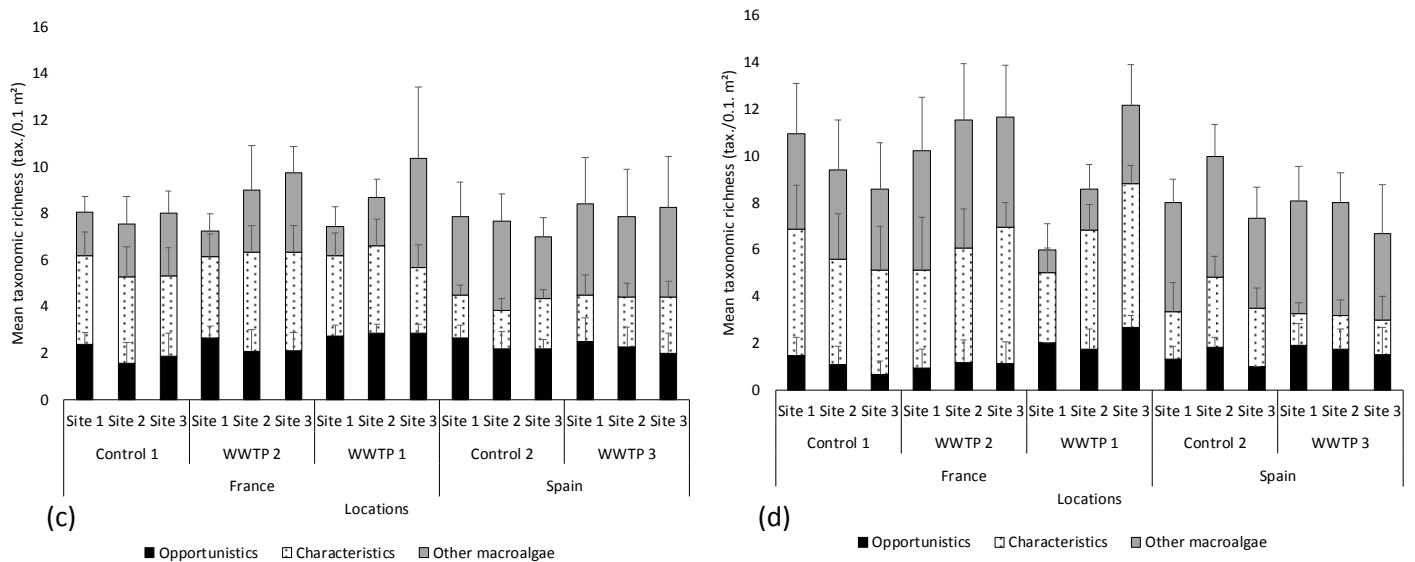


Fig. 5: Mean taxonomic richness of macroalgae of each impacted and control locations (a, b) and of each site (i.e. each distance) within locations (c, d) classed into functional groups (opportunistics in black, characteristics in white with black points and others in grey) according to Ar Gall & Le Duff (2016) for French locations and Juanes (2008) for Spanish ones for (a and c) upper and (b and d) lower midlittoral zones.

## 2.4 Ecological quality

462 The quality index was calculated, per location for controls, and per distance from the outfall (i.e. site)  
 463 for impacted locations (Table 7). In France, sites from all locations were ranked as “Good” except the  
 464 closest site from the outfall in ‘WWTP 1’. In Spain, all final scores were ranked as “Moderate” in the  
 465 impacted location and as “Good” in the control one.

466 *Table 7: Metrics calculated using the Water Framework Directive (WFD) protocol for each control*  
 467 *location and each distance of impacted locations.*

	Max. points	Control 1	WWTP 1			WWTP 2			Control 2	WWTP 3		
			Site 1	Site 2	Site 3	Site 1	Site 2	Site 3		Site 1	Site 2	Site 3
Global cover of macroalgae [C]	0.4	0.306	0.306	0.306	0.306	0.306	0.306	0.306	0.306	0.306	0.306	0.306
Occurrence of characteristic species [N]	0.3	0.15	0.075	0.25	0.2	0.15	0.2	0.25	0.2	0.15	0.1	0.1
Total cover of opportunistic species [O]	0.3	0.2	0.075	0.1	0.15	0.2	0.15	0.2	0.15	0.125	0.15	0.1
Final score	1	0.656	0.456	0.656	0.656	0.656	0.656	0.756	0.656	0.581	0.556	0.506
<b>Ecological quality</b>		<b>Good</b>	<b>Moderate</b>	<b>Good</b>	<b>Good</b>	<b>Good</b>	<b>Good</b>	<b>Good</b>	<b>Good</b>	<b>Moderate</b>	<b>Moderate</b>	<b>Moderate</b>

468  
 469

### 470 3. Discussion

471 The present study aimed to assess the effects of wastewater treatment plant (WWTP) discharges on  
 472 rocky benthic intertidal assemblages (macroalgae and macroinvertebrates) of the French and Spanish  
 473 Basque coast (southeastern Bay of Biscay). The results from this research show significant differences  
 474 in the composition and abundances of taxa, including those sensitive to pollution, between the three  
 475 studied WWTP and their respective controls for both midlittoral zones (upper and lower). Significant  
 476 differences in the composition and abundance of assemblages were also found at a lower spatial  
 477 scale (sites corresponding to the three distances from the outfalls). Regarding mean taxonomic  
 478 richness, no evident differences were found, especially for macrofauna.

479 When detecting impacts due to pollution in the marine environment, the study of benthic  
 480 communities provides several advantages, among which the bioindicator nature of some species  
 481 (opportunists vs. sensitives) should be highlighted (Díez et al., 2009). In the present study, three  
 482 macroalgae taxa (*Ceramium* spp., *Corallina* spp. and *Halopteris scoparia*) were identified as  
 483 significant contributors (Ct (%) > 10) to the dissimilarity between the three WWTP and their  
 484 respective controls. *Ceramium* spp., a corticated filamentous red alga that includes diverse



485 opportunistic species tolerant to pollution (Díez et al., 1999; Juanes et al., 2008), showed higher  
486 abundance in WWTP locations for both midlittoral zones. In the upper midlittoral zone, *Corallina* spp.  
487 showed high abundances in both controls and WWTP locations being one of the most frequent  
488 macrophyte forming a distinctive belt in the intertidal zone at the southeastern Bay of Biscay  
489 (Gorostiaga et al., 2004). Nevertheless, its abundance was higher in WWTP comparing to the  
490 controls. This genus is considered as characteristic and is formed by articulated calcareous algae  
491 which show certain tolerance to moderated polluted environments (Díez et al., 1999; Díez et al.,  
492 2009; Gorostiaga et al., 2004; Mangialajo et al., 2008; Pellizzari et al., 2017). *Halopteris scoparia*, with  
493 a terete corticated thallus and considered as a characteristic species, was more abundant in the  
494 lower midlittoral of control locations. This species has been already reported in locations with good  
495 environmental conditions (Arévalo et al., 2007; Díez et al., 2012, 1999). Therefore, its lower  
496 abundance or even its absence (in Spanish WWTP location) could suggest an effect of discharges.  
497 Nevertheless, considering this species was also present in French WWTP locations, the impact of the  
498 discharges might not be considered as elevated.

499 Apart from these high contributors, other species appeared to be responsible for the difference  
500 between WWTP and control locations. For instance, in the upper midlittoral zone of French area,  
501 *Laurencia obtusa* and *Osmundea pinnatifida* were more abundant in the control location than in  
502 'WWTP 1' and 'WWTP 2'. In fact, *Osmundea pinnatifida* was not present in 'WWTP 1'. These  
503 rhodophytes are typical intertidal species related to clean waters (Díez et al., 2009). In the lower  
504 midlittoral zone of the same area, the leathery species, *Cystoseira tamariscifolia*, also showed higher  
505 abundances in the control location comparing to 'WWTP 1' and 'WWTP 2'. It is well known the  
506 sensitiveness of the species of the genus *Cystoseira* to anthropogenic impact, and they are thus  
507 considered as indicators for good water quality in the European Directive (Duarte et al., 2018; García-  
508 Fernández and Bárbara, 2016; Valdazo et al., 2017). However, similar to that described for *Halopteris*  
509 *scoparia* above, *Laurencia obtusa* and *Cystoseira tamariscifolia* were also present (with lower

510 abundances) in WWTP locations and, therefore, the potential impact of WWTP discharges might be  
511 considered as moderate.

512 WWTP and control locations also presented high variability in terms of taxa composition and  
513 abundance at the site scale (i.e. distance from the outfall) for both midlittoral zones and for French  
514 and Spanish areas. Within French WWTP locations, among the significant contributors (Ct (%) > 10), it  
515 should be highlighted the increase in the abundance of the sensitive species (*Halopteris scoparia*)  
516 and the decrease of *Caulacanthus ustulathus* from Site 1 (closest to the outfall) to Site 3 (furthest to  
517 the outfall) in 'WWTP 1'. The latter red macroalga was described as a more abundant species close  
518 the outfall in a study carried out in an inlet on the Basque coast (Díez et al., 2013). In 'WWTP 2', the  
519 abundance of *Ceramium* spp. and *Corallina* spp. decreased towards Site 3, whereas *Laurencia obtusa*  
520 increased. In this location *Cystoseira tamariscifolia* and *Halopteris scoparia* showed their highest  
521 values in Site 3 (the furthest one from the outfall). Within the Spanish WWTP location, similar trends  
522 were detected with some sensitive species being more abundant in Site 3 and opportunistic species  
523 in Site 1. For instance, *Chondria coerulescens*, a species related to high levels of sedimentation and  
524 tolerant to moderate pollution levels (Gorostiaga et al., 2004) decreased towards Site 3. Taking into  
525 account these trends of bioindicator macroalgae within WWTP locations, it might be deduced a  
526 gradient of the effect of the outfall on benthic intertidal assemblages. However, looking at Sites 1  
527 and 3 within control locations, separated 200 m but in the absence of any gradient, results were  
528 somewhat similar. In this regard, sensitive species, such as *Cystoseira tamariscifolia* or *Halopteris*  
529 *scoparia*, dominated in Site 3, whereas opportunistic taxa, such as *Ceramium* spp and *Enteromorpha*  
530 spp., were more abundant in Site 1. Chlorophytes like the genus *Enteromorpha* are also common in  
531 non-polluted areas and their higher presence could be explained by the effect of other factors such  
532 as sediments accumulation (Littler et al., 1983) or grazing pressure (Hay, 1981). In relation to this  
533 taxa composition and abundance approach, it should be highlighted that some species were  
534 aggregated for the analysis at the genus level. This fact might have supposed a decrease of the  
535 bioindicator nature of some species. For example, two species from the same genus may have

536 different sensitivity (e.g. *Gelidium pusillum* less sensitive to pollution than other species from this  
537 same genus such as *Gelidium corneum*) (Díez et al., 1999).

538 An environmental stress such as eutrophication or anthropogenic disturbances can result in a loss of  
539 richness (Amaral et al., 2018; Simboura and Zenetos, 2002). Therefore, the mean taxonomic richness  
540 (MTR) was assessed to detect changes caused by WWTP discharges because this metric could be also  
541 used as a criterion of ecological quality (Amaral et al., 2018; Simboura and Zenetos, 2002; Wells et  
542 al., 2007). However, using the macrofauna MTR, no detectable effect of WWTP discharges was  
543 highlighted due to very low values (<1 in France and <5 in Spain) and high variability compared to  
544 macroalgae (Fig. 4; Fig. 5) for which rocky platforms constitute a suitable habitat for their  
545 colonization (Guinda et al., 2014). Macrofauna settlement was not as favorable because the lack of  
546 canopy-forming macroalgae (Díez et al., 2014), the uniform geomorphology, high exposure to a  
547 strong hydrodynamic regime (Abadie et al., 2005) and the competitive advantage of the macroalgae  
548 in the lower levels of the intertidal zone (especially in the case of the caespitose vegetation).  
549 Therefore, it was only possible to highlight general trends, such as a higher macrofauna MTR in the  
550 Spanish side and in the upper midlittoral zone. Furthermore, results of macrofauna patterns would  
551 be probably quite different if outfalls were located in an intertidal boulder field providing hiding  
552 places for high macrofauna diversity (Bernard, 2012; Huguenin et al., 2018).

553 Macroalgae MTR appeared not to be really affected by discharges at the location scale. Indeed, no  
554 difference was highlighted between impacted and control locations (except in the upper zone in the  
555 Spanish side). However, the ratio between characteristic and opportunistic taxa was significantly  
556 affected in the three WWTP locations at both levels (one exception was the upper level of 'WWTP  
557 3'). Between sites within impacted locations (i.e. between the three distances from the outfall), the  
558 only one significant MTR increase (from Site 1 to 3) was in 'WWTP 1' in the lower zone.

559 Thus, similarly to other works (Simboura and Zenetos, 2002; Vinagre et al., 2016a), our results show  
560 the difficulty to make accurate predictions of the effect of WWTP discharges on the MTR (especially

561 on macrofauna). By contrast, multivariate analysis appeared as more appropriate because it allows  
562 to integrate all benthic assemblages (i.e. species composition and abundance of macroalgae as well  
563 as macrofauna). This may also be explained by the fact that the MTR does not consider the relative  
564 abundance of the species neither other relevant traits of the taxa (life cycle and morphology). Thus,  
565 only strong impacts could potentially influence the MTR. Some authors had already mentioned that  
566 such metrics are not universally relevant to study the effect of this type of disturbance (Harper and  
567 Hawksworth, 1994; Magurran, 2004) and that they could be often affected by sampling effort (Clarke  
568 and Warwick, 2001b). Average cover parameter could be thus probably more useful to detect  
569 impacts.

570 In this study, macroalgae and macrofauna communities were considered to assess potential effects  
571 of wastewater discharges as recommended by some studies (Archambault et al., 2001; Bishop et al.,  
572 2002; Underwood, 1996) and to fulfill European Directives requirements (WFD and MSFD). Indeed,  
573 these communities are playing a key role in water quality for the conservation status and functional  
574 aspects of the environment (Casamajor (de) et al., 2016). Vinagre et al. (2016a) even suggest that  
575 macrofauna might be considered as an indicator of disturbance in intertidal rocky shores as good as  
576 the macroalgae.

577 Using the quality index, all sites within 'WWTP 2' were ranked as *Good*, while all sites within the  
578 Spanish location 'WWTP 3' were ranked as *Moderate*. In 'WWTP 1', only the proximate site from the  
579 outfall was ranked as *Moderate*, while site 2 and 3 were ranked as *Good*. A study achieved in  
580 compliance with the WFD along the French Basque coast (Casamajor (de) et al., 2016) ranked two  
581 other locations (considered as not impacted and representative of the whole water body) as *Good*  
582 (with values between 0.706 and 0.732). This is entirely in line with indices calculated on 'Control 1'  
583 and sites away from the outfall on impacted locations, which seems to be less impacted and have a  
584 better ecological quality. Moreover, in Spain, the WWTP location was moderately impacted whatever  
585 the distance from the outfall. But, it is important to note that the ratio was calculated according to  
586 the list initially established for the French Basque coast (Ar Gall et al., 2016; Casamajor et al., 2010). A

587 Spanish list was anyway defined by Juanes et al. (2008) for the calculation of WFD metrics, but the  
588 number of opportunistic and characteristic species was much lower than the French one. Thus,  
589 scores assigned to each metric would have been not really significant and the ecological quality  
590 would have been underestimated. If we had wanted to calculate the Spanish CFR index (Guinda et  
591 al., 2008) with our data, this would not have been possible due to the differences of sampling designs  
592 (transects vs. random quadrats). In addition, despite the fact that the Basque coast has only two algal  
593 belts, it seemed preferable to stratify the protocol according to these two belts (quadrats in each  
594 belt) rather than to perform a transect covering the two belts.

#### 595 **4. Conclusion**

596 The present work established the assessment of the potential impact of WWTP discharges on  
597 intertidal rocky benthic assemblages in the southeastern Bay of Biscay. Even if the importance to  
598 consider both communities was proved, it suggests that benthic macroalgae constitute the best  
599 relevant organisms to assess the effect of this pressure on the intertidal rocky platform habitat in the  
600 study area. The results from the present study do not evidence a clear impact of the WWTP  
601 discharges on the rocky benthic intertidal assemblages. Taking into account the presence of some  
602 sensitive taxa in WWTP locations and that a Good ecological status has been ranked in French WWTP  
603 locations, only the existence of a moderate impact associated with discharges could be concluded. In  
604 the Spanish side the ecological quality ratio offered lower values than those expected for the control  
605 and impacted locations. The use of the complementary metric "mean taxonomic richness" was not  
606 helpful to discriminate the potential impacts due to the absence of clear trends. Finally, multivariate  
607 analyses appeared thus to be more efficient than other biological and ecological metrics although  
608 certain difficulties emerge when discriminating between changes associated to natural variability and  
609 those caused by anthropic activity. For this reason, it is necessary to deepen on the bioindicator  
610 character of the different macroalgae. These results will enable several MSFD descriptors to be  
611 supported, such as "Biodiversity", "Non-indigenous species", "Eutrophication", "Sea-floor integrity"

612 and "Contaminants" whilst also bridging deficiencies emphasized by Directives on the response of  
613 biological indicators to various pressures and the biocenosis of the southeastern Bay of Biscay.

614

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627

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Table 1: General WWTP features

	'WWTP 1'	'WWTP 2'	'WWTP 3'
Location	France	France	Spain
Population equivalent (PE)	78 217	45 000	27 500
Nominal flow (m <sup>3</sup> /day)	10 450	7 350	5 930
Outfall location	Intertidal zone	Intertidal zone	Intertidal zone

Table 2: Ecological quality according to the CFR index

Score	Ecological quality
0.80 - 1	Very good
0.60 - 0.79	Good
0.40 - 0.59	Moderate
0.20 - 0.39	Poor
0 - 0.19	Bad

Table 3: Summary of PERMANOVA results testing for effects of presence of sewage discharges on benthic assemblages between impacted and control locations ('WWTP 1'/Control 1' (a), 'WWTP 2'/Control 1' (b), 'WWTP 3'/Control 2' (c)) in both midlittoral zones

(a)	'WWTP 1'/Control 1'	Df	MeanSqs	F.Model	R2	Pr(>F)	Significance
Upper midlittoral zone							
	Locations	1	2.93519	27.8767	0.19025	1.00E-04	***
	Locations/Sites	4	0.59622	5.6626	0.15458	1.00E-04	***
	Residuals	96	0.10529	0.65517			
Lower midlittoral zone							
	Locations	1	2.3794	13.3604	0.11347	1.00E-04	***
	Locations/Sites	4	0.50673	2.8453	0.09666	1.00E-04	***
	Residuals	93	0.17809	0.78986			
(b)	'WWTP 2'/Control 1'	Df	MeanSqs	F.Model	R2	Pr(>F)	Significance
Upper midlittoral zone							
	Locations	1	4.1775	34.2	0.19055	1.00E-04	***
	Locations/Sites	4	0.7719	6.319	0.14084	1.00E-04	***
	Residuals	120	0.1221	0.66861			
Lower midlittoral zone							
	Locations	1	4.6721	23.5977	0.15924	1.00E-04	***
	Locations/Sites	4	0.3758	1.8983	0.05124	0.0011	**
	Residuals	117	0.198	0.78952			

(c) 'WWTP 3'/'Control 2'	Df	MeanSqs	F.Model	R2	Pr(>F)	Significance
Upper midlittoral zone						
Locations	1	1.05628	8.1187	0.12313	1.00E-04	***
Locations/Sites	4	0.31941	2.4551	0.14893	2.00E-04	***
Residuals	48	0.1301	0.72795			
Lower midlittoral zone						
Locations	1	2.41452	17.8223	0.22095	1.00E-04	***
Locations/Sites	4	0.50256	3.7096	0.18396	1.00E-04	***
Residuals	48	0.13548	0.59509			

Table 4: Summary of pairwise post hoc results testing for effects of presence of sewage discharges on benthic assemblages between sites within each location ('WWTP 1' (a), 'WWTP 2' (b), 'WWTP 3' (c), 'Control 1' (d), 'Control 2' (e) in both midlittoral zones.

(a)	Upper midlittoral zone		WWTP 1	
			Site 1	Site 2
	WWTP 1	Site 2	<b>0.0015</b>	-
		Site 3	<b>0.0015</b>	<b>0.004</b>
	Lower midlittoral zone		WWTP 1	
			Site 1	Site 2
WWTP 1	Site 2	<b>0.0015</b>	-	
	Site 3	<b>0.0015</b>	<b>0.017</b>	
(b)	Upper midlittoral zone		WWTP 2	
			Site 1	Site 2
	WWTP 2	Site 2	<b>0.001</b>	-
		Site 3	<b>0.001</b>	<b>0.001</b>
	Lower midlittoral zone		WWTP 2	
			Site 1	Site 2
WWTP 2	Site 2	0.479	-	
	Site 3	<b>0.006</b>	<b>0.019</b>	
(c)	Upper midlittoral zone		WWTP 3	
			Site 1	Site 2
	WWTP 3	Site 2	0.284	-
		Site 3	<b>0.042</b>	0.112
	Lower midlittoral zone		WWTP 3	
			Site 1	Site 2
WWTP 3	Site 2	0.327	-	
	Site 3	<b>0.003</b>	<b>0.01</b>	

(d)

Upper midlittoral zone		Control 1	
		Site 1	Site 2
Control 1	Site 2	<b>0.0015</b>	-
	Site 3	<b>0.0015</b>	0.2967
Lower midlittoral zone		Control 1	
		Site 1	Site 2
Control 1	Site 2	0.21	-
	Site 3	0.21	0.27

(e)

Upper midlittoral zone		Control 2	
		Site 1	Site 2
Control 2	Site 2	0.094	-
	Site 3	<b>0.015</b>	<b>0.033</b>
Lower midlittoral zone		Control 2	
		Site 1	Site 2
Control 2	Site 2	<b>0.002</b>	-
	Site 3	<b>0.002</b>	<b>0.002</b>

Table 5: Summary of global dissimilarities between 2 groups from SIMPER analyses (i.e. between impacted and control locations and between sites within each location) for both midlittoral zones (upper and lower)

		Global dissimilarity (%)	
		Upper midlittoral zone	Lower midlittoral zone
<b>WWTP 1' vs. 'Control 1'</b>		<b>51.12</b>	<b>63.58</b>
<b>WWTP 2' vs. 'Control 1'</b>		<b>53.81</b>	<b>71.14</b>
'WWTP 1'	S1 vs. S2	43.62	52.00
	S2 vs. S3	39.45	41.41
	S1 vs. S3	52.74	56.96
'WWTP 2'	S1 vs. S2	49.57	56.88
	S2 vs. S3	49.17	55.08
	S1 vs. S3	52.93	57.36
'Control 1'	S1 vs. S2	42.41	56.27
	S2 vs. S3	40.95	61.04
	S1 vs. S3	41.89	57.44
<b>WWTP 3' vs. 'Control 2'</b>		<b>42.00</b>	<b>52.54</b>
'WWTP 3'	S1 vs. S2	31.16	33.44
	S2 vs. S3	33.08	50.08
	S1 vs. S3	36.56	51.28
'Control 2'	S1 vs. S2	32.65	30.99
	S2 vs. S3	40.26	46.28
	S1 vs. S3	37.47	45.78

*Table 6: List of species/taxa identified into quadrats in control locations ('Control 1' and 'Control 2') and in impacted locations ('WWTP 1', 'WWTP 2' and 'WWTP 3'). Mean abundances (ind./0.1m<sup>2</sup>) and total mean taxonomic richness for each location are shown. Macroalgae were classed into taxonomic groups (red, brown and green) and functional groups (characteristic, opportunistic) according to Ar Gall et al. (2016) for French locations and Juanes et al. (2008) for Spanish ones. Macroalgae were assigned to one of two Ecological Status Groups (ESG) according to morphological and functional characteristics (Ar Gall et al., 2016; Gaspar et al., 2012; Neto et al., 2012; Orfanidis et al., 2011, 2001; Vinagre et al., 2016a). ESG I corresponded to late successional or perennial to annual taxa and ESG II to opportunistic or annual taxa. Macrofauna species were assigned to one of five Ecological Groups (EG I–V) according to their responses to natural and man-induced changes in water quality: the higher the group, the higher the tolerance to pollution (Borja et al., 2000). Significance codes: M: Macroalgae; ESG: Ecological Status Groups for macroalgae species; EG: Ecological groups for macrofauna; L = Lower midlittoral zone; U = Upper midlittoral zone. Sampling fluctuations were described by their standard deviation (SD)*



<i>Scinia furcellata</i>	M	Rhodophyta	<b>0.01</b> (SD=0.08)	-	-	-	-	-	-	I
<i>Tenarea tortuosa</i>	M	Rhodophyta	-	-	<b>0.02</b> (SD=0.14)	-	-	-	√ (L+U)	
<i>Vertebrata fruticulosa</i>	M	Rhodophyta	-	-	-	√ (L+U)	<b>0.03</b> (SD=0.17)	-	-	II
<i>Cladostephus spongiosus</i>	M	Ochrophyta	-	-	-	-	<b>0.56</b> (SD=0.88)	-	√ (L+U)	I
<i>Colpomenia peregrina</i>	M	Ochrophyta	<b>0.36</b> (SD=0.48)	<b>0.22</b> (SD=0.42)	<b>0.33</b> (SD=0.55)	√ (L+U)	<b>0.03</b> (SD=0.17)	<b>0.58</b> (SD=0.50)	-	II
<i>Cutleria adspersa</i>	M	Ochrophyta	-	-	<b>0.11</b> (SD=0.34)	-	<b>0.06</b> (SD=0.33)	<b>0.29</b> (SD=0.46)	-	
<i>Cutleria multifida</i>	M	Ochrophyta	-	-	<b>0.01</b> (SD=0.10)	-	-	-	-	
<i>Cystoseira tamariscifolia</i>	M	Ochrophyta	<b>0.37</b> (SD=0.89)	-	<b>0.10</b> (SD=0.39)	-	-	-	-	I
<i>Dictyota dichotoma</i>	M	Ochrophyta	<b>0.05</b> (SD=0.22)	<b>0.02</b> (SD=0.13)	<b>0.23</b> (SD=0.49)	√ (L)	-	-	-	II
<i>Ectocarpales/Ectocarpus</i>	M	Ochrophyta	<b>0.01</b> (SD=12)	-	<b>0.01</b> (SD=0.10)	√ (L+U)	<b>0.14</b> (SD=0.35)	<b>0.22</b> (SD=0.42)	√ (L+U)	II
<i>Ralfsia verrucosa</i>	M	Ochrophyta	-	-	-	-	<b>0.03</b> (SD=0.17)	<b>0.07</b> (SD=0.26)	-	I
<i>Scytosiphon lomentaria</i>	M	Ochrophyta	-	-	-	-	-	<b>0.08</b> (SD=0.28)	-	
<i>Halopteris scoparia</i>	M	Ochrophyta	<b>1.41</b> (SD=1.66)	<b>0.58</b> (SD=1)	<b>0.08</b> (SD=0.39)	√ (L)	<b>0.69</b> (SD=0.86)	<b>0.01</b> (SD=0.12)	√ (L+U)	II
<i>Taonia sp.</i>	M	Ochrophyta	<b>0.04</b> (SD=0.20)	<b>0.03</b> (SD=0.18)	<b>0.23</b> (SD=0.49)	-	<b>0.03</b> (SD=0.17)	<b>0.15</b> (SD=0.36)	-	
<i>Zonardinia typus</i>	M	Ochrophyta	-	-	<b>0.05</b> (SD=0.29)	-	-	-	-	
<i>Chaetomorpha spp.</i>	M	Chlorophyta	-	-	<b>0.01</b> (SD=0.10)	-	-	<b>0.13</b> (SD=0.33)	√ (L+U)	
<i>Cladophora spp.</i>	M	Chlorophyta	<b>0.01</b> (SD=0.12)	-	<b>0.04</b> (SD=0.19)	-	-	<b>0.01</b> (SD=0.12)	√ (L+U)	II
<i>Codium adaerens</i>	M	Chlorophyta	<b>0.33</b> (SD=0.86)	<b>0.32</b> (SD=0.75)	<b>0.30</b> (SD=0.76)	√ (L)	<b>0.42</b> (SD=0.77)	<b>0.57</b> (SD=0.85)	-	II (spp.)
<i>Codium decorticatum</i>	M	Chlorophyta	-	-	-	-	-	<b>0.08</b> (SD=0.28)	-	II (spp.)
<i>Codium fragile</i>	M	Chlorophyta	-	<b>0.02</b> (SD=0.13)	-	-	<b>0.17</b> (SD=0.38)	<b>0.08</b> (SD=0.28)	-	II (spp.)
<i>Derbesia tenuissima</i>	M	Chlorophyta	-	-	<b>0.01</b> (SD=0.10)	-	-	-	-	
<i>Enteromorpha spp.</i>	M	Chlorophyta	<b>0.64</b> (SD=0.83)	<b>0.67</b> (SD=0.68)	<b>0.48</b> (SD=0.73)	√ (L+U)	<b>0.53</b> (SD=0.61)	<b>0.33</b> (SD=0.47)	√ (L+U)	II
<i>Pterosiphonia spp.</i>	M	Chlorophyta	<b>0.33</b> (SD=0.78)	<b>0.02</b> (SD=0.13)	<b>0.12</b> (SD=0.43)	-	<b>0.03</b> (SD=0.17)	-	-	II ( <i>P. complanata</i> )
<i>Ulva spp.</i>	M	Chlorophyta	<b>1.55</b> (SD=1)	<b>1.47</b> (SD=0.60)	<b>1.11</b> (SD=0.78)	√ (L+U)	<b>0.28</b> (SD=0.51)	<b>0.39</b> (SD=0.52)	√ (L+U)	II
Macroalgae mean taxonomic richness			<b>8.79</b> (SD=3.19)	<b>8.38</b> (SD=2.39)	<b>9.90</b> (SD=3.22)	-	<b>7.97</b> (SD=1.58)	<b>7.88</b> (SD=2.46)	-	
<i>Balanus sp.</i>	SM	Crustacea	-	<b>0.05</b> (SD=0.39)	-	-	<b>1.72</b> (SD=3.58)	<b>0.53</b> (SD=1.14)	-	I
<i>Chthamalus spp.</i>	SM	Crustacea	<b>0.02</b> (SD=0.25)	-	-	-	<b>1.33</b> (SD=1.82)	<b>1.04</b> (SD=1.44)	-	I
<i>Mytilus spp.</i>	SM	Mollusca	<b>0.02</b> (SD=0.25)	<b>0.30</b> (SD=0.91)	<b>0.22</b> (SD=0.79)	-	<b>1.53</b> (SD=2.67)	<b>0.92</b> (SD=2.11)	-	III
<i>Rocellaria dubia</i>	SM	Mollusca	-	-	<b>0.03</b> (SD=0.21)	-	-	-	-	I
<i>Serpula sp.</i>	SM	Annelida	-	-	<b>0.02</b> (SD=0.19)	-	-	-	-	I
<i>Spirobranchus spp.</i>	SM	Annelida	-	-	-	-	-	<b>0.04</b> (SD=0.35)	-	II
<i>Porifera</i>	SM	Porifera	-	-	<b>0.28</b> (SD=1.56)	-	-	-	-	
Sessile macrofauna mean taxonomic richness			<b>0.01</b> (SD=0.12)	<b>0.12</b> (SD=0.32)	<b>0.16</b> (SD=0.39)	-	<b>1.06</b> (SD=1.24)	<b>0.79</b> (SD=0.85)	-	
<i>Acanthochitona spp.</i>	MM	Mollusca	-	<b>0.02</b> (SD=0.13)	<b>0.01</b> (SD=0.10)	-	-	-	-	I
<i>Actinia equina</i>	MM	Cnidaria	<b>0.01</b> (SD=0.08)	-	-	-	-	-	-	I
<i>Actinothoe sphyrodeta</i>	MM	Cnidaria	-	-	-	-	<b>0.03</b> (SD=0.17)	-	-	
<i>Anemonia viridis</i>	MM	Cnidaria	<b>0.01</b> (SD=0.12)	-	-	-	-	-	-	
<i>Annelida</i>	MM	Annelida	-	-	<b>0.02</b> (SD=0.14)	-	-	-	-	
<i>Bittium reticulatum</i>	MM	Mollusca	<b>0.02</b> (SD=0.19)	-	<b>0.07</b> (SD=0.43)	-	-	-	-	I

<i>Cerithium spp.</i>	MM	Mollusca	<b>0.02</b> (SD=0.19)	-	-	-	-	II
<i>Chiton spp.</i>	MM	Mollusca	<b>0.01</b> (SD=0.08)	-	<b>0.01</b> (SD=0.10)	-	-	II
<i>Diodora gibberula</i>	MM	Mollusca	-	-	<b>0.01</b> (SD=0.10)	-	-	
<i>Eulalia viridis</i>	MM	Annelida	-	<b>0.07</b> (SD=0.25)	<b>0.03</b> (SD=0.17)	<b>0.11</b> (SD=0.40)	<b>0.90</b> (SD=2.46)	II
<i>Melarhappe neritoides</i>	MM	Mollusca	-	-	<b>0.14</b> (SD=1.44)	-	-	II
<i>Ocenebra edwardsii</i>	MM	Mollusca	<b>0.04</b> (SD=0.22)	-	<b>0.03</b> (SD=0.17)	-	-	II (sp.)
<i>Pachygrapsus marmoratus</i>	MM	Crustacea	<b>0.01</b> (SD=0.12)	-	-	-	-	II
<i>Paguridae spp.</i>	MM	Crustacea	<b>0.13</b> (SD=0.58)	<b>0.02</b> (SD=0.13)	<b>0.01</b> (SD=0.10)	-	-	II ( <i>Pagurus sp.</i> )
<i>Paracentrotus lividus</i>	MM	Echinodermata	-	-	-	-	<b>0.10</b> (SD=0.30)	I
<i>Patella spp.</i>	MM	Mollusca	<b>0.12</b> (SD=0.68)	<b>0.12</b> (SD=0.67)	<b>0.03</b> (SD=0.21)	<b>3.08</b> (SD=4.44)	<b>3.81</b> (SD=3.81)	I
<i>Porcellana platycheles</i>	MM	Crustacea	-	-	-	-	<b>0.21</b> (SD=1.10)	I
<i>Steromphala cineraria</i>	MM	Mollusca	<b>0.02</b> (SD=0.19)	-	-	-	-	I
<i>Steromphala pennanti</i>	MM	Mollusca	<b>0.45</b> (SD=1.32)	<b>0.03</b> (SD=0.18)	<b>0.03</b> (SD=0.17)	-	-	I
<i>Steromphala umbilicalis</i>	MM	Mollusca	<b>0.16</b> (SD=0.62)	-	<b>0.02</b> (SD=0.14)	-	-	I
<i>Stramonita haemastoma</i>	MM	Mollusca	-	-	<b>0.01</b> (SD=0.10)	-	-	
<i>Tritia incrassata</i>	MM	Mollusca	-	-	<b>0.03</b> (SD=0.21)	-	-	II
Mobile macrofauna mean taxonomic richness			<b>0.52</b> (SD=0.88)	<b>0.18</b> (SD=0.50)	<b>0.25</b> (SD=0.55)	<b>0.67</b> (SD=0.63)	<b>1.15</b> (SD=0.82)	
Total mean taxonomic richness			<b>9.29</b> (SD=3.26)	<b>8.68</b> (SD=2.46)	<b>10.31</b> (SD=3.26)	<b>9.69</b> (SD=2.29)	<b>9.82</b> (SD=2.98)	



Table 7: Metrics calculated using the Water Framework Directive (WFD) protocol for each control location and each distance of impacted locations

	Max. points	'Control 1'	'WWTP 1'			'WWTP 2'			'Control 2'	'WWTP 3'		
			Site 1	Site 2	Site 3	Site 1	Site 2	Site 3		Site 1	Site 2	Site 3
Global cover of macroalgae [C]	0.4	0.306	0.306	0.306	0.306	0.306	0.306	0.306	0.306	0.306	0.306	0.306
Occurrence of characteristic species [N]	0.3	0.15	0.075	0.25	0.2	0.15	0.2	0.25	0.2	0.15	0.1	0.1
Total cover of opportunistic species [O]	0.3	0.2	0.075	0.1	0.15	0.2	0.15	0.2	0.15	0.125	0.15	0.1
Final score	1	0.656	0.456	0.656	0.656	0.656	0.656	0.756	0.656	0.581	0.556	0.506
<b>Ecological quality</b>		<b>Good</b>	<b>Moderate</b>	<b>Good</b>	<b>Good</b>	<b>Good</b>	<b>Good</b>	<b>Good</b>	<b>Good</b>	<b>Moderate</b>	<b>Moderate</b>	<b>Moderate</b>

**Highlights:**

- Detectable effects of discharges were highlighted on assemblage structure
- Macroalgae constituted a relevant biotic component to study impact of WWTP discharges
- 24 contributors responsible for differences (impacted vs. control) were identified
- The pseudo-EQR ratio was sensitive to the WWTP pressure