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1 An active biomonitoring approach using three-spined stickleback (*Gasterosteus aculeatus, L.*) to
2 assess the efficiency of a constructed wetland as tertiary treatment of wastewater.

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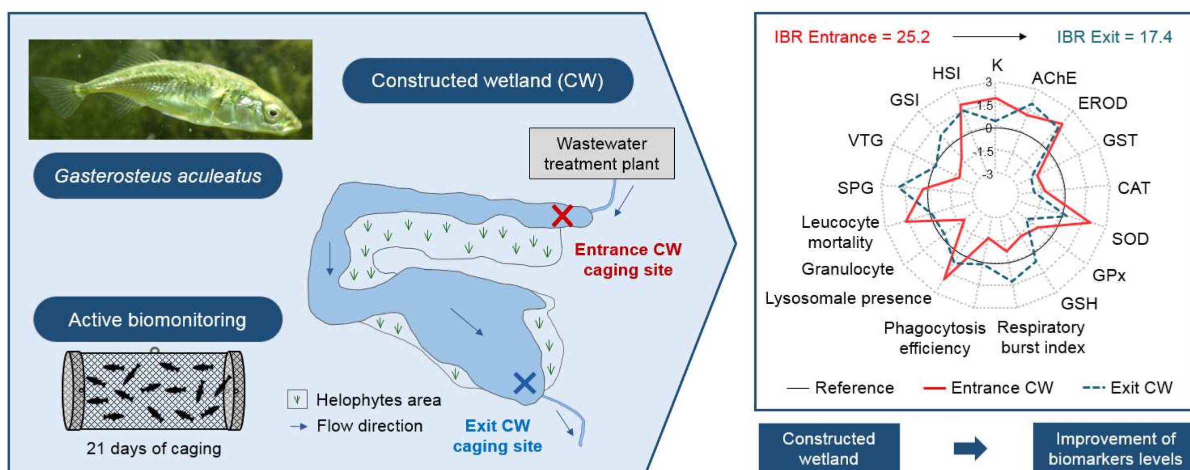
12

ABSTRACT

The present work aimed to assess the efficiency of a constructed wetland as tertiary treatment on urban wastewater with a multibiomarker approach using caged three-spined sticklebacks (*Gasterosteus aculeatus L.*). Fish were caged on three sites: at the entrance of the Constructed Wetland (CW), directly inside the wastewater effluent, at the exit of the CW, and in a weakly impacted site considered as a reference. After 21 days of caging, sticklebacks state of health was assessed using several biomarkers representing some biological functions such as innate immune and antioxidant systems, biotransformation enzymes, reproduction parameters and synaptic transmission. A strong inhibition of the innate immune system, an induction of EROD activity and an alteration of the hepatosomatic index were observed in fish caged at the entrance of the CW compared to those caged in the reference site. In addition, wastewater effluent induced a decrease of antioxidant system without induced oxidative damage on cell membranes. No improvement of these biomarkers was observed for antioxidant parameters at the exit of the CW. However, in fish caged at the exit of the CW, the EROD induction observed at the entrance was reduced and the innate immune system presented the same level compared to fish caged in the reference site, underlying the beneficial effect of the CW for these parameters. Integrated Biomarker response (IBR) was equal to 25.2 at the entrance of the CW and 17.4 at the exit of the CW which highlighted the global positive effect of the CW on water quality based on fish biomarker measurement.

GRAPHICAL ABSTRACT



KEYWORDS

Biomonitoring, Integrated Biomarker Response, fish, wastewater, wetland

38 1. Introduction

39 Human activities generate large volumes of wastewater that must be treated before being
40 released into the environment in order to limit the potential negative effects on ecosystems and human
41 health. However, for several decades, the presence of micropollutants in aquatic environments has
42 become a widespread problem of increasing concern. These micropollutants, also known as "emerging
43 contaminants", include many different molecules such as pharmaceuticals, personal care products,
44 natural and synthetic hormones, pesticides, and industrial chemicals such as polychlorinated biphenyl
45 (PCB) or polycyclic aromatic hydrocarbons (PAH) (Luo et al., 2014). Wastewater treatment plants
46 (WWTPs) are not designed to treat all these contaminants and WWTPs effluents concentrate these
47 molecules which are released into the natural environment where they can be found in concentrations
48 ranging from ng/L to µg/L (Blum et al., 2018). This complex mixture of xenobiotics may have
49 deleterious effects on living organisms downstream of effluents discharge area. Indeed, many studies
50 highlight estrogenic effects, induction of oxidative stress, modification of immune system parameters
51 and histological damage in caged fish downstream of WWTPs (Cazenave et al., 2014; Jasinska et al.,
52 2015; McGovarin et al., 2018; Pérez et al., 2018).

53 To improve wastewater treatment of WWTPs, new technologies have emerged such as
54 membrane bioreactors, activated carbon absorption, ultra- or nano- filtration, reverse osmosis,
55 ozonation or other advanced oxidation processes (Gorito et al., 2017). However, even if these
56 technologies are effective, they are often expensive. Another alternative may be to set constructed
57 wetland (CW) downstream of WWTPs. CW allows self-purification of water through natural process
58 involving wetland vegetation, soil, and their associated microbial assemblage to treat effluent or other
59 water source (US EPA 2000). This solution has the advantage of being financially more accessible,
60 both in terms of implementation and maintenance (Ayaz and Akca, 2000).

61 Several authors have studied the efficiency of some different types of CW to improve
62 physicochemical parameters. Their results have shown that efficiency depends on the installation type
63 and the season. However, in all cases, CWs are efficient to decrease suspended solids, nitrogen
64 concentration, chemical and biological oxygen demand and water contamination by some pathogens
65 such as coliforms (removal higher than 90%) (Ahmed et al., 2008). Other studies have also highlighted
66 the effectiveness of CWs in reducing pesticides (Anderson et al., 2013), human and veterinary drugs
67 (Anderson et al., 2013; Hsieh et al., 2015), industrial chemicals (Síma et al., 2013; Toro-Vélez et al.,
68 2016) as well as antibiotics and antibiotic-resistant bacteria (Chen et al., 2015). Some authors have
69 also focused on the effects of endocrine disruption in fish and reported a decrease in estrogenic effects
70 downstream of the CW in male fathead minnow (Pimephales promelas) (Hemming et al., 2001;
71 Bringolf and Summerfelt, 2003).

72 All studies conducted to assess CWs efficiency used chemical or physicochemical parameters. 73 However, chemical analyses do not consider the bioavailability of compounds and the real exposure 74 and risk for living organisms. Moreover, physicochemical analyses do not allow the assessment of 75 health status of living organisms in theses ecosystems. Over the past decades, some tools have been 76 proposed to link chemical contamination to biological responses of exposed individuals. Among them, 77 biomarkers are defined as observable and measurable changes on organisms (ranging from molecular 78 to individual level and from biochemical to behavioral responses) following a pollution exposure or an 79 environmental modification (Van der Oost et al., 2003). The biomarker approach is particularly useful 80 to assess environmental impact of local pollution point source (Sanchez et al., 2012) and, by 81 extension, to assess the effectiveness of process that improves the water quality.

82 The present work aims to assess for the first time the effects of a constructed wetland built 83 downstream of a wastewater treatment plant using a multi-biomarker approach in three-spined 84 stickleback (*Gasterosteus aculeatus*) with an active biomonitoring strategy. Caged fish are 85 increasingly used in environmental risk assessment, especially in case of WWTP's effluent 86 contamination (Cazenave et al., 2014; McGovarin et al., 2018; Pérez et al., 2018). Caging is 87 particularly useful when the sentinel species is absent in a study site. Even if the species is present, this 88 approach prevents the risk associated with the capture of endangered species. Moreover, the stress 89 caused by predation is reduced and the sampling is facilitated compared to passive sampling (Oikari, 90 2006). Several biotic (number of individuals, size, sex, age, etc.) and abiotic parameters (distance with 91 the pollution source, exposure duration, etc.) can be standardized through caging approach (Oikari, 92 2006). The use of well calibrated individuals allows a thorough biomarker comparison between sites 93 according to the chemical contamination. In this study, the three-spined stickleback was chosen as 94 sentinel species because of its large geographical repartition in northern Europe and its resistance to 95 pollution and environmental changes (temperature, salinity), which allows caging in many 96 hydrosystems. Moreover, sticklebacks have a small size (from four to eight centimeters) (Wootton, 97 1984) and are easy to handle which facilitates caging (Le Guernic et al., 2016). Biomarkers measured 98 in this study are representative of some important physiological functions frequently assessed in 99 organism downstream WWTP such as innate immune responses, antioxidant system, 100 biotransformation enzymes, reproduction parameters and synaptic transmission (Cazenave et al., 2014; 101 Jasinska et al., 2015; McGovarin et al., 2018; Pérez et al., 2018). The assessment of a great number of 102 physiological functions allow to integrate the effect of a complex mixture of contaminants as in a 103 WWTP's effluent. Finally, biomarkers were integrated in an Integrated Biomarker Response index 104 (IBRv2) developed by Sanchez et al. (2013) that will be used to synthetize the global biomarker 105 response of fish.

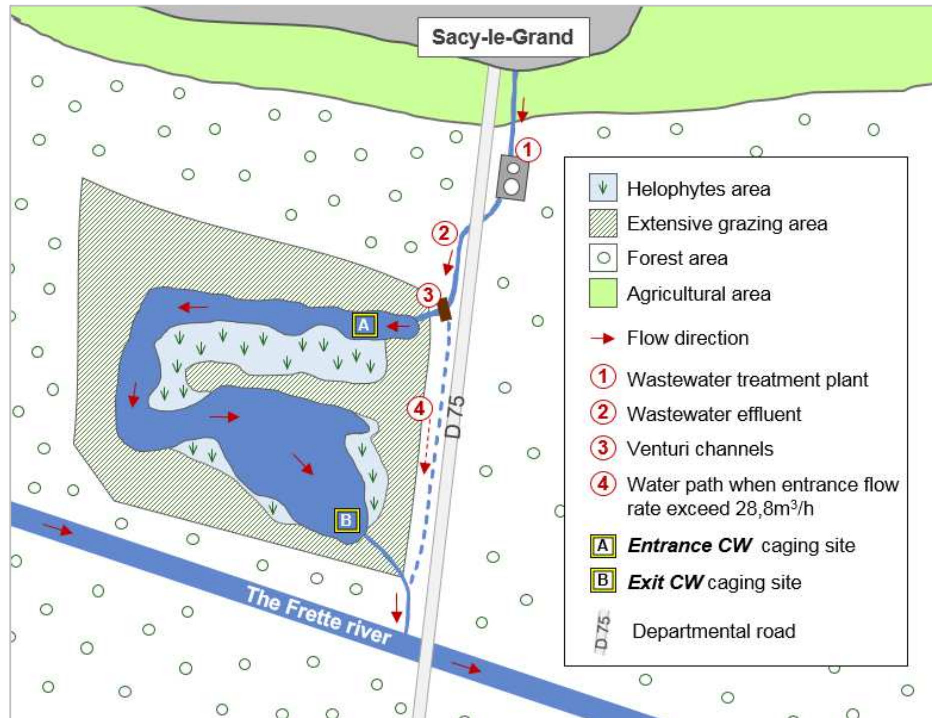
106

107 2. <u>Material and methods</u>

108 ### *2.1. Description of the study area*

109 The city of Sacy-le-Grand is located inside a huge wet and peaty area of 1000 hectares which
110 includes a Natura 2000 and Ramsar site with a surface area of 245 hectares (i.e. Les Marais de Sacy).
111 The WWTPs of Sacy-le Grand is a small plant that collects and treats wastewater from this town and
112 surrounding villages and is designed for 10,000 population equivalents. In addition to the primary
113 treatment, this WWTP is equipped with a phosphate and nitrate removal treatment. The CW is located
114 downstream the WWTP and is used as tertiary treatment: the effluent that enters the CW presents a
115 multi-contamination related to municipal water (effluent from WWTP), and potential runoff from the
116 agricultural plain (pesticides) and from the road nearby (hydrocarbons). This CW was built to improve
117 the water quality before releasing it into the protected area (49°20'40.5"N, 2°32'49.1"E).

118 Inside the CW, the vegetation has grown naturally, and a large part of the shallow waters has
119 been colonized by native vegetation. Thus, 94 different plant species have been identified around and
120 inside the wetland. Among them, the helophytes play the major role in the cleanup process and
121 particularly the broadleaf cattail (*Typha latifolia*) and rush (*Juncus effusus* and *Juncus inflexus*) that
122 have been frequently inventoried in this area. The environment is maintained open by extensive
123 grazing of water buffalo (*Bubalus bubalis*) during Spring and Summer. The studied wetland is an
124 artificial lake of 8500m² with a maximum depth of 1.90 m and a maximum capacity of 3900 m^3. Water
125 enters the wetland through a Venturi channel with a variation of flow rate from 10.8 m^3/h to 28.8 m^3/h
126 which corresponds to a residence time of 5 to 15 days. Beyond 28.8 m^3/h, the excess water runs into
127 the ditch without going through the wetland (Fig. 1).



128

129 *Fig. 1 : Drawing of the constructed wetland of Sacy-le-Grand and surroundings*

130

131 *2.2. Experimental design*

132 The cylindrical cages used are 630 mm high and 270 mm diameter (volume = 36 L). Adult
133 three-spined sticklebacks (42.1 ± 2.8 mm) came from the breeding facilities of the French National
134 Institute for Industrial Environment and Risks (INERIS). They were maintained in outdoor ponds free
135 from chemical contamination under ecosystem conditions for several months before the start of the
136 experiment.

137 Thirty sticklebacks were caged at the entrance (*Entrance CW* site), directly at the WWTP
138 effluent inlet, and thirty others were caged at the exit of the CW (*Exit CW* site) (Fig. 1). At the same
139 time, 15 sticklebacks were caged in a site considered as reference: the Ladrancourt channel
140 (*Ladrancourt* site). Indeed, this site was far from the contamination induced by the WWTP effluent
141 and did not seem to be under any major anthropic pressure. Moreover, the water of the Ladrancourt
142 channel comes directly from the groundwater table by an artesian well, which suggests a relatively
143 clean water. All cages were placed at 50 cm depth. This experiment was carried out in winter (from
144 January 31th to February 20th), outside of the stickleback breeding period, and the exposure lasted 21
145 days during which the fish received no external food intake. pH, oxygen and conductivity were
146 recorded at all sites five times during the study. Temperature was monitored continuously throughout
147 the exposure.

148 *2.3. Organ and blood sampling*

149 After the 21-days exposure, sticklebacks were anesthetised by balneation in MS222 (Tricaine
150 methanesulfonate, 100 mg/L, Sigma-Aldrich, USA) and then were sacrificed by cervical dislocation.
151 This experiment was conducted in accordance with the European directive 2010/63/UE on the
152 protection of animals used for scientific purposes at INERIS facilities (registration number D60-769-
153 02). Standard length, total weight, liver and gonad weights were recorded to calculate physiological
154 indices. Sex was determined at the same time. The general well-being of fish was evaluated with
155 Fulton's condition index (K) (Fulton, 1902).

156 $$K = \frac{Total\ body\ weight}{Standard\ length^{\ 3}} * 100$$

157 Blood samples (5 µL) were then collected and placed in 45 µL of phosphate buffered saline
158 solution (Fisher Scientific, Belgium) supplemented with 30 % heparin and 20 % glycerol. These
159 samples were stored at -80 °C before vitellogenin analysis.

160 The spleen was recovered and gently smashed through sterilized nylon mesh (40 µm, Sigma-
161 Aldrich, USA) with Leibovitz 15 medium (L15, Sigma-Aldrich, USA) modified with heparin lithium
162 (100 mg/L, Sigma-Aldrich, USA), penicillin (500 mg/L, Sigma-Aldrich, USA) and streptomycin (500
163 mg/L, Sigma-Aldrich, USA) (Bado-Nilles et al., 2014a). The leucocytes suspension was stored at 4° C
164 for 18 hours before analysis to prevent bias due to grinding stress.

165 The kidney was recovered and placed in 200 µL of denaturation buffer (Tris-HCl 100 mM,
166 EDTA 10 mM, urea 8 M, SDS 2 %, β-mercapto-ethanol 200 mM). The samples were then frozen in
167 liquid nitrogen and stored in freezer at -80 °C until further analysis.

168 The liver was recovered; weighted and placed in 400 µL of potassium phosphate buffer (0.1
169 M; pH 7.4, Sigma-Aldrich, USA) modified with glycerol (20 %, Sigma-Aldrich, USA) and
170 phenylmethylsulfonyl fluoride (PMSF, 2 µM, Sigma-Aldrich, USA) as protease inhibitor. A piece of
171 tail muscle (20 ± 5 mg) was recovered too and placed in 800 µL of the same buffer. The samples were
172 then frozen in liquid nitrogen and stored in freezer at -80 °C until further analysis.

173 The gonads and livers were weighted to calculate the gonado-somatic index (GSI) and the
174 hepatosomatic index (HSI) respectively (Slooff et al., 1983).

175 $$GSI = \frac{Gonad\ weight}{Total\ body\ weight} * 100$$

176 $$HSI = \frac{Liver\ weight}{Total\ body\ weight} * 100$$

177 *2.4. Biomarkers analysis*

178 All analyses were performed on individual fish.

179 *2.4.1. Innate immune capacities*

180 The leucocytes suspension was used for innate immune biomarkers analysis. To have
181 comparable results, sample leucocyte concentration was then adjusted to 10^6 cell/mL in L15 medium
182 with Malassez haemocytometer (Marienfeld, Germany) to perform analysis by flow cytometry.
183 Analyses were carried out on whole leucocytes, using a CyAn™ ADP (Beckman coulter, USA) flow
184 cytometer using 96 well microplates containing 200 µL of leucocytes suspension. A total of 10,000
185 events per sample were analysed after cell excitation by 488 nm argon laser.

186 The cellular mortality percentages (necrosis and apoptosis) leucocyte distribution (lymphocyte
187 and granulocyte-macrophage), lysosomale presence (LMP), phagocytic capacity and efficiency and
188 leucocyte respiratory burst were assessed following protocols described by Bado-Nilles et al. (2013,
189 2014b) and Gagnaire et al. (2015). All immune biomarkers were expressed in percentage except LMP
190 which was expressed in unit of fluorescence and respiratory burst index which was expressed without
191 unit.

192 *2.4.2. Synaptic transmission, antioxidant and metabolic detoxication enzymes activities*

193 Muscle and liver samples were grinded with glass beads (diameter of 1 mm) using a
194 Precellys24® homogenizer (Bertin Instruments, France) and centrifuged at 10,000 g for 15 min at 4
195 °C. The supernatant of each sample (post-mitochondrial fraction, S9) was placed in 1 mL microtubes.
196 All assays described below were adapted on three-spined stickleback by Sanchez et al. (2005, 2007,
197 2008a).

198 Protein concentration was assessed in both liver and muscle S9 fraction using the Bradford
199 method (Bradford, 1976).

200 Synaptic transmission was assessed with measurement of acetylcholinesterase activity (AChE)
201 on S9 muscle fractions following the method of Ellman et al. (Ellman et al., 1961). Results are
202 expressed in U/g of total protein.

203 Total glutathione (GSH) (Vandeputte et al., 1994), superoxide dismutase activity (SOD)
204 (Paoletti et al., 1986), catalase activity (CAT) (Babo and Vasseur, 1992), glutathione peroxidase
205 activity (GPx) (Paglia and Valentine, 1967), and lipid peroxidation with thiobarbituric acid reactive
206 substance method (TBARS) (Ohkawa et al., 1979) were analysed on S9 liver fractions using a liquid
207 handling automaton (Freedom EVO®, Tecan, Switzerland) and a microplate reader (Synergy™ H4
208 Hybrid, BioTek, USA). GPx, SOD and CAT were expressed in U/g of total protein, while TBARS and
209 GSH amounts were measured in nmol and µmol/g of total protein, respectively.

210 The activity of two enzymes involved in metabolic detoxication were analysed on S9 liver
211 fractions: ethoxyresorufin-O-deethylase (EROD) and glutathione-S-transferase (GST) following
212 Flammarion et al. (2002) and Habig et al., (1974) methods respectively. Results of EROD and GST
213 are expressed in pmol/min/mg of total protein and U/g of total protein.

214 *2.4.3. Endocrine disruption biomarkers*

215 Vittelogenin (VTG) was measured in male blood samples according to the method described
216 by Sanchez et al. (2007). VTG concentration was expressed as ng/mL of fish blood.

217 Spiggin (SPG) was measured in kidney after a dissolution process (grind in boiling water) by
218 specific competitive ELISA as described by Katsiadaki et al. (2002) and Sanchez et al. (2008b).
219 Results for spiggin were expressed as U/g of total fish weight.

220 *2.4.4. Statistical analyses*

221 All statistical analyses were performed with R software version 3.3.2 (Foundation for
222 *Statistical* Computing, Vienna, Austria).

223 One-way ANOVA was performed to analyse physicochemical data (α = 5 %). Normality of
224 residuals (Shapiro-Wilk's tests, α = 5 %) and homoscedasticity (Bartlett's test, α = 5 %) between
225 groups were tested in order to validate the use of an ANOVA. If these criteria were respected,
226 ANOVA was performed and was followed by a Tukey test (α = 5 %). Otherwise the data were log-
227 transformed, and if normality and homoscedasticity of residuals were not obtained despite the log-
228 transformation of the data, ANOVA was replaced by Kruskal-Wallis tests (α = 5 %) followed by
229 Nemenyi's tests (α = 5 %).

230 Biomarker results were first analysed through a Principal Component Analysis (PCA). Some
231 biomarkers were suppressed by grouping or deleting to avoid the redundancy of given information. In
232 this way, leucocyte necrosis and apoptosis rates were summed as a single parameter called "leucocyte
233 mortality". For oxidative burst parameters, only oxidative burst index was kept for PCA whereas ROS
234 B (Basal Reactive Oxygen Species) and ROS A (Reactive Oxygen Species when cells are Activated)
235 were not taken into consideration for PCA construction. In the same way, only Fulton's condition
236 index (K) was considered whereas fish standard length and weight were not taken into consideration.
237 Finally, phagocytic capacity was not considered, and only phagocytic efficiency was kept. TBARS
238 content, which was below the detection limit for all sites, was not take into consideration for PCA.

239 Then, two-way ANOVAs were applied for each biomarker to determine the impacts of "Sex"
240 and "Exposure area" factors. Theses analyses were realized as previously described. Residue
241 normality and homoscedasticity between each group were systematically verified using Shapiro-Wilk

242 and Bartlett's tests ($\alpha = 0.05$). In case of non-compliance with normality, ANOVAs were replaced by
243 Kruskal-Wallis tests ($\alpha = 0.05$). In this case, interaction of both factors was not considered. ANOVAs
244 and Kruskal-Wallis tests were followed respectively by post-hoc tests of Tukey or Nemenyi (α =
245 0.05). When significant difference was detected between gender, data for male and female stickleback
246 were analysed separately for the concerned biomarker. Globally, 25 biomarkers were tested and, with
247 a first order risk equal to 5%, from 0 to 4 false positive results are expected (IC 95%, binomial
248 distribution).

249 The data set was used to calculate an integrated biomarker response index according to the
250 method described by Sanchez et al. (2013). This index, called IBRv2, indicates the global difference of
251 a studied sites compared to a reference value. Biomarker measurement in fish caged in Ladrancourt
252 channel have been used as reference values for the present study. As for the PCA analysis, the
253 redundant information were suppressed by deleting or grouping the considered biomarkers and
254 TBARS was not considered. Briefly, sites averages were calculated for each biomarker (X_i). The
255 calculated averages of the Ladrancourt site were considered as the reference values (X_0). Then,
256 averages of the three sites (Ladrancourt, entrance and exit of wetland) were divided by the reference
257 values (X_0) and a log transformation was applied ($Y_i = \log(X_i/X_o)$). A deviation index was calculated
258 by dividing the site values by the general standard deviation (s) of the log transformed ratio ($A = Yi/s$).
259 This allowed to create the basal line and to represent biomarker variation from the reference values
260 (Ladrancourt). At each exposure sites, the calculated A values were reported in a star plot representing
261 the deviation of each biomarker from the reference level. A value above 0 reflects a biomarker
262 induction while a value below 0 indicates a biomarker inhibition. Finally, the absolute values of these
263 index were summed to obtain the overall IBRv2 of each site ($IBRv2 = \sum |A|$).

264 ## 3. Results

265 ### *3.1. Water physical and chemical parameters*

266 Results of physicochemical parameters are presented in Table 1. Significant differences were
267 highlighted for water temperature between the three sites with warmer water at Ladrancourt and cooler
268 water at the exit of the wetland. Conductivity and oxygen rate at the entrance of the wetland were
269 respectively higher and lower compare to Ladrancourt but these differences disappeared at the exit of
270 the wetland.

271 *Table 1: Physical and chemical parameters of studied sites. Results are expressed with*
272 *arithmetical mean ± standard deviation. Statistical differences between sites are expressed with*
273 *letters according to Tukey or Nemenyi Tests.*

	Ladrancourt				Entrance CW				Exit CW			
	mean	±	sd		mean	±	sd		mean	±	sd	
Temperature (°C)	8.9	±	0.8	**c**	7.6	±	1.2	**b**	5.2	±	1.7	**a**

pH	7.5	±	0.1		7.3	±	0.3		7.6	±	0.1	
Conductivity (µS/cm)	534.6	±	160.4	**a**	913.4	±	56.8	**b**	697	±	81.1	**ab**
Oxygen rate (%)	95.6	±	5.4	**b**	56.4	±	3.9	**a**	74.8	±	22.3	**ab**

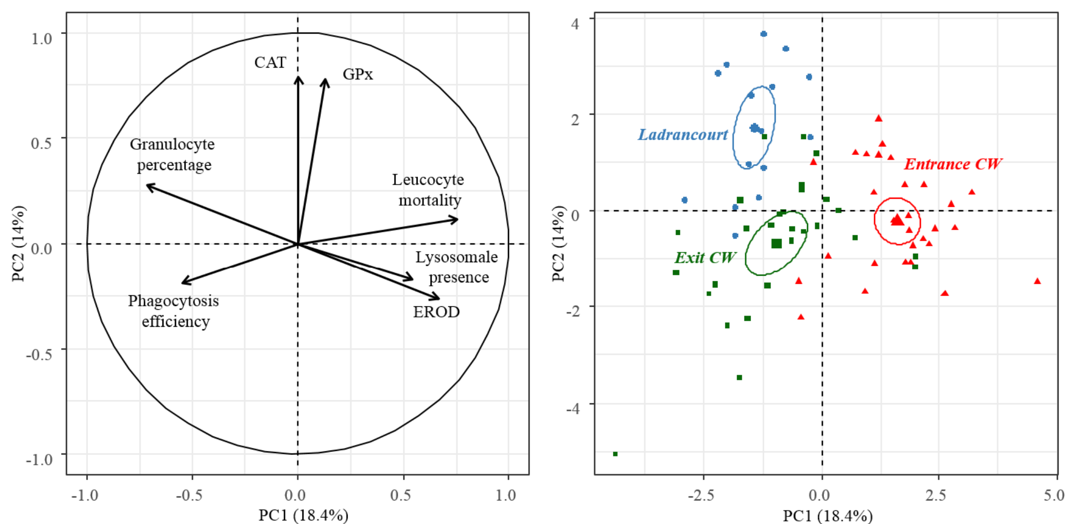
274

275 *3.2. Stickleback biomarkers*

276 No mortality was observed during the whole experiment. Sticklebacks showed no external
277 sign of stress nor injury.

278 The first two principal components of PCA (PC1 and PC2) explained 32.4 % of the overall
279 variance (Fig. 2). Groups of fish from the different sites are well separated along axis 1 and 2.
280 "Ladrancourt" and "Exit of CW" are separated along PC2 (14 % of overall variance) which was
281 mainly formed by GPx and CAT activities (Table 2). "Ladrancourt" and "Exit of CW" are principally
282 separated from "Entrance of CW" along PC1 (18.4 % of overall variance) which was mainly built by
283 some innate immune biomarkers and EROD activity (Table 2). Fish caged at the entrance of the CW
284 seems to present a decrease of innate immune system (elevated leucocytes mortality, lower phagocytic
285 activity and granulocyte percentage) as well as an increase in EROD activity.

286 More generally, "Exit of CW" and "Ladrancourt" groups are closer than "Entrance of CW"
287 group. Biomarker levels from caged fish at the exit of the wetland appeared to be closer to biomarker
288 levels in fish caged in Ladrancourt.



289

Fig. 1: Results of PCA analysis. Variables graphical is presented to the left. Only variable which contributes for more than 10% to the building of axis are indicated. Individuals graphical is presented to the right. Each dot represents one fish and the ellipses correspond to the 95% confidence ellipses around the barycenter of the different groups. GPx : glutathione peroxidase; CAT : catalase; EROD : 7-ethoxyresorufin-O-deethylase.

290

291

292 *Table 2: Contribution of each biomarker to the construction of the two main components (%).*
293 *CAT : catalase; GPx : glutathione peroxidase; EROD : 7-ethoxyresorufin-O-deethylase; GST :*
294 *glutathione-S-transferase; AChE : Acetylcholinesterase; SOD : superoxide dismutase; GSH :*
295 *total glutathione.*

	PC1	PC2	Sum of total contribution (%)
CAT activity	0.00	26.46	26.46
GPx activity	0.54	25.55	26.09
Granulocyte percentage	16.50	3.23	19.73
Leucocyte mortality	18.17	0.59	18.76
EROD activity	14.02	2.89	16.91
Phagocytosic efficiency	9.65	1.47	11.12
Lysosomale presence	9.33	1.18	10.51
Oxidative burst index	3.57	6.05	9.62
Fulton's condition Index	4.77	3.88	8.65
GST activity	1.26	7.07	8.33
AChE activity	0.23	7.77	8.00
SOD activity	4.30	3.43	7.73
Hepatosomatic index	6.78	0.94	7.72
Spiggin	3.00	3.49	6.49
Gonadosomatic index	0.13	4.97	5.10
GSH content	3.96	1.02	4.98
Vitellogenin	3.78	0.02	3.79

296

297 *3.3. Statistical analysis of biomarkers responses*

298 Levels of biomarkers and statistical results are expressed in Table 3. Some biomarkers
299 increased in fish caged at the entrance of wetland such as male HSI, leucocyte apoptosis and necrosis,
300 and lysosomale presence, whereas some other decreased such as phagocytic activityf (capacity and
301 efficiency), granulocyte rate and GSH content. These changes at the entrance of the wetland were no
302 longer detectable at the exit of the wetland where these biomarkers presented the same levels as in fish
303 caged in Ladrancourt site. Female GPx activity and catalase activity for both sexes presented a
304 significant decrease in fish caged at the entrance and at the exit of the CW. EROD was significantly
305 increased in fish caged at the entrance and at the exit of CW. This induction was higher in male (×10.5
306 in entrance and ×7.8 in exit) than in female fish (×7.1 in entrance and ×5.1 in exit). AChE activity was
307 higher in fish caged at the exit of wetland than in the other sites. Finally, spiggin in female fish was
308 higher at the exit of the CW than in the two other sites. TBARS content was under the detection limit
309 for all sites and was thus not shown.

310

311

Table 3: Biomarker responses in three-spined stickleback caged in the three sites. Values are means ± standard deviation. Statistical difference between groups are expressed with letters according to Tukey Test or Nemenyi Test. GSH : total glutathione; GPx : glutathione peroxidase; CAT : catalase; SOD : superoxide dismutase; EROD : 7-ethoxyresorufin-O-deethylase; GST : glutathione-S-transferase; AChE : Acetylcholinesterase; MFI : Mean fluorescence intensity; FU : Fluorescence unit.

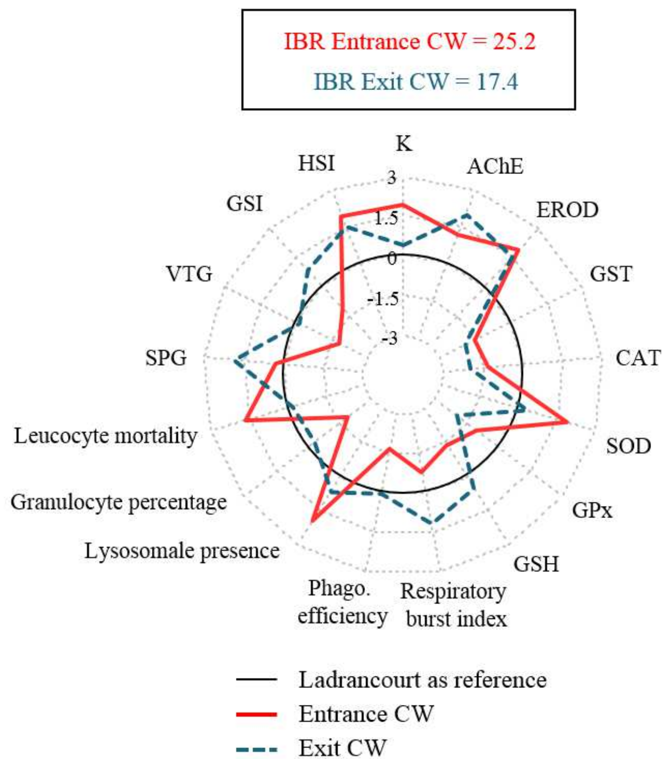
Biomarkers		Ladrancourt				Entrance CW				Exit CW			
		mean	±	Sd		mean	±	Sd		mean	±	Sd	
Sex repartition (number of ♂ / number of ♀)		6/9				13/17				12/18			
General condition (organism)	Length (mm)	42.5	±	3.0		42.0	±	2.7		42.1	±	2.8	
	Weight (mg)	1053.3	±	226.3		1098.3	±	234.5		1035.0	±	194.9	
	Condition Index ♂	1.44	±	0.10		1.53	±	0.16		1.4	±	0.08	
	Condition Index ♀	1.31	±	0.13		1.42	±	0.13		1.36	±	0.12	
	Hepatosomatic index ♂	2.2	±	0.6	**a**	5.1	±	1.3	**b**	3.1	±	0.8	**a**
	Hepatosomatic index ♀	3.0	±	0.8	**a**	5.9	±	2.2	**b**	5.6	±	1.6	**b**
Innate immune system (spleen)	Leucocyte apoptosis (%)	6.2	±	2.3	**a**	10.5	±	4.2	**b**	6.4	±	3.4	**a**
	Leucocyte necrosis (%)	2.6	±	1.0	**ab**	3.2	±	1.3	**b**	2.0	±	0.7	**a**
	Lymphocytes among leucocytes (%)	58.8	±	6.3	**a**	80.5	±	9.3	**b**	64.1	±	8.9	**a**
	Lysosomal presence (MFI)	3.2	±	0.7	**a**	3.8	±	0.6	**b**	3.4	±	0.5	**a**
	Phagocytic capacity (%)	58.9	±	4.9	**b**	51.9	±	4.9	**a**	59.1	±	6.5	**b**
	Phagocytic efficiency (%)	23.8	±	5.0	**b**	17.9	±	4.4	**a**	23.6	±	7.0	**b**
	Basal oxidative activity (FU)	672.5	±	428.1		575.3	±	285.4		635.2	±	313.4	
	Active oxidative activity (FU)	795.2	±	460.6		708.5	±	331.6		755.4	±	337.9	
	Respiratory burst index	1.3	±	0.3		1.3	±	0.2		1.3	±	0.8	
Antioxydant system (liver)	GSH content (µmol/g of total protein)	7.3	±	2.7	**ab**	4.7	±	4.6	**a**	8.6	±	5.3	**b**
	GPx activity ♂ (U/g of total protein)	43.6	±	4.0		50.1	±	20.8		36.5	±	17.5	
	GPx activity ♀ (U/g of total protein)	106.4	±	32.4	**b**	80.3	±	28.8	**ab**	64.1	±	28.5	**a**
	CAT activity ♂ (U/mg of total protein)	865.0	±	215.6	**b**	576.2	±	133.6	**a**	494.5	±	116.8	**a**
	CAT activity ♀ (U/mg of total protein)	1074.3	±	180.2	**b**	755.3	±	153.0	**a**	633.1	±	168.6	**a**
	SOD activity (U/mg of total protein)	13.4	±	5.0		16.9	±	4.8		13.6	±	4.7	
Metabolic detoxification (liver)	GST activity (U/g of total protein)	7382	±	1026		6796	±	1372		6646	±	2246	
	EROD activity ♂ (pmol/min/mg of total protein)	2.87	±	3.67	**a**	30.19	±	17.03	**b**	22.36	±	18.23	**b**
	EROD activity ♀ (pmol/min/mg of total protein)	2.77	±	2.24	**a**	19.67	±	15.13	**b**	14.17	±	8.84	**b**
Synaptic transmission (muscle)	AChE (U/g of total protein)	31.2	±	7.4	**a**	39.6	±	12.9	**a**	48.6	±	15.4	**b**
Reproduction (gonad, blood and kidney)	Gonadosomatic index ♂	0.6	±	0.2		0.7	±	0.3		0.7	±	0.2	
	Gonadosomatic index ♀	5.9	±	1.6		5.7	±	2.9		6.8	±	3.1	
	Vitellogenin ♂ (log ng/mL blood)	2.1	±	0.9			< DL			2.2	±	0.8	
	Spiggin ♀ (log U/g of total weight)	0.3	±	0.2	**a**	0.4	±	0.4	**a**	1.1	±	0.3	**b**

3.4. *Integrated biomarker response (IBRv2)*

Results of IBR calculations and star plots are presented in Fig.3. IBR results for the CW entrance was 25.2 against 17.4 for the CW exit.

As expected, all biomarkers which were highlighted to be significantly different from the Ladrancourt site present a strong deviation index from the basal line (deviation index > 1.5 or < -1.5). Moreover, some biomarkers with non-significant difference with the Ladrancourt site present a strong

324 deviation index. This can be observed for Oxidative burst index, VTG in male, SOD and GST activity
325 at the entrance of the CW as well as in Oxidative burst index, GSI and GST activity at the exit of the
326 CW. Hence, the star plots emphasized some trends in induction or inhibition of biomarker which were
327 not significant with statistical approach. This is probably due to the high inter-individual variability of
328 these biomarkers.



329

330 *Fig. 3 : Result of IBRv2 calculations and star plot of deviation index from Ladrancourt station. K: Fulton's*
331 *condition index; AChE: Acetylcholinesterase; EROD: 7-ethoxyresorufin-O-deethylase; GST: glutathione-S-*
332 *transferase; CAT: catalase; SOD: superoxide dismutase; GPx: glutathione peroxidase; GSH: total glutathione;*
333 *Phago. efficiency: phagocytosis efficiency; SPG: Spiggin; VTG: Vitellogenin; GSI: Gonadosomatic index; HSI:*
334 *Hepatosomatic index.*

335

336

337 4. Discussion

338 The improvement of the water quality is highlighted and summarized by the Integrated Biomarker
339 Response index which was equal to 25.2 at the entrance of the constructed wetland and decreased to
340 17.4 at the exit of the constructed wetland. Considering the entrance of the CW as the theorical
341 maximum IBR, the IBR at Ladrancourt was reduced by 31 % which attests the efficiency of the CW in
342 improving the water quality on a biological point of view. The seeming induction/inhibition of some
343 biomarkers (i.e. Oxidative burst index, SOD, GST) which have not presented a significant difference
344 with the reference site can be explained by the IBR calculation method. In fact, it is based on the mean
345 of biomarker in each site and does not consider the interindividual variation inside groups. The result
346 of IBR calculation is in accordance with ACP analysis which has highlighted the greatest proximity
347 between the fish caged at the reference site and those caged at the exit of the wetland.

348 More precisely, the most important improvement in biomarker levels is measured for the
349 innate immune system which was impaired in fish caged at the entrance of the CW (increase of
350 leucocyte apoptosis and necrosis, reduce of granulocyte percentage and decrease of phagocytosis
351 activity). This immune impairment was totally absent in fish caged at the exit of the wetland. The
352 immunosuppressive effects of WWTP effluents are known and already observed in literature (Kakuta,
353 1997; Ménard et al., 2010). In fact, municipal wastewater can contain a variety of organic (i.e.
354 polycyclic aromatic hydrocarbons, pesticides, etc.) and inorganic substances (i.e. arsenic, cadmium,
355 chromium, lead, etc.) that are known to modulate immunity in fish (Ahmed, 2000; Bols et al., 2001).
356 The immunosuppressive effects observed at the entrance of the CW suggest the presence of a
357 contamination that modulated the immune response in stickleback. This contamination seems to be
358 totally removed after the CW where fish do not present any immunosuppression which highlights the
359 improvement of water quality by the CW.

360 Fish caged at the exit of the CW have presented higher AChE activity (48.6 ± 15.4 U/g of
361 protein) in comparison to those caged at the reference site (31.2 ± 7.4 U/g of protein) and at the
362 entrance of the CW (39.6 ± 12.9 U/g of protein). The measured AChE activity at the exit of wetland
363 corresponds to the normal range (from 44 to 49 U/g of total protein) in control condition outside the
364 breeding period which was observed in a previous study in three-spined stickleback (Catteau et al.,
365 2019). In comparison, activities recorded at the Ladrancourt and the entrance of CW sites in the
366 present study are low, which could indicate the presence of some organophosphorus contaminants
367 known as cholinesterases inhibitors (Sturm et al., 1999). This hypothesis is supported by the
368 agricultural context around the Marais-de-Sacy area which could have contaminated the Ladrancourt
369 site and the entrance of the CW with pesticides and therefore organophosphorus compounds. The
370 constructed wetland seems to reduce chemical contamination and allow the return of AChE activity to
371 a normal range. The discussion about the potential contamination of Ladrancourt site underlines the
372 difficulty to find real contamination free reference sites. One way to solve this difficulty would be to

373 measure biomarker levels in fish before exposure to use these values as reference (Vieira et al., 2017)
374 or to establish generic reference values from control conditions (Marchand et al., 2019).

375 Concerning the antioxidant system, the decrease of CAT and female GPx activities in entrance
376 and exit of CW could be explain by an inhibition of antioxidant defense induced by the WWTP
377 effluent. However, another hypothesis could be that the seeming contamination of the Ladrancourt
378 channel have induced the CAT and female GPx activities in fish caged in this site. Anyway, results of
379 the present study are rather different to those found in literature. Indeed, most studies have shown an
380 increase of antioxidant systems (Cazenave et al., 2014; Jasinska et al., 2015; Pérez et al., 2018) and
381 even an induction of oxidative damages (Cazenave et al., 2014; McGovarin et al., 2018; Pérez et al.,
382 2018) in organisms downstream of WWTP. However, these observations seem to be dependent of
383 considered species and organs. In the present study, because of the likely contamination of the
384 reference site, it's difficult to highlight the effect of the WWTP effluent on antioxidant system.
385 However, the only difference between entrance and exit of the CW is found in GSH measurement,
386 which is slightly higher in the fish caged at the exit of the CW. Surprisingly despite the observed
387 modulations of antioxidant defenses, TBARS content is below the detection limit in both sites which
388 indicates the absence of oxidative damages. This result is contrary to the results highlighted in
389 Sanchez et al. (2008) and in Catteau et al. (2019) which have shown quantifiable basal values of
390 TBARS in the three-spined stickleback at other times of the year. This suggests the existence of a
391 seasonal cycle for the TBARS content in the three-spined stickleback, which seems to be reduced in
392 winter with the low temperature. Finally, all the antioxidant modulations showed in this study were
393 not strong or long enough to be deleterious for fish.

394 The induction of EROD activity in fish caged in the entrance and in the exit of the CW was
395 gone along with the induction of the HSI. It is known that an increase of HSI can be linked with the
396 induction of some hepatic enzyme activities (Slooff et al., 1983). Indeed, some authors noticed a
397 significant increase in HSI downstream of WWTP simultaneously with an increase in EROD activity
398 (Corsi et al., 2003; Mdegela et al., 2010). This induction of EROD activity was slightly reduced but
399 still measured at the exit of the CW in both male and female fish. Considering the EROD activity at
400 the entrance of the CW as a theorical maximum induction activity, the EROD activity decreased only
401 of 26% and 28% in male and female sticklebacks caged at the exit of the CW. Numerous compounds
402 can bind to the AhR receptor and consequently induce CYP1A and EROD activities, especially
403 dioxin-like compounds (i.e. polychlorinated dibenzo-p-dioxins, polychlorinated dibenzofurnas,
404 polychlorinated biphenyls, etc.) or PAH compounds (Whyte et al., 2000). These xenobiotics are
405 known to pass into the aquatic compartment through atmospheric deposition (Sakurai et al., 1998),
406 runoff from agricultural area (Masunaga et al., 2001) or WWTPs effluents (Moon et al., 2008). Some
407 induction of EROD activity downstream of WWTP have been already reported in literature, which
408 tends to confirm the hypothesis of a regular contamination of WWTPs effluents by dioxine-like and/or

409 PAH compounds (Corsi et al., 2003; McCallum et al., 2017). Even if some studies have highlighted
410 the efficiency of CW to remove PAHs (Yi et al., 2016; Lamichhane, 2017), the major way to remove
411 these compounds seems to be the direct adsorption to the sediment or to any suspended solid before
412 sedimentation (Fountoulakis et al., 2009; Wojciechowska, 2013). Consequently, free water surface
413 wetlands with horizontal flow (like the studied CW studied) seems to be less efficient to remove PAHs
414 compared to a subsurface water wetland or a CW with vertical flow, which have more contact with
415 solid matters (Fountoulakis et al., 2009). Finally, the results highlight a weak improvement of EROD
416 activity by the CW, which suggest a weak capacity of this facility to remove this type of
417 contamination.

418

419 Finally, despite the improvement induced by the CW for some parameters, results for
420 endocrine disruption are not as encouraging as for other biomarkers. Indeed, a strong induction of
421 spiggin was measured in female fish caged at the exit of the wetland (× 3.5 log unit) compared to the
422 other fish. This induction was very strong compared to the induction factor found in wild female
423 stickleback previously measured in Rhonelle and Escaut rivers (respectively × 1.22 and × 1.34 log unit
424 in comparison with the reference site) by Sanchez et al. (2007). Although the androgen-like
425 compounds are known to be present in WWTP effluents (Chang et al., 2011), the female sticklebacks
426 caged at the entrance of the wetland did not show any spiggin production induction. One possible
427 hypothesis to explain the absence of induction at the entrance of the CW could be that the complex
428 mixture of the effluent induced some interactions which masked the effect of androgen-like
429 compounds by a decrease of its bioavailability (i.e. adsorption to organic particles). A potential
430 reduction of organic matter by the CW can increase the bioavailability of androgens which can explain
431 the measured spiggin induction at the exit site only. Other investigations are necessary in order to
432 furtherly understand this spiggin induction.

433 The link between the physico-chemical parameters and the biomarker responses can also be
434 discussed especially for temperature which is known to have an important role in the regulation of
435 metabolism activity in poikilotherm organisms such as fish. Water temperature is more elevated in
436 Ladrancourt site than in the CW. Water in Landrancourt comes directly from the groundwater and is
437 consequently very stable throughout the year and never drops below 7°C. The water temperature
438 seemed to decrease in the CW to reach the coolest level at the exit (mean from 7.6°C at the entrance to
439 5.2 °C at the exit). These temperatures are at the bottom of the tolerance range of the stickleback (from
440 0 to 34.6°C) (Wootton, 1984) and the differences between the three sites are only of a few degrees. In
441 our study, observed differences of temperature do not seem to play a major role in the biomarkers
442 modulation in the wetland. Indeed, the IBR index is the highest at the entrance of the CW, compared
443 to the reference site whereas temperatures are of the same order. On the contrary, the variations of

conductivity and oxygen rate were exactly in the same way than some biomarkers responses, especially for innate immune biomarkers. Indeed, conductivity and oxygen rate were respectively higher and lower at the entrance of the CW compared to Ladrancourt site. These modulations were no longer observable at the exit of the CW where the oxygen and conductivity were at the same level compared to Ladrancourt. This highlights the beneficial of the constructed wetland to improve the physico-chemical quality of water. Few information is available in literature about the modulation of biomarker levels according to conductivity. However, the role of oxygen rate is more documented, especially in the induction of oxidative stress in case of hyperoxia (Martínez-Álvarez et al., 2005) and in decreasing of immune system in case of hypoxia (Bowden, 2008). Consequently, hypoxic conditions as at the entrance of the CW could be partly responsible for the immunosuppression measured in fish at the entrance of the wetland. So, to reduce the result misinterpretation, the confounding factors must not be neglected and should be integrated in the result interpretation.

5. Conclusion

In the present study, the efficiency of the constructed wetland of Sacy-le-Grand to improve the water quality was investigated by using an active biomonitoring approach with three-spined sticklebacks. Some physiological functions were assessed such as antioxidant and innate immune systems, metabolic detoxication, synaptic transmission and reproduction parameters. The integration of all the biomarker results in an integrated biomarker response index (IBRv2) has highlighted the benefic effect of this constructed wetland on water quality based on fish biomarkers assessment. This tool has helped to successfully discriminate the entrance and the exit of the wetland on the basis of water quality without use of chemical analysis. Even if few effects on the antioxidant system and the metabolic detoxification as well as an induction of spiggin were observed at the exit of the CW, the most part of biomarker levels impacted by the WWTP effluent were improved at the exit of the CW. More precisely, fish caged at the exit of the constructed wetland did not present any immunosuppressive effect and AChE activity returned to a normal range value in fish caged at the exit of the CW.

The chemical composition of effluents is variable according to the type of wastewater received by the plant (municipal, industrial, hospital), the treatment performed by the plant and the season (temperature and precipitation). In addition, the constructed wetland functioning is depending on the type of CW but also on the season. The results of a multi-biomarker approach only give an ad hoc measurement of the water quality. The tools should be deployed several times at other times of the year to confirm the observed results.

The present work has demonstrated the efficiency of the multi-biomarker approach using caged stickleback to characterize the adverse effects of a contamination by a WWTP effluent and to assess improvement induced by a constructed wetland. The caging approach associated with the

479 integration of the results in an IBR allowed to make a robust environmental diagnosis of the water
480 quality. This tool could be spread out on a larger scale to assess the impact of wastewater treatment
481 plants on the water quality of rivers.

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