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

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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
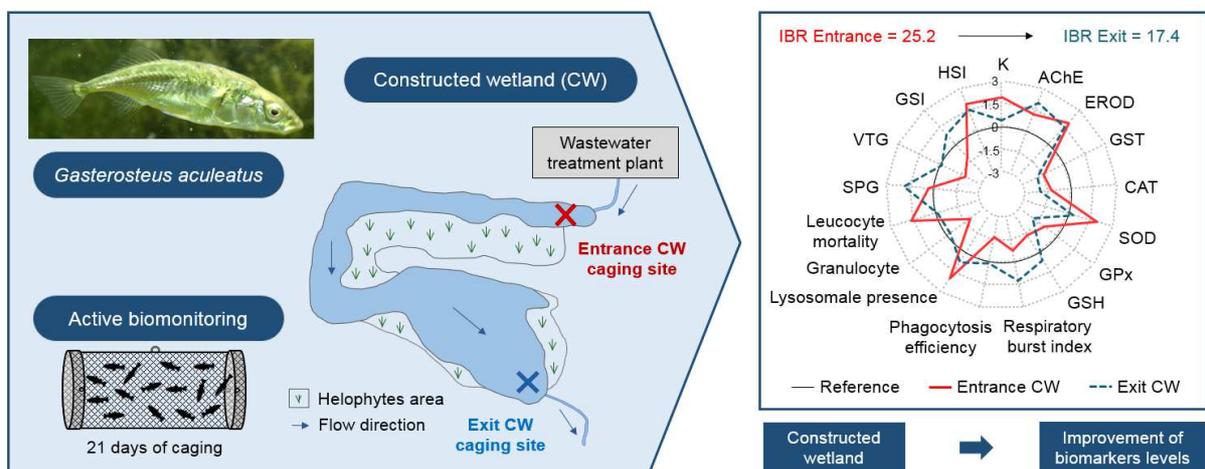
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1. Introduction

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

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

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

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72   All studies conducted to assess CWs efficiency used chemical or physicochemical parameters. However, chemical analyses do not consider the bioavailability of compounds and the real exposure and risk for living organisms. Moreover, physicochemical analyses do not allow the assessment of health status of living organisms in theses ecosystems. Over the past decades, some tools have been proposed to link chemical contamination to biological responses of exposed individuals. Among them, biomarkers are defined as observable and measurable changes on organisms (ranging from molecular to individual level and from biochemical to behavioral responses) following a pollution exposure or an environmental modification (Van der Oost et al., 2003). The biomarker approach is particularly useful to assess environmental impact of local pollution point source (Sanchez et al., 2012) and, by 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 downstream of a wastewater treatment plant using a multi-biomarker approach in three-spined stickleback (*Gasterosteus aculeatus*) with an active biomonitoring strategy. Caged fish are increasingly used in environmental risk assessment, especially in case of WWTP's effluent contamination (Cazenave et al., 2014; McGovarin et al., 2018; Pérez et al., 2018). Caging is particularly useful when the sentinel species is absent in a study site. Even if the species is present, this approach prevents the risk associated with the capture of endangered species. Moreover, the stress caused by predation is reduced and the sampling is facilitated compared to passive sampling (Oikari, 2006). Several biotic (number of individuals, size, sex, age, etc.) and abiotic parameters (distance with the pollution source, exposure duration, etc.) can be standardized through caging approach (Oikari, 2006). The use of well calibrated individuals allows a thorough biomarker comparison between sites according to the chemical contamination. In this study, the three-spined stickleback was chosen as sentinel species because of its large geographical repartition in northern Europe and its resistance to pollution and environmental changes (temperature, salinity), which allows caging in many hydrosystems. Moreover, sticklebacks have a small size (from four to eight centimeters) (Wootton, 1984) and are easy to handle which facilitates caging (Le Guernic et al., 2016). Biomarkers measured in this study are representative of some important physiological functions frequently assessed in organism downstream WWTP such as innate immune responses, antioxidant system, biotransformation enzymes, reproduction parameters and synaptic transmission (Cazenave et al., 2014; Jasinska et al., 2015; McGovarin et al., 2018; Pérez et al., 2018). The assessment of a great number of physiological functions allow to integrate the effect of a complex mixture of contaminants as in a WWTP's effluent. Finally, biomarkers were integrated in an Integrated Biomarker Response index (IBRv2) developed by Sanchez et al. (2013) that will be used to synthetize the global biomarker response of fish.

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

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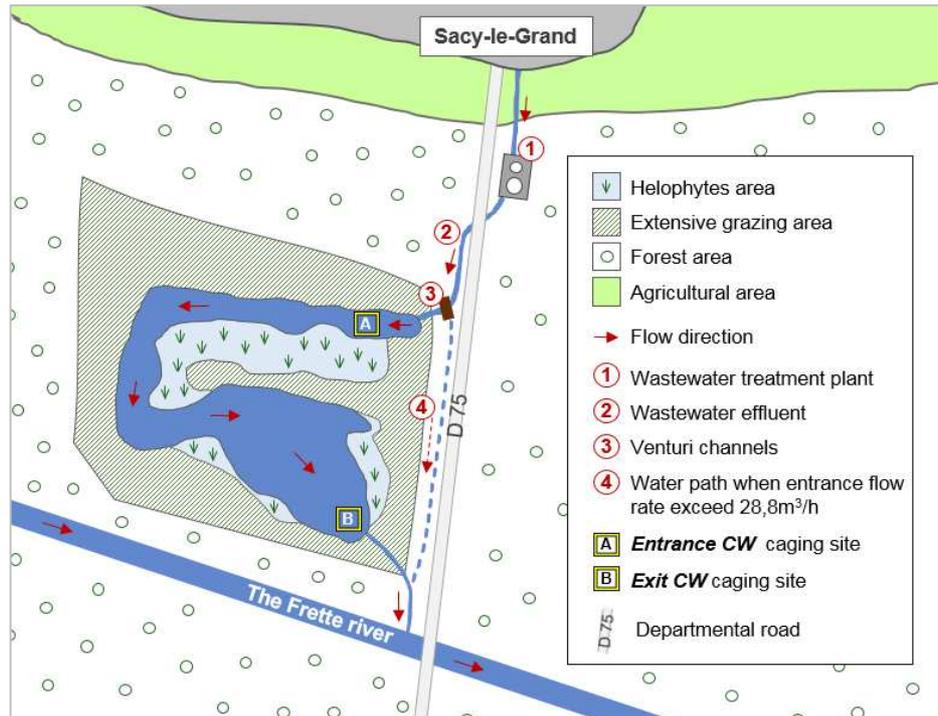
Fig. 1 : Drawing of the constructed wetland of Sacy-le-Grand and surroundings

2.2. Experimental design

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

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

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2.3. Organ and blood sampling

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

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

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

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

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

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

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

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

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

2.4. Biomarkers analysis

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178   All analyses were performed on individual fish.

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.

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.

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

2.4.3. Endocrine disruption biomarkers

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

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

2.4.4. Statistical analyses

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

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

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

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

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

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

3. Results

3.1. Water physical and chemical parameters

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

Table 1: Physical and chemical parameters of studied sites. Results are expressed with arithmetical mean ± standard deviation. Statistical differences between sites are expressed with 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

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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.

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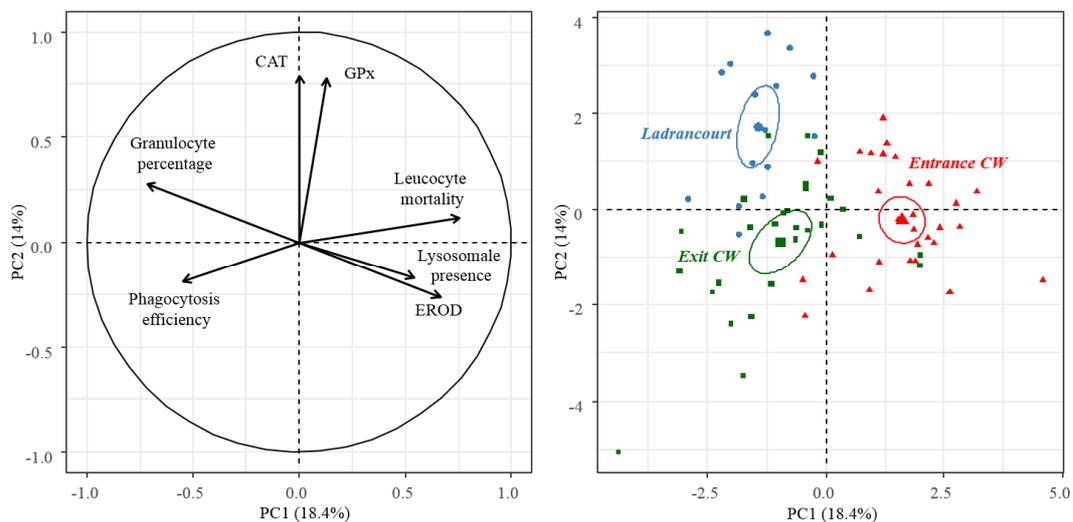
Fig. 1: Results of PCA analysis. Variables graphical is presented to the left. Only variable which contributes for more than 10% to the building of axis are indicated. Individuals graphical is presented to the right. Each dot represents one fish and the ellipses correspond to the 95% confidence ellipses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

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

3.3. Statistical analysis of biomarkers responses

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

|	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

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3.4. *Integrated biomarker response (IBRv2)*

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

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

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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.
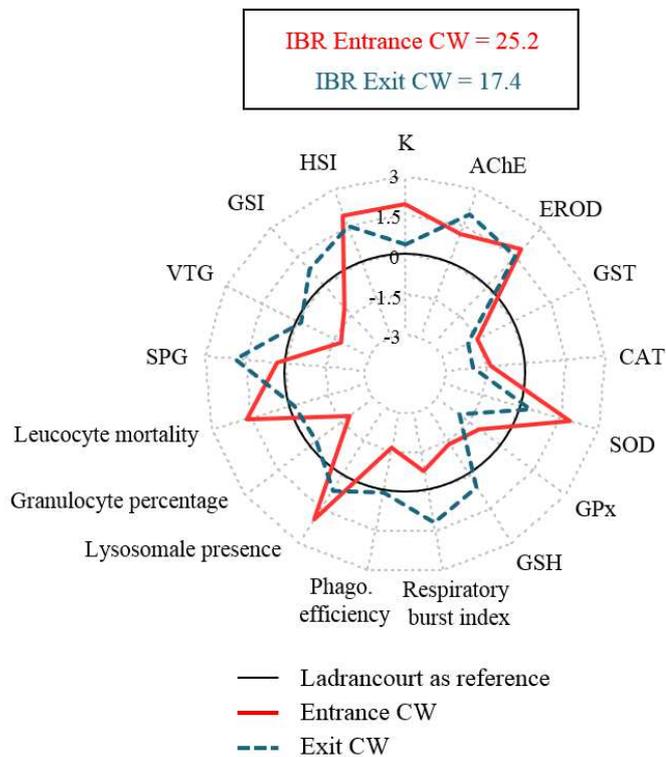
329

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.*

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4. <u>Discussion</u>

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

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

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

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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

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

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

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

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

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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.

482 <u>Acknowledgments</u>

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