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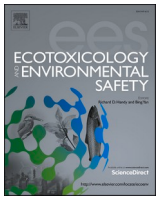
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Water quality of the Meuse watershed: Assessment using a multi-biomarker approach with caged three-spined stickleback (*Gasterosteus aculeatus L.*)

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ABSTRACT

The use of a multi-biomarker approach with three-spined sticklebacks (*Gasterosteus aculeatus*) through an active biomonitoring strategy appears to be a promising tool in water quality assessment. The present work proposes to assess the efficiency of these tools in the discrimination of some sites in a large scale on the Meuse basin in Europe. The study was part of an EU program which aims to assess water quality in the Meuse across the French-Belgian border. Sticklebacks were caged 21 days upstream and downstream from the wastewater treatment plants (WWTPs) of Namur (Belgium), Charleville-Mézières (France), Bouillon (Belgium) and Avesnes-sur-Helpe (France). First, the state of a variety of physiological functions was assessed using a battery of biomarkers that represented innate immunity (leucocyte mortality and distribution, phagocytosis activity, respiratory burst), antioxidant system (GPx, CAT, SOD and total GSH content), oxidative damages to the membrane lipids (TBARS), biotransformation enzymes (EROD, GST), synaptic transmission (AChE) and reproduction system (spiggin and vitellogenin concentration). The impacts of the effluents were first analysed for each biomarker using a mixed model ANOVA followed by post-hoc analyses. Secondly, the global river contamination was assessed using a principal component analysis (PCA) followed by a hierarchical agglomerative clustering (HAC). The results highlighted a small number of effects of WWTP effluents on the physiological parameters in caged sticklebacks. Despite a significant effect of the "localisation" factor (upstream/downstream) in the mixed ANOVA for several biomarkers, post-hoc analyses revealed few differences between upstream and downstream of the WWTPs. Only a significant decrease of innate immune responses was observed downstream from the WWTPs of Avesnes-sur-Helpe and Namur. Other biomarker responses were not impacted by WWTP effluents. However, the multivariate analyses (PCA and HAC) of the biomarker responses helped to clearly discriminate the different study sites from the reference but also amongst themselves. Thus, a reduction of general condition (condition index and HSI) was observed in all groups of caged sticklebacks, associated with a weaker AChE activity in comparison with the reference population. A strong oxidative stress was highlighted in fish caged in the Meuse river at Charleville-Mézières whereas sticklebacks caged in the Meuse river at Namur exhibited weaker innate immune responses than others. Conversely, sticklebacks caged in the Helpe-Majeure river at Avesnes-sur-Helpe exhibited higher immune responses. Furthermore, weak defence capacities were recorded in fish caged in the Semois river at Bouillon. This experiment was the first to propose an active biomonitoring approach using three-spined stickleback to assess such varied environments. Low mortality and encouraging results in site discrimination support the use of this tool to assess the quality of a large number of water bodies.

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1. Introduction

Nowadays, chemical contamination of aquatic environment is observed around the world. The presence of micropollutants in aquatic environments has become an increasing concern for several decades. These micropollutants include many different molecules such as pharmaceuticals, personal care products, natural and synthetic hormones, pesticides and industrial chemicals (i.e. polychlorinated biphenyl, polycyclic aromatic hydrocarbons, etc.) and come from multiple sources. In order to assess, limit and finally reduce the global contamination of aquatic environments, several laws have been promulgated across different countries. At the European scale, the most important legal framework for aquatic environments protection is the Water Framework Directive (WFD, 2000/60/CE and 2012/39/UE) (EU Commission, 2013, 2000) which aims at improving continental water quality thereon achieving good ecological and chemical status of water bodies by 2027. However, several methodological limitations can be observed in the recommended tools for ecological and chemical states assessment in the WFD. Ecological quality assessment is based on the study of biological communities (i.e. benthic invertebrate fauna, fish fauna) but does not include early indicators and can neither determine the origin of a disruption. Chemical quality assessment is currently based on monitoring of 45 chemicals only (or chemical families), defined as "priority chemicals" in the WFD. This approach is thus not exhaustive. According to the context of contamination, more specific analysis could be considered but these measures are often expensive to do. In addition, the limit of quantification can be higher than the concentration with effect on ecosystems. Over the past decade, several tools were proposed to fill the lack of predictivity and exhaustiveness with an increasing interest in a multi-biomarker approach.

Biomarkers have been defined in several manners (Kroon et al., 2017) but the most frequently used definition is the one by Peakall and Walker (1994) who define biomarkers as a "change in biological response (ranging from molecular through cellular and physiological responses to behavioural changes) which can be related to the exposure of a sentinel organism to some chemicals or to the toxicant effect of these chemicals". Aquatic organisms are often exposed to a wide range of pollutants. Thus, studying sets of biomarkers allows risk assessors to integrate the overall contamination to assess the global state of a study area through the assessment of the health status of individuals. Batteries of biomarkers representative of major physiological functions are increasingly used for environmental monitoring programs (Flammarion et al., 2002; Le Guernic et al., 2016a; Sanchez et al., 2008b).

Two strategies of biomonitoring have been developed. The passive biomonitoring approach, which is based on the biomarker measurement using native individuals, was successfully used in the past (Galloway et al., 2004; Hinck et al., 2006; Sanchez et al., 2008b; Dalzochio et al., 2016). However, the biomarker responses in the case of passive sampling can be driven by confounding factors (i.e. length of individuals, sex ratio, food quantity and quality, migration, adaptation to a chronic contamination) (Oikari, 2006; Dalzochio and Gehlen, 2016). Moreover, the spatial monitoring can be limited by the presence of the sentinel species in the studied area (Conti and Cecchetti, 2001). To overcome the limitations induced by the passive sampling, an active biomonitoring approach based on the caging of a sentinel species in the studied sites has been developed. With this approach, individuals can be selected according to the same characteristics (sex, age and size) which can help to limit the variability induced by these confounding factors. Moreover, variability of the response can also be limited by controlling abiotic factors such as distance from pollution source, depth of cage, season, and duration of caging. Caging is particularly useful for comparisons of the effective chemical toxicity between different study sites and can be used when the sentinel species is absent in a study site. Even if the species is present, this approach prevents the risk of capturing endangered species. This active approach can be used with various aquatic species such as bivalves, crustaceans or fish (Besse et al., 2013; Cappello et al., 2013; Dey et al., 2016). Among fish, the three-spined stickleback is a species that has gained interest in biomonitoring for several years and is particularly used in the active approach. Its small size enables easy handling and caging and its tolerance to salinity and temperature variation (Wootton, 1984) allows its caging in a large geographical area in many hydrosystems. This fish was also found to be relatively tolerant to pollution (Pottinger et al., 2002; Sanchez et al., 2008b). Moreover, stickleback is known as a model species for endocrine disruption, especially through the assessment of vitellogenin and spiggin proteins (Katsiadaki et al., 2002). Many biomarkers representative of physiological functions not directly associated with endocrine disruption were developed on this species (antioxidant defences, innate immune responses, biotransformation, synaptic transmission) and used both in passive (Sanchez et al., 2008b) and active biomonitoring (Le Guernic et al., 2016a, 2016b). Finally, effects of caging conditions (density, impacts of transport, food access restriction and confinement) have been well characterised on sticklebacks biomarkers (Le Guernic et al., 2016c; Catteau et al., 2019) which help limit the biomarker responses variability and thus improve interpretation of the results. For all these reasons, the study of a well-known biomarker set, in three-spined stickleback, using an active approach seems to be a promising tool for environmental quality assessment.

For the first time, the present work proposes to assess the efficiency of these tools to discriminate different sites in the Meuse watershed in Europe. To assess the impacts of municipal wastewater on the physiological responses of fish, study sites have been chosen upstream and downstream of the discharge points into the Meuse and its tributaries of some wastewater treatment plants (WWTP).

2. Material and methods

2.1. Description of the study sites

This work was conducted within the framework of a cross-border research program (Interreg DIADeM program) which aims to assess the water quality in the Meuse river basin on both sides of the French-Belgian border. The Meuse basin is influenced by important urban, industrial and agricultural activities but also by many activities linked to tourism (especially during the high season between May and September). With the aim to integrate the diversity of hydrosystems present in this watershed, four study sites, each having a WWTP, have been selected. The river Meuse (average flow of 230 m^3/s) was studied around the WWTP of Charleville-Mézières (49°45'55.3"N 4°43'43.5"E, France) which presents the highest capacity of 117 000 population equivalent (PE) and around the WWTP of Namur (50°28'51.2"N 4°57'18.1"E, Belgium) with a capacity of 93 100 PE. Two tributaries were also investigated, namely the river Helpe Majeure (average flow of 3.86 m^3/s) around the WWTP of Avesnes-sur-Helpe (19 830 PE, 50°07'48.5"N 3°55'13.6"E, France) and the river Semois (average flow of 26 m^3/s) around the WWTP of Bouillon (7500 PE, 49°47'24.0"N 5°03'00.0"E, Belgium) (Fig. 1). All of the studied WWTPs use activated sludge treatment. The Namur WWTP has also specific nitrogen and phosphorus treatments (chemical and biological). The WWTP of Bouillon is in an area with important tourist activity and is therefore endowed with tertiary UV treatment.

Oxygen concentration, pH and conductivity were recorded at three different moments in the different sites during the study and temperature was monitored continuously. Moreover, water samples were collected and the concentrations of 47 molecules were measured. These molecules were 40 pharmaceuticals (antibiotics, analgesics, anti-inflammatories, diuretics, anti-ulcer and neuroleptics), 3 hormones (Estriol, Estrone, Progesterone), 2 hospital activity tracers (iodinated contrast media Iomeprol and Iopromide) and 2 domestic activity tracers (Caffeine, Cotinine). The pharmaceuticals, contrast agents and domestic activity tracers concentrations measured in the Meuse at Namur were higher than those at Charleville-Mézières which is located upstream. For

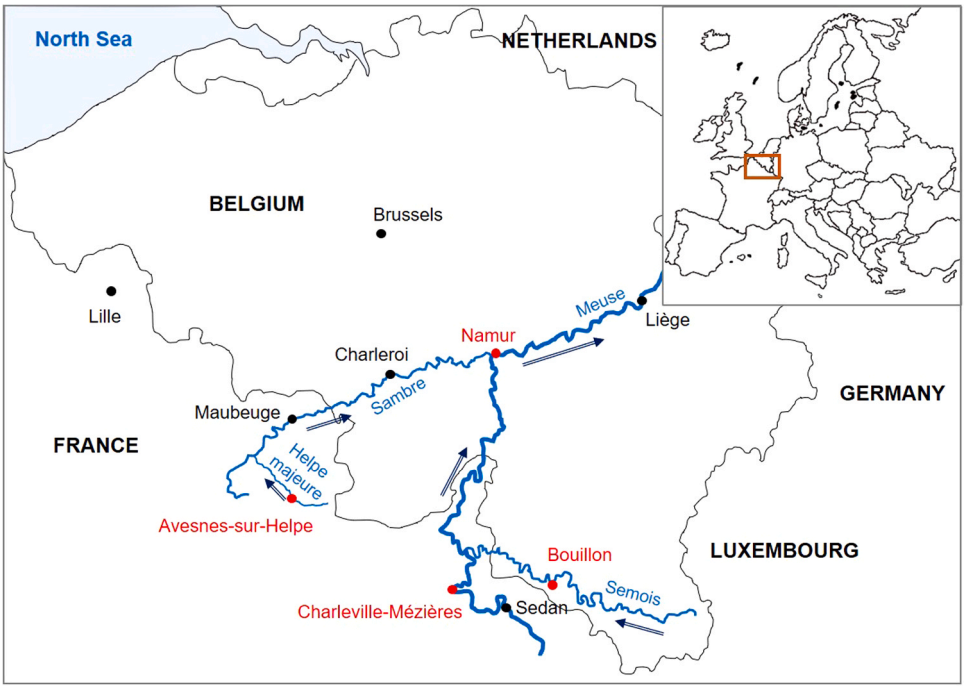


Fig. 1. Localisation of studied sites in France and Belgium along the Meuse basin. Arrows indicate the flow direction.

these three categories of compounds, the concentrations measured in the tributaries are similar to those measured at Charleville-Mézières. However, an important increase of the concentrations was observed downstream of the WWTP at Bouillon. Higher concentrations were also recorded downstream of the discharge points at Namur and Charleville-Mézières (only for the pharmaceuticals). No significant influence of the WWTP was observed at Avesnes-sur-Helpe. Physicochemical parameters and chemical concentrations measured at the 4 sites studied are presented in Table 1. The detailed method and results of chemical analyses are available in supp.data 1 and 2.

2.2. Experimental design

The three-spined sticklebacks used during this study come from a well-characterised population used since several years in the French National Institute for Industrial Environment and Risks (INERIS) (49°16'20.8"N 2°30'16.5"E, Oise, France). Fish were maintained throughout the year in outdoor ponds with natural vegetation and macro-invertebrate communities. Each 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 E60-769-02).

The experiment was conducted in the Autumn 2018 as previously recommended to limit the impact of the reproductive status (Catteau et al., 2019). One week before the start of the experiment, the sex of 240 adult sticklebacks (1-year-old; 4.40 ± 0.45 cm ; 1.07 ± 0.34 g) was determined using the head morphology model (De Kermoysan et al., 2013) to obtain an equilibrated sex ratio of the population. Male and female fish were then maintained in separate tanks until the start of the experiment.

At the start of the experiment, fish were transported in specific tanks to limit stress. After measuring fish length and weight, duplicate cages containing 15 fish sex mixed were placed upstream and downstream of each WWTP to achieve numbers of 15 males and 15 females in each site. All cage deposits were realised during the same period (from the 24th September to the 8th October). The fish fed on natural prey during all

Table 1
Physicochemical parameters and chemical concentrations measured at the 4 sites studied. The physicochemical parameters, results are expressed by an arithmetical Mean ± Sd (standard deviation). For the pharmaceuticals, the mean was calculated by taking the arithmetical mean of the sum of the concentrations measured for the 45 compounds monitored (pharmaceuticals including hormones and hospital activity tracers). For domestic activity tracers, the mean of the sum of the concentration measured for caffeine and cotinine was calculated.

			Bouillon		Avesnes-sur-Helpe		Namur		Charleville-Mézières	
			Up.	Down.	Up.	Down.	Up.	Down.	Up.	Down.
Physicochemical parameters	**Temperature (°C)**	**Mean**	13.1	13.4	12.5	12.6	15	15.2	13.5	13.6
		Sd	1.7	1.6	1.5	1.5	0.9	1	1.6	1.6
	Conductivity (µS/cm)	**Mean**	310	314	505.8	379	685.3	707	606.6	599.4
		Sd	26.4	26.7	122.2	20.5	46.4	39.6	30.3	11.5
	pH	**Mean**	8.6	8.6	8	7.9	8.1	8	8.1	8.1
		Sd	0.5	0.3	0.4	0.2	0.2	0.2	0.2	0.1
	Oxygen rate (mg/L)	**Mean**	11.7	11.5	9.1	9	9.9	9.9	9.7	9.7
		Sd	1.2	0.8	0.9	0.9	0.4	0.5	0.3	0.1
Chemical analyses	**Number of samples**		2	2	2	2	7	7	3	3
	Pharmaceuticals	**Mean**	388.6	1628.8	440.3	425.9	2040.6	4337.3	535.3	731.7
		CV	57.0	96.1	19.2	13.1	110.7	46.6	47.9	23.6
	Domestic activity tracers	**Mean**	100.3	169.8	170.7	167.4	637.0	614.0	172.0	164.0
		CV	15.2	32.7	61.8	63.4	62.9	61.6	40.3	41.1

the experiment and received no supplementary food. Thirty fish from the initial population were kept in the outdoor ponds to be considered as the control condition for this study.

2.3. Biological samples recovery and biomarker analyses

After 21 days of exposure, sticklebacks were anaesthetised by balneation with MS222 (Tricaine methanesulfonate, 100 mg/L, Sigma-Aldrich, USA) before cervical dislocation. The sacrifice and biological samples recovery were realised directly *in situ* to avoid transport stress. All dissections (including the control ones) occurred from the 16th October to the 30th October. The protocols for biological samples recovery and biomarker analyses in the three-spined stickleback are detailed in Catteau et al. (2019, 2020). All analyses were performed individually.

Blood samples (5 μL) were placed in 45 μL of phosphate buffered saline solution (Fisher Scientific, Belgium) supplemented with 30% heparin and 20% glycerol. Kidneys were 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). Pieces of muscle (20 ± 5 mg) and livers were sampled and placed in 800 μL and 400 μL of potassium phosphate buffer respectively (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. Livers were weighed to determine the hepatosomatic index (HSI). All these biological samples were frozen in liquid nitrogen and stored in a freezer at − 80 °C until further analyses.

Muscles and livers were ground with glass beads (diameter of 1 mm) using a FastPrep-24™ 5G (Millipore, France) and then centrifuged at 10,000*g* for 15 min at 4 °C. The supernatant of each sample (post-mitochondrial fraction, S9) was recovered for biochemical biomarker analyses. Muscle supernatants were used for assessing the acetylcholinesterase activity (AChE), as a neurotoxicity marker. Liver supernatants were used to assess the total glutathione concentration (GSH), the superoxide dismutase activity (SOD), the glutathione peroxidase activity (GPx), the catalase activity (CAT) and the thiobarbituric reactive substance concentration (TBARS), as biomarkers of oxidative stress. Moreover, the ethoxyresorufin-O-deethylase activity (EROD) and the glutathione-S-transferase activity (GST) were also assessed, as metabolic biotransformation markers. All these biomarkers were expressed by the protein concentration, measured using the Bradford method. Finally, the vitellogenin concentration (VTG) was assessed in blood samples of male sticklebacks and the spiggin concentration (SPG) was measured in kidneys after dissolution process (ground in boiling water). These biomarkers are respectively representative of oestrogenic and androgenic effects and are measured by specific competitive ELISA tests. All these biochemical biomarkers (oxidative stress, metabolic biotransformation and endocrine perturbation) were validated for three-spined sticklebacks by Sanchez et al. (2005, 2008a, 2008b).

The spleen was also recovered, gently pressed through sterilised nylon mesh (40 μm, Sigma-Aldrich, USA) and the cells obtained were stored in Leibovitz 15 medium (L15, Sigma-Aldrich, USA) modified with penicillin (500 mg/L, Sigma-Aldrich, USA) and streptomycin (500 mg/L, Sigma-Aldrich, USA) at 4 °C for 18 h. The leucocytes suspensions obtained were used for innate immune capacities determination following the protocols initially developed and described by Bado-Nilles et al. (2014) and Gagnaire et al., 2015. All analyses were carried out using a flow cytometer (MACSQuant X, Miltenyi Biotec, USA) with 96 well microplates and 200 μL of leucocytes suspension. A total of 10,000 events per sample were analysed after cell excitation by 488 nm argon laser. Sample concentrations were adjusted to 10^6 cell/mL before analyses. Several parameters were measured, the cellular mortality percentage (apoptosis and necrosis), the leucocyte distribution (percentage of granulocytes and lymphocytes among leucocytes), the phagocytosis efficiency and the respiratory burst capacity.

2.4. Statistical analysis

All statistical analyses were performed with R software version 3.3.2 (R Core Team, 2014).

To assess the difference between the biomarker's levels upstream and downstream of the WWTPs, a mixed model ANOVA was performed (Package *nlme*, function *lme*) on each biomarker with "Site" (Bouillon, Avesnes, Namur or Charleville), "Localisation" (upstream or downstream) and "Sex" (male or female) as fixed factors and "Cage" as random factor. If a significant effect of the "Sex" factor ($p \leq 0.05$) was found for a biomarker, the ANOVA was applied on each gender separately for the biomarker concerned. Normality of residuals (Shapiro-Wilk's tests, $p \leq 0.05$) and homoscedasticity (Levene's test, $p \leq 0.05$) were checked in order to validate the use of ANOVA with raw data. If these criteria were not met, analyses were performed on the log-transformed data if the criteria were met. When "Localisation" factor was found to be significant for a biomarker, the mixed ANOVA was followed by post-hoc analyses adapted for mixed models to identify in which site the upstream/downstream differences were significant. The post-hoc analyses used were the least square means for multiple comparisons with a "Tukey" adjustment method for the p-value (*lsmeans* function of R package *lsmeans*).

In a second step, a Principal Component Analysis (PCA) followed by a Hierarchical Agglomerative Clustering (HAC) using Ward's criterion were performed. This second analysis aims to gather the individuals into clusters characterised by the active variables (the individual biomarker responses in this study) and was conducted with the R package *FactoMineR* with the *HCPC* function. The clusters were described according to the percentage of individuals of each site that are included in it but also with the biomarkers that contribute to the construction of the clusters. For the description with the biomarker values, the analysis gives the average of the biomarkers in the cluster ("Mean in category"), the average of the biomarkers for the whole data ("Overall mean"), the associated standard deviation and a v.test associated with a p value. This v.test corresponds to the test of the following hypothesis: "the mean of the category is equal to the overall mean". An absolute value of the v.test greater than 1.96 corresponds to a p-value lower than 0.05; the sign of the v.test indicates whether the biomarker mean value in the cluster is lower or greater than the overall mean (Husson et al., 2010).

3. Results

A slight mortality was observed with one (Avesnes-sur-Helpe, Bouillon upstream, Charleville-Mézières) or two fish per cage (Bouillon downstream, Namur) which corresponds to a minimal survival rate of 93.3%. Furthermore, at the end of the experiment, no injury nor significant weight-loss were observed. However, a significant decrease in Fulton condition index was measured in 8 of the 16 cages, especially upstream of Namur and downstream of Avesnes-sur-Helpe (Supp.data 3). As expected, using the model based on head morphology, the sex-ratio was relatively close to 50/50 except at Charleville-Mézières where female sticklebacks were more represented than males (70.7% against 29.3%) (Supp.data 4).

3.1. Effects of the WWTP on biomarker responses

The "localisation" factor (upstream/downstream) was significant for many biomarkers, namely the HSI, the EROD activity and the leucocyte necrosis rate in male fish as well as the GST activity, the granulocyte-macrophage subpopulation and the phagocytosis activity (capacity and efficiency) in both sexes (Supp.data 5). In addition, the interaction between "site" and "localisation" factors was significant for AChE and SOD activities as well as the leucocyte apoptosis rate and the respiratory burst index. However, post-hoc analyses revealed few differences between upstream and downstream of the different WWTP. Only a significant increase of the leucocyte apoptosis rate in male individuals can

be observed downstream of Avesnes WWTP in comparison with the upstream level (4.53 ± 3.62 in upstream; 17.55 ± 10.22 in downstream; p-value = 0.027) (Fig. 2). The variability in responses between the different cages (integrated into the model as a random factor) may explain the lack of significant differences between downstream and upstream. However, some p-values were close to the critical p-value (α = 0.05), namely the leucocyte necrosis (4.96 ± 3.29% upstream; 17.59 ± 10.28% downstream; p-values = 0.055) and the granulocyte-macrophage subpopulation in Namur (37.19 ± 10.73% upstream; 23.29 ± 7.99% downstream; p-values = 0.053), which suggests an increase of leucocyte necrosis downstream of the Namur WWTP as well as a modification in the leucocyte subpopulations. All these results show that little differences can be found in the global physiological response of sticklebacks caged upstream and downstream of the different WWTPs. This observation is consistent with the ACP and HAC results. As illustrated with the PCA, the WWTP effluents have only a slight impact on the biomarker responses (Fig. 3) compared to other sources of variability. Indeed, the cluster composition has highlighted that fish caged upstream and downstream in one site are globally grouped in the same cluster (Table 2). Overall, the results have indicated low impacts of the WWTP effluents whatever the characteristics (size, flow, urbanization…) of the river considered.

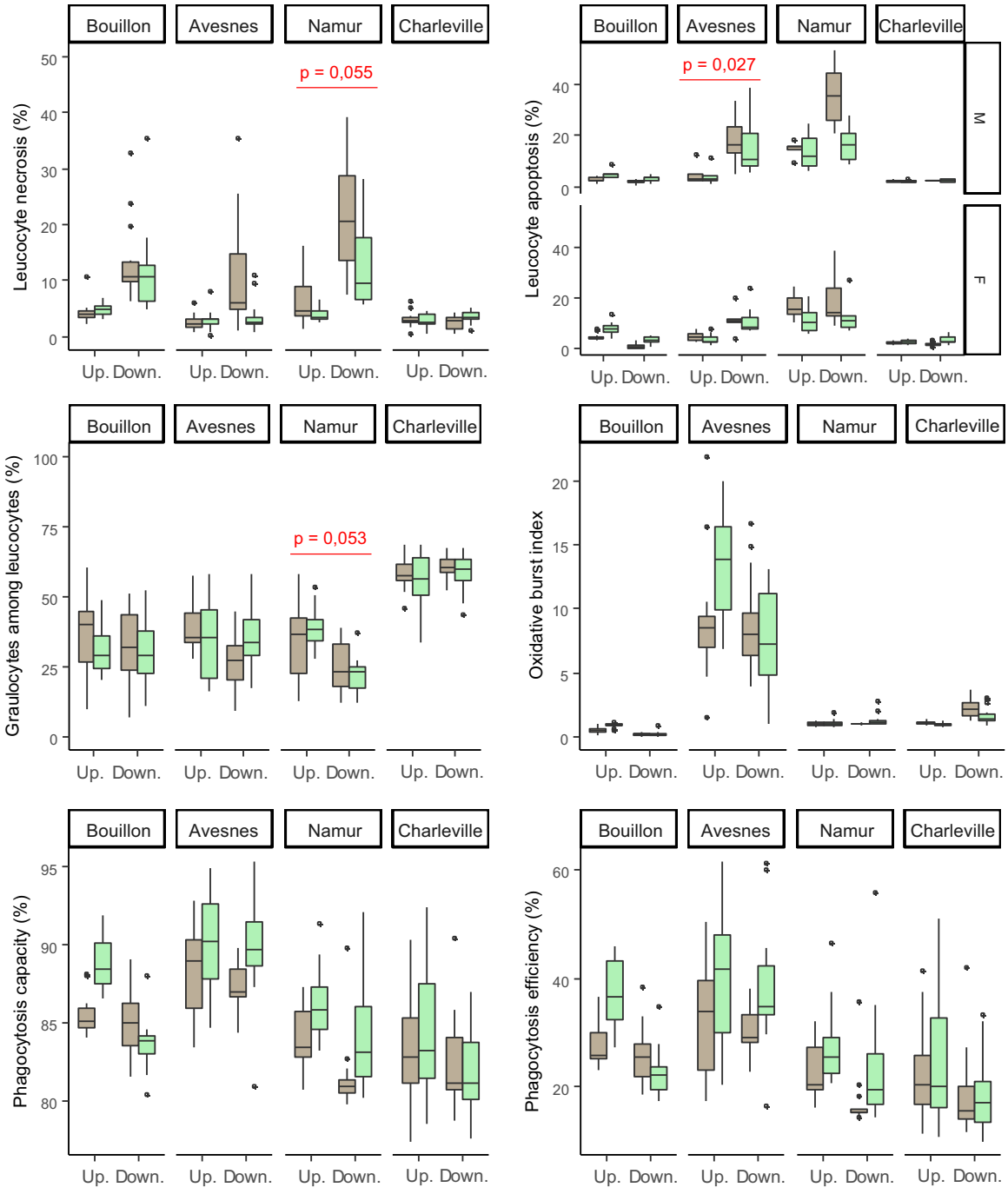


Fig. 2. Innate immune responses in the three-spined sticklebacks caged upstream and downstream of each WWTP. Boxplots (grey and green) represent the two cages at each localisation. P-values of post-hoc tests (Least Square Means for Multiple Comparisons with a "Tukey" adjustment method for p-value) comparing upstream and downstream < 0.1 were reported in red for the sites concerned. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

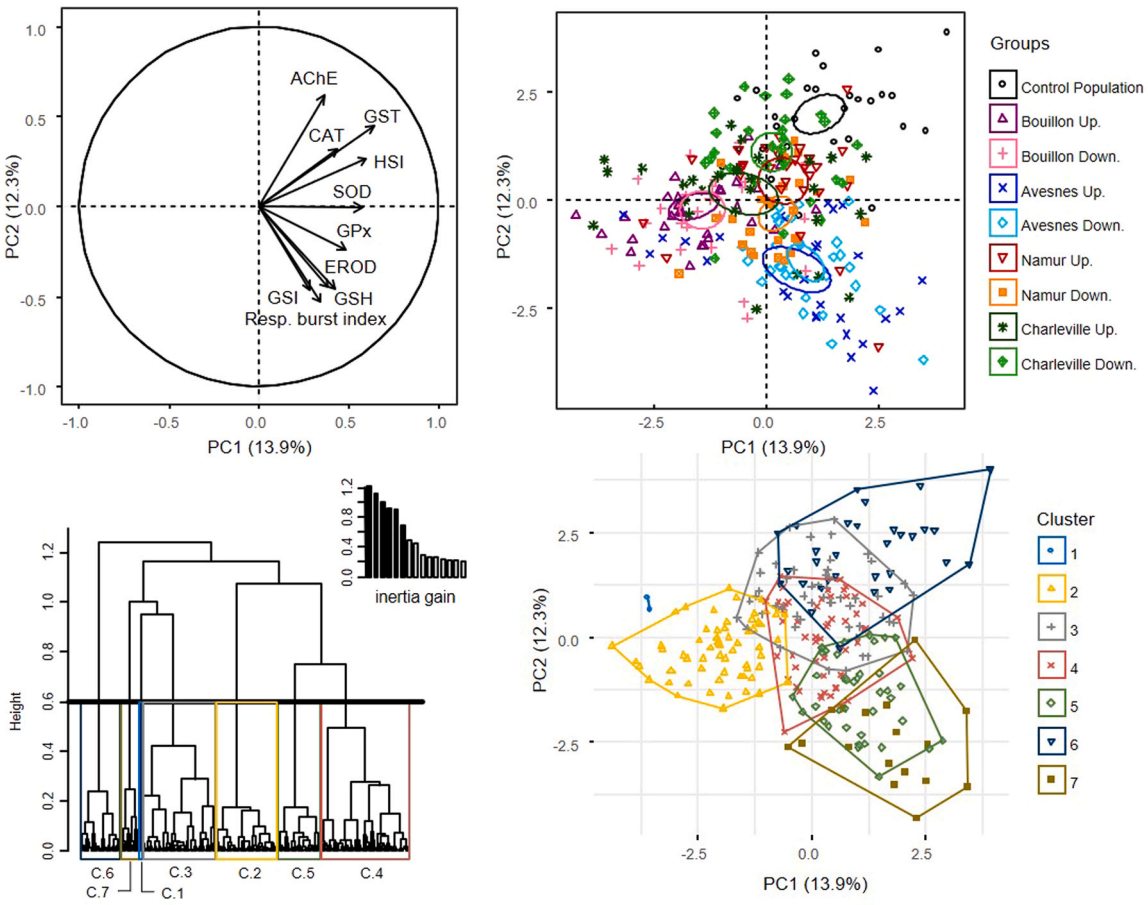


Fig. 3. Results of the principal component analysis (PCA) and of the hierarchical agglomerative clustering (HAC). Top left: Variable graphical. Only variable which contributes for more than 10% to the building of axis are indicated. Top right: Individual graphical. Bottom left: Dendrogram generated by the AHC. Bottom right: Individual graphical with cluster. GSI: Gonadosomatic index; Resp. burst index: Respiratory burst index; EROD: 7-ethoxyresorufin-O-deethylase; GSH: total glutathione; GPx: Glutathione peroxidase; SOD: Superoxide dismutase; HSI: Hepatosomatic index; GST: Glutathione-S-transferase; CAT: Catalase, AChE: Acetylcholinesterase; Up.: Upstream; Down.: Downstream.; C: Cluster.

Table 2
Composition of the clusters. Results are presented in percentage of individuals in each site which are included in the corresponding cluster. Up.: Upstream; Down.: Downstream.

	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	Cluster 6	Cluster 7
Control population		3.3				93.3	3.3
Avesnes Up.		20.7			62.1		17.2
Avesnes Down.		3.4		13.8	69.0		13.8
Bouillon Up.		75.9		20.7		3.4	
Bouillon Down.		75.0		21.4			3.6
Namur Up.		10.7	28.6	42.9		10.7	7.1
Namur. Down.		7.1		92.9			
Charleville Up.	6.9	13.8	65.5			6.9	6.9
Charleville Down.		6.9	93.1				
Total number of individuals	2	62	54	54	38	34	15

3.2. Effects of site specificity on biomarkers

The "site" factor was significant for most of the biomarkers studied with p-values lower than 0.001 (Supp.data 5). This result indicates that, despite the low impacts of effluents on stickleback's biomarker responses, the biomarker levels underlined differences between the caging sites. This result is consistent with the HAC, which highlighted differences in the global biomarker responses between the sites but also with the reference population. In fact, sticklebacks caged in the same site can be gathered in one cluster which is associated with the site studied (except for Namur as discussed below). The sticklebacks from the reference population were clearly discriminated from caged sticklebacks.

Cluster 2 (n = 62) gathered most of the individuals caged in Bouillon. More specifically, this cluster is respectively composed of 75.9% and 75.0% of the fish caged in the Semois upstream and downstream of Bouillon's WWTP (Table 2). This cluster was described by lower values for a high number of biomarkers in comparison with the overall mean (from the most to the least important in the cluster building): SOD, GST, HSI, TBARS, CAT, GSH, AChE, GPx, respiratory burst index, EROD and GSI (Table 3). The biomarkers impacted are mainly those that play a role in the defence systems (antioxidant and biotransformation). Oxidative damages are also lower in this group.

Most of the sticklebacks caged in the Helpe Majeure at Avesnes-sur-

Table 3
Contribution of each biomarker to the building of the clusters in the HAC. This table is a summary of Supp.data 5. The numbers correspond to the v.test calculated for each biomarker and each cluster. Only significant v.test are presented in the table. For all |v.test| > 3.3, p.value < 0.001. The v.tests of the variable contributing the most (|v.test| ≥ 5) are represented in bold type. K: Fulton's condition index; HSI: Hepatosomatic index; Phago.: Phagocytosis; Resp. burst index: Respiratory burst index; GSH: total glutathione; GPx: Glutathione peroxidase; CAT: Catalase; SOD: Superoxide dismutase; TBARS: Thiobarbituric acid reactive substances; EROD: 7-ethoxyresorufin-O-deethylase; GST: glutathione-S-transferase; AChE: Acetylcholinesterase; GSI: Gonadosomatic index; VTG: Vitellogenin; SPG: Spiggin.

Physiological function	Biomarker	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	Cluster 6	Cluster 7
General condition	K				−3.1	−2.2	3.5	2.3
	HSI		**−5.9**				**8.5**	
Innate immune responses	Leuc. Mortality			−4.2	**9.1**		−3.1	
	Granulocyte-macrophage subpopulation	2.4		**9.6**	**−6.5**	−2.3		
	Phago. Efficiency			**−5.8**	−3.6	4.7	4.4	3.6
	Resp. burst index		−3.7	−2.8	−3.2	**12.9**	−3.0	2.0
Antioxidant system	GSH content		**−5.2**	3.7		2.8	−4.6	4.7
	GPx activity		−4.7	2.4				**7.3**
	CAT activity		**−5.2**		**5.5**		4.2	
	SOD activity		**−9.6**	**6.1**	2.0	3.0		
Oxidative damages	TBARS content		**−5.4**	**6.6**				
Biotransformation	EROD activity		−3.6	−3.3		**6.1**		
	GST activity		**−8.3**	2.7	2.0	2.8	4.8	−3.3
Synaptic transmission	AChE activity		**−5.0**			−2.6	**11.2**	
Reproductive system	GSI		−2.4				−2.1	**−3.3**
	VTG (log)	**15.5**						
	SPG (log)			4.7			−3.0	−2.5

Helpe are gathered in cluster 5 (n = 38), which respectively contained 62.1% of upstream and 69.0% of downstream individuals (Table 2). This group was mainly characterised by a very high respiratory burst index as well as a higher phagocytosis efficiency which suggests an induction of immune responses, despite the slight change in leucocyte composition to the detriment of granulocytes. This cluster is also defined by higher defence capacities (EROD, SOD, and GST activities, GSH content) and a lower AChE activity and K index (Table 3).

The sticklebacks caged in the Meuse at Namur, especially downstream of the WWTP, are split in two clusters (3 and 4). Cluster 4 (n = 54) gathered the majority of the fish caged downstream of the WWTP of Namur (92.9%), 42.9% of the fish caged upstream as well as most of the individuals from the Semois which were not included in cluster 2 (Table 2). Cluster 4 was mainly described by a strong inhibition of immune capacity compared with other groups. High leucocyte mortality and a change in leucocyte distribution (to the detriment of granulocytes) were reported for this cluster associated with a low phagocytosis efficiency and a low respiratory burst index. The mean catalase, SOD and GST activities were also higher for the individuals included in this cluster (Table 3).

Cluster 3 (n = 54) was mainly composed of fish caged in the Meuse at Charleville-Mézières. This group gathers respectively, 65.5% and 93.1% of the sticklebacks caged upstream and downstream of Charleville-Mézières' WWTP. This cluster is also composed of 28.6% of the fish caged upstream of Namur's WWTP (Table 2). This group exhibited a lower leucocyte mortality, phagocytosis efficiency and respiratory burst index as well as a switch in leucocyte distribution to the benefit of granulocytes compared with the overall mean. In addition, the individuals of this cluster exhibited higher levels of antioxidant defences (SOD and GPx activities, GSH content). Higher oxidative damages were also recorded which suggests a high oxidative stress on these individuals (Table 3).

Finally, nearly all the sticklebacks from the reference population (93.3%) were included in cluster 6 (n = 34) (Table 2). This cluster was defined by a higher AChE activity and high HSI which highlighted an inhibition of AChE activity for all caged fish compared with the reference group associated with a decrease in HSI. Cluster 6 was also characterised by lower leucocyte mortality, respiratory burst index, GSH content, GSI and SPG concentrations in females and high phagocytosis efficiency, CAT and GST activities (Table 3).

Two other clusters were defined. Cluster 1 contained only two individuals from the Meuse caged upstream Charleville-Mézières's WWTP. These individuals were male fish which were not in the same cluster as the others because of their high VTG concentration and a change in the leucocyte distribution to the benefit of granulocytes (Table 2 and Table 3). Cluster 7 (n = 15) contained individuals from all sites, even 31.0% of them were sticklebacks caged in the Helpe Majeure at Avesnes-sur-Helpe. These individuals exhibited higher GSI and GPx activity. The fish in this group were all female sticklebacks which had probably not quite completed their breeding (Table 2 and Table 3).

4. Discussion

The present work aimed to assess the water quality of the Meuse watershed using tools that have proven their reliability through other case studies, namely the multi-biomaker approach with three-spined stickleback as model species (Bado-Nilles et al., 2014; Le Guernic et al., 2016c; Sanchez et al., 2008b). The innovative aspect of this work is based on the use of an active approach with this species. The caging approach was already successfully used in lentic environments (Le Guernic et al., 2016a, 2016b) and in little river and wetland (Catteau et al., 2020) well suited to the life of sticklebacks. The environments studied in the present work were large rivers (case of the Meuse) and/or rivers with high flow rates, which are less favourable environments for the sticklebacks. Despite this, visual inspection of the stomach contents revealed very satisfactory access to food (copepods, chironomids, crustaceans, gastropod shell, data not shown), even when the cages were positioned directly in the water column (Namur). In addition, the high survival rates (> 90%) and the absence of significant weight loss, highlight the good general condition of the fish and the feasibility of the approach in environments less suited to the life preferences of the species.

The caging approach has helped to successfully discriminate the different sites studied. In the Meuse at Charleville-Mézières, the majority of the fish were in one cluster and exhibited an oxidative stress highlighted by a strong activity of the antioxidant system and a high level of oxidative damage to the membrane lipids (TBARS) in comparison with others. This higher activity of antioxidant enzymes could be linked to the imbalance of the initial distribution of fish in the cages on this site, where females were more represented than males (70/30). Even if the experiment was conducted outside of the reproductive period to limit physiological modulations related to reproductive hormones, some biomarkers of oxidative stress (mainly catalase and GPx) are known to be more expressed in female sticklebacks, even during sexual rest periods (Catteau et al., 2019, 2020). However in the studies cited, TBARS content has not been demonstrated to be related to sex. This tends to

confirm the existence of oxidative stress, despite the bias related to the sex ratio. According to chemical analyses, the Meuse at Charleville-Mézières seems to be a site lowly contaminated by the compounds measured. However, the oxidative stress demonstrated in this study could be linked to the presence of pollutants which were not assessed during this study, such as pesticides, metals or other organic compounds (Lushchak, 2011; Sevcikova et al., 2011; Stoliar and Lushchak, 2012) which are widely found in surface waters and sediments. This oxidative stress in caged sticklebacks is accompanied by a modulation of immune response, especially visible by an increase of the proportion of granulocytes. An induction of vitellogenin production in two male individuals (cluster 1) was also measured, suggesting exposure to oestrogenic compounds which was not highlighted by the chemical analysis. The effect may be caused by oestrogenic compounds which were not measured in the water samples or to the fact that biological effects can occur at concentrations lower than the limit of quantification. This deleterious effect observed in the Meuse at Charleville could be linked to unknown punctual pollution sources or to a diffuse pollution linked, amongst other things, an urban contamination from the cities through which the river flows before reaching Charleville-Mézières (Verdun 18 k inhabitants and Sedan 17 k inhabitants). Even if sticklebacks in Namur were caged in the same hydrosystem as in Charleville-Mézière, they did not present the same response pattern. Indeed, fish caged in the Meuse at Namur (mostly grouped in cluster 4) were characterised by a general immunotoxicity, mostly represented by a high leucocyte mortality rate and a switch in leucocyte distribution in detriment of granulocytes. This immunotoxicity is mainly found in sticklebacks caged downstream from the WWTP effluent (even if the effect is not strictly significant) which constitutes almost half of the individuals of this cluster (48%). This highlighted that effluent effects on biomarkers were in accordance with chemical measurements which show an increase of contaminant's concentration downstream of the WWTP. This type of immunotoxic effect downstream from WWTP effluents has already been shown in literature (Kakuta, 1997; Ménard et al., 2010; Catteau et al., 2020). A variety of organic (i.e. polycyclic aromatic hydrocarbons) and inorganic substances (i.e. metals) not measured in this study can be found in municipal wastewater and are known to modulate immunity in fish (Bols et al., 2001). In addition to these WWTP's effluent-related effects, it can also be hypothesised that a supplement of contaminants brought by the Sambre could be the cause of this difference in Namur compared to Charleville. Indeed, high concentrations of the compounds monitored in this study have been measured in the Sambre just upstream from the confluence (mean 3 577 ng/l, CV 60%, maximum 7 894 ng/l, for the 45 pharmaceutical compounds, data not show). The Sambre river flows through two urban areas (Maubeuge, 29k inhabitants and Charleroi, 204k inhabitants) as well as areas with agricultural pressure which contribute to the deterioration of water quality downstream from the confluence (Van Vliet and Zwolsman, 2008). This highlights the ability of the multibiomarker approach with caging strategy to effectively discriminate two sites located on the same river. Similarly, the tools deployed helped to distinguish the two tributaries and sub-tributaries. Sticklebacks caged in the Semois at Bouillon (mainly in cluster 2) presented a general weakening of defence systems (antioxidant and biotransformation) compared to the overall level. These responses are surprising with respect to the chemical analyses which have shown that the Semois upstream of the WWTP was not very contaminated. One hypothesis could be that biomarkers were impacted by molecules which have not been monitored in this study. However, this decrease in all defence systems may also be linked to physico-chemical parameters of the Semois River. Indeed, the average conductivity measured at Bouillon was much lower than at other sites whereas concentrations of dissolved oxygen were much higher. No information was found in the literature about modulations of biomarkers according to conductivity. As for oxygenation, hypoxia effects are more documented than hyperoxia. However, Lushchak et al. (2001) showed that in goldfish tissues, hyperoxia could induce a rapid and transient induction of lipid peroxidation which decreased after a few hours to reach levels below the level of the controls. Unlike the present study, the authors showed an induction of antioxidant systems (GST and CAT after returning to a state of normoxia). However, the previous study was carried out for only a few hours of exposure and it is difficult to compare with an exposure time of 21 days. Oxidative stress biomarkers are known for their very rapid and transient responses (Sanchez et al., 2005), which makes their interpretation difficult in a context of long-term multi-contamination. On the contrary to the inhibition of general defence systems in Bouillon, the sticklebacks caged in the Helpe-Majeure (mostly grouped in cluster 5) presented an induction of innate immune responses due to increase of phagocytosis efficiency and respiratory burst index, which corresponds to the oxygen-dependent route of elimination of phagocyted substances (Bols et al., 2001). At the same time, an induction of EROD activity was recorded, which can be linked with the increase of respiratory burst index. The parallel with ROS production by macrophages and EROD induction in presence of polycyclic aromatic compounds was demonstrated by Reynaud et al. (2002). However, in cluster 5, induction of EROD was very slight (2.5 ± 1.3) compared to the overall mean (1.4 ± 1.2). This variation was very low in comparison with previous measured inductions (from 15- to 88-fold) in fish exposed to β-naphtoflavone and prochloraz, known as CYP1A1 inducers (Morrow et al., 2004; Sanchez et al., 2008c). Thus, values recorded in fish caged in Avesnes-sur-Helpe can be considered as a moderate variation of EROD activity. The increase of innate immune response could be linked with a pathogen contamination, maybe due to the agricultural and farming context around the study area. In addition to this general pollution effect, fish caged downstream the WWTP in Avesnes-sur-Helpe were the only ones to present a significant increase in male leucocyte necrosis in comparison with fish caged upstream the WWTP. This effect on leucocyte integrity by a WWTP effluent has already been highlighted in a previous study using caged three-spined sticklebacks (Catteau et al., 2020). Finally, despite this increase in leucocyte necrosis downstream of the effluent at Avesnes-sur-Helpe and a slight immunotoxicity induced by the effluent in Namur, few effects of WWTPs effluents were demonstrated in the present study.

Despite a number of site-specific biomarker responses, a common feature in all caged fish groups was global inhibition of acetylcholinesterase, illustrated by the fact that almost all fish from the reference population were grouped in one cluster with higher acetylcholinesterase activity. This general inhibition can be an indicator of neurotoxicity in caged sticklebacks. In fact, cholinesterase enzymes are responsible for the degradation of the neurotransmitter acetylcholine from the synaptic cleft. This neurotransmitter is involved in the transmission of nervous messages. A modification in cholinesterase activities can induce adverse effects on the normal nervous system function (Fulton and Key, 2001). The measured inhibition of AChE activity in sticklebacks was higher in Bouillon and Avesnes-sur-Helpe (from 65% to 75%) than in Namur and Charleville-Mézières (from 40% to 60%). As the majority of biomarker responses, the AChE activity is modulated by several confounding factors, namely the fish size, the temperature, or the season (Sanchez et al., 2008b; Durieux et al., 2011; Nimet et al., 2017). However, in the present study, stickleback's length was calibrated between 40 mm and 50 mm, the temperature variation was very low (between 12 °C and 15 °C) and all the fish were recovered at the same period. Organophosphates and carbamates are known inhibitors of cholinesterase activity. Unfortunately, pesticides were not assessed in this study and the general exposure to pesticides can only be a hypothesis. Payne et al. (1996) have also shown AChE inhibitions in brown trouts (*Salmo trutta*) sampled in rivers with urban contamination. Authors have suggested that other compounds than pesticides could be responsible of AChE inhibitions, namely detergent and combustion-type hydrocarbons (from exhaust soots, used engine oils…). The inhibition of AChE activity in caged sticklebacks could thus be due both to an exposition to pesticides and to a cocktail of unknown chemicals inhibitors from urban effluents.

Associated with all the effects on the physiological functions previously described, caged sticklebacks exhibited weaker condition index and hepatosomatic index than the reference population. It was shown that this decrease was not related to caging when it occurred at non-breeding periods (Catteau et al., 2019). These decreases of physiological parameters can thus be linked with a general weakening of individuals caused by the general contamination of the rivers. The general contamination along the rivers (even upstream of the WWTPs effluents) could explain the global absence of difference in physiological condition of sticklebacks upstream and downstream of the WWTPs effluents. The effluents contribute to the general contamination and the effect could be undetectable. The exposure time could also have played a role in the present results, being too short or too long to measure some biomarker modulations. Moreover, some physiological functions potentially impacted by chemicals have not been assessed (i.e. genomic integrity, energy metabolism). The use of global approaches (metabolomics, proteomics) could allow greater exhaustiveness in the functions assessed and therefore avoid missing some effects. Additionally, as previously highlighted, the biomarkers studied can be modulated by factors other than chemical contamination, namely the physicochemical parameters. However, modulations induced by the main physicochemical parameters are not completely known and mastered which can induce interpretation biases. An accurate characterisation of the effects of physicochemical parameters on the biomarker responses could ensure better interpretation of the results.

In conclusion, this study has demonstrated the possibility and the pertinence of using an active biomonitoring approach with adult three-spined sticklebacks in environments that do not correspond to the stickleback's normal habitat. The effects of WWTP effluents showed to be low which indicates that the WWTPs do not significantly impact the fish health. However, the tools deployed have effectively helped to discriminate the sites studied from the reference but also amongst themselves. The study of a large set of biomarkers, representative of several physiological functions, appeared to be a very relevant approach to identify the functions of living organisms that are impacted and to highlight differences in the global contamination of various sites. The multivariate analysis has enabled to clearly identify the physiological functions impacted for each site. Active biomonitoring and multi-biomarker approaches have demonstrated their efficiency to distinguish sites in the environment and could be considered as promising tools to assess the quality of water and to identify the effects of contamination on fish physiology.

CRediT authorship contribution statement

Audrey Catteau: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Original Draft, Writing - review & editing, Visualization. **Anne Bado-Nilles:** Investigation, Resources, Writing - review & editing. **Rémy Beaudouin:** Formal analysis. **Cleo Tebby:** Formal analysis, Writing - review & editing. **Sandrine Joachim:** Investigation, Writing - review & editing. **Olivier Palluel:** Investigation, Resources, Writing - review & editing. **Cyril Turiès**: Investigation, Resources, Writing - review & editing. **Nina Chrétien:** Investigation, **Katherine Nott:** Investigation, Resources, Writing - review & editing. **Sébastien Ronkart:** Investigation. **Alain Geffard:** Supervision, Project administration, Funding acquisition, Writing - review & editing. **Jean-Marc Porcher:** Conceptualization, Methodology, Investigation, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2020.111407.

References

Bado-Nilles, A., Jolly, S., Porcher, J.-M., Palluel, O., Geffard, A., Gagnaire, B., Betoulle, S., Sanchez, W., 2014. Applications in environmental risk assessment of leucocyte apoptosis, necrosis and respiratory burst analysis on the European bullhead, Cottus sp. Environ. Pollut. 184, 9–17. https://doi.org/10.1016/j.envpol.2013.07.049.

Besse, J.-P., Coquery, M., Lopes, C., Chaumot, A., Budzinski, H., Labadie, P., Geffard, O., 2013. Caged Gammarus fossarum (Crustacea) as a robust tool for the characterization of bioavailable contamination levels in continental waters: towards the determination of threshold values. Water Res. 47, 650–660. https://doi.org/10.1016/j.watres.2012.10.024.

Bols, N.C., Brubacher, J.L., Ganassin, R.C., Lee, L.E.J., 2001. Ecotoxicology and innate immunity in fish. Dev. Comp. Immunol. 25, 853–873. https://doi.org/10.1016/S0145-305X(01)00040-4.

Cappello, T., Maisano, M., D'Agata, A., Natalotto, A., Mauceri, A., Fasulo, S., 2013. Effects of environmental pollution in caged mussels (Mytilus galloprovincialis). Mar. Environ. Res. 91, 52–60. https://doi.org/10.1016/j.marenvres.2012.12.010.

Catteau, A., Bado-Nilles, A., Beaudouin, R., Joachim, S., Palluel, O., Turiès, C., Galet, C., Geffard, A., Porcher, J.-M., 2020. An active biomonitoring approach using three-spined stickleback (Gasterosteus aculeatus, L.) to assess the efficiency of a constructed wetland as tertiary treatment of wastewater. Ecol. Indic. 114, 106238 https://doi.org/10.1016/j.ecolind.2020.106238.

Catteau, A., Le Guernic, A., Marchand, A., Hani, Y.M.I., Palluel, O., Turiès, C., Bado-Nilles, A., Dedourge-Geffard, O., Geffard, A., Porcher, J.-M., 2019. Impact of confinement and food access restriction on the three-spined stickleback (Gasterosteus aculeatus, L.) during caging: a multi-biomarker approach. Fish Physiol. Biochem. 45, 1261–1276. https://doi.org/10.1007/s10695-019-00670-1.

Conti, M.E., Cecchetti, G., 2001. Biological monitoring: lichens as bioindicators of air pollution assessment — a review. Environ. Pollut. 114, 471–492. https://doi.org/10.1016/S0269-7491(00)00224-4.

Dalzochio, T., Gehlen, G., 2016. Confounding factors in biomonitoring using fish. Ecotoxicol. Environ. Contam. 11, 53–61. https://doi.org/10.5132/eec.2016.01.08.

Dalzochio, T., Rodrigues, G.Z.P., Petry, I.E., Gehlen, G., da Silva, L.B., 2016. The use of biomarkers to assess the health of aquatic ecosystems in Brazil: a review. Int. Aquat. Res. 8, 283–298. https://doi.org/10.1007/s40071-016-0147-9.

De Kermoysan, G., Pery, A., Porcher, J.-M., Beaudouin, R., 2013. A non-invasive method based on head morphology to sex mature three-spined stickleback (Gasterosteus aculeatus L.) in rearing conditions. Math. Biosci. 244, 148–153. https://doi.org/10.1016/j.mbs.2013.05.001.

Dey, S., Samanta, P., Pal, S., Mukherjee, A.K., Kole, D., Ghosh, A.R., 2016. Integrative assessment of biomarker responses in teleostean fishes exposed to glyphosate-based herbicide (Excel Mera 71). Emerg. Contam. 2, 191–203. https://doi.org/10.1016/j.emcon.2016.12.002.

Durieux, E.D.H., Farver, T.B., Fitzgerald, P.S., Eder, K.J., Ostrach, D.J., 2011. Natural factors to consider when using acetylcholinesterase activity as neurotoxicity biomarker in Young-Of-Year striped bass (Morone saxatilis). Fish Physiol. Biochem. 37, 21–29 https://doi.org/10.1007/s10695-010-9412-9.

EU Commission, 2013. Directive n°2013/39/UE du Parlement européen et du Conseil du 12 août 2013 modifiant les directives 2000/60/CE et 2008/105/CE en ce qui concerne les substances prioritaires pour la politique dans le domaine de l'eau Texte présentant de l'intérêt pour l'EEE [WWW Document]. URL http://data.europa.eu/eli/dir/2013/39/oj/eng. (Accessed 8 August 2019).

EU Commission, 2000. Directive n° 2000/60/CE du 23/10/00 établissant un cadre pour une politique communautaire dans le domaine de l'eau | AIDA [WWW Document]. URL http://www.ineris.fr/aida/consultation_document/995#Annexe_V. (Accessed 5.19.17).

Flammarion, P., Noury, P., Garric, J., 2002. The measurement of cholinesterase activities as a biomarker in chub (Leuciscus cephalus): the fish length should not be ignored. Environ. Pollut. 120, 325–330. https://doi.org/10.1016/S0269-7491(02)00137-9.

Fulton, M.H., Key, P.B., 2001. Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorus insecticide exposure and effects. Environ. Toxicol. Chem. 20, 37–45. https://doi.org/10.1002/etc.5620200104.

Gagnaire, B., Bado-Nilles, A., Betoulle, S., Amara, R., Camilleri, V., Cavalié, I., Chadili, E., Delahaut, L., Kerambrun, E., Orjollet, D., Palluel, O., Sanchez, W., 2015. Former uranium mine-induced effects in caged roach: a multiparametric approach for the evaluation of in situ metal toxicity. Ecotoxicology 24, 215–231. 10.1007/s10646-014-1374-8.

Galloway, T.S., Brown, R.J., Browne, M.A., Dissanayake, A., Lowe, D., Jones, M.B., Depledge, M.H., 2004. A multibiomarker approach to environmental assessment. Environ. Sci. Technol. 38, 1723–1731. https://doi.org/10.1021/es030570.

Hinck, J.E., Schmitt, C.J., Blazer, V.S., Denslow, N.D., Bartish, T.M., Anderson, P.J., Coyle, J.J., Dethloff, G.M., Tillitt, D.E., 2006. Environmental contaminants and biomarker responses in fish from the Columbia River and its tributaries: spatial and temporal trends. Sci. Total Environ. 366, 549–578. https://doi.org/10.1016/j.scitotenv.2005.11.008.

Husson, F., Josse, J., Pages, J., 2010. Principal component methods - hierarchical clustering - partitional clustering: why would we need to choose for visualizing data? 17.

Kakuta, I., 1997. Effect of sewage on blood parameters and the resistance against bacterial infection of goldfish, Carassius auratus. Environ. Toxicol. Water Qual. 12, 43–51. https://doi.org/10.1002/(SICI)1098-2256(1997)12:1<43::AID-TOX7>3.0.CO;2-8.

Katsiadaki, I., Scott, A.P., Mayer, I., 2002. The potential of the three-spined stickleback (Gasterosteus aculeatus L.) as a combined biomarker for oestrogens and androgens in European waters. Mar. Environ. Res. 54, 725–728. https://doi.org/10.1016/S0141-1136(02)00110-1.

Kroon, F., Streten, C., Harries, S., 2017. A protocol for identifying suitable biomarkers to assess fish health: a systematic review. PLoS One 12, e0174762.

Le Guernic, A., Sanchez, W., Bado-Nilles, A., Palluel, O., Turies, C., Chadili, E., Cavalié, I., Delahaut, L., Adam-Guillermin, C., Porcher, J.-M., Geffard, A., Betoulle, S., Gagnaire, B., 2016a. In situ effects of metal contamination from former uranium mining sites on the health of the three-spined stickleback (Gasterosteus aculeatus, L.). Ecotoxicology 25, 1234–1259. https://doi.org/10.1007/s10646-016-1677-z.

Le Guernic, A., Sanchez, W., Palluel, O., Bado-Nilles, A., Floriani, M., Turies, C., Chadili, E., Vedova, C.D., Cavalié, I., Adam-Guillermin, C., Porcher, J.-M., Geffard, A., Betoulle, S., Gagnaire, B., 2016b. Acclimation capacity of the three-spined stickleback (Gasterosteus aculeatus, L.) to a sudden biological stress following a polymetallic exposure. Ecotoxicology 25, 1478–1499. https://doi.org/10.1007/s10646-016-1699-6.

Le Guernic, A., Sanchez, W., Palluel, O., Bado-Nilles, A., Turies, C., Chadili, E., Cavalié, I., Adam-Guillermin, C., Porcher, J.-M., Geffard, A., Betoulle, S., Gagnaire, B., 2016c. In situ experiments to assess effects of constraints linked to caging on ecotoxicity biomarkers of the three-spined stickleback (Gasterosteus aculeatus L.). Fish Physiol. Biochem. 42, 643–657. https://doi.org/10.1007/s10695-015-0166-2.

Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. Aquat. Toxicol. 101, 13–30. https://doi.org/10.1016/j.aquatox.2010.10.006.

Lushchak, V.I., Lushchak, L.P., Mota, A.A., Hermes-Lima, M., 2001. Oxidative stress and antioxidant defenses in goldfish Carassius auratus during anoxia and reoxygenation. Am. J. Physiol. - Regul. Integr. Comp. Physiol. 280, R100–R107..

Ménard, L., Escarné, R., Marcogliese, D.J., Cyr, D., Fournier, M., Gagné, F., 2010. The impacts of urban pollution on the immune system of spottail shiners. Fresenius Environ. Bull. 19, 1369–1374.

Morrow, M.D., Higgs, D., Kennedy, C.J., 2004. The effects of diet composition and ration on biotransformation enzymes and stress parameters in rainbow trout, Oncorhynchus mykiss. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 137, 143–154.

Nimet, J., Guimarães, A.T.B., Delariva, R.L., 2017. Use of muscular cholinesterase of Astyanax bifasciatus (Teleostei, Characidae) as a biomarker in biomonitoring of rural streams. Bull. Environ. Contam. Toxicol. 99, 232–238. https://doi.org/10.1007/s00128-017-2111-9.

Oikari, A., 2006. Caging techniques for field exposures of fish to chemical contaminants. Aquat. Toxicol. 78, 370–381. https://doi.org/10.1016/j.aquatox.2006.03.010.

Payne, J.F., Mathieu, A., Melvin, W., Fancey, L.L., 1996. Acetylcholinesterase, an old biomarker with a new future? Field trials in association with two urban rivers and a paper mill in Newfoundland. Mar. Pollut. Bull. 32, 225–231. https://doi.org/10.1016/0025-326X(95)00112-Z.

Peakall, D.B., Walker, C.H., 1994. The role of biomarkers in environmental assessment (3). Vertebrates. Ecotoxicology 3, 173–179. https://doi.org/10.1007/BF00117082.

Pottinger, T.G., Carrick, T.R., Yeomans, W.E., 2002. The three-spined stickleback as an environmental sentinel: effects of stressors on whole-body physiological indices. J. Fish. Biol. 61, 207–229. https://doi.org/10.1111/j.1095-8649.2002.tb01747.x.

R Development Core Team, 2014. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. R Foundation for Statistical Computing, Vienna.

Reynaud, S., Marionnet, D., Taysse, L., Duchiron, C., Deschaux, P., 2002. The effects of 3-methylcholanthrene on macrophage respiratory burst and biotransformation activities in the common carp (Cyprinus carpio L.). Fish Shellfish Immunol. 12, 17–34. https://doi.org/10.1006/fsim.2001.0350.

Sanchez, W., Goin, C., Brion, F., Olsson, P.E., Goksøyr, A., Porcher, J.M., 2008a. A new ELISA for the three-spined stickleback (Gasterosteus aculeatus L.) spiggin, using antibodies against synthetic peptide. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 147, 129–137. https://doi.org/10.1016/j.cbpc.2007.08.007.

Sanchez, W., Katsiadaki, I., Piccini, B., Ditche, J.-M., Porcher, J.-M., 2008b. Biomarker responses in wild three-spined stickleback (Gasterosteus aculeatus L.) as a useful tool for freshwater biomonitoring: a multiparametric approach. Environ. Int. 34, 490–498. https://doi.org/10.1016/j.envint.2007.11.003.

Sanchez, W., Palluel, O., Meunier, L., Coquery, M., Porcher, J.-M., Aït-Aïssa, S., 2005. Copper-induced oxidative stress in three-spined stickleback: relationship with hepatic metal levels. Environ. Toxicol. Pharmacol. 19, 177–183. https://doi.org/10.1016/j.etap.2004.07.003.

Sanchez, W., Piccini, B., Porcher, J.-M., 2008c. Effect of prochloraz fungicide on biotransformation enzymes and oxidative stress parameters in three-spined stickleback (Gasterosteus aculeatus L.). J. Environ. Sci. Health Part B 43, 65–70. https://doi.org/10.1080/03601230701735151.

Sevcikova, M., Modra, H., Slaninova, A., Svobodova, Z., 2011. Metals as a cause of oxidative stress in fish: a review. Vet. Med. 56, 537–546. https://doi.org/10.17221/4272-VETMED.

Stoliar, O.B., Lushchak, V.I., 2012. Environmental Pollution and Oxidative Stress in Fish. INTECH Open Access Publisher.

Van Vliet, M.T.H., Zwolsman, J.J.G., 2008. Impact of summer droughts on the water quality of the Meuse river. J. Hydrol. 353, 1–17. https://doi.org/10.1016/j.jhydrol.2008.01.001.

Wootton, R.J., 1984. A Functional Biology of Sticklebacks. University of California Press.