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1 **Multi-biomarker approach in wild European bullhead, *Cottus* sp., exposed to**
2 **agricultural and urban environmental pressures: practical recommendations for**
3 **experimental design**

4
5 Sabrina Jolly^{1,2}, Anne Bado-Nilles^{1,2}, Florent Lamand³, Cyril Turies¹, Edith Chadili¹, Jean-
6 Marc Porcher¹, Stéphane Betouille², Wilfried Sanchez^{1*}

7
8 1. Institut National de l'Environnement Industriel et des Risques (INERIS), Unité
9 d'Ecotoxicologie in vitro et in vivo, BP2, 60550 Verneuil-en-Halatte, France

10

11 2. Université de Reims Champagne-Ardenne, Unité de Recherche Vignes et Vins de
12 Champagne - Stress et Environnement EA 2069, Laboratoire Ecologie-Ecotoxicologie, BP
13 1039, 51687 Reims Cedex 2, France

14

15 3. Office National de l'Eau et des Milieux Aquatiques (ONEMA), Délégation Inter-Régionale
16 Nord-Est, 57155 Marly, France

17

18

19 * Corresponding author. Tel : +33 (0)3 44 61 81 21 ; Fax: +33 (0)3 44 55 67 67

20 E-mail address : Wilfried.Sanchez@ineris.fr (W. Sanchez)

21

22 **Abstract**

23 In freshwater ecosystems, a large number of chemical substances are able to disturb
24 homeostasis of fish by modulating one or more physiological functions including the immune
25 system. The aim of this study was to assess multi-biomarker responses including
26 immunotoxicity induced by urban and agricultural pressure in European bullheads living in a
27 small French river basin. For this purpose, a set of biochemical, immunological, physiological
28 and histological parameters was measured in wild bullheads from five locations characterized
29 by various environmental pressures. Moreover, to address effects of physiological status and
30 contamination level variation on biomarker responses, fish were sampled during three periods
31 (April, July and October). Results revealed a clear impact of environmental pressure on fish
32 health and particularly on immunological status. An increase of EROD activity was recorded
33 between upstream and downstream sites. Upstream sites were also characterized by
34 neurotoxicological effects. Fish exhibited upstream/downstream variations of immunological
35 status but strong differences were observed according to sampling season. Conversely,
36 regarding biochemical and immunological effects, no significant response of physiological
37 indexes was recorded related to environmental pressures. According to these results, the
38 European bullhead appears as a valuable model fish species to assess adverse effects in
39 wildlife due to urban and agricultural pressures.

40

41 **Keywords:** Fish, Immunotoxicity, European bullhead, Seasonal variability, Multi-biomarker
42 approach

43 **1. Introduction**

44 Adverse effects induced by several environmental pollutants, including widely used pesticides
45 such as organophosphate, organochlorine or metals in aquatic ecosystems were assessed by
46 many authors (Krzystyniak et al., 1995; Brousseau et al., 1997). Since these chemicals are
47 known to induce disturbance of immune function in wild organisms, and especially in fish
48 (Thomas, 1995; Dautremepuits et al., 2004), some immune parameters are proposed as
49 ecotoxicological biomarkers. Currently, innate immune response was used as attractive
50 biomarker for immunotoxicological risk assessment studies on mammals (Luster et al., 1988).
51 This innate immune response is part of the first-line defense in the immune system of
52 organisms acting against pathogens without prior exposure to any particular microorganism.
53 Among them, respiratory burst, a crucial reaction of phagocytes to degrade internalized
54 particles and bacteria (Huber et al., 2006), and lysozyme activity, a major parameter in the
55 nonspecific immune defense (Magnadottir et al., 2006), are described as relevant indicators of
56 toxicant exposure in aquatic organisms (Fournier et al., 2000; Bols et al., 2001; Reynaud and
57 Deschaux, 2006).

58 Moreover, immune biomarkers appear as attractive effect-based monitoring tools due to their
59 capacities to predict population disturbances by modification of disease susceptibility
60 (Zelikoff, 1998; Bols et al., 2001). However, ecotoxicological status of water bodies cannot
61 be reduced to measurement of immunomarkers while, i) immunotoxic pollutants are able to
62 disturb other physiological functions such as reproduction (Kime, 1995) or xenobiotic
63 metabolism (Whyte et al., 2000), and/or ii) many other pollutants are involved in
64 environmental pollution. Also, immune parameters must be integrated in a multi-biomarker
65 approach based on measurement of a set of complementary parameters. These parameters
66 must be linked to several physiological processes able to reflect the effects of global

67 contamination in investigated organisms (Flammarion et al., 2002; Sanchez et al., 2008a),
68 such as biotransformation, oxidative stress and/or neurotoxic disturbances.

69 For this purpose, a set of complementary biomarkers involved in fish immunity (i.e.
70 respiratory burst and lysozyme activity) but also in organic compounds biotransformation (i.e.
71 7-ethoxyresorufin-O-deethylase, EROD; cytochrome P4503A activity, CYP3A, and
72 glutathione-S-transferase, GST activities), protection against oxidative stress (i.e. glutathione
73 peroxidase, GPx,) and neurotoxic disturbances (i.e. acetylcholinesterase activity, AChE) was
74 measured to assess effects of field multi-contamination in wild European bullheads, *Cottus*
75 sp., exposed to agricultural and urban environmental pressures. This fish was selected due to
76 its wide repartition in French and European freshwater ecosystems. Moreover, this small
77 benthic fish is characterized by a strong sedentary behavior (Reyjol et al., 2009) that allows
78 assessing local environmental disturbances conversely to migratory or mobile fish species.

79 Some studies have documented the physiological and biochemical responses of this species
80 after field or laboratory chemical exposure and highlighted the bullhead as a relevant model
81 fish species in ecotoxicology (Bucher et al., 1992, 1993; Dorts et al., 2011a, 2011b). This
82 work was completed by histological analysis of gonads. Indeed, histology offers a powerful
83 tool for sex determination, identifying the stage of gonad development, documenting presence
84 of intersex and other abnormalities. Histological analysis of reproductive tissues can provide
85 predictive information related to the fitness of organisms and their reproduction potential
86 (Blazer, 2002). Gross indices such as Liver- and Gonado-Somatic Index (LSI and GSI,
87 respectively) and Condition Factor (CF) were also determined. These integrative indicators
88 serve to provide information on energy reserve, possible disease and general condition of fish
89 (Mayer et al., 1992). The effect-based monitoring approach deployed in this study allows
90 integrating effects of environmental pollutants in various biological levels (i.e. biochemical,
91 histological, physiological and morphological). Three different seasons were also chosen:

92 spring (April), summer (July) and autumn (October). In fact, environmental conditions, which
93 are directly (temperature, photoperiod, precipitation, pathogens) or indirectly (anthropogenic
94 pressures) controlled by the season, can affect fish physiology and health (Bowden et al.,
95 2007).

96

97 **2. Materials and methods**

98 **2.1. Site descriptions**

99 This study was performed in the Vesle River basin located in Champagne-Ardenne region
100 (Northeastern France). Five sites were investigated: Bouy, Prunay and Muizon located on the
101 Vesle River; Courtagnon and Serzy in the tributary Ardre River (Fig. 1). The Bouy and
102 Prunay sites, located upstream from the city of Reims, were highly influenced by intensive
103 cereal farming and by agricultural (sugar beets, alfalfa, peas and potatoes; 70% of land use)
104 and viticultural practices (5.5% of land use), respectively. Muizon station was chosen due to
105 its urbanized area location (downstream from the city of Reims), where water quality has been
106 classified as “bad” by the French Water Quality Monitoring Program (RNB) due to high
107 concentration of Diuron, associated with high levels of nitrogen and phosphorous (Seine-
108 Normandie Water Agency, 2007). In the Ardre River, the upstream Courtagnon site was
109 located in a forested sector without direct environmental inputs of chemicals, whereas Serzy
110 station, situated downstream, was located in an intensive wine-growing area with “bad” water
111 quality due to high concentrations of isoproturon, aminotriazole, glyphosate, atrazine and
112 MCPA (Seine-Normandie Water Agency, 2007). Due distance between investigated sites and
113 sedentary character of bullhead, investigated populations were isolated in each of the five
114 stations.

115

116 **2.2. Fish collection and tissue sampling**

117 For each location, 12-20 adult European bullheads of both sexes (mean length 82 ± 6 mm)
118 were caught by electrofishing, which seems to have no effect on immune parameters tested
119 (Vanderkooi et al., 2001) , in April, July and October 2010. Fish were immediately killed by
120 spinal dislocation, weighed, measured and sexed. The liver, the gonads and approximately 10
121 mg of muscle were collected and weighed. The liver and the muscle were stored at -80 C for
122 biochemical analysis, whereas the gonads were maintained in Bouin's fluid for histological
123 analysis. Trunk kidneys were also removed under aseptic conditions and cut in two parts to
124 measure immunological parameters. A piece of trunk kidney was stored in liquid nitrogen and
125 another piece was pressed through sterilized nylon mesh (40 μ m, Dutscher) with Leibovitz 15
126 Medium (Sigma) containing heparin lithium (10 U.mL⁻¹, Sigma), penicillin (500 U.mL⁻¹,
127 Biochrom AG) and streptomycin (500 μ g.mL⁻¹, Biochrom AG) to obtain leucocyte
128 suspension (Secombes et al., 1990). Leucocytes were stored for 12 hours at $5 \pm 1^\circ\text{C}$ before
129 measuring immune cellular responses.

130

131 **2.3. Morphometric indices**

132 As described by Janssen et al. (1995), CF were calculated as $[100 \times \text{body weight (g)}] / \text{length}^3$
133 (cm)]. The LSI was calculated as the $[100 \times \text{liver wet weight (g)}] / \text{total body weight (g)}$
134 (Sloof et al., 1983). GSI was calculated as $[100 \times \text{gonad weight (g)}] / \text{fish weight (g)}$ (Lofts et
135 al., 1966).

136

137 **2.4. Biochemical analysis**

138 Both liver and muscle were homogenized in an ice-cold potassium phosphate buffer (0.1M,
139 pH 7.5) supplemented with 20% glycerol and 0.2 mM phenylmethylsulfonyl fluoride (PMSF)
140 in order to inhibit proteolysis. Homogenates were centrifuged at 10,000 g, + 4°C for 15 min
141 and the resulting post-mitochondrial fraction was used for biomarker measurements. Protein

142 concentration was determined using the method of Bradford (1976) with bovine serum
143 albumin (Sigma Aldrich chemicals, France) as the standard. Hepatic activities of EROD,
144 CYP3A, GST, GPx were assessed. Briefly, EROD activity was measured according the
145 method developed by Flammarion et al. (1998a). The diluted samples at 25 to 100 μ g of
146 proteins were added to phosphate buffer containing 8 μ M of 7-ethoxyresorufin and 0.5 mM of
147 NADPH. Formed resorufin was quantified by fluorimetric measurement with 530 nm
148 wavelength excitation and 590 nm wavelength emission. Resorufin was used as standard, and
149 results were expressed in pmol of resorufin/min/mg of proteins. CYP3A activity was
150 measured according to Miller et al. (2000). The post-mitochondrial fraction at 60 μ g of
151 proteins was added to 200 mM of 7-benzyloxyfluoromethylcoumarin and 2 mM of NADPH.
152 The formation of 7-hydroxy-4-trifluoromethylcoumarin (HFC) is monitored by fluorescence
153 using an excitation wavelength of 410 nm and an emission wavelength of 510 nm. Data were
154 expressed as pmol of HFC formed/min/mg of proteins using HFC as standard. GST activity
155 assay was conducted according to Habig et al. (1974). The diluted samples at 12.5 μ g of
156 proteins were mixed with 1 mM of chloro dinitro benzene and 1 mM of reduced glutathione.
157 Enzymatic reaction was monitored spectrophotometrically at 340 nm and results were
158 expressed in U of GST/g of proteins. The diluted samples at 56 μ g of proteins were used to
159 determine GPx activity according to the method of Paglia and Valentine (1967). Cumene
160 hydroperoxide was used as substrate and enzymatic activity was assessed at 340 nm. Results
161 were expressed in U of GPx/g of proteins.

162 AChE activity was measured in muscle according to the method developed by Ellman et al.
163 (1961). The formation of 5-thio-2-nitro-benzoic acid generated by the sample diluted at 5 μ g
164 of proteins or by the standard of AChE from electric eel (Sigma-Aldrich Chemicals), was
165 monitored at 405 nm. The results were expressed as U of AChE/g of proteins.

166

167 **2.5. Immunological parameters**

168 To determine lysozyme concentration, the piece of kidney stored at -80 °C, was homogenized
169 in an ice-cold potassium phosphate buffer (0.05 M, pH 6.2). Homogenates were centrifuged at
170 3,450 g, + 4 °C for 30 min and the resulting supernatant was used for immune analysis. The
171 protein concentration was determined and the lysozyme activity was measured by a slight
172 modification of the turbidimetric assay described by Studnicka et al. (1986). Sample or
173 lysozyme standard (Sigma-Aldrich) was added to *Micrococcus lysodeikticus* suspension
174 (0.2 g/L) and the decrease in absorbance was recorded at 450 nm by spectrophotometry. The
175 results were expressed as U of lysozyme/g of proteins.

176 Concerning determination of respiratory burst activity (Reactive oxygen species (ROS)
177 production), leucocyte suspension samples were centrifuged for 10 min at 400 g at 4°C. The
178 supernatant was eliminated and the leucocytes were re-suspended in 1 mL of L15 medium
179 supplemented with heparin, penicillin and streptomycin. Viable cells were counted by trypan
180 blue exclusion and the cell suspension was diluted with L15 medium to obtain 60,000 viable
181 cells per well. Determination of respiratory burst activity (ROS production) was performed
182 using a modification of the chemiluminescence method of Stave et al. (1983). Each assay well
183 was filled with a volume of 160 µl containing cell suspension in L15 medium (60,000 cells),
184 luminol (20 µL at 2×10^{-5} M in L15 medium) and PMA (20 µL at 125 ng.mL⁻¹). Controls
185 were carried out with L15 medium. Cellular baseline was determined at this zero time, PMA
186 was immediately added and the level of intracellular luminescence was measured in both
187 unstimulated and stimulated cells. Chemiluminescence was determined at 20°C with a
188 microplate luminometer (VICTOR², 1420 Multilabel counter). Data were collected every
189 1.30 min for 45 min. The results were expressed in Relative Luminescence Units.

190

191 **2.6. Gonad histology**

192 Dissected tissues were fixed in Bouin's fluid for 24 hours, dehydrated through a series of
193 ethanol, cleared with toluene and finally embedded in paraffin. Sections of 5 μ m in thickness
194 were cut using a model RM2245 microtome (Leica Microsystems, Wetzlar, Germany) and
195 stained with hematoxylin and eosin (HE). Gonad sections were then examined under a Zeiss
196 Axioimager z₁ light microscope (Zeiss GmbH, Göttingen, Germany) and gonadal
197 development stages were determined and adapted from the classification described by Jafri
198 (1990). Oocyte maturation was divided into four different stages according to the cell size, the
199 morphology and the extent of vitellus accumulation: primary oocytes (stage 1), pre-
200 vitellogenic oocytes (stage 2), secondary oocytes (stage 3) and degenerated oocytes (stage 4).
201 Four spermatogenic stages were differentiated according to the morphology and the size of
202 the cells: spermatogonia (stage 1), spermatocytes (stage 2), spermatids (stage 3) and
203 spermatozooids (stage 4). As several stages coexist in a given gonad, the most advanced stage
204 occurring inside the gonad was chosen to characterize the sexual maturity of fish. The
205 occurrence of abnormalities such as intersex, fibrosis and necrosis were noted.

206

207 **2.7. Statistical analysis**

208 All data are reported as mean \pm standard deviation and the SPSS 17.0 software was used for
209 statistical analysis. Normal distribution of data was verified using Kolmogorov-Smirnov test.
210 Since data sets did not have a normal distribution and homogeneity of variance, the data was
211 log-transformed using $F(x) = \log(1+x)$, prior to parametric analysis. A two-way analysis of
212 variance (ANOVA) was performed for each parameter using sites and gender as factors.
213 When sites by gender interactions were significant ($\alpha = 0.05$), male and female data were
214 treated separately. However, male and female LSI and GSI data were also treated separately
215 due to physiological differences between genders. Biomarker responses measured at each

216 sampling period were compared using one-way ANOVA followed by the Sidak test ($\alpha =$
217 0.05).

218

219 **3. Results**

220 **3.1. Morphometric indices**

221 In April, length (81.3 ± 7.1 mm), weight (7.1 ± 3.2 g) and CF (1.3 ± 0.2 g/cm³) were
222 measured in adult European bullheads at each station and no significant differences were
223 recorded among investigated sites. However, statistical analysis of seasonal variations (data
224 not presented) showed that in July and October, Courtagnon's fish possessed a weak length
225 and weight compared to other sites. Moreover, in July, the Vesle downstream site of Muizon
226 had bigger bullheads than other stations, having the highest CF (1.6 ± 0.9 g/cm³).

227 Among the investigated gross indices, LSI and GSI showed significant gender differences
228 with high values in female fish compared to male. Assessment of seasonal variations showed
229 that LSI values were lower in October compared to April and July whereas GSI data were
230 lower in July than in April and October. Inter-site comparisons showed a lack of variation for
231 LSI and GSI during the year. Nevertheless, statistical analysis of seasonal variations (data not
232 presented) showed an increase of LSI in fish from Serzy collected either in April and October
233 or in July, in fish located at Serzy, Bouy and Muizon. Concerning GSI, in April, only at the
234 Vesle upstream sites of Serzy and Prunay female values were significantly higher than other
235 stations tested; and in October, the male data were lower in Vesle than Ardre River (Table 1).

236

237 **3.2. Biochemical and immune biomarkers**

238 Only the three biotransformation enzymes tested (EROD, CYP3A and GST) and the
239 lysozyme concentrations were gender dependent. In fact, high EROD, CYP3A and GST and
240 low lysozyme activity were detected in male bullheads compared to female (Table 2 and 3).

241 Moreover, in this study, all selected biomarkers were influenced by seasonal variation. In
242 July, a decrease of biotransformation enzyme activities was recorded in both genders for
243 EROD, CYP3A activities and in male for GST activity. This decrease of GST activity was
244 also observed in October in both male and female bullheads. Conversely, high AChE and
245 respiratory burst values were exhibited for bullheads caught in the same period. An increase
246 of lysozyme and GPx activities was also noticed in July (Table 2 and 3).
247 Independent of seasonal variations, some modulations in biomarker responses were detected
248 at the sites. For biochemical biomarkers (Table 2), in April and July, a gradient of EROD
249 activity was observed in the Vesle and Ardre Rivers with significantly low values measured in
250 fish from the agriculturally-contaminated site of Prunay. When CYP3A values were almost
251 equal at each station, higher GST values were detected in April for Bouy's fish. In the same
252 way, GPx values were higher in Bouy station from April to July. AChE activity was also
253 modified with inhibition recorded in July for Vesle and Ardre upstream sites of Serzy, Prunay
254 and Bouy stations and in October only for Serzy area. Finally, concerning immunomarkers
255 (Table 3), an immunostimulation (respiratory burst and lysozyme concentration) was detected
256 in July at Prunay station. Respiratory burst activity was also characterized by a decreasing
257 trend along the Vesle River in October, with a minimal value of 4530 RLU max in Muizon.
258 Conversely, bullheads sampled in April shown a trend of lysozyme increase along the Vesle
259 River with a maximal concentration of 14.7 U/g at the Vesle downstream site of Muizon.

260

261 **3.3. Histological analysis of gonads**

262 For each gender and season, the forest site of Courtagnon site possessed specific stages of
263 gonad development compared to others stations sampled where a similar repartition of
264 testicular/ovary development was observed at each sampling date (Fig. 2) and without
265 intersex (data not shown). Moreover, except for Courtagnon, several histological anomalies,

266 such as fibrosis and necrosis, were also observed in bullhead gonads at all sites with a
267 significant gender difference, i.e. female gonads exhibited more anomalies than male gonads
268 (Table 4).

269 More specifically for male gonads, in April, approximately 30 % of spermatids and 70 % of
270 spermatozooids were detected in the Serzy, Bouy, Prunay and Muizon areas, whereas 100 % of
271 spermatozooids were shown at the Courtagnon station. In July, sites were characterized by the
272 presence of spermatogonia (approximately 35 %) and spermatocytes (approximately 52 %).

273 At this time, only two sites, Serzy and Prunay, still possessed fish with few spermatozooids
274 (approximately 31 %) in their testes. Moreover, the Courtagnon station was clearly dissimilar,
275 with absence of spermatocytes and with a higher percentage of spermatozooids (57 %) than
276 spermatogonia (43 %). Finally, in October, fish testicles were all in the spermatocyte stage
277 and only Bouy and Muizon possessed some organisms in the spermatid stage
278 (approximately 57 %) (Fig. 2). Moreover, during the entire year, a higher percentage of fish
279 with parasperm (> 50 %) was detected at each sampling area with a maximum values (> 80
280 %) in April and October (data not shown).

281 Concerning female gonads, in April, ovary atresia was recorded. However, Prunay possessed
282 some fish (63 %) with secondary oocyte stages; and Bouy was clearly different with the
283 presence of bullhead with only the first three gonad development stages. In July, the
284 Courtagnon area again showed 100 % of fish with ovary atresia, whereas at the four other
285 sites each stage of gonad development was detected. Pre-vitologenic (approximately 52 %) and
286 secondary oocytes (approximately 48 %) were specifically detected in female fish
287 sampled in October. However, some variations were observed in fish from Courtagnon that
288 exhibited gonads with only secondary (approximately 60 %) and degenerated oocytes
289 (approximately 40 %) (Fig. 2).

290

291 **4. Discussion**

292 **4.1. Basal values and reference selection for multi-biomarker data analysis**

293 Two strategies based on absolute reference definition or on upstream/downstream approach
294 are commonly used to analyze biomarker responses. Both interpretation methods are
295 considered complementary due to the level of information supported by these strategies
296 (Sanchez et al., 2010). In this work, the site of Courtagnon could be considered a valuable
297 study area for establishing an absolute reference in the Vesle River basin. Indeed, this site is
298 located in the forested upper area of a Vesle tributary and is not exposed to point source
299 chemical pollutants. However, examination of the reproductive cycle of Courtagnon's
300 bullhead, which resulted from a relationship between variations of bullhead reproductive
301 status and histological examination, showed a clear disturbance of this parameter, with
302 presence of fish exhibiting gonads with secondary or degenerated oocytes in all sampling
303 seasons (Fig. 2). This result could be explained by the specific hydrological status of the
304 Courtagnon site. The site is located near the source of the Ardre River and could be
305 characterized by a lack of temperature variation as observed in other similar sampling
306 locations such as the Vallon du Vivier (Sanchez et al., 2008b) where signs of bullhead
307 reproduction were recorded throughout the year (ONEMA, personal communication). In other
308 stations investigated in this study, the reproductive cycle of bullheads appeared to be in
309 accordance with data reported in the scientific literature describing a spawning period
310 between April and July (Zbinden et al., 2004). Before spawning period (April), male gonads
311 possess spermatids, spermatozooids and parasperm and, after spawning period (July), the
312 gametogenesis began again at all sites with the presence of spermatogonia and/or
313 spermatocytes and a reduction of parasperm presence (data not shown). In the same way,
314 secondary oocytes or atresia were recorded in female ovaries in April and July and a new
315 reproductive cycle was begun with the presence of primary and pre-vitellogenic oocytes. Due

316 to the interaction between reproduction and other physiological functions investigated in this
317 study, such as immunity (Harris and Bird, 2000) and biotransformation (Kirby et al., 2007),
318 fish from Courtagnon cannot be used to establish reference values for bullhead biomarkers in
319 the Vesle River basin. To bridge this gap, a relative reference system based on an
320 upstream/downstream approach was proposed in this study to analyze multi-biomarker
321 responses. In this context, classification of real induction or inhibition seems to be difficult
322 but this strategy allows for integrating the effects induced by pollution and other
323 environmental factors in the upstream area.

324

325 **4.2. Relationship between environmental pressure and fish responses**

326 A noteworthy fact was that the biomarker response patterns noticed along the Vesle River
327 showed a clear difference between upstream sites characterized by agricultural pressure
328 (Prunay and Bouy) and the downstream site of Muizon affected by urban pressure (Table 2),
329 reflecting adverse effects of environmental pollution on wild fish health. This statement is
330 particularly true in April for biotransformation enzymes and in July and October for AChE
331 activity.

332 A gradient effect was recorded for the two immunobiomarkers tested, including respiratory
333 burst and lysozyme activities (Table 3), with a decrease of responses between upstream and
334 downstream. As described by several authors (Fournier et al., 2000; Reynaud and Deschaux,
335 2006), this immunosuppressive effect was correlated with exposure to various chemicals
336 discharged in the river, such as heavy metals, PAHs, PCBs and pesticides. The diminution of
337 lysozyme activity could induce an inhibition of their hydrolysis catalyzation of 1,4- β -linkages
338 between N-acetylmuramic acid and N-acetyl-D-glucosamine residues (Yano, 1996). This
339 action highlighted a direct decrease of pathogen membrane destruction. Moreover, a reduction
340 of phagocytosis capacities was indirectly proved by a decrease of respiratory burst, a crucial

341 reaction to degraded internalized pathogens (Huber et al, 2006), and lysozyme activity, which
342 is an activator of phagocytes and acts as an opsonine (Yano, 1996). Therefore, this
343 disturbance of fish immunity denoted a reduction of pathogen destruction and an increase of
344 fish susceptibility to pathogen aggressions.

345 Concerning biochemical biomarkers, a similar response profile was observed for each
346 parameter tested (EROD, CYP3A, GST, GPx and AChE). In the present work, in wild
347 bullheads an inhibition of EROD activity was suggested at upstream sites. As described
348 previously, this recognized biomarker of exposure to dioxin-like compounds can be inhibited
349 by several pollutants widely used in agricultural activities, such as copper (Flammarion et al.,
350 1996; Viarengo et al., 1997) or pesticides (Flammarion et al., 1998b), and is strongly detected
351 in the Vesle River due to viticultural and agricultural pressures (ONEMA, personal
352 communication). In addition to the destabilization of catalytic activity, phases I and II of
353 pollutant detoxification and hepatic steroid catabolism were also modified in downstream
354 stations compared to upstream sites, due to a trend of modulation of CYP3A and GST
355 activities, respectively. Moreover, GST is known to be involved in the cellular defense
356 against oxidative stress by conjugating electrophilic compounds to glutathione (GSH) (Habig
357 et al., 1974). Since, oxidative stress could be induced by some pollutants, such as copper and
358 pesticides (Dautremepuits et al., 2004; Ahmad et al., 2005), an increase of antioxidant
359 enzymes, such as GPx, could be detected at downstream stations. These enzymes permit the
360 reduction of intermediate products to maintain cellular viability (Reischl et al., 2007) and to
361 decrease oxidative species produced during pollutant metabolization in order to restrain
362 cytochrom P450 decline (Della Torre et al., In Press). Finally, a decrease of AChE activity in
363 bullheads from upstream sites was also observed. The AChE activity is frequently described
364 as a relevant and sensitive parameter to assess exposure to neurotic pollutants and particularly
365 pesticides in wild fish (Payne et al., 1996). Moreover, as obtained in bullhead from sites

366 receiving agricultural run-off in the Vesle and Ardre Rivers, an association between induction
367 of oxidative stress and inhibition of AChE activity in fish exposed to various pesticides such
368 as chlorpyrifos, diazinon and glyphosate (Modesto and Martinez, 2010; Oruç, 2010) was
369 demonstrated.

370 To complete biochemical and immunological analysis, physiological indicators including LSI,
371 GSI and CF were measured in wild bullheads. These parameters are classically used as
372 integrative indicators that provide valuable information on fish health and reproductive status.
373 However, due to influence of biotic and environmental factors on these parameters, they are
374 considered as bad discriminators (Sanchez et al., 2008a). In the present work, few significant
375 inter-site variations were recorded for these physiological indexes and observed differences
376 were between fish from both sites of Ardre river and between both Ardre and Vesle rivers.
377 Also, these results could be explained by hydromorphological variations previously
378 described. To our knowledge, no data is available on basal level of somatic indexes and CF in
379 the European bullhead. Consequently, it is not easy to address accurately these physiological
380 changes.

381

382 **4.3. Seasonal variability: a complex effect**

383 Seasonal effects on biomarker responses are well described in fish (Sanchez et al., 2008b).
384 Indeed, this knowledge is indispensable for a better application and analysis of biomarkers in
385 environmental risk assessment. The present work provided relevant data related to interaction
386 between fish biochemical responses and biotic or environmental factors. A noteworthy fact
387 was that seasonal and also gender effects recorded in the bullhead are in accordance with data
388 available in the scientific literature for other model fish species. A clear gender effect was
389 observed for biotransformation enzyme responses with high values in male fish. This
390 extensively described result is explained by the negative effect of endogenous estradiol on

391 cytochrome P-450 catalytic activity (Arukwe and Goksøyr, 1997). This effect is most
392 pronounced during breeding periods. As phase I biotransformation enzymes, GST activity
393 seems to be correlated to spawning period with lower values recorded in fish collected in
394 October. The same pattern was reported in *Anguilla anguilla* and *Mugil cephalus* (Gorbi et al.,
395 2005) but the involved mechanisms are not understood. A seasonal variation was also
396 measured for GPx activity. If less information is available on seasonal changes of the
397 antioxidant enzymes in fish, any studies reported a similar seasonal pattern in fish such as the
398 three-spined stickleback and explained this effect by the involvement of GPx in steroid
399 metabolism (Sanchez et al., 2007). In light of this data, several authors recommend
400 measurement of biomarkers outside the reproductive period to decrease the number of
401 sampled fish and to increase statistical power due to a low variability of investigated
402 responses (Flammarion and Garric, 1997; Sanchez et al., 2008a). However, in the present
403 study, more inter-site differences were recorded in April than in July and October especially
404 for EROD activity, oxidative stress parameters and neurotoxicity biomarkers. A decrease of
405 respiratory burst was observed in fish collected in October compared to July. A number of
406 reports have shown that abiotic factors (e.g. water temperature, photoperiod, pollution, diet)
407 as well as biotic factors (e.g. pathogens) directly or indirectly controlled by the season cycle,
408 affect the immune function of fish (Saha et al., 2002). These response profiles could be
409 explained by the seasonality of pollution due to agricultural practices in the investigated area
410 since viticulture prophylactic treatments are applied in spring. Also, this study argues for a
411 design of freshwater risk assessment studies based on variability of environmental pressures.
412 Several sources of pollutants such as agriculture, but also urban activities (Hemming et al.,
413 2004; Miège et al., 2006), are known to exhibit strong temporal variability. In this context, a
414 monitoring design based only on fish physiological cycles appears as not relevant and could
415 lead to erroneous diagnosis of contamination effects on wildlife. However, a better knowledge

416 of model fish species physiology must be considered as a major requirement for biomarker
417 deployment.

418

419 **5. Conclusions**

420 To summarize, the present study was designed to assess multi-biomarker responses including
421 immunotoxicity induced by urban and agricultural pressure in European bullheads living in a
422 small French river basin, the Vesle watershed. Results revealed various kinds of biological
423 effects due to environmental pressure on fish health and particularly on immunological status.
424 This study also highlighted the high variability of biomarker responses between sites
425 (downstream/upstream effect) and seasons (environmental condition impact). Some cautions
426 about selection of reference sites and seasons to determine freshwater risk assessment were
427 also given:

428 i) Reference site must be established according to the function of chemical and biological
429 qualities of area, but in a river basin monitoring context, an upstream/downstream approach
430 appears as a powerful methodology to analyze multi-biomarker responses and integrate the
431 complexity of contamination;

432 ii) Historically, measurement of multi-biomarker responses was performed according to fish
433 physiological cycles. However, seasonal variability of environmental pressure cannot be
434 ignored in the design of monitoring programs.

435

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441 Ethical considerations: Procedures described in the present paper were conducted in
442 accordance with laws and regulations controlling animal experiments in France. All
443 experimental protocols were approved by the Ethical Committee of the French National
444 Institute of Industrial Environment and Risks.

445

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615

616 **Figure Legends**

617

618 **Fig. 1.** Sampling sites on Vesle river system, with A = localization of sites in France and B =

619 localization of the selected sampling sites in Vesle basin.

620

621 **Fig. 2.** Distribution of gonadal development stages in male and female bullheads sampled in

622 April, July and October 2010 from Courtagnon, Serzy, Bouy, Prunay and Muizon.

623 In male bullhead: stage 1 = spermatogonia, stage 2 = spermatocytes, stage 3 = spermatids and

624 stage 4 = spermatozooids.

625 In female bullhead: stage 1 = primary oocytes, stage 2 = pre-vitellogenic oocytes, stage 3 =

626 secondary oocytes and stage 4 = degenerated oocytes.

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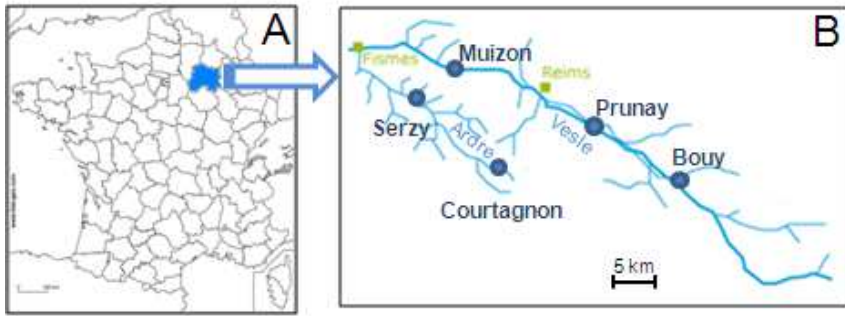
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643 **Fig. 1.**

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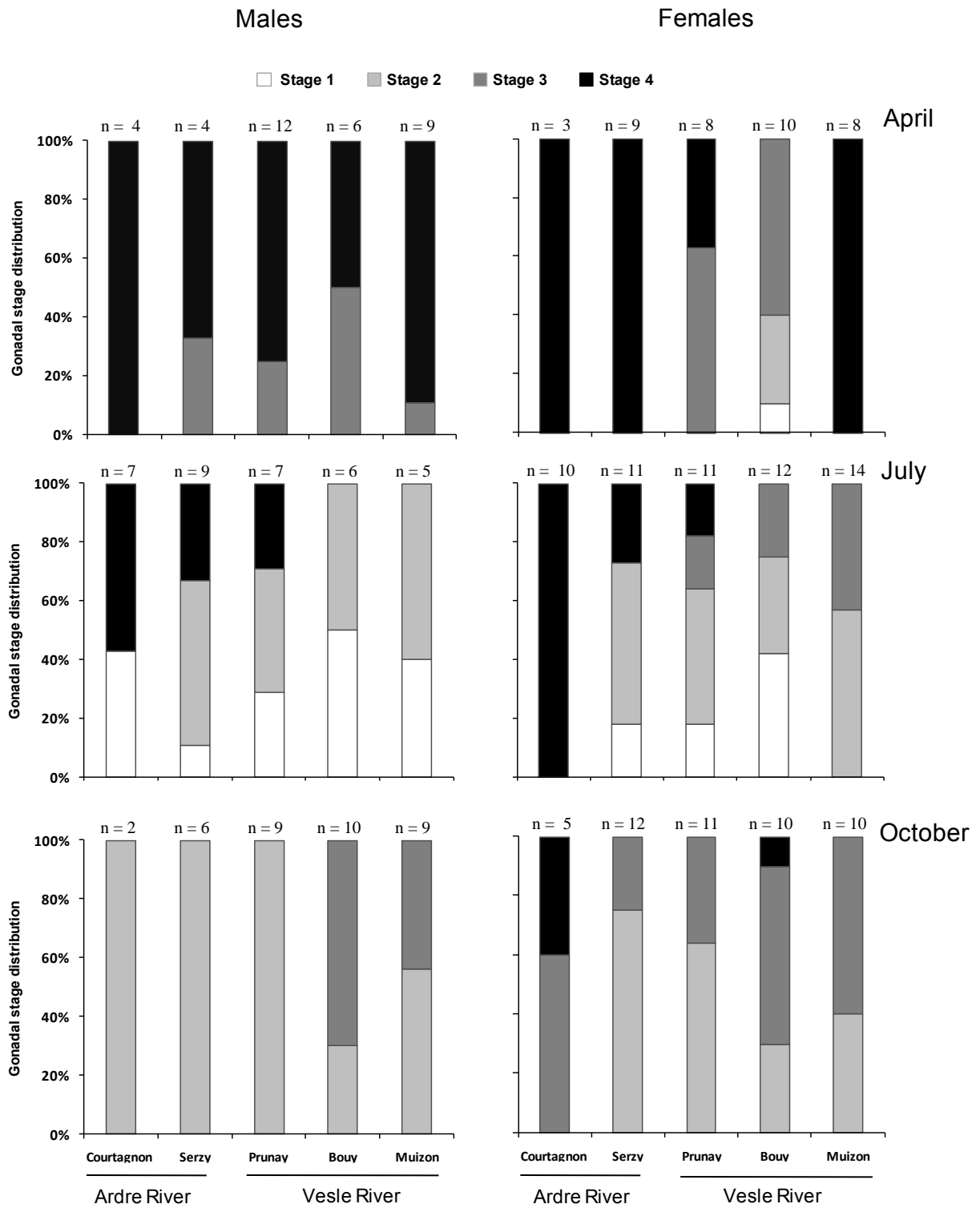
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664 **Fig. 2.**