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Whole-transcriptome response to wastewater treatment plant and stormwater effluents in the Asian clam, *Corbicula fluminea*

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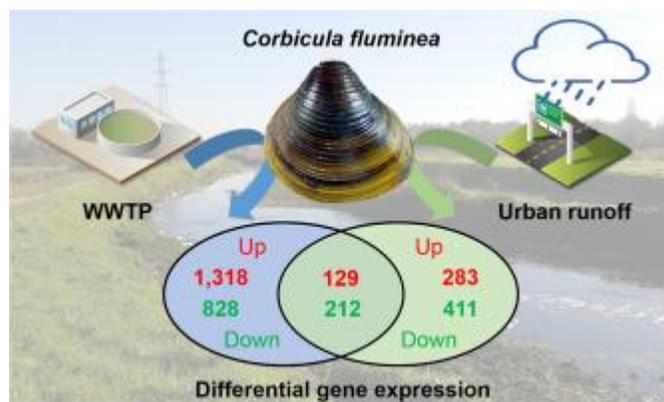
Abstract :

The increase in human population and urbanization are resulting in an increase in the volume of wastewater and urban runoff effluents entering natural ecosystems. These effluents may contain multiple pollutants to which the biological response of aquatic organisms is still poorly understood mainly due to mixture toxicity and interactions with other environmental factors. In this context, RNA sequencing was used to assess the impact of a chronic exposure to wastewater treatment plant and stormwater effluents at the whole-transcriptome level and evaluate the potential physiological outcomes in the Asian clam *Corbicula fluminea*.

We de-novo assembled a transcriptome from *C. fluminea* digestive gland and identified a set of 3,181 transcripts with altered abundance in response to water quality. The largest differences in transcriptomic profiles were observed between *C. fluminea* from the reference site and those exposed to wastewater treatment plant effluents. On both anthropogenically impacted sites, most differentially expressed transcripts were involved in signaling pathways in relation to energy metabolism such as mTOR and FoxO, suggesting an energy/nutrient deficit and hypoxic conditions. These conditions were likely responsible for damages to proteins and transcripts in response to wastewater treatment effluents whereas exposure to urban runoff might result in immune and endocrine disruptions.

In absence of comprehensive chemical characterization, the RNAseq approach could provide information regarding the mode of action of pollutants and then be useful for the identification of which parameters must be studied at higher integration level in order to diagnose sites where the presence of complex and variable mixtures of chemicals is suspected.

Graphical abstract



Highlights

► Clams were caged for 3 months in a river receiving WWTP and stormwater effluents. ► We de-novo assembled a transcriptome from clams' digestive glands. ► 3181 genes were differentially expressed between sites. ► Water pollution induced disorders to energy metabolism and immunity. ► RNAseq allowed the identification of novel early-warning molecular markers.

Keywords : Transcriptomics, Wastewater treatment plant, Urban runoff, Water quality, In-situ exposure, *Corbicula fluminea*

44 1. Introduction

45 Aquatic ecosystems are threatened by the rapid growing of human population and the parallel increase
46 in urbanization that are driving the increase in the volume of wastewater and urban runoff worldwide
47 (Muñoz et al., 2008; Sato et al., 2013). Direct and indirect effects of chronic exposure to effluents
48 from Wastewater Treatment Plant (WWTP) and urban runoff effluents and the consequences on
49 ecosystem functioning are still poorly understood.

50 Wastewater treatment plants effluents are characterized by inorganic and organic materials such as
51 metals, biocides, polycyclic aromatic hydrocarbons (PAHs), chlorinated solvents, pharmaceuticals and
52 personal care products (Arlos et al., 2015; Miège et al., 2009; Muñoz et al., 2008). For both
53 pharmaceuticals and personal care products, wastewater is the main route of emission to the
54 environment (Ternes et al., 1999). Negative effects of WWTP effluents were documented on aquatic
55 organisms (including bivalves), from the molecular to the community level (Morris et al., 2017).

56 WWTP effluent exposure were often associated with endocrine disruption and altered reproduction
57 (Fuzzen et al., 2015; Mezghani-Chaari et al., 2015), immune response disturbances (Jasinska et al.,
58 2015) and oxidative stress (Gillis et al., 2014). Urban runoff (also known as stormwater), the
59 precipitation-related discharge of impervious surfaces, is considered another major source of water
60 pollution (Huber et al., 2016). Major pollutants found in urban runoff include both organic and
61 inorganic substances, such as nutrients, organic and particulate matter, PAHs (Krein and Schorer,
62 2000), metals such as Pb, Zn and Cu and pathogens (Sidhu et al., 2012). *Salmonellae* that is found in
63 humans, pets, farm animals, and wild life feces is the most common pathogen. Municipal sewage,
64 agriculture pollution, and storm water runoff are the main sources in natural waters (Cabral, 2010). A
65 list of 25 selected stormwater priority pollutants was proposed by Eriksson *et al.* (Eriksson et al.,
66 2007). These pollutants may have multiple origin and a high level of qualitative and quantitative
67 variability. The characteristics of runoff waters (physical, chemical or microbial) are dependent on the
68 type of surfaces encountered and their use (Göbel et al., 2007). However, main contamination sources
69 are related to traffic, surrounding land use, atmospheric contamination, meteorology and other
70 environmental factors (Huber et al., 2016). Moreover, the concentration of each single constituent,

71 likely to represent a risk for human, animals or plants, may vary from one precipitation event to the
72 other and from site to site (Kayhanian et al., 2008; Kayhanian Masoud et al., 2003).

73 Transcriptomics (thanks to the development of next generation - high throughput - sequencing), like
74 other “omics”, can provide a global investigation of the potential toxicity of contaminated water, as
75 well as the discrimination of clean and polluted sites. Indeed, mRNA sequencing (RNAseq) data may
76 depict the alteration of several genes and molecular pathways simultaneously in non-model organisms
77 in response to stressful conditions (Gonzalez and Pierron, 2015). This method is more and more often
78 used in a multi-stress context (Baillon et al., 2015; Bertucci et al., 2017; Pierron et al., 2011; Poynton
79 et al., 2008; Regier et al., 2013).

80 The Asian freshwater clam *Corbicula fluminea* is an invasive species, widely distributed in rivers,
81 lakes and estuaries throughout Europe (Sousa et al., 2008). This suspension feeder has been used as a
82 bioindicator to assess the impact of different contaminants, such as metals (Arini et al., 2014) or
83 organic molecules (Bebianno et al., 2016). In the present study, we investigated the transcriptome-
84 wide response of field caged specimens to wastewater treatment plant and stormwater runoff effluents
85 in order to get insights on the impacted metabolic pathways and to evaluate the potential use of
86 transcriptomics as early diagnostic tool of water quality *in-situ*.

87

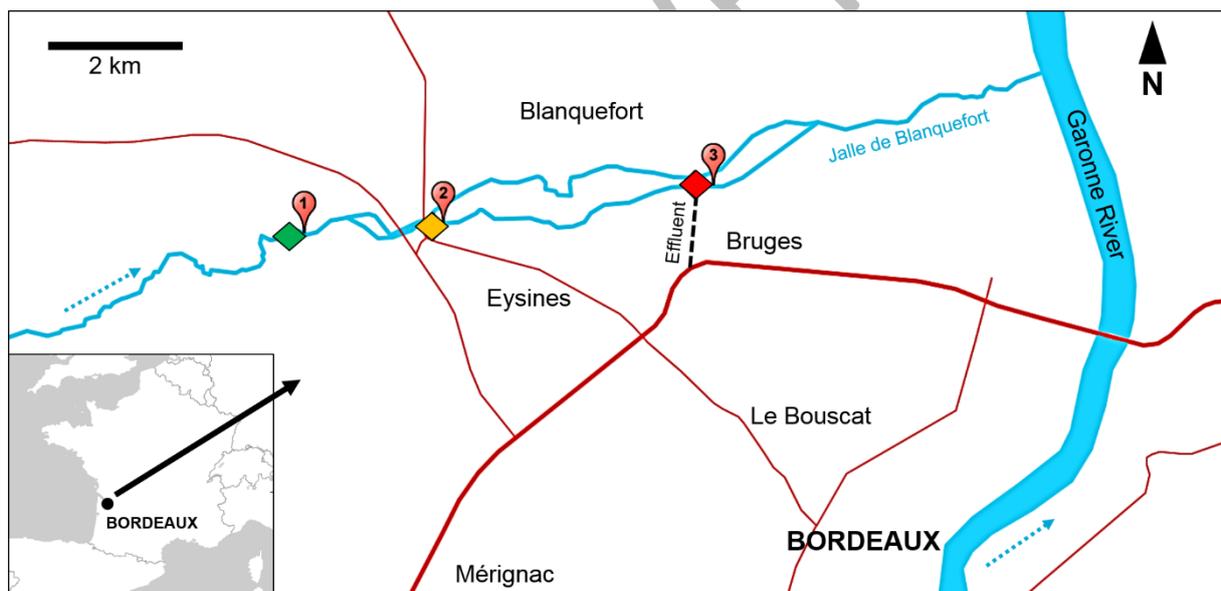
88 **2. Materials and methods**

89 **2.1. Study organism**

90 The freshwater clam (Bivalvia: Corbiculoidea) *Corbicula fluminea* (Müller, 1774) is an invasive
91 species original from Asia that has spread through most Europe and America. This benthic species is
92 considered a good model organism due to its wide distribution, abundance, ease of collection and
93 maintenance under laboratory or *in-situ* conditions. Moreover, it filters large volumes of water for
94 respiratory and nutritive purposes. It was used in ecotoxicological studies to evaluate the effects of a
95 variety of pollutants such as metals, flame retardants, pesticides and pharmaceuticals. Adult specimens
96 (approximately 2 cm long) were originally from a reference site on the Isle River (45°0.878'N,
97 0°9.820'E).

98 **2.2. Study sites**

99 Three field sites located along the Jalle d'Eysines River (also known as the Jalle de Blanquefort) close
100 to Bordeaux, France (Figure 1) were selected. Site 1 ($44^{\circ}53.652'N$, $0^{\circ}41.534'W$), located upstream,
101 was chosen as reference site owing to its use as a source of drinking water. Intermediate Site 2
102 ($44^{\circ}53.732'N$, $0^{\circ}39.916'W$) is located 100 metres downstream of a wastewater treatment plant. The
103 most downstream site, Site 3 ($44^{\circ}54.035'N$, $0^{\circ}36.803'W$) receives inlet of urban runoff water from
104 surrounding area and from Bordeaux's ring road. Three plastic racks, each containing 20 clams were
105 transplanted at each site (Salazar and Salazar, 2005) for 3 months (14th December 2015 – 14th March
106 2016). These dates are outside of the reproduction period of *C. fluminea* (i.e. May to October;
107 (Mouthon, 2001)). In December 2015 to March 2016, rainfall events were registered on 71 days with a
108 monthly average precipitation of 127 mm (www.infoclimat.fr). Sites 1, 2 and 3 will be referred to as
109 Reference, WWTP exposure and urban runoff exposure, respectively.
110



111
112 Figure 1: Overview of the study area. The sites are figured with red paddles numbered 1-3. Green,
113 yellow and red diamonds indicate the reference site, wastewater treatment plant and urban runoff
114 effluent, respectively. Main localities are indicated. Major roads are in red.

115

116 2.3. Physico-chemical parameters and pollution analysis

117 Turbidity, temperature, pH, conductivity, RedOx potential and dissolved oxygen (DO) concentration

118 were measured once a day on each site during the entire exposure period with a multiparameter probe.
119 The collection of water samples for pollutants analysis was only performed episodically on 5 days
120 scattered in February and March 2016. For trace metal analysis, water samples were collected in acid
121 pre-cleaned (72 h in 1.5 N HNO₃, and rinsed with ultrapure water) polypropylene bottles. All samples
122 were immediately filtered on-site through 0.2 µm Sartorius® polycarbonate filters. Filtrates were
123 collected in pre-cleaned 30 ml polypropylene bottles, acidified (0.5%; HNO₃ PlasmaPur Ultrex®) and
124 stored at 4°C until analysis. Samples were analyzed by ICP-MS (Xserie2, Thermo Scientific) under
125 standard conditions. The applied analytical methods were continuously quality checked by analysis of
126 international certified reference materials. Accuracy was within 10% of the certified values and the
127 analytical error lower than 5 % (r.s.d.) for concentrations ten times higher than detection limits.
128 Water samples for pesticide analysis were collected with HDPE bottles (Nalgene). Considering the
129 slight amounts of suspended matter in the river (usually between <2 and 10 ng.L⁻¹) and the polar
130 nature of the monitored pesticides, samples were filtrated over 0.7 µm glass filters from Wattman
131 (Fisher Bioblock Scientific, Illkirch, France), and analysis were performed only on dissolved phase.
132 Waters were then stored at -18°C until extraction. Glyphosate and aminomethylphosphonic acid
133 (AMPA) were extracted following the protocol described by Fauvelle et al. (2015). Glyphosate-
134 ¹³C₂¹⁵N and AMPA-¹³C<sub>15N were introduced in samples (3 ng) to play role of internal standard. 40
135 µL of the final extract were injected in LC-MS/MS (HPLC 1290 and triple quadrupole 6460 from
136 Agilent Technologies (Santa Clara, CA, USA). Separation was performed at 0.6 mL.min⁻¹ over a
137 reverse C18 phase Acquity BEH column (50 x 2.1mm; 1.7µm, Waters) at 35°C with ultrapure water
138 and ammonium acetate 5mM added with ammonium acetate until pH 9, and MeOH as mobile phases.
139 Acquisition was performed using electrospray in negative mode in dynMRM. Fipronil was
140 characterized following the method described by Le Coadou et al. (2017), with on-line SPME coupled
141 with an Agilent 7890 gas chromatography system and a 7000C tandem mass spectrometer from
142 Agilent-Technologies (Santa Clara, CA, USA). 9 mL of sample were transferred into SPME auto
143 sampler vial, spiked with 0.3 ng of fipronil-¹³C₁₅N as internal standard. Extraction was performed for
144 30 min using a PDMS/DVB fiber (65µm), at 50°C under agitation (250 rpm). Analytes were desorbed
145 for 10 min in the injector of the chromatograph set at 250°C in pulsed splitless mode (25 psi, 1 min).</sub>

146 Separation was performed using an Agilent J&W GC column HP 5 MS UI (30 m x 0.25 mm x 0.25
147 μm film thickness; 5% phenylmethylsiloxan). Diuron, hexazinone, isoproturon, metolachlor,
148 metolachlor OA and terbutryn were extracted by solid phase extraction, following the protocol
149 described by Belles et al. (2014) concerning polar pesticides. The extraction was performed on 100
150 mL of sample, acidified at pH 2 with chloridric acid. 5 ng of diuron-d6, hexazinone-d6, isoproturon-
151 d6, metolachlor-d6 and terbutryn-d5 were added in samples to play role of internal standard. 5 μL of
152 the final extracts were injected in LC-MS/MS (HPLC 1290 and triple quadrupole 6460 from Agilent
153 Technologies (Santa Clara, CA, USA). Separation was performed at 0.5 mL.min⁻¹ over a reverse C18
154 phase Kinetex column (100 x 2.1 mm; 1.7 μm , Phenomenex) at 35°C with ultrapure water (+5mM
155 ammonium acetate and 0.1% acetic acid) and MeOH as mobile phases. Acquisition was performed
156 using electrospray in positive mode in dynMRM. Performance of each protocols were evaluated for
157 each batch with the extraction of artificial samples based on mineral water spiked with known amount
158 of studied molecules. Quantification limits and quantification yields were calculated on these samples
159 to ensure the performances of the different protocols. Blanks were also extracted in parallel to control
160 the potential contamination introduced during extraction procedures and analysis.

161 **2.4. Clams collection**

162 Eight individuals were collected randomly and immediately dissected at each site at the end of the
163 exposure period, leading to a total number of 24 individuals. Soft (body) tissues and shells were
164 weighted in order to determine the condition index (CI) with the following formula: Tissue (g, fresh
165 weight) / Shell (g, dry weight) * 100 (Quayle and Newkirk, 1989). The digestive glands were then
166 dissected for RNA extraction. In bivalves, the digestive gland functions both as a site for nutrients /
167 pollutants uptake and as an important reservoir for contaminant storage. Samples were collected in
168 RNA-later solution and kept on ice until return to the lab where they were placed at 4°C overnight
169 before storage at -20°C until RNA extraction.

170 **2.5. RNA Extraction, library construction and sequencing**

171 Total RNA was isolated from digestive glands tissue using the SV Total RNA isolation system kit
172 (Promega) according to the manufacturer's instructions. RNA-seq libraries were prepared and
173 sequenced by Genotoul (France) with the Illumina HiSeq 3000 technology. The RNA-seq raw reads

174 used in this study have been deposited in the NCBI Gene Expression Omnibus (Edgar et al., 2002)
175 under GEO Accession GSE104933.

176 **2.6. Bioinformatics workflow**

177 The read quality of the RNA-seq libraries was evaluated using FastQC
178 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Reads were cleaned and filtered using
179 the DRAP pipeline (Cabau et al., 2017) and *de novo* assembled with the Trinity assembler version
180 2.0.6 (Grabherr et al., 2011). Assembled contigs were filtered in order to keep only those with at least
181 one fragment per kilobase of transcript per million reads (FPKM). Reads have been realigned back to
182 contigs with bwa (Li and Durbin, 2009) (version 0.7.12). The resulting files were compressed, sorted
183 and indexed with samtools (Li et al., 2009) (version 1.1). The contig transcription counts have been
184 generated with samtools (version 1.1). The alignment files have then been filtered for duplicates with
185 samtools (version 1.1). This Transcriptome Shotgun Assembly project has been deposited at
186 DDBJ/EMBL/GenBank under the accession GFYR000000000. The version described in this paper is
187 the first version, GFYR010000000. The assembled contigs were aligned with NCBI blast (Camacho et
188 al., 2009) (version 2.2.26) on Refseq protein, Swissprot and Ensembl protein reference files from
189 *Crassostrea gigas*, *Lottia gigantea* and *Lingula anatina* to retrieve the corresponding annotations. The
190 contigs were also processed with rnammer (Lagesen et al., 2007) (version 1.2) to find ribosomal
191 genes, with repeatmasker (Smith et al.) (version open-4-0-3) to list the contained repeats and with
192 interproscan (Quevillon et al., 2005) (version 4.8) for gene ontology (GO) and structural annotation.
193 GO annotations were obtained with an E-value and a homology threshold set to 10^{-6} and 50%,
194 respectively. Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathways assignments
195 were performed with the the GhostKOALA server (Kanehisa et al., 2016). Detailed statistic
196 information of *de novo* assembly are listed in Table 1. Moreover, all the results have been uploaded in
197 a RNAbrowse instance (Mariette et al., 2014) (Sigenae web address available upon publication).
198 Transcriptome completeness was assessed with BUSCO v3 (Simão et al., 2015) against the Metazoa
199 orthologs dataset.

200 **2.7. Real-time PCR**

201 Specific primers amplifying approximately 100 bp were designed for a set of 18 genes using the
202 software primer3 (Rozen and Skaletsky, 2000) (Details are given in Appendix A). Expected lengths of
203 the amplicons were checked by agarose gel electrophoresis after regular PCR amplification using the
204 GoTaq DNA Polymerase (Promega). Primer efficiencies were determined using standard curve
205 analysis ($E = 10^{(-1/\text{slope})}$) with a dilution series of pooled cDNA from all conditions and ranged from 90
206 to 99 %. Transcript level quantification was performed using the GoTaq qPCR mastermix from
207 Promega and a Startagene Mx3000P system. PCR conditions were as follows: 1X GoTaq qPCR
208 mastermix (Promega), 200 nM primers and 10 ng of cDNA in a total volume of 25 μ L. PCR
209 parameters were: 95 °C for 2 min, followed by 40–45 cycles of 15 s at 95 °C, 60 s at 60 °C and a
210 dissociation curve step (60–95 °C) to confirm the absence of nonspecific products. The dissociation
211 curves showed a single amplification product and no primer dimers. Three contigs were selected as
212 control genes based on their stability in the RNA-seq data set. The relative quantification for each gene
213 was normalized by the geometric mean of control genes and relative to its expression in the reference
214 site. Fold expression values were calculated as $(E^{\text{dCT}})_{\text{target}} / (E^{\text{dCT}})_{\text{control}}$ where E is the amplification
215 efficiency for each pair of primers and $\text{dCT} = \text{CT}_{\text{site}} - \text{CT}_{\text{reference}}$.

216 2.8. Statistical analyses

217 The comparison of CIs amongst the three sites was performed by analysis of variance (ANOVA), after
218 checking assumptions of normality and homoscedasticity, followed by a Tukey HSD test. As the
219 assumptions of the parametric ANOVA were not met for physico-chemical parameters we used the
220 Scheirer-Ray–Hare (SHR) non-parametric ANOVA to test the differences amongst sites. Pairwise
221 comparisons within the 3 sites were carried out using Mann-Whitney U-tests. Computations were
222 performed with the R package “Stats”. Differences were considered significant at the level $p < 0.05$.
223 Since the number of measures didn’t allow proper statistical comparison of micropollutants, minimal
224 and maximal values only were presented.

225 Differential gene expression was calculated with the R package DESeq2 (Love et al., 2014). *P*-values
226 were corrected using the Benjamini and Hochberg method, and a false discovery rate (FDR) threshold
227 of 0.05 was used. To avoid large fold changes in low abundance transcripts, only contigs with more
228 than 2 counts per million (cpm) in at least one condition were considered in subsequent analyses

229 (Appendix C). GO-enrichment analyses were carried out with R package topGO (Alexa and
230 Rahnenfuhrer, 2016). In order to reduce redundancy in GO terms and ease the interpretation, we used
231 the REVIGO web server with a value of C=0.5 (Supek et al., 2011). KEGG enrichment was calculated
232 with a Fisher exact test. Both GO and KEGG analyses were performed with a p-value ≤ 0.05 .

233

234 **3. Results and discussion**

235 **3.1. Transcriptome assembly**

236 RNA sequencing generated 795 million paired-end 150 bp reads. We assembled a total of 58,291
237 contigs of which 23,758 (40.76 %) were annotated. The assembly statistics are presented in Table 1.
238 The quality of our assembly is evidenced by the high abundance of Metazoa benchmarking universal
239 single-copy orthologues (BUSCO summary: C:98.7% [S:92.5%,D:6.2%],F:0.2%,M:1.1%,n:978). The
240 percentage of reads mapping the transcriptome ranged from 95.8 to 99.2 %. The oyster *Magallana*
241 *gigas* (previously *Crassostrea gigas*) accounted for 55.84 % (10,793 sequences) of the best BLAST
242 homology. The functional annotation showed that 13,608 (57.3 %) and 9,427 (36.7 %) of these contigs
243 were associated to GO and KEGG terms, respectively (details are given in Appendix B).

244

Statistics				
Sequencing and mapping	Total	Reference	WWTP	Runoff
Total number of Pair-End reads	794,791,848	291,118,523	245,734,147	257,939,178
Number of mapped reads	777,208,882	284,107,241	241,459,784	251,641,857
Min. number of reads per sample		30,314,069	20,657,826	23,463,430
Max. number of reads per sample		43,582,589	40,380,849	50,218,485
Assembly				
Number of contigs	58,291			
Total contigs length (bases)	98,530,638			
Min. contig length (bases)	207			
Max. contig length (bases)	53,892			
Mean contig size (bases)	1,690			
N50 (bases)	2,401			
Mean GC %	41.4			
Annotation				
Number of contigs with BLAST hits	23,758			
Number of contigs with GO terms	13,608			
Number of contigs with KEGG ortholog	9,427			

245

246 Table 1: *Corbicula fluminea*'s digestive gland transcriptome assembly statistics.

247

248 **3.2. Water physicochemistry and pollutants analysis**

249 Significant differences amongst sites were detected for conductivity, pH, Redox potential, DO and

250 temperature (Table 2). However, variations were relatively small and unlikely to produce much

251 metabolic modifications since they correspond to normal range of values and seasonal variations

252 experienced by clams in similar environments (Vidal et al., 2002). For instance, temperature variation

253 was less than 1°C and pH values at each site were in the range of the High Ecological Status class of

254 the European Water Framework Directive (WFD, 2000/60/EC; EC, 2000), *i.e.* from 6.5 to 8.2. On the

255 contrary, DO appeared as the most variable parameter with an average of 10.13 mg/l (89.39%

256 saturation), 7.47 mg/l (67.18%) and 5.51 mg/l (49.34%) on reference, WWTP and runoff sites,

257 respectively. WWTP and urban runoff could hence be classified as good and moderate, respectively

258 whilst the reference site was of high ecological status (EC, 2000). Trace metal showed no differences

259 amongst sites. Amongst the nine pesticides we measured, the largest differences were observed for

260 glyphosate and its primary degradation product, AMPA (Aminomethylphosphonic acid), and for

261 terbutryn that were higher in the WWTP and urban runoff effluents.

		Reference	WWTP	Runoff
Physicochemistry	Turbidity (FNU)	14.09 ± 3.04	25.47 ± 6.77	26.38 ± 11.46
	Conductivity (µS/cm)	330.50 ± 11.39 ^a	396.22 ± 22.82 ^b	420.88 ± 17.97 ^b
	pH	7.26 ± 0.07 ^a	6.89 ± 0.03 ^b	7.13 ± 0.03 ^a
	Redox potential (mV)	414.82 ± 4.86 ^a	403.85 ± 3.84 ^a	453.99 ± 1.71 ^b
	Dissolved O₂ (mg/l)	10.13 ± 0.08 ^a	7.47 ± 0.47 ^b	5.51 ± 0.33 ^c
	Temperature (°C)	9.77 ± 0.14 ^a	10.57 ± 0.16 ^b	10.38 ± 0.14 ^b
Metal (min-Max µg/L)	Al	101.9 - 277.7 (3)	82.1 - 261.2 (3)	84.1 - 140.3 (3)
	Cd	0.02 - 0.03 (3)	0.02 - 0.03 (3)	0.01 - 0.03 (3)
	Co	0.09 - 0.57 (3)	0.10 - 0.49 (3)	0.11 - 0.31 (3)
	Cr	0.54 - 0.64 (3)	0.49 - 0.64 (3)	0.50 - 0.58 (3)
	Cu	0.72 - 1.20 (3)	1.24 - 1.39 (3)	1.16 - 1.59 (3)
	Fe	197.3 - 279.1 (3)	168.2 - 283.0 (3)	183.3 - 226.5 (3)
	Ni	0.56 - 1.09 (3)	0.57 - 1.20 (3)	0.55 - 1.15 (3)
	Pb	0.16 - 1.07 (3)	0.14 - 0.29 (3)	0.14 - 0.27 (3)
	V	0.98 - 1.10 (3)	1.03 - 1.26 (3)	1.08 - 1.19 (3)
	Zn	4.25 - 8.37 (3)	4.88 - 8.33 (3)	6.44 - 11.09 (3)
Pesticides (min-Max ng/L)	AMPA	52.3 (1)	93.2 (1)	141.2 - 230.3 (2)
	Diuron	2.6 - 5.9 (4)	3.8 - 7.3 (4)	4.3 - 14.1 (5)
	Fipronil	0.5 - 0.8 (2)	1.0 - 1.8 (3)	1.0 - 1.2 (4)
	Glyphosate	29.1 (1)	27.1 (1)	57.9 - 77.2 (2)
	Hexazinone	2.3 - 17.8 (4)	2.0 - 16.5 (4)	1.6 - 14.9 (5)
	Isoproturon	1.5 (1)	0.2 - 1.5 (2)	0.3 - 1.7 (3)
	Metolachlor	8.9 - 20.1 (4)	8.3 - 17.7 (4)	5.7 - 16.3 (5)
	Metolachlor OA	317.3 - 353.0 (4)	274.3 - 501.7 (4)	188.6 - 469.9 (5)
	Terbutryn	0.3 - 0.8 (4)	0.6 - 5.2 (4)	0.6 - 5.4 (5)

263 Table 2: Physico-chemical parameters and micropollutants concentrations at each site. Physico-
264 chemical parameters are indicated as mean \pm SE during the whole exposure period. Letters indicate
265 statistical differences according to the Scheirer-Ray-Hare test followed by Wilcoxon-Mann-Whitney
266 tests when differences were detected (p value ≤ 0.05). Micropollutants concentrations are indicated as
267 minimum and maximum values measured (the number of measures is indicated within brackets).

268

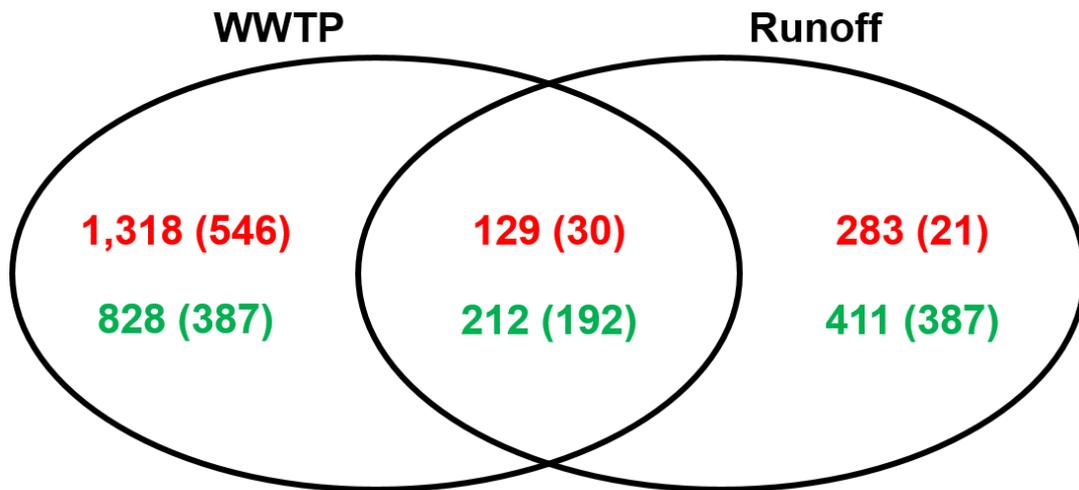
269 **3.3. Effects on the Condition Index**

270 At the end of the caging period, means \pm SE in reference, WWTP exposure and urban runoff exposure
271 were 51.67 ± 2.09 , 53.65 ± 1.14 and 55.37 ± 2.65 , respectively, and no significant difference was
272 detected (ANOVA, p -value = 0.46). The significance of other factors such as natural variability, age of
273 the individuals, diet, mixture of pollutants or hydrology is difficult to predict (Petrie et al., 2015) and
274 would require a substantial increase in sampling and analytical effort. Aquatic toxicity is often
275 undertaken under controlled laboratory conditions over short time period / acute exposure to
276 pollutants. Field experiments, on the contrary, allow to determine the impact of mixtures and chronic
277 exposure, hence providing more environmentally realistic, though less reproducible, information.

278 **3.4. Gene transcription analyses**

279 A total of 3,181 Differentially Expressed Genes (DEGs, $FDR \leq 0.05$) were identified in WWTP
280 exposure and urban runoff exposure compared to the reference site. In details, 1,447 and 412 were up-
281 regulated and 1,040 and 623 were down-regulated in WWTP exposure and urban runoff exposure,
282 respectively (Figure 2 and Appendix C). To validate the sequencing data, the transcription of 15 genes
283 that showed variations in their expression levels was measured by the RT-qPCR method. RNAseq and
284 RT-qPCR gave consistent results with a R^2 values of 0.91 and 0.82 for WWTP exposure *vs.* Reference
285 and urban runoff exposure *vs.* Reference comparisons, respectively (Appendix A).

286



287

288 Figure 2: Venn diagram and number of differentially expressed genes. Number of up- (red) and down
 289 (green) regulated genes in individuals caged downstream of the WWTP and downstream of the urban
 290 runoff effluent. The number of contigs with a BLAST hit ($1e-5$) is indicated between brackets.

291

292 There was little overlap amongst the transcripts differentially expressed in the two sites compared to
 293 the reference site. Fold change values of the regulated genes were relatively small, ranging from 0.51
 294 to 2.38 in WWTP exposure and from 0.56 to 2.20 in urban runoff exposure (Table 3 and Appendix C).

295 The number of DEG is sometimes viewed as a proxy of the level of stress experienced by the
 296 organisms. However, the nature and the number of affected biological processes, as well as the
 297 magnitude of changes certainly provide more relevant information regarding the nature of the stress
 298 and the associated biological consequences of water quality. Such detailed information regarding the
 299 biological processes in which each DEG was involved were provided by their functional annotation,
 300 *i.e.* GO (Table 4) and KEGG orthology (Table 5).

301

Contig name	Description	FC WWTP	FC runoff	Putative function
Xenobiotic detoxification				
Reg_ADHX.2.2	Alcohol dehydrogenase class III	1.51	1.76	Formaldehyde detoxification
Reg_LOC108269088.1.3	Alcohol dehydrogenase class III	1.77	1.65	Formaldehyde detoxification
Reg_LOC108269088.2.3	Alcohol dehydrogenase class III	1.85	1.55	Formaldehyde detoxification
Reg_LOC108269088.3.3	Alcohol dehydrogenase class III	1.93	1.60	Formaldehyde detoxification
Reg_ADHX.1.2	Alcohol dehydrogenase class III	---	1.75	Formaldehyde detoxification
Reg_LOC106174241.2.2	Alcohol dehydrogenase class III-like	---	1.69	Formaldehyde detoxification
Energy metabolism				
Reg_LOC105326051.4.4	Phosphoenolpyruvate carboxykinase, cytosolic [GTP]-like	1.83	1.69	Gluconeogenesis
Reg_LOC105326362	Pyruvate carboxylase, mitochondrial-like	1.91	1.36	Gluconeogenesis
Reg_LOC105326051.3.4	Phosphoenolpyruvate carboxykinase, cytosolic [GTP]-like	1.69	1.59	Gluconeogenesis
Reg_LOC105347001	Neutral cholesterol ester hydrolase 1-like	---	0.63	Lipid catabolism
Reg_LOC105344840.2.2	Complement C1q tumor necrosis factor-related protein 4-like	2.38	---	Regulator of glucose and lipid metabolism
Immune response				
Reg_LOC102197942	C-type lectin domain family 4 member E-like	0.54	---	Pathogen recognition
Reg_Fcer2.1.3	Low affinity immunoglobulin epsilon Fc receptor	0.58	---	Immune response
Reg_SAA2.2.2	Serum amyloid A-2 protein [Homo sapiens]	1.84	1.60	Response to inflammation
Reg_LOC105347518	Interferon-induced protein 44-like	1.89	---	Response to virus
Reg_ifi44	Interferon-induced protein 44 [Mus musculus]	1.89	---	Response to virus
Cell signaling				
Reg_LOC106880299.2.2	Serine/threonine-protein kinase WNK1-like	---	0.56	Regulation of cell signaling, survival, and proliferation
Reg_LOC105321825	Probable G-protein coupled receptor 139	0.58	0.59	Signal transduction
Reg_LOC105338118	Ski oncogene-like	---	0.62	Repressor of TGF-beta signaling
Reg_contig_05776	Receptor-type tyrosine-protein phosphatase T	---	1.63	Signal transduction
Ion transport				
Reg_LOC101856164.2.2	Solute carrier family 23 member 1-like	0.56	---	Sodium/ascorbate cotransporter
Reg_LOC105334363	Monocarboxylate transporter 9-like	0.57	---	Transport
Reg_contig_33484	Zinc transporter ZIP1	0.60	0.72	Transport, endogenous zinc uptake
Reg_LOC106156136	Stromal interaction molecule homolog	0.72	0.62	Calcium transport
Miscellaneous				
Reg_LOC105335663.1.2	Lysosomal-trafficking regulator-like	---	0.62	Intracellular protein trafficking
Reg_LOC105338935	Homeodomain-interacting protein kinase 2-like	---	0.62	Cellular response to hypoxia
Reg_LOC105345002.3.3	Cat eye syndrome critical region protein 5-like	0.51	0.60	Chromatin remodelling
Reg_LOC101857055	Rab5 GDP/GTP exchange factor-like	0.53	---	Membrane trafficking of recycling endosomes
Reg_LOC105340685	Neuronal PAS domain-containing protein 4-like	0.54	---	Neuron plasticity
Reg_LOC105330779.3.4	EMILIN-2-like	0.59	---	Cell adhesion
Reg_VKT2	Kunitz serine protease inhibitor Pr-mulgin 2	0.64	0.62	Endopeptidase inhibitor
Reg_LOC105319937	Beta-TrCP-like	0.69	0.62	Regulation of protein translation, cell growth and survival
Reg_LOC100633144	Zinc finger BED domain-containing protein 1-like	1.77	2.20	Transcription factor
Reg_LOC105317854.5.5	Baculoviral IAP repeat-containing protein 7-A-like	1.90	---	Apoptosis regulation
Reg_LOC105348304.2.2	Heat shock protein 70 B2-like	1.98	---	Protein folding

302 Table 3: List of the most up- and down-regulated genes in WWTP and runoff compared to reference.
303 The 10 most up-regulated (indicated in red) and the 10 most down-regulated (indicated in green) were
304 identified in each site compared to the reference site. (FC, fold-change)

305

306 **3.5. Common transcriptomic response of *C. fluminea* from both sites**

307 Genes involved in xenobiotic detoxification were amongst the most up-regulated genes (Table 3),
308 particularly many isoforms of class III alcohol dehydrogenases (ADH) that are responsible for
309 formaldehyde detoxification by metabolizing glutathione adducts (Haseba et al., 2006). This enzyme
310 involved in Phase I xenobiotics metabolism was previously shown to be up-regulated in the digestive
311 gland of bivalves exposed to various organic pollutants, particularly pharmaceuticals (Bebianno et al.,
312 2016; Schmidt et al., 2014). These genes also participate to the KEGG pathway ‘Degradation of
313 aromatic compounds’ in Table 4, suggesting the presence of organic pollutants in WWTP and runoff
314 waters. This hypothesis is partly confirmed by higher concentrations of some pesticides presented in
315 Table 2. Repressed contigs were linked to cell signalling and ion transport. These results were
316 confirmed by GO (Table 4) and KEGG (Table 5) enrichment analysis. Details are given in Table 3 and
317 Appendix B. The mTOR (mammalian target of rapamycin), the FoxO (forkhead box O) and the
318 AMPK (5’ AMP-activated protein kinase) pathways (Table 5) are highly conserved across metazoans
319 and intimately cross-talk in response to metabolic stress in order to maintain cell homeostasis. Under
320 stress, it is expected that the mTOR pathway is inhibited leading to a reduction in protein and lipid
321 biosynthesis and an increased rate of autophagy whereas FoxO and AMPK pathways are activated to
322 promote glucose / fatty acid metabolism and autophagy too (Hay, 2011; Mendoza et al., 2011; Zhao et
323 al., 2017). This metabolic dialogue aims to reduce cell growth and proliferation and to sustain
324 anabolic processes such as energy production by providing fatty acid and amino acids to the
325 mitochondria (Laplante and Sabatini, 2009). In a stressful environment, these processes might be used
326 in the digestive gland as an alternative source of energy production by mitochondrial oxidation in
327 order to maintain ATP/ADP homeostasis and the energy demand of other organs. Here, we observed a
328 higher expression of genes involved in energy metabolism (glycolysis, gluconeogenesis and fatty acid
329 degradation) in clams exposed to either WWTP- or urban runoff-impacted waters, which suggests the

330 activation of FoxO (and a repression of mTOR) (Zhao et al., 2017). Yet, genes involved in all the
331 aforementioned pathways were downregulated. We can hypothesize that these signaling disorders and
332 metabolic changes might have been caused directly by the presence of organic pollutants (Regoli and
333 Giuliani, 2014). However, other parameters might affect energy metabolism of which adjustment
334 plays a central role in adaptation and tolerance to environmental stress in aquatic invertebrates
335 (Sokolova et al., 2012). Cellular hypoxia could inhibit mTOR (Laplante and Sabatini, 2009), but
336 appears unlikely as it was evidenced that even under reduced environmental O₂ concentration as
337 observed in WWTP and runoff effluents, *C. fluminea* is able to maintain a remarkably stable level of
338 oxygen in its internal milieu without change in its ventilatory activity (Tran et al., 2000). In the same
339 manner, starvation could be ruled out as stressor since we observed no difference amongst CI after 3
340 months compared to our reference site. Moreover, while food deprivation would inhibit mTOR, it
341 would activate foxO and AMPK, which was not observed here at the transcriptional level.
342 Temperature is also a crucial variable that determines metabolic rate of poikilothermic organisms but
343 as stated previously, temperature differences amongst sites were small (Table 2).

344 **3.6. Transcriptomic response to WWTP effluent**

345 Amongst the most responsive genes in WWTP exposure, it is particularly relevant in stressful
346 environment to find homologues of genes involved in the inhibition of apoptosis, such as BIRC7
347 (Baculoviral IAP repeat-containing protein 7 ; Gyrd-Hansen and Meier, 2010), and protein folding,
348 such as a Heat Shock Protein (HSP) 70 isoform (Table 3). Members of the 70 kDa chaperone family
349 are found in nearly all subcellular compartments of nucleated cells. HSP70 rapidly accumulates after
350 exposure to environmental stress and high cytosolic levels are known to protect from apoptotic cell
351 death (Hartl and Hayer-Hartl, 2002). We also found contigs related to immune response, inflammation
352 and response to pathogens that were either up- or down-regulated almost 2 fold. A potential impact of
353 WWTP exposure on protein metabolism was supported by the significant enrichment of the GO term
354 “protein folding” carried out by 21 up-regulated genes (Table 4) and the KEGG category “protein
355 processing in endoplasmic reticulum (ER)” represented by 26 genes (Table 5). This up-regulation of
356 genes involved in protein processing and folding was further coupled with a higher level of RNA
357 processing (GO:006396, Table 4) and aminoacyl-tRNA biosynthesis (*i.e.* bounding of a transfer RNA

358 with its corresponding amino acid, Tables 4 and 5). The functional analysis of DEG also suggested a
359 reduction in auto- and mitophagy (Table 5). Protein synthesis and reduced autophagy generally result
360 from the activation of the mTOR signaling pathway (Mendoza et al., 2011) while, as stated in the
361 previous chapter, the maintenance of cellular homeostasis requires FoxO activation and mTOR
362 inhibition (Zhao et al., 2017). That reinforces the hypothesis of severe metabolic disorders and/or an
363 impairment in signal transduction from the environment to the gene machinery. These pathways were
364 identified as a potential marker for chronic environmental challenge in the Pacific oyster, *Crassostrea*
365 *gigas*, indicating tension between cell renewal and apoptosis (Clark et al., 2013).
366 The exposure to WWTP effluent could also be responsible for a higher level of misfolded proteins as
367 suggested by the over-representation of genes involved in proteolysis (GO:0051603, Table 4) amongst
368 DEGs. A comprehensive look at the KEGG orthology revealed that some of these genes were involved
369 in proteasome formation (17 up-regulated genes) and that part of the ER-associated genes were
370 responsible for ER-associated degradation (ERAD, Appendix D). Protein degradation might also
371 supply amino acids for gluconeogenesis and be an evidence of severe energy need / deficit in the
372 organisms exposed to WWTP waters. This impairment of protein synthesis, which can cause cell
373 malfunction, deterioration or even cell death, might also result from modification of RNA and
374 disturbance of the translational process as suggested by the stimulation of genes involved in mRNA
375 surveillance and RNA degradation (Table 5).
376 Similar to what we found above, genes involved in energy metabolism (8 and 6 DEGs respectively
377 linked to fatty acid metabolism and pyruvate metabolism, Table 5) showed an increased level of
378 transcription. However, WWTP is the only site to exhibit signs of molecular and cellular damages
379 ultimately impacting longevity. This confirms a higher level of stress as previously suggested by the
380 greater number of DEGs.

	GO ID	Description		FDR	n DEG
common to both sites	GO:0006094	Gluconeogenesis	▲	3.45E-2	3
Enriched in WWTP	GO:0006457	Protein folding	▲	2.19E-11	21
	GO:0051603	Proteolysis involved in cellular protein catabolism	▲	5.59E-4	12
	GO:0006487	Protein N-linked glycosylation	▲	4.60E-2	2
	GO:0043039	tRNA aminoacylation	▲	1.55E-6	12
	GO:0006396	RNA processing	▲	5.59E-4	17
	GO:0016071	mRNA metabolic process	▲	3.60E-2	7
	GO:0006779	Porphyrin-containing compound biosynthesis	▲	4.60E-2	3
	GO:0006637	Acyl-CoA metabolic process	▲	2.63E-2	4
	GO:0045454	Cell redox homeostasis	▲	2.63E-2	7
		GO:0007165	Signal transduction	▼	1.80E-5
	GO:0010468	Regulation of gene expression	▼	4.15E-2	24
Enriched in runoff	GO:0006468	Protein phosphorylation	▼	4.17E-04	17
	GO:0035556	Intracellular signal transduction	▼	6.93E-7	28

381

382 Table 4: List of significantly enriched Gene Ontology terms. (n DEG, number of associated differentially expressed genes ; FDR, false discovery rate Fisher

383 exact test). Red and green arrows indicate up-regulated and down-regulated pathways, respectively.

	KEGG pathway	Description		WWTP			Runoff			function
				pvalue	up	down	pvalue	up	down	
Common to both sites	map00071	Fatty acid degradation	▲	1.53E-3	11	-	3.38E-2	6	2	energy metabolism
	map00010	Glycolysis / Gluconeogenesis	▲	1.33E-2	9	1	2.80E-2	9	-	
	map01220	Degradation of aromatic compounds	▲	1.87E-2	4	-	3.09E-4	6	-	detoxification
	map04150	mTOR signaling pathway	▼	1.90E-4	5	13	3.62E-4	-	17	signaling pathways
	map04068	FoxO signaling pathway	▼	5.84E-4	4	12	3.33E-3	3	11	
	map04072	Phospholipase D signaling pathway	▼	1.35E-2	2	11	8.88E-7	-	21	
	map04152	AMPK signaling pathway	▼	2.41E-2	5	7	3.50E-4	3	13	
	map04071	Sphingolipid signaling pathway	▼	2.86E-2	-	11	1.20E-3	-	14	
	map04012	ErbB signaling pathway	▼	9.35E-3	1	8	5.93E-6	-	14	
	map01521	EGFR tyrosine kinase inhibitor resistance	▼	2.70E-2	1	7	5.91E-4	-	11	
map04630	Jak-STAT signaling pathway	▼	2.75E-2	2	4	2.36E-2	-	6		
Enriched in WWTP	map03040	Spliceosome	▲	3.11E-9	33	-	NS	-	-	gene expression
	map00970	Aminoacyl-tRNA biosynthesis	▲	1.13E-5	14	-	NS	-	-	
	map03015	mRNA surveillance pathway	▲	3.75E-3	11	2	NS	-	-	
	map03018	RNA degradation	▲	3.10E-2	8	3	NS	-	-	
	map04141	Protein processing in endoplasmic reticulum	▲	2.60E-6	26	2	NS	-	-	protein processing
	map03050	Proteasome	▲	5.55E-12	17	-	NS	-	-	
	map00510	N-Glycan biosynthesis	▲	9.35E-3	8	1	NS	-	-	energy metabolism
	map01212	Fatty acid metabolism	▲	3.32E-2	8	-	NS	-	-	
	map00620	Pyruvate metabolism	▲	4.32E-2	6	1	NS	-	-	
	map04140	Autophagy - animal	▼	1.81E-2	3	13	NS	-	-	Cell death
map04137	Mitophagy - animal	▼	9.35E-3	2	7	NS	-	-		
map04211	Longevity regulating pathway	▼	2.57E-2	2	7	NS	-	-		
Enriched in runoff	map04010	MAPK signaling pathway	▼	NS	-	-	8.27E-4	-	23	signaling pathways
	map04070	Phosphatidylinositol signaling system	▼	NS	-	-	3.68E-2	-	14	
	map04144	Endocytosis	▼	NS	-	-	1.83E-2	-	22	immunity
	map04062	Chemokine signaling pathway	▼	NS	-	-	5.41E-6	-	18	
	map04666	Fc gamma R-mediated phagocytosis	▼	NS	-	-	5.00E-4	-	15	
	map04664	Fc epsilon RI signaling pathway	▼	NS	-	-	2.93E-5	-	12	
	map04920	Adipocytokine signaling pathway	▼	NS	-	-	4.31E-3	3	6	
	map04670	Leukocyte transendothelial migration	▼	NS	-	-	4.67E-2	-	9	
	map04912	GnRH signaling pathway	▼	NS	-	-	1.41E-2	-	14	endocrine system
	map04915	Estrogen signaling pathway	▼	NS	-	-	3.70E-2	-	13	
map03320	PPAR signaling pathway	▼	NS	-	-	3.38E-2	3	5		
map04540	Gap Junction	▼	NS	-	-	1.16E-3	-	10	Misc.	
map00562	Inositol phosphate metabolism	▼	NS	-	-	1.23E-2	-	9		

385 Table 5: List of significantly enriched metabolic pathways based on KEGG orthology. Red and
386 green arrows indicate globally up-regulated and down-regulated pathways, respectively. Number
387 of up-regulated and down-regulated contigs involved in each pathway are indicated in red and
388 green, respectively. Enrichment was tested with a Fisher exact test ($p\text{value} \leq 0.05$). Only pathways
389 represented by >5 contigs were considered.

390

391 **3.7. Transcriptomic response to urban runoff**

392 The down-regulation of a homeodomain-interacting protein kinase 2 (HIPK2) homologue (Table 3) is
393 particularly relevant in the most hypoxic site (Table 2). HIPK2 was found to repress the transcription
394 of Hypoxia Inducible Factor (HIF) 1 α (Nardinocchi et al., 2009). As a transcription factor, HIF1
395 targets genes involved in glucose metabolism and cell survival in response to low O₂ (Ke and Costa,
396 2006). This cellular response to hypoxia might lead *C. fluminea* to maintain its homeostasis and
397 ventilatory activity (Tran et al., 2000).

398 In urban runoff exposure, the repression of DEGs involved in signalling pathways (GO:0035556,
399 Table 4) was also observed. Thanks to KEGG orthology, these pathways could be grouped into
400 immunity and endocrine system related processes (Table 5 and Appendices B and C). The MAPK
401 (Mitogen-activated protein kinases) pathway is amongst the most ancient signal transduction pathways
402 and is widely used throughout evolution in many physiological processes (Cargnello and Roux, 2011;
403 Morrison, 2012). The classical MAPK pathway (also referred to as the ERK1/2 module) is part of
404 several aforementioned pathways and contigs encoding proteins involved in this module were down-
405 regulated at both sites. However, in clams exposed to urban runoff, genes involved in the JNK and p38
406 modules were also downregulated (Appendix E). These modules are activated in response to various
407 cellular stresses, including heat shock, oxidative stress, cytokines, hypoxia, interleukin-1 (IL-1),
408 lipopolysaccharide (LPS) and growth factor deprivation (Bogoyevitch et al., 2010; Cuadrado and
409 Nebreda, 2010). While the JNK plays an important role in the apoptotic response to cellular stresses
410 (Dhanasekaran and Reddy, 2008), the p38 plays a critical role in normal immune and inflammatory
411 responses. A major function of the p38 pathway is to produce proinflammatory cytokines such as
412 TNF- α , IL-1, IL-6 and IL-12, which are essential for the eradication of infectious microorganisms

413 (Arthur and Ley, 2013; Cuadrado and Nebreda, 2010; Zhang and Dong, 2005). In addition, the
414 ERK1/2 pathway has an important role in macrophages, regulating cytokine production (Arthur and
415 Ley, 2013). Hence, the inhibition of MAPK pathways could result in severe reduction of the immune
416 response in organisms from urban runoff exposure unlike WWTP exposure where the ability to
417 respond to virus was suggested by the presence of immune-related contigs amongst the most up-
418 regulated (Table 3). This inhibition might explain the observed reduction of several immune processes
419 in the present study like endocytosis, phagocytosis and response to and production of chemokines. In
420 bivalves, cellular response is carried out by circulating hemocytes (Allam and Pales Espinosa, 2016;
421 Song et al., 2010). Such effects are similar to what was observed in fish for instance (Segner et al.,
422 2011). In bivalves, different classes of environmental pollutants like metals, pharmaceuticals, biocides,
423 polychlorinated biphenyls (PCBs) and PAHs were shown to affect immunity (Galloway and Depledge,
424 2001). The hypothesis that urban runoff impacts bivalve immunity should be confirmed in future
425 studies by the measurement of hemocytes-related variables such as type counts, size, and production
426 of reactive oxygen species (*e.g.* Lassudrie et al., 2016). This phenomenon is of particular interest
427 since, in addition to metal and organic pollutants, pathogens can find their way into surface water
428 through leaking sewer systems, sewer pumping station overflows, seepage from septic systems,
429 agricultural runoff and discharge of treated wastewater into aquatic environments. High numbers of
430 enteric viruses, bacteria and protozoa have been reported in stormwater runoff (Sidhu et al., 2012).
431 Such contamination is hence more likely to occur on urban runoff site following heavy rainfall than on
432 WWTP where no leakage was noticed over the studied period. It is interesting to note that MAPKs are
433 targeted by pharmaceuticals such as anti-cancer and anti-rheumatic drugs, and by some pathogens as
434 an immune evasion strategy (Arthur and Ley, 2013). This is the case, for instance, for *Vibrio*
435 *parahaemolyticus* and *Salmonella enterica*, both responsible for gastroenteritis, that is a particularly
436 prevalent disease during the study period, *i.e.* winter months. Immune system could also be altered by
437 both generalized stress and changes in PPAR pathway (DeWitt et al., 2009). Peroxisome proliferator-
438 activated receptors (PPARs) regulate inflammation, lipid metabolism and energy utilization. Changes
439 in PPAR pathway might result from endocrine disrupting chemicals (Casals-Casas et al., 2008). In
440 fish, such changes were documented in organisms exposed to anthropogenic pollutants like organotins,

441 oil and nonylphenol (Bilbao et al., 2010; Cocci et al., 2013; Pavlikova et al., 2010). Other disturbances
442 affected GnRH and estrogen signaling pathways. Numerous chemicals, including pharmaceuticals,
443 pesticides, plastics feedstocks and additives, and phytochemicals, can act as estrogen agonists or
444 antagonists, mimicking or blocking the natural effects of estrogens in invertebrates (Keay and
445 Thornton, 2009). Though WWTP are important sources, estrogens and other aforementioned
446 endocrine disruptors can also be found in runoff and surface waters (Adeel et al., 2017). Here, we
447 were only able to measure a limited number of pesticides including glyphosate, AMPA and terbutryn
448 that showed higher concentrations in runoff. Glyphosate is currently the most widely used herbicide in
449 the world. In fish, that received most of the interest amongst aquatic organisms, glyphosate showed
450 potential effects on reproduction through the disruption of steroid hormone synthesis and increased
451 oxidative stress (e.g. Uren Webster et al., 2014). Bivalves received little interest, however recent
452 studies in *Mytillus galloprovincialis* evidenced effects of glyphosate on hemocytes, membrane
453 transport, energy metabolism and neurotransmission (Matozzo et al., 2018; Milan et al., 2018). A more
454 comprehensive chemical analysis of the effluent water looking at various class of compounds would
455 be necessary to confirm the biological outcomes on bivalve's physiology.

456 **3.8. Insights into the nature of environmental stress**

457 Taken together, our results suggest that exposure to organic contaminants and hypoxia could lead to
458 energy impairments, cellular damages, immune disorders and endocrine disruption through
459 disturbances in signaling pathways. We observed no difference in CI of organisms from the three sites
460 at the end of the exposure period. In bivalves, it has been shown that environmental stress may affect
461 physiology without impacting CI (e.g. Riascos et al., 2012), then the changes in transcription we
462 observed might represent early warning markers occurring before observable deleterious consequences
463 at the whole-organism level (Forbes et al., 2006).

464 These outcomes at the gene expression level are compatible with pollution generally associated with
465 urban waters. The presence of emerging contaminants in the environment is mainly attributed to the
466 discharge of treated wastewater from WWTP. Highly prescribed therapeutic substances like anti-
467 inflammatory drugs, β -blockers, antidepressants and antiepileptics, together with personal care
468 products, are the most ubiquitous chemicals to influent wastewaters due to their incomplete removal

469 during wastewater treatment (Muñoz et al., 2008; Petrie et al., 2015). Urban runoff is a relatively
470 recent concern. It carries multiple pollutants such as metals (mainly Cd, Pb, Zn and Cu), pesticides,
471 PCBs, PAHs, organic material (pet waste, leaves, litter, *etc*) of which the decay consume oxygen, and
472 bacteria from sewage, pets and urban wildlife (Dong and Lee, 2009; Huber et al., 2016; McLellan et
473 al., 2015; Revitt et al., 2014). Despite being considered as a major source (Heberer, 2002), effects of
474 pharmaceuticals were not observed downstream of the WWTP. We can hypothesize that the effects of
475 the pollutants appeared or were reinforced by additional input in urban runoff. Indeed, studies have
476 shown that mixtures of chemicals exhibit greater effects than the pollutants individually (Cedergreen,
477 2014). However, cautions must be taken in the prediction and interpretation of biological
478 consequences of the stress associated with both sources. Variability of stormwater quality is high and
479 depends on the accumulation time on diverse impervious surface between two precipitation events
480 (Zgheib et al., 2011) whereas treated wastewater flux and composition are more constant and
481 predictable over a three-month period.

482 Solely based on a transcriptomic approach and a limited number of chemicals, our study still provided
483 some valuable first clues regarding the nature of the stress experienced by *C. fluminea* and the
484 identification of initiating molecular events of toxicological outcomes. Nevertheless, the complexity of
485 metabolic pathways and their interplay (illustrated by the many signaling pathways cited previously),
486 as well as the diversity and variability in the chemical composition of the two types of effluents make
487 difficult the identification of a specific initiating event and the prediction of the adverse biological
488 consequences. To counter this limitation, the chemical characterization of the pollution, as well as the
489 presence of pathogens appear necessary for future studies in order to confirm or clarify transcriptomic
490 results. In the same manner, the integration of multi-omics technologies, such as the coupling of
491 transcriptomic and metabolomic information (Santos et al., 2010), has the potential to better predict
492 toxicological outcomes and gain further insight into the mechanistic aspects of chemical effects. For
493 instance, such approach could confirm whether transcriptional changes translate into metabolic or
494 physiological adjustments and/or impacts. Freshwater bivalves are amongst the most threatened
495 molluscs worldwide (Lydeard et al., 2004). Such an integrative approach could then be applied to
496 assess the impacts of polluted water in endangered native species.

497

498 **4. Conclusions**

499 Our results provide insights in the molecular impact of low quality waters in Asian clams, *C. fluminea*.
500 The chemical characterization of WWTP and urban runoff exposures over a 3-month period is hardly
501 achievable in field experiments. However, RNA sequencing could be useful for diagnosing sites where
502 the level and nature of contamination are unknown as it allows the investigation of a large collection
503 of biological processes without *a priori*. Our results revealed the up-regulation of genes involved in
504 detoxification, suggesting the presence of organic pollutants on both sites. The down-regulation of
505 genes involved in intracellular signaling pathways suggested metabolic disorders likely to impact
506 energy metabolism, protein metabolism and cell survival in response to WWTP effluent, and
507 endocrine and immune systems in response to urban runoff exposure. These findings could then allow
508 the identification of relevant biological parameters to survey in similar future studies, such as energy
509 metabolism, endocrine disorders or immunity that should be performed at a higher level of integration,
510 such as histological or enzymatic assays. Moreover, this work highlights the necessity of a
511 comprehensive chemical characterization of the pollution in order to better predict the toxicological
512 outcomes, particularly if applied to endangered freshwater bivalve species.

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519

520 **Competing Interests Statement**

521 The authors declare no competing interests.

522

523 **Appendices**

524 Appendix A: Summary of quantitative real-time PCR validation.

525 Appendix B: Complete annotation of the *de-novo* transcriptome of *C. fluminea*'s digestive gland.

526 Appendix C: Results of DESeq2 differential expression analysis.

527 Appendix D: Details of the KEGG pathway 04141.

528 Appendix E: Details of the KEGG pathway 04010.

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