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Identification and expression of microRNAs in european eels *Anguilla anguilla* from two natural sites with different pollution levels

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Abstract :

MicroRNAs (miRNAs) are a class of small non-coding RNA that control multiple biological processes through negative post-transcriptional regulation of gene expression. Recently a role of miRNAs in the response of aquatic organisms to environmental toxicants emerged. Toxicant-induced changes in miRNA expression might then represent novel biomarkers to evaluate the health status of these organisms. In this study, we aimed to identify the miRNA repertoire in the liver of the European eel Anguilla anguilla and to compare their differential expression between a polluted site located in the Gironde Estuary and a pristine site in Arcachon Bay (France).

A total of 299 mature miRNAs were identified. In polluted water, 19 miRNAs were up-regulated and 22 were down-regulated. We predicted that these differentially expressed miRNAs could target 490 genes that were involved in ribosome biogenesis, response to hormones, response to chemical and chromatin modification. Moreover, we observed only few examples (29) of negative correlation between the expression levels of miRNAs and their targets suggesting that, in the system studied, miRNAs might not only regulate gene expression directly by degrading mRNA but also by inhibiting protein translation or by regulating other epigenetic processes.

This study is the first example of in situ investigation of the role of miRNAs in the response of a fish species to water quality. Our findings provide new insights into the involvement of epigenetic mechanisms in the response of animals chronically exposed to pollution and pave the way for the utilization of miRNAs in aquatic ecotoxicology.

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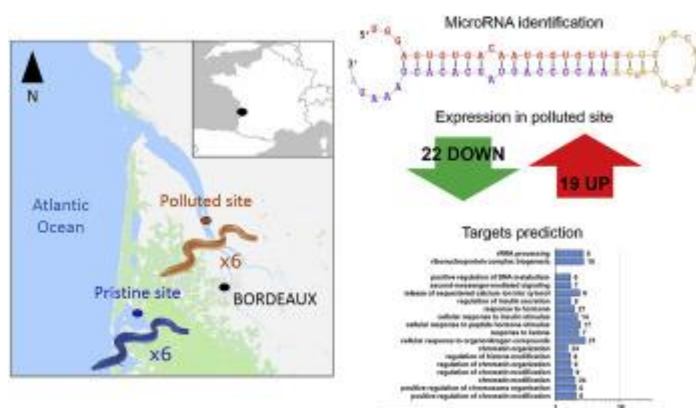
Graphical abstract



Highlights

► 299 MicroRNAs were characterized in the endangered European eel *Anguilla anguilla.* ► The expression levels of 41 microRNAs were altered in fish from a polluted site. ► Differentially expressed microRNAs might regulate important biological processes.

Keywords : MicroRNA, Anguilla Anguilla, Aquatic ecotoxicology, Epigenetics, Biomarker

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47 **Introduction**

48 MicroRNAs (miRNAs) are a class of small non-coding RNA [1,2]. These 20-24 nucleotides-long
49 sequences associate with the 3'-untranslated region (3'-UTR) of target messenger RNAs (mRNAs),
50 and post-transcriptionally regulate the expression of numerous genes by mediating translational
51 repression or mRNA degradation [3,4]. Owing to this ability to regulate gene expression without
52 involving DNA sequence changes, miRNAs are hence considered as epigenetic factors like histone
53 modification and DNA methylation. In mammals, more than 50% of mRNAs are predicted to be the
54 subject of miRNA-mediated control [5]. One miRNA may regulate hundreds of target mRNAs, and one
55 mRNA may contain multiple binding sites for multiples miRNAs [6], thus resulting in a complex
56 regulatory network. Although miRNAs are involved in regulation of almost all cellular processes, such
57 as development, growth, apoptosis, immunity and maintenance of tissue-specific function [7],
58 mechanistic aspects of this regulation are not fully understood. In mammals, the aberrant expression of
59 miRNAs has been linked to various diseases and cancer [8,9] and to the inheritance of stress-induced
60 phenotypes across generations [10]. Toxic environmental factors such as nanoparticles, organic
61 pollutants and metals can alter miRNA expression [11,12]. These changes in miRNAs expression may be
62 part of the adaptive responses of organisms to pollutants and of the mechanisms of toxicity and disease
63 aetiology.

64 Advances and accessibility of sequencing technologies, coupled with the development of microRNAs
65 dedicated tools are improving the sensitivity of analyses and the ability to detect lowly abundant small
66 RNAs [13–16] even in non-model species. The latest release of the miRNA database (miRBase 21)
67 contains 28,645 hairpin precursors from 223 species [17]. In aquatic organisms, the role of miRNAs has
68 been investigated in response to various environmental stresses, such as immune challenge, metals,
69 salinity, hypoxia or acidic water [18–24].

70 As a catadromous fish species, the European eel *Anguilla anguilla* (Anguillidae; Teleostei) exhibits a
71 complex life cycle including marine (spawning, larval phase and sexual maturation) and continental
72 (somatic growth) environments. Moreover, *A. anguilla* is a panmictic species and its unique life cycle
73 makes it particularly vulnerable to pollution. After spawning in the Sargasso Sea, eel larvae drift back

74 toward European and North African continental waters following oceanic currents such as the Gulf
75 Stream and the North Atlantic Drift. There, they first metamorphose into glass eel (unpigmented)
76 before reaching the juvenile growth phase (yellow eel) in continental water bodies where they remain
77 up to 15-20 years, often moving across different habitat types. This stage ends with a second
78 metamorphosis and maturing eels (silver eels) will migrate back to the Sargasso Sea to reproduce and
79 die [25,26]. For their unusual life cycle and vulnerability to pollution, eels are considered as sentinel
80 species in ecotoxicology [27,28]. Moreover, the species has been considered as critically endangered since
81 2008 (IUCN red list) [29]. Its catadromous migratory behaviour, long life, serious habitat reduction,
82 migration barriers, pollution, human-introduced diseases, overfishing, as well as climatic events may
83 be amongst the causes of the catastrophic collapse of the European eel population observed over the
84 past decades [30–32]. A better understanding of the biology of this species may come from the use of
85 high-throughput (next generation) sequencing technologies that have recently provided transcriptomic
86 [33–36] and genomic [37–39] information. However, like in many non-model species, the non-coding portion
87 of these data has been overlooked in spite of the emerging importance of their role in gene expression
88 regulation. So far, in *A. anguilla*, microRNAs were only predicted from *de novo* assembled
89 transcriptome [33] while in the related species *A. marmorata* (marbled eel), microRNAs were
90 specifically sequenced and their expression pattern assessed in response to salinity [20].
91 In previous studies, the responses of wild immature yellow eels to pollution was investigated in 8
92 locations presenting a broad contamination gradient in France and Canada [34,40–42]. These studies
93 identified Arcachon Bay and the Gironde Estuary as the cleanest and the most contaminated French
94 sites, respectively. We used samples from these two highly contrasted sites in order i) to identify
95 evolutionarily conserved as well as novel miRNAs in the European eel *Anguilla anguilla* by using
96 next generation sequencing, ii) to compare their expression between a pristine and a polluted site and
97 iii) to link miRNA and mRNA levels. Comprehension of gene regulation by miRNAs may give new
98 insights into a neglected mechanism of response to environmental pollution in aquatic organisms.
99

100 **Material and Methods**

101 **Sample collection –** European eels *Anguilla anguilla* (Linnaeus, 1758) were collected from two
102 sampling sites with a contrasted contamination profile. The Gironde estuary (GE, 45° 12.110'N, 0°
103 43.579'W) is considered as a highly impacted site while Arcachon Bay (AB, 44° 41.300'N, 1°
104 1.650'W) represents a pristine environment. Six individuals were collected on each site. The liver was
105 dissected, transferred in RNA-later solution and placed at 4°C overnight before storage at -20°C until
106 RNA extraction. These samples were part of a previous study carried out by our group [34].
107 **Condition indices, tissue composition and trace metal analyses –** Information concerning the 12
108 individuals used in the present study (biometry, tissue composition and trace metals) were extracted
109 from previously published data [34,41,43,44]. Fulton index and HSI were calculated as (total weight /
110 length^3) x 100 and as (liver weight / total weight) x 100, respectively (weight is in grams and length is
111 in centimeters). The silvering status of the eels was assessed by the calculation of the ocular index (OI)
112 according to the Pankhurst method [45]. Statistical differences between the two sites were assessed either
113 with Student t tests or Wilcoxon tests after checking assumptions of normality and homoscedasticity.
114 **Small RNA isolation and deep sequencing –** Total RNA were isolated from individual liver tissues
115 following the Trizol™ Reagent (Invitrogen™) protocol. MicroRNA were then purified by using the
116 PureLink™ MiRNA Isolation kit (Invitrogen™) according to the manufacturer's instructions. Total
117 RNA and MicroRNA concentration and quality were assessed by measuring the absorbance at 260nm
118 and 280nm, and migration on a TBE-urea 12% polyacrylamide gel. The NEBNext® Small RNA
119 Library Prep Set for Illumina® (New Englan BioLabs®) was used to prepare the sequencing libraries.
120 Fragment size (around 150 bp) and quality of the individual libraries were checked on a 2100
121 Bioanalyzer® (Agilent ®) with a High Sensitivity chip. The 12 individually tagged libraries were then
122 sequenced using a HiSeq 4000 sequencer at the GenomeEast microarray and sequencing platform
123 (Illkirch, France).
124 **MicroRNA discovery analysis –** The 50bp raw reads from each of the 12 samples were quality
125 filtered ($Q > 30$) and adaptors (5' AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC 3') were
126 removed. The *Anguilla anguilla* genome [37] (GenBank accession number: AZBK00000000.1) was used
127 as a reference genome. High quality, adaptor processed reads were aligned against the eel reference
128 genome using the miRDeep2 package [14,46] with default parameters. Our strategy was similar to the one

129 used for the discovery and characterization of miRNA in the salmon *Salmo salar* [47]. Shortly,
130 miRDeep2 provides a list of putative miRNA precursors with their corresponding mature and star
131 sequences. Each precursor is associated with a score that is a measurement of the posterior probability
132 that a predicted sequence is a true miRNA gene. Each score corresponds to a signal-to-noise ratio that
133 estimates total miRNA reported/mean estimated false positive miRNA over 100 rounds of permutated
134 controls. We used a miRDeep2 score of 1 that yielded a signal-to-noise ratio of 10:1 as a cut-off
135 threshold. MiRNAs over this score were further compared by BLAST searches to all known stem-loop
136 sequences from all species deposited in miRBase, release 21 [17]. Only miRNA with reads matching
137 both harms (5p and 3p) of their precursors were considered. MicroRNA with an E-value < 1.e-6 were
138 annotated as a true *Anguilla anguilla* miRNA and named accordingly to its ortholog in miRBase.
139 Sequences with no significant match in miRBase were used as queries in a BLAST analysis against
140 the nr/nt and refseqRNA databases in GenBank [48], the functional small RNA database [49] and the small
141 RNA families in Rfam [50] to exclude other kind of small RNA. Finally, the remaining miRNAs were
142 aligned with the reference genome. Sequences with an E-value < 1e-6 against >5 loci were considered
143 as repeats and discarded. Final sequences were then considered as novel miRNAs. The nomenclature
144 rules used for the miRNA naming were in agreement with guidelines from miRBase [51,52].
145 **Transcriptome annotation –** The transcriptome assembly was previously annotated based on
146 similarity (E value ≤ 1e-10) with known proteins from the swissprot and nr protein databases using the
147 BLASTx algorithm [34]. For the present work, we enriched this annotation with Gene Ontology (GO)
148 assignment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis that were
149 performed using the BLAST2GO software [53,54] and the GhostKOALA server [55], respectively.
150 Enrichment tests were performed with the Fisher exact test procedure and a correction for multiple
151 testing using the Benjamini and Hochberg method. A Pvalue threshold of 0.01 was used. Redundancy
152 in GO terms was reduced by the GO Trimming method [56] using a uniqueness threshold of 50%. For a
153 matter of clarity, only GO and KEGG categories containing ≥5 sequences were considered.
154 **Differential expression of transcripts –** Differences in transcription levels between the two study
155 sites were assessed with the RNAseq data extracted from Baillon *et al* [34] for the exact same individual

156 eels. Normalization and expression analysis were performed using the DESeq2 package [57]. *P*-values
157 for differential gene expression were corrected for multiple testing using the Benjamini and Hochberg
158 method, and a false discovery rate (FDR) threshold of 0.05 was used.
159 **Expression analysis of Eel miRNAs –** The copy number of known and novel mature miRNAs were
160 counted with the quantifier module in miRDeep2 (additional files 1 and 3). Normalization and
161 expression analysis were performed using the DESeq2 package [57]. The false discovery rate (FDR)
162 threshold was 0.05. In order to validate these results, the expression profiles of 10 mature miRNAs
163 were assessed using qRT-PCR (additional file 4). Reverse transcriptions were performed by using the
164 miScript II RT system (Qiagen®). Real Time PCR Analyses were performed on a Roche LightCycler®
165 480 Instrument II by using the miScript PCR Starter Kit (Qiagen®). Cycling conditions were 95°C for
166 15 seconds followed by 40 cycles of 15 seconds at 94°C, 30 seconds at 55°C and 30 seconds at 70°C.
167 **Target prediction –** The sequences and annotation of protein-coding transcripts were retrieved from
168 Baillon *et al* [34]. The target genes of mature eel miRNAs were predicted using the miRanda software [58]
169 that is based on the local alignment between the query miRNA sequences and the reference
170 transcriptome and on the thermodynamic stability of miRNA/mRNA duplexes (parameters used were
171 score $\geq$ 160, free energy $\leq$ -25kcal/mol).
172
173 **Results**
174 **Trace metals analysis –** Differences between individuals collected in both sites are presented in Table
175 1. First, AB and GE sites differed in salinity and dissolved oxygen concentrations. We observed no
176 difference in Fulton condition factor between the two sites. However, the Hepato-Somatic Index (HSI)
177 of AB eels was higher than GE eels. No difference was detected in OI and with a value below the
178 threshold value of 6.5 [45], all the individuals were indeed at the yellow eels stage. As expected from
179 previous results [41], trace metal concentrations were significantly higher in the liver of eels from GE,
180 with the exceptions of Cr and Zn that showed no difference and Ni that was higher in AB. Cadmium
181 showed the largest difference with a concentration more than 200-times higher in the liver of GE
182 individuals in comparison to fish from AB.

183 **Small RNA sequencing –** The sequencing of the 12 sRNA libraries led to a total of 362,831,354 raw
184 reads out of which 318,968,039 (87.91%) were kept for the following analyses after quality filtering
185 (Q > 30) and adaptor removal (detailed statistics are given in Table 2). With the exception of the
186 sample AB03 (59.35%), between 72.12% (AB06) and 89.40% (GE02) of these reads were 21-23
187 nucleotides (nt) in length (Figure 1). Another population of 28-30 nt was identified, representing on
188 average 12.99% and 2.36% of the reads in AB and GE samples, respectively. These two short reads
189 populations represented between 81.70% and 90.76% of the total high-quality reads. Up to 87.73% of
190 these reads could be mapped against the reference genome for miRNA prediction (Table 2). The raw
191 sequencing data from this study were submitted to the NCBI Gene Expression Omnibus [59] under GEO
192 Accession GSE109689.
193 **Identification of known and novel miRNAs in *A. anguilla* –** A total of 820 putative miRNA
194 precursors were predicted by the miRDeep2 analysis (additional file 1) amongst which 474 had a score
195 above 1 and reads on both harms, *i.e.* were associated with both a 3p and a 5p sequence (Figure 2).
196 Amongst those, 230 pre-miRNA provided a match against a stem-loop sequence in miRBase (E-value
197 < 1e-6) and were considered *Anguilla anguilla* orthologs of an evolutionary conserved miRNA gene
198 and named in accordance with the nomenclature rules. Most of these conserved miRNAs (181 out of
199 230) matched with miRNAs from other fish species in miRBase. They corresponded to 158 unique
200 mature miRNAs from 96 evolutionary conserved families. The remaining 244 putative precursors
201 were further analyzed by BLAST searches against the reference genome and other RNA databases.
202 That allowed us to identify 145 potential novel pre-miRNAs that were named based on their genome
203 location. Redundancy was lower since 141 unique novel mature miRNAs were identified. Taken
204 together we thus identified a total of 375 miRNA precursors with their respective mature and star
205 sequences. The total number of unique mature miRNAs was 299 (detailed list provided in additional
206 file 2). In all libraries, aan-mir-122 was on average 14 times more expressed than the second most
207 abundant mature miRNA (ranging from ~4 in AB06 to ~25 times in GE05). Other abundant miRNAs
208 included aan-mir-21, aan-mir-22 and aan-let-7a (additional file 3).
209 **Prediction of transcriptomic targets –** In order to determine the biological processes potentially
210 regulated by the 299 mature microRNAs we identified, we predicted their target genes in the

211 *anguilla*'s transcriptome assembly [34]. The *A. anguilla* transcriptome contains 18,113 contigs with high
212 homology with known sequences (BLASTx, Evalue ≤ 1e-10). Although 3'UTR regions are the most
213 important regulatory sites for post-transcriptional regulation of gene expression by miRNAs, we
214 considered whole length transcripts. 242 miRNAs showed a potential to hybridize within the
215 transcriptome wherein 3,637 contigs were identified as potential targets (additional file 4). As
216 expected, one miRNA could target several transcripts and one transcript could be targeted by several
217 miRNAs. For instance, the novel miRNA aan-scaffold-2762 could hybridize with up to 271 targets
218 and the transcript contig_15454, encoding a homologue of E3 ubiquitin-protein ligase UBR2 (Uniprot
219 accession number Q8IWV8), was potentially the target of 8 different miRNAs. The entire set of
220 predicted mRNA targets was further functionally categorized through GO annotation and KEGG
221 pathway analysis. No GO term, nor KEGG pathway, was significantly enriched in the list of target
222 genes compared to the transcriptome background, suggesting that miRNAs might affect any biological
223 process.
224 **Differentially expressed mature miRNAs between the two sites –** One objective of this study was
225 to evaluate the response of miRNAs to differing levels of contamination. A multidimensional scaling
226 of the count data showed that despite inter-individual variability, samples are well separated according
227 to their site of origin (additional file 5). We identified 41 mature miRNAs with differential expression
228 (DE) in GE compared to the reference site (AB). Nineteen (19) miRNAs were up-regulated, while 22
229 were down-regulated (Table 3). The most up-regulated and down-regulated miRNAs were
230 respectively mir-29a (2.77 times more expressed in GE) and mir-462 (3.97 times less expressed in
231 GE). These results were validated by qRT-PCR ($r^2 = 0.88$, additional file 5). Amongst all the DE
232 miRNAs, 35 were likely to target 490 sequences in *A. Anguilla*'s transcriptome (Table 3). The targets
233 of up-regulated miRNAs were functionally enriched in transcripts encoding proteins involved in
234 ribosome biogenesis. This process is hence expected to decrease in GE. Biological processes affected
235 by down-regulated miRNAs were related to the response to hormones (*e.g.* GO:0016568), response to
236 chemicals (e.g. GO:0071417) and chromatin organisation (*e.g.* GO:0006325) (Figure 3). These
237 processes are then expected to increase in GE.

238 **Links between DE miRNAs and DE transcripts –** Based on previous data [34], we identified 1,554
239 down-regulated and 1,343 up-regulated genes in the Gironde Estuary compared to the Arcachon Bay
240 (additional file 7). Down-regulated genes contained a significant proportion of sequences related to
241 lipid metabolism (GO:0006629 and GO:0044255), response to chemical (GO:0001101 and
242 GO:0009410), primary metabolism, development (GO:0051216 and GO:0032836) and hormone
243 metabolism (GO:0042445). Up-regulated transcripts were involved in processes such as transport
244 (GO:0006810, GO:0051649 and GO:0032940), development (GO:0007423, GO:0007389,
245 GO:0048840 and GO:0035889) and response to stimulus (GO:0010035 and GO:0071216) (Figure 3).
246 Due to the action mode of miRNAs, their expression patterns generally showed an inverse relationship
247 with those of their mRNA targets. We compared the list of DE genes with the lists of DE miRNAs and
248 their targets. Out of the 35 DE miRNAs that had putative targets in the transcriptome, 20 of them were
249 able to hybridize to 49 (out of 490) DE mRNAs targets, forming 52 miRNA-mRNA pairs with both
250 positive and negative relationships in their expression values (log_2FC). In detail, there were 29 (out of
251 52) negative miRNA-mRNA interactions (Spearman's ρ = -0.69, Pvalue = 3.46e-05) involving 18 DE
252 miRNAs and 29 DE mRNAs. Whilst a single DE miRNA could target up to 4 different DE transcripts,
253 each DE transcript was targeted by only one DE miRNA. Many of these DE transcripts were involved
254 in biological processes cited above, such as immune response and lipid metabolism (Table 4).
255

256 **Discussion**

257 The increasing evidence that the expression of miRNAs is affected by several known toxicants as well
258 as oxidative and other forms of cellular stress certainly suggests an important role of miRNAs in
259 toxicology, which could provide a link between environmental influences and gene expression [60]. The
260 use of miRNAs in environmental toxicology is expanding, particularly in human health [8,11,12]. This
261 interest mainly results from the strong potential of miRNAs to become robust, minimally invasive and
262 non-expensive biomarkers for various diseases [61]. In the same manner, examples of miRNA-related
263 work are more frequent in aquatic organisms exposed to environmental stressors. One can cite some
264 recent publication in bivalves [22,62,63], crustaceans [64,65] or teleost fish [20,21,66,67] for instance. To our

265 knowledge, our work is the first *in situ* study using miRNA in an aquatic organism. MicroRNAs are
266 being characterized in an increasing number of teleost fish species where they repress target mRNA
267 abundance or translation in a highly conserved manner across most eukaryotes [68].
268 In this study, we conducted a comprehensive identification of the microRNA repertoire in the liver of
269 the European eel *A. anguilla* and its response to environmental pollution. This organ was chosen
270 owing to its role in pollutant metabolism, accumulation and toxicity. Only trace metal concentrations
271 were reported here since they were measured in the liver. However, one should note that eels from the
272 Gironde Estuary also showed higher levels of persistent organic pollutants (Poly-Chlorinated
273 Biphenyls, Organo-Chlorine Pesticides, and Poly-Brominated Diphenyl Ethers) in their muscle
274 compared to eels from the Arcachon Bay, as evidenced by previous analyses [34,43]. Owing to panmixia
275 and proximity between the two sites, this contrast is most likely due to water quality rather than
276 population differences.
277 The size distribution of the small RNA libraries revealed that 22-23 nt microRNAs were dominant,
278 followed by small RNAs of 28-29 nt in length (Figure 1). This distribution is similar to what was
279 found in the sister species *A. marmorata* [20]. It is interesting to note that, in both eel species, the relative
280 abundance of 22-23 nt and 28-29 nt varies depending on environmental conditions, which could
281 represent a new path of investigation in the future. These 28-29 nt sRNA might correspond to piRNA
282 (piwi-interacting RNA that have a length of 24-32 nt [1,69]), another smallRNA silencing system that
283 plays a role in germline specification, gametogenesis, transposon silencing, genome integrity, and
284 stem cell maintenance across the animal phylogeny [70].
285 We identified a total of 299 mature miRNA in eel liver, of which 158 were known and 141 were
286 novel. This number is comparable to what is currently available in the miRBase database for the
287 model species *Danio rerio* (375 mature sequences). This identification benefited from the availability
288 of a sequenced genome of *A. anguilla* [37]. Only mature miRNAs were considered in our analysis since
289 they eventually regulate gene expression at the post-transcriptional level. In all libraries, mir-122 was
290 the most abundant mature sequence. This was not surprising since it is the most abundant liver
291 miRNA in vertebrates (>70% of the total hepatic miRNA expression) with exquisite tissue specificity.

292 Mir-122 is mainly involved in lipid homeostasis [71]. Its expression level increased by 2.34-fold in
293 polluted water (Table 3). Such accumulation is associated to injured hepatocytes and is a biomarker of
294 liver toxicity in zebrafish [72] and mammals [73]. The alteration of lipid metabolism was also evidenced by
295 prevalence of related Gene Ontology terms amongst down-regulated transcripts (Figure 3). In farmed
296 tilapia (GIFT, *Oreochromis niloticus*), the level of mir-122 was negatively correlated to Cd stress and
297 promotes hepatic antioxidant defence [74]. This discrepancy with the present work, where Cd was the
298 metal showing the largest difference between the two sites, could either be due to the action of other
299 stressors or to taxa specific mechanisms of miRNA regulation. The two other abundant miRNAs, mir-
300 21 and mir-22, were also shown to play a role in the development of hepatic diseases [75,76]. Liver
301 damage was confirmed by a series of transcriptomic changes described previously [34]. This first set of
302 results shows the high tissue specificity of our approach and confirms the potential of miRNAs in
303 aquatic ecotoxicology.
304 We chose to study two contrasted sites both in terms of organic and metallic contaminants [34,40]. These
305 sites also differed in salinity (19.9‰ and 7.78‰ for Arcachon Bay and Gironde Estuary, respectively)
306 and oxygen percentage (60% *vs.* 86.5%) whereas the temperature was relatively similar (21.9°C *vs.*
307 21.3°C) (Table 1). With a dissolved oxygen concentration of 4.68 mg/l, site AB faces moderate
308 hypoxia according to the European Water Framework Directive (WFD, 2000/60/EC). In such a
309 complex context, it is obviously challenging to identify causing agents. However, since contamination
310 of aquatic ecosystems typically includes many pollutants that present different mechanisms of action
311 likely interacting together and under the influence of natural factors, this situation might provide more
312 realistic information as well as a first insight into the role of miRNAs in a polluted environment. In
313 fish, salinity and hypoxia were previously shown to affect the miRNA regulatory machinery [20,21,77]. In
314 our study, only 5 miRNAs (miR-19b, miR-139, miR99a, miR-454 and miR-221) and 3 miRNAs (miR-
315 125a, miR-181c and miR-30b) were found to respond to salinity and hypoxia, respectively, in the
316 same way than in the literature cited above. Moreover, eels were shown to be able to cope with much
317 more severe hypoxia, down to about 30% O_2 saturation [78].Then, the differential expression of the
318 majority of the miRNA we identified could most likely be linked to the presence of pollutants. The

319 expression level of 19 miRNAs increased in the polluted site while the expression of 22 miRNAs was
320 higher in AB. This relatively small number of DE miRNA is similar to what was found in other fish
321 species exposed to environmental stressors [19–21,24] and could be due to the relatively low number of
322 samples per site and inter-individual variability (additional file 5) that should be taken into account in
323 future studies. The most expressed known miRNAs in AB (>3 fold) were miR-462, miR-15e and miR-
324 23a, and the most up-regulated known miRNAs in GE (>2 fold) were miR-29a, miR-122 (discussed
325 above) and miR-203. As previously, the fold change values measured here are in agreement with what
326 can be found for Teleosts in the literature [19–21,24]. The role of these miRNAs could be inferred from
327 previous studies.
328 Despite the highly conserved mode of action of miRNAs, caution must be taken when comparing fish
329 and mammals as only a small fraction of miRNA-mRNA interactions (~10%) is conserved [79] and
330 identification of conserved targets between species remains challenging [80]. For instance, in fish, miR-
331 462 was shown to be upregulated after hypoxia treatment in liver [81,82] or virus infection [18,83]. MiR-462
332 is only described in teleost fish and is actually an orthologue of the human miR-191 that plays a role in
333 cell cycle regulation, but not in viral infection [83]. The role of miR-23a instead seems to be conserved.
334 It was identified as an essential regulator of adipocyte differentiation of which increased expression
335 might reduce lipid accumulation and triglyceride content in adipocytes [84]. This role was confirmed in
336 our study by the identification of a transcript encoding a very long-chain acyl-CoA synthetase
337 (Uniprot accession O14975, additional file 5) as a putative target of miR-23a. In human, miR-29a is
338 known to regulate cell proliferation, differentiation and apoptosis as several of its targets are
339 oncogenes or anti-apoptotic genes [85]. Amongst the predicted mRNA targets of miR-29a, we identified
340 a thyroid receptor-interacting protein homologue (Uniprot accession Q15643, additional file 5) of
341 which degradation could reduce gene transcription in response to thyroid hormone while miR-122
342 predicted targets included transcripts encoding proteins involved in lipid synthesis, energy generation,
343 platelet activation, apoptosis and transcription regulation. The role of miR-203 remains unclear as its
344 overexpression might promote both apoptosis and tumor growth, as well as host-virus interactions [86].
345 This difference in lipid metabolism is not confirmed by our observation as fish from the two sites

346 showed no significant difference regarding their muscular lipid content (table 1), contrary to previous
347 studies that showed that eels exposed to Cd [87] and organic pollutants [88] increased their fat consumption
348 and/or reduced their energy reserves. We can here hypothesize that miRNA regulation in the liver
349 contributes to maintain a significant production of triglycerides and their export to the main site of
350 lipid storage in eels, *i.e.* the muscle [87]. At the opposite, the lower HSI values in fish from the estuary
351 could indicate an impairment in the accumulation of energy reserves under the form of carbohydrate
352 such as glycogen in hepatocytes.
353 More comprehensive information was gained from the functional analysis of miRNA targets in *A.*
354 *Anguilla*'s transcriptome (Figure 3). Based on gene ontology of these targets, changes in miRNA
355 expression might result in reduced ribosome biogenesis and increased response to hormone and
356 chemical, and chromatin modification. The effect on ribosome biogenesis could be evidenced by a
357 significantly reduced level of total proteins in the liver of GE eels (Table 1). One could expect to find
358 some similar functions in the predicted targets of up-regulated miRNAs and down-regulated
359 transcripts, and conversely. In spite, we found no relation between enriched gene ontology in miRNA
360 targets and mRNA, as well as a small number of miRNA-mRNA pairs with negative correlation
361 (Table 4). This situation was also observed in a comparable study carried out in *Pelteobagrus vachelli*
362 exposed to hypoxia [21] in which the authors identified only 97 negative correlations out of 308 miRNA-
363 mRNA pairs. In the same study, and in the same manner as our results, most of the DE mRNA (43 out
364 of 60) were target by a single DE miRNA. This highlights that the identification of functional miRNA-
365 targets interaction is still challenging and/or false positives might still be frequent with *in silico*
366 analyses [89]. MicroRNAs are obviously not the only mode of regulation of gene expression, however
367 based on the lack of correspondence between miRNA targets and DE transcripts, we could
368 hypothesize that the degradation of messenger RNA was not the major mode of action of miRNAs
369 here and that they act more likely by inhibiting mRNA translation into proteins, instead. This
370 inhibition might be reinforced by the targeting of mRNA involved in ribosome assembly and function.
371 This result is in accordance with those obtained in juvenile zebrafish exposed to the antibacterial agent
372 triclosan where "structural constituent of ribosome" and "ribosome biogenesis" are amongst the
373 significantly enriched annotation of differentially expressed miRNAs target genes [90]. Which might

374 suggest a conserved mode of action of miRNA in response to pollutants in fish. Another way by which
375 miRNA could regulate gene expression is through chromatin modification, as suggested by the
376 presence of 28 epigenetic regulators in miRNA targets (additional file 8). In human, miRNA and
377 epigenetic regulation are not entirely separable and a strong interplay exists, but the mechanisms of
378 cooperation are still unclear [91,92]. Compared to mammals, the study of epigenetics in teleost fish has
379 received less attention so far [67]. Pollutant-induced changes in epigenetic marks were already
380 documented [42,93,94]. For instance, transcriptomic changes in the liver of wild yellow perch (*Perca*
381 *flavescens*) exposed to cadmium and copper may be driven through histone modifications [95]. The
382 present work suggests that similar effects in eels could be mediated by miRNA regulation.
383 The European eel spends most of its lifetime (about 8-15 years) in European estuarine and freshwater
384 basins in a stage known as yellow eels. During this period, eels accumulate a considerable amount of
385 lipid reserves, which are fundamental for the success of the long oceanic migration since during this
386 period they do not feed. Before initiating their oceanic migration to reproduce in the Sargasso Sea,
387 they metamorphose into silver eel [25,26]. Laboratory and field studies have shown that eels can
388 bioaccumulate high concentrations of environmental contaminants, which have been shown to cause
389 mortality, growth delay, reproductive alterations, tumours, malformations, nervous and endocrine
390 disturbances and immunological changes [28]. In the present work, we evidenced a role of miRNAs in
391 the regulation of genes involved in all these processes, reinforcing the potential of miRNAs as
392 biomarkers of yellow eel's health status in a polluted environment. MiRNAs involved in lipid
393 metabolism (like miR-122) and endocrine function might be of particular interest owing to the critical
394 importance of these biological processes in the reproductive success of *A. anguilla*. Although lipid
395 storage might not be absolutely necessary to initiate metamorphosis (silvering [96]), a sufficient energy
396 reserve is needed to cover the migratory needs, ensure gonadal maturation and reproduction.
397 Moreover, morphological and physiological changes that take place during the silvering phase are
398 initiated and regulated by hormonal changes [28].
399 The present study was carried out on wild organisms, in a multi-stress context. In such conditions, it is
400 difficult to establish significant relationships between the expression level of one particular miRNA

and one particular contaminant. Further experiments carried out with more individuals, in controlled conditions, different organs and/or at different developmental stages are thus needed to fully reveal the potential of miRNAs as biomarkers.

Conclusion

To the best of our knowledge, this study is the first example of high-throughput sequencing of microRNAs in wild aquatic organisms. We were able to identify both conserved and putative novel miRNAs and their targets at the transcriptomic level in the liver of European eels from two sites with contrasted pollution profiles. We showed that some miRNA were differentially expressed in organisms experiencing chronic exposure to pollution and that these changes have the potential to affect important biological processes such as lipid and hormone metabolism or protein synthesis. Moreover, our work also revealed promising aspects of epigenetics in teleost fish, as miRNAs might regulate gene expression by interacting with other epigenetic mechanisms such as chromatin modification. Part of the biological processes affected by miRNAs for which expression levels were altered were similar to previous transcriptome studies, confirming the potential role of miRNAs as early molecular markers for monitoring stress responses in exposed animals.

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634
635
636 **Supporting information**

637 Appendix S1: miRNAs prediction by miRDeep2.
638 Appendix S2: List of the 375 conserved and novel miRNA in *A. Anguilla*.
639 Appendix S3: Table of raw microRNA-seq reads count.
640 Appendix S4: Potential microRNA target prediction by MiRanda.
641 Appendix S5: Multidimensional scaling plot of the gene expression profiles obtained with the DESeq2
642 package for RNA-seq and miRNA-seq data.
643 Appendix S6: RT-qPCR validation of DEseq2 results.
644 Appendix S7: Anguilla anguilla's transcriptome annotation and expression analysis.
645 Appendix S8: Example of down-regulated miRNA targeting transcripts involved in chromatin
646 modification.

		Arcachon Bay	Gironde Estuary	
Environmental variables	**Temperature (°C)**	21.9	21.3	
	Salinity (‰)	19.90	7.78	
	O_2 (%)	60.0	86.5	
	O_2 (mg/l)	4.68	7.33	
Condition indices	**Fulton**	0.156 ± 0.013	0.163 ± 0.014	
	HSI	1.20 ± 0.11	0.85 ± 0.10	*
	OI	5.39 ± 1.42	5.28 ± 0.48	
Tissue composition	**Liver proteins (mg/g ww)**	138.43 ± 6.41	117.66 ± 4.93	*
	Muscle lipids (% dw)	35.3 ± 5.29	49.76 ± 8.72	
Metals (µg/g dw)	**Ag**	0.14 ± 0.07	1.16 ± 0.45	**
	As	6.31 ± 1.82	8.40 ± 1.24	*
	Cd	0.05 ± 0.01	11.55 ± 5.29	**
	Cr	1.24 ± 0.88	0.87 ± 0.89	
	Cu	65.07 ± 20.89	95.71 ± 19.90	*
	Ni	0.40 ± 0.25	0.15 ± 0.08	*
	Pb	0.26 ± 0.13	1.68 ± 0.76	**
	Se	13.42 ± 6.54	58.01 ± 9.23	***
	Zn	212.05 ± 51.79	230.11 ± 31.69	
	Hg	0.22 ± 0.08	1.45 ± 0.78	**

647

648 Table 1. Environmental variables, condition indices, tissue composition, and metal concentrations in
649 the liver (mean ± SE, n=6) of European eels sampled in Arcachon Bay and Gironde Estuary. HSI:
650 hepato-somatic index, OI: ocular index, ww: wet weight, dw: dry weight. (*, ** and *** symbols are
651 Pvalue ≤0.05, ≤0.01 and ≤0.001, respectively)

Sample	Average read size (bp)	N. raw reads	Adaptor dimers (%)	Bases >Q30 (%)	Adaptor-clipped reads (%)	N. clean reads	Mapped reads (%)
AB01	152	26 358 156	2.78	97.18	89.95	22 858 432	78.67
AB02	153	33 128 781	1.90	97.35	91.85	29 568 983	72.95
AB03	154	31 170 977	2.09	97.19	92.32	27 946 975	57.50
AB04	153	23 589 174	2.00	97.17	86.17	19 630 656	72.24
AB05	154	32 383 114	1.45	97.21	86.25	27 176 296	74.61
AB06	154	36 886 852	2.00	97.13	82.04	29 121 827	72.89
GE01	154	27 871 224	2.61	97.42	92.59	24 925 687	87.73
GE02	155	30 141 909	1.13	97.44	94.47	27 999 536	87.45
GE03	155	26 984 661	0.79	97.36	92.49	24 603 024	85.79
GE04	156	32 705 673	0.83	97.41	91.15	29 347 359	87.53
GE05	157	28 617 828	0.70	97.43	91.55	25 872 244	87.14
GE06	156	32 993 005	0.77	97.37	91.95	29 917 020	86.65
Total		**362 831 354**				**318 968 039**	

652

653 Table 2: Summary of samples sequenced for discovery of *Anguilla anguilla* miRNA genes. AB, Arcachon Bay; GE, Gironde Estuary.
654

655

miRNA name	FC in GE	n targets	miRNA name	FC in GE	n targets
aan-mir-462	-3.97	2	aan-mir-29a	2.77	2
aan-mir-15e	-3.44	25	aan-scaffold_1	2.69	0
aan-mir-23a	-3.41	1	aan-mir-122	2.34	15
aan-scaffold_951	-3.38	70	aan-scaffold_3557	2.31	8
aan-mir-125a	-2.75	38	aan-mir-203	2.04	1
aan-mir-221	-2.7	17	aan-mir-101a	1.87	0
aan-mir-23b	-2.65	0	aan-mir-101b	1.81	1
aan-scaffold_3084	-2.57	0	aan-mir-194	1.78	2
aan-mir-181c	-2.32	16	aan-mir-139	1.78	19
aan-mir-16b	-2.3	25	aan-mir-19c	1.69	0
aan-mir-16a	-2.29	21	aan-let-7c	1.66	13
aan-mir-454	-2.26	4	aan-mir-15b	1.65	83
aan-mir-150	-2.17	36	aan-mir-17	1.53	10
aan-mir-30b	-2.02	1	aan-mir-21	1.51	2
aan-mir-15d	-1.87	27	aan-mir-152a	1.5	2
aan-mir-92a	-1.85	12	aan-mir-19b	1.49	1
aan-scaffold_11090	-1.77	17	aan-mir-19a	1.42	1
aan-mir-451a	-1.72	0	aan-mir-99	1.34	8
aan-scaffold_8003	-1.7	1	aan-mir-722	1.32	1
aan-mir-30a	-1.68	6			
aan-mir-15c	-1.58	3			
aan-mir-22a	-1.29	27			

656

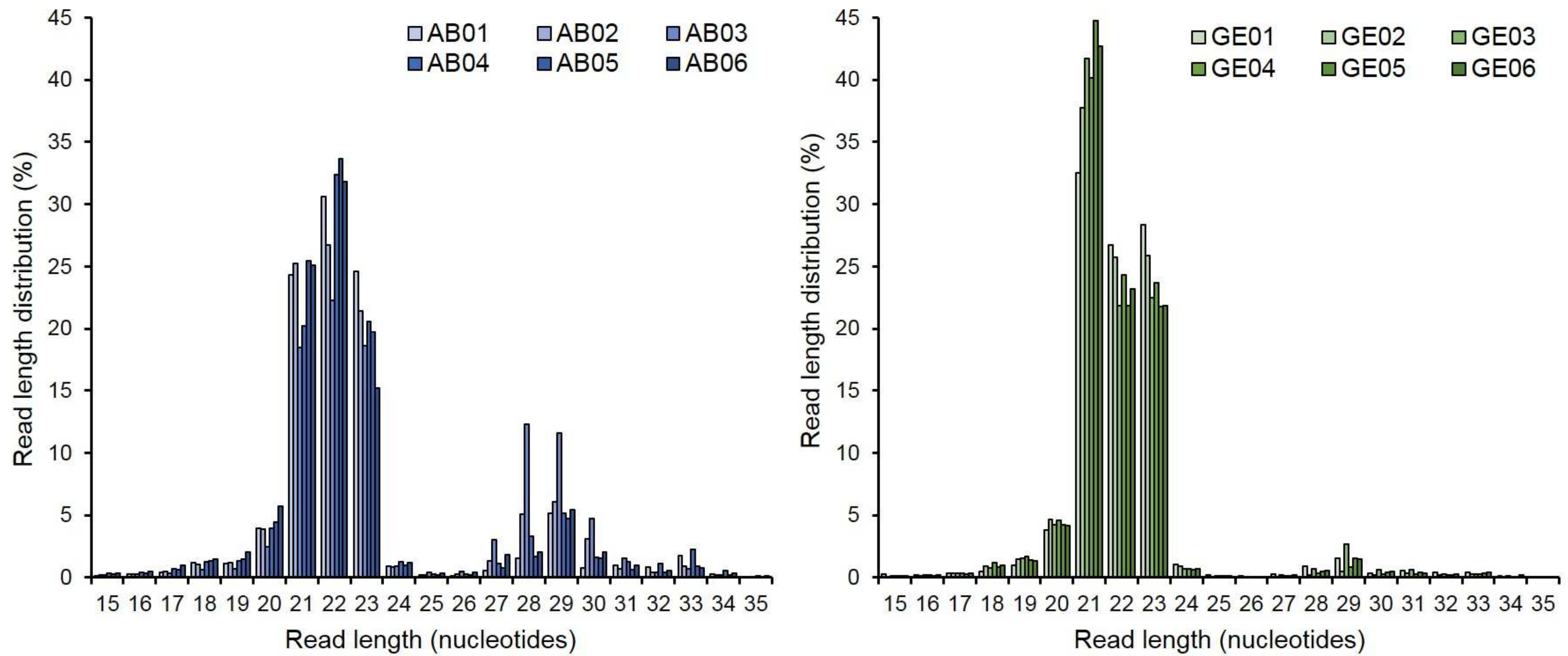
657 Table 3: Relative expression and number of target transcripts of differentially expressed miRNA
658 (FDR≤0.05) in GE compared to AB groups.

miRNA		Target					
Name	**log_2FC**	**Name**	**log_2FC**	**Hit**	**Description**	**Evalue**	**Metabolic pathway**
aan-let-7c	0.73	contig_15984	-1.78	P31643	Sodium- and chloride-dependent taurine transporter	2e-98	Neurotransmitter transporter
aan-mir-122	1.23	contig_21530	-0.89	Q7YQL6	Oligophrenin-1	0	Membrane trafficking
aan-mir-139	0.84	contig_17347	-1.23	P17453	Bactericidal permeability-increasing protein	3e-59	Immune response
		contig_22852	-1.00	Q7ZXB8	Polyadenylate-binding protein 2-B	1e-71	mRNA surveillance pathway
		contig_9114	-1.53	Q5RDH6	Transferrin receptor protein 1	2e-20	Positive regulation of T and B cell proliferation
aan-mir-15b	0.73	contig_16649	-2.39	P12276	Fatty acid synthase	0	Fatty acid metabolism
		contig_20278	-0.79	P26038	Moesin	0	Tight junction
		contig_35159	-0.93	A1L1F4	Sister chromatid cohesion protein PDS5 homolog A	0	Chromatin organisation
aan-mir-17	0.62	contig_27803	-1.20	Q0P4F7	Acyl-CoA synthetase family member 2, mitochondrial	1e-145	Lipid biosynthesis
aan-scaffold_3557	1.19	contig_35648	-0.69	Q6PEI3	Phosphatase and actin regulator 4	6e-84	Cytoskeleton regulation
aan-mir-125a	-1.46	contig_18202	0.86	Q5ZIK9	Coatomer subunit epsilon	3e-127	Membrane trafficking
aan-mir-150	-1.12	contig_23625	0.93	Q9D9K3	Cell death regulator Aven	6e-23	Cell growth and death
		contig_6617	0.86	Q96A54	Adiponectin receptor protein 1	2e-72	Glucose and lipid metabolism
		contig_15851	1.98	Q8K4T3	STE20-related kinase adapter protein beta	6e-84	Signal transduction
aan-mir-15e	-1.77	contig_39346	0.93	Q9CZV8	F-box/LRR-repeat protein 20	0	Ubiquitin system
aan-mir-16a	-1.20	contig_15454	0.56	Q8IWV8	E3 ubiquitin-protein ligase UBR2	0	Ubiquitin system
aan-mir-16b	-1.20	contig_18374	0.70	P46940	Ras GTPase-activating-like protein IQGAP1	1e-152	Cytoskeleton regulation
		contig_21720	1.52	A5PJN2	ERO1-like protein alpha	0	Protein processing in endoplasmic reticulum
aan-mir-181c	-1.21	contig_22779	0.94	Q7ZTU9	T-box transcription factor TBX2b	0	Signal transduction
aan-mir-221	-1.44	contig_18895	1.65	Q9H2F3	3 beta-hydroxysteroid dehydrogenase type 7	4e-122	Lipid metabolism
aan-mir-22a	-0.37	contig_7998	0.89	Q4R372	F-box only protein 25	1e-170	Signal transduction
aan-mir-30a	-0.75	contig_14191	0.85	P27544	Ceramide synthase 1	9e-112	Lipid metabolism
aan-mir-92a	-0.89	contig_5968	0.99	Q6EDY6	Leucine-rich repeat-containing protein 16A	9e-108	membrane trafficking
aan-scaffold_11090	-0.83	contig_21680	0.75	Q2L969	Metaxin-2	9e-95	Mitochondrial biogenesis
		contig_35030	1.12	Q9QZR5	Homeodomain-interacting protein kinase 2	0	Cell growth and death

aan-scaffold_951	-1.71	contig_14686	0.77	Q9NYF5	Protein FAM13B	2e-135	Signal transduction
		contig_8899	1.42	P60467	Protein transport protein Sec61 subunit beta	5e-21	Secretion system
		contig_16299	1.13	A5PN09	Ubiquitin carboxyl-terminal hydrolase 20	9e-128	Ubiquitin system
		contig_38623	0.76	A2AQ19	RNA polymerase-associated protein RTF1 homolog	6e-167	Transcription machinery

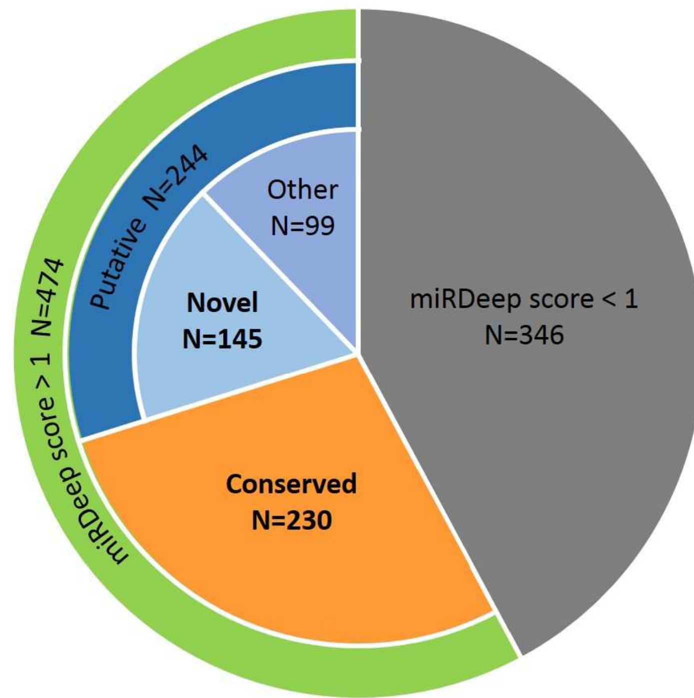
659

660 Table 4: Negative relationship between the relative expression of DE miRNA and their DE targets. Up-regulated and Down-regulated genes (FDR≤0.05) in
661 GE vs. AB are indicated in red and green, respectively.



662

663 Figure 1: Length distribution of high-quality reads in Illumina libraries. Left panel: Arcachon Bay (AB), Right panel: Gironde Estuary (GE).



664

665 Figure 2: Pie chart showing the distribution of the 820 miRNA sequences predicted by miRDeep2.
666 N=number of sequences in each category.

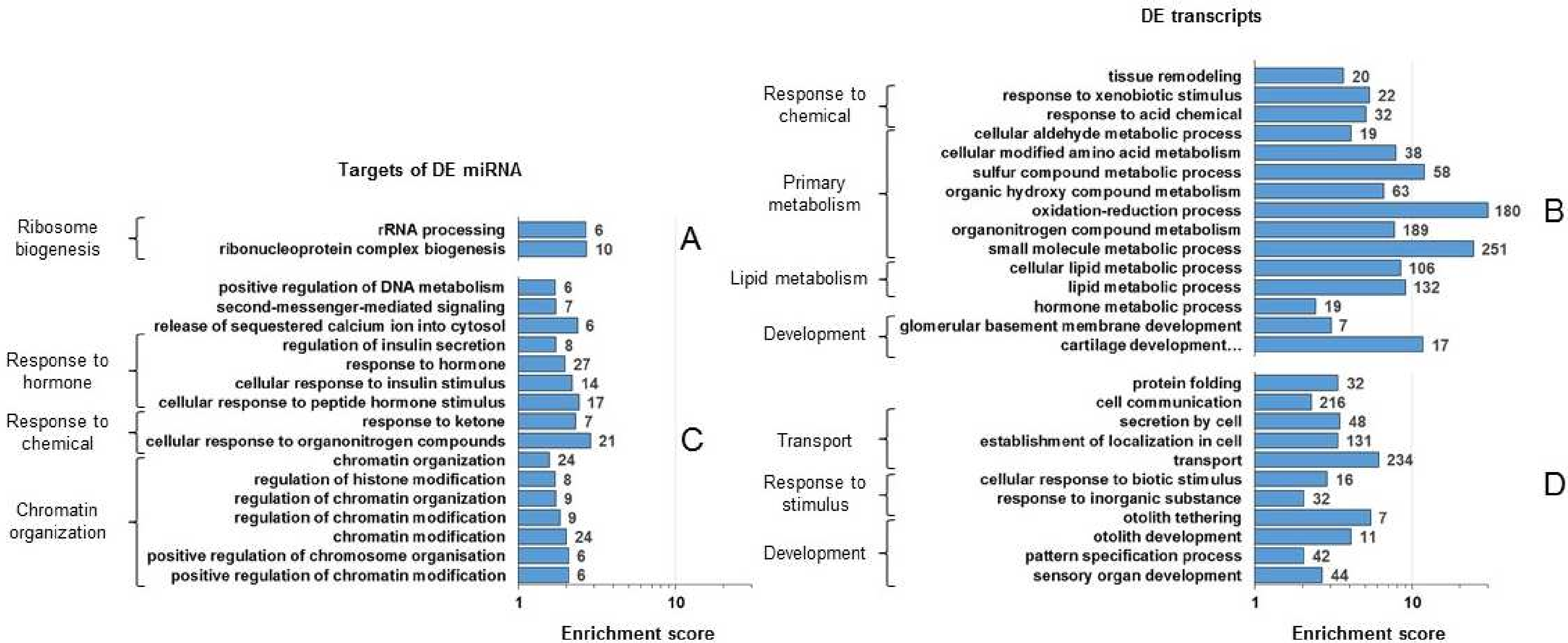


Figure 3. GO enrichment analysis (p-value ≤ 0.01 ; ≥ 5 sequences) of the transcript targets of differentially expressed miRNAs (Left panel) and differentially expressed mRNAs (Right panel), **A:** targets of up-regulated miRNAs. **B:** down-regulated mRNAs, **C:** targets of down-regulated miRNAs, **D:** up-regulated mRNAs. X-axis, enrichment score ($-\log_{10}$ Pvalue) ; Y-axis, Biological processes. The number of sequences in each category is presented on the right of the corresponding bar.

667
668 Figure 3: GO enrichment analysis (p-value ≤ 0.01; ≥ 5 sequences) of the transcript targets of differentially expressed miRNAs (Left panel) and differentially
669 expressed mRNAs (Right panel), A: targets of up-regulated miRNAs. B: down-regulated mRNAs, C: targets of down-regulated miRNAs, D: up-regulated
670 mRNAs. X-axis, enrichment score (-log10 Pvalue); Y-axis, Biological processes. The number of sequences in each category is presented on the right of the
671 corresponding bar.

N

Atlantic Ocean

Polluted site

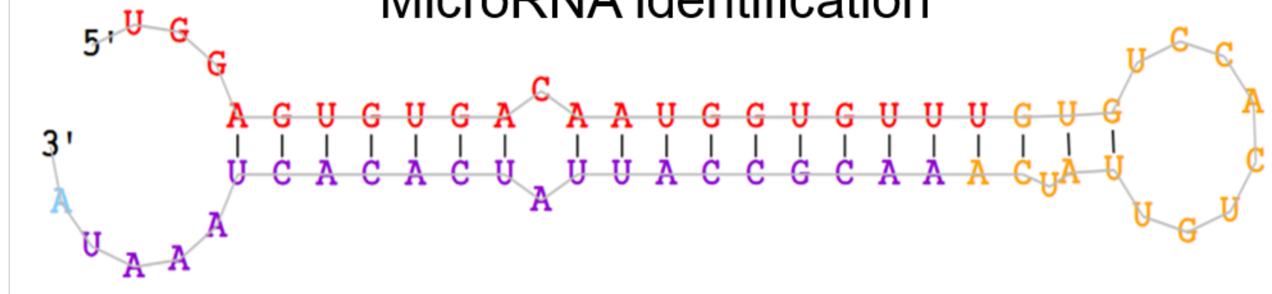
x6

Pristine site

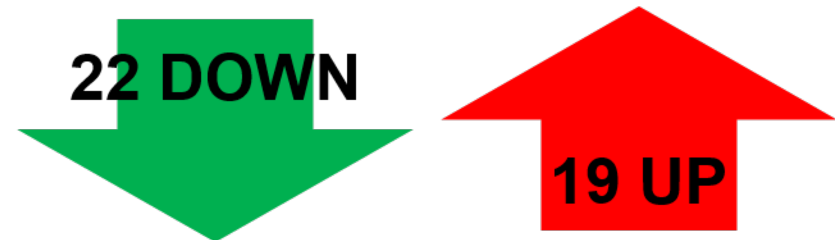
x6

BORDEAUX

MicroRNA identification



Expression in polluted site



Targets prediction