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Title

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.

Capsule

This work explores the repertoire of small non-coding RNAs (microRNAs) and its response to water pollution in the endangered European eel.

Keywords

MicroRNA; *Anguilla anguilla*; Aquatic ecotoxicology; Epigenetics; Biomarker

Introduction

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

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

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

toward European and North African continental waters following oceanic currents such as the Gulf Stream and the North Atlantic Drift. There, they first metamorphose into glass eel (unpigmented) before reaching the juvenile growth phase (yellow eel) in continental water bodies where they remain up to 15-20 years, often moving across different habitat types. This stage ends with a second metamorphosis and maturing eels (silver eels) will migrate back to the Sargasso Sea to reproduce and die [25,26]. For their unusual life cycle and vulnerability to pollution, eels are considered as sentinel species in ecotoxicology [27,28]. Moreover, the species has been considered as critically endangered since 2008 (IUCN red list) [29]. Its catadromous migratory behaviour, long life, serious habitat reduction, migration barriers, pollution, human-introduced diseases, overfishing, as well as climatic events may be amongst the causes of the catastrophic collapse of the European eel population observed over the past decades [30–32]. A better understanding of the biology of this species may come from the use of high-throughput (next generation) sequencing technologies that have recently provided transcriptomic [33–36] and genomic [37–39] information. However, like in many non-model species, the non-coding portion of these data has been overlooked in spite of the emerging importance of their role in gene expression regulation. So far, in *A. anguilla*, microRNAs were only predicted from *de novo* assembled transcriptome [33] while in the related species *A. marmorata* (marbled eel), microRNAs were specifically sequenced and their expression pattern assessed in response to salinity [20].

In previous studies, the responses of wild immature yellow eels to pollution was investigated in 8 locations presenting a broad contamination gradient in France and Canada [34,40–42]. These studies identified Arcachon Bay and the Gironde Estuary as the cleanest and the most contaminated French sites, respectively. We used samples from these two highly contrasted sites in order i) to identify evolutionarily conserved as well as novel miRNAs in the European eel *Anguilla anguilla* by using next generation sequencing, ii) to compare their expression between a pristine and a polluted site and iii) to link miRNA and mRNA levels. Comprehension of gene regulation by miRNAs may give new insights into a neglected mechanism of response to environmental pollution in aquatic organisms.

Material and Methods

Sample collection – European eels *Anguilla anguilla* (Linnaeus, 1758) were collected from two sampling sites with a contrasted contamination profile. The Gironde estuary (GE, 45° 12.110'N, 0° 43.579'W) is considered as a highly impacted site while Arcachon Bay (AB, 44° 41.300'N, 1° 1.650'W) represents a pristine environment. Six individuals were collected on each site. The liver was dissected, transferred in RNA-later solution and placed at 4°C overnight before storage at -20°C until RNA extraction. These samples were part of a previous study carried out by our group [34].

Condition indices, tissue composition and trace metal analyses – Information concerning the 12 individuals used in the present study (biometry, tissue composition and trace metals) were extracted from previously published data [34,41,43,44]. Fulton index and HSI were calculated as (total weight / length^3) x 100 and as (liver weight / total weight) x 100, respectively (weight is in grams and length is in centimeters). The silvering status of the eels was assessed by the calculation of the ocular index (OI) according to the Pankhurst method [45]. Statistical differences between the two sites were assessed either with Student t tests or Wilcoxon tests after checking assumptions of normality and homoscedasticity.

Small RNA isolation and deep sequencing – Total RNA were isolated from individual liver tissues following the Trizol™ Reagent (Invitrogen™) protocol. MicroRNA were then purified by using the PureLink™ MiRNA Isolation kit (Invitrogen™) according to the manufacturer's instructions. Total RNA and MicroRNA concentration and quality were assessed by measuring the absorbance at 260nm and 280nm, and migration on a TBE-urea 12% polyacrylamide gel. The NEBNext® Small RNA Library Prep Set for Illumina® (New Englan BioLabs®) was used to prepare the sequencing libraries. Fragment size (around 150 bp) and quality of the individual libraries were checked on a 2100 Bioanalyzer® (Agilent ®) with a High Sensitivity chip. The 12 individually tagged libraries were then sequenced using a HiSeq 4000 sequencer at the GenomeEast microarray and sequencing platform (Illkirch, France).

MicroRNA discovery analysis – The 50bp raw reads from each of the 12 samples were quality filtered (Q > 30) and adaptors (5' AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC 3') were removed. The *Anguilla anguilla* genome [37] (GenBank accession number: AZBK00000000.1) was used as a reference genome. High quality, adaptor processed reads were aligned against the eel reference genome using the miRDeep2 package [14,46] with default parameters. Our strategy was similar to the one

used for the discovery and characterization of miRNA in the salmon *Salmo salar* [47]. Shortly, miRDeep2 provides a list of putative miRNA precursors with their corresponding mature and star sequences. Each precursor is associated with a score that is a measurement of the posterior probability that a predicted sequence is a true miRNA gene. Each score corresponds to a signal-to-noise ratio that estimates total miRNA reported/mean estimated false positive miRNA over 100 rounds of permutated controls. We used a miRDeep2 score of 1 that yielded a signal-to-noise ratio of 10:1 as a cut-off threshold. MiRNAs over this score were further compared by BLAST searches to all known stem-loop sequences from all species deposited in miRBase, release 21 [17]. Only miRNA with reads matching both harms (5p and 3p) of their precursors were considered. MicroRNA with an E-value < 1.e-6 were annotated as a true *Anguilla anguilla* miRNA and named accordingly to its ortholog in miRBase. Sequences with no significant match in miRBase were used as queries in a BLAST analysis against the nr/nt and refseqRNA databases in GenBank [48], the functional small RNA database [49] and the small RNA families in Rfam [50] to exclude other kind of small RNA. Finally, the remaining miRNAs were aligned with the reference genome. Sequences with an E-value < 1e-6 against >5 loci were considered as repeats and discarded. Final sequences were then considered as novel miRNAs. The nomenclature rules used for the miRNA naming were in agreement with guidelines from miRBase [51,52].

Transcriptome annotation – The transcriptome assembly was previously annotated based on similarity (E value ≤ 1e-10) with known proteins from the swissprot and nr protein databases using the BLASTx algorithm [34]. For the present work, we enriched this annotation with Gene Ontology (GO) assignment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis that were performed using the BLAST2GO software [53,54] and the GhostKOALA server [55], respectively. Enrichment tests were performed with the Fisher exact test procedure and a correction for multiple testing using the Benjamini and Hochberg method. A Pvalue threshold of 0.01 was used. Redundancy in GO terms was reduced by the GO Trimming method [56] using a uniqueness threshold of 50%. For a matter of clarity, only GO and KEGG categories containing ≥5 sequences were considered.

Differential expression of transcripts – Differences in transcription levels between the two study sites were assessed with the RNAseq data extracted from Baillon *et al* [34] for the exact same individual

eels. Normalization and expression analysis were performed using the DESeq2 package [57]. *P*-values for differential gene expression were corrected for multiple testing using the Benjamini and Hochberg method, and a false discovery rate (FDR) threshold of 0.05 was used.

Expression analysis of Eel miRNAs – The copy number of known and novel mature miRNAs were counted with the quantifier module in miRDeep2 (additional files 1 and 3). Normalization and expression analysis were performed using the DESeq2 package [57]. The false discovery rate (FDR) threshold was 0.05. In order to validate these results, the expression profiles of 10 mature miRNAs were assessed using qRT-PCR (additional file 4). Reverse transcriptions were performed by using the miScript II RT system (Qiagen®). Real Time PCR Analyses were performed on a Roche LightCycler® 480 Instrument II by using the miScript PCR Starter Kit (Qiagen®). Cycling conditions were 95°C for 15 seconds followed by 40 cycles of 15 seconds at 94°C, 30 seconds at 55°C and 30 seconds at 70°C.

Target prediction – The sequences and annotation of protein-coding transcripts were retrieved from Baillon *et al* [34]. The target genes of mature eel miRNAs were predicted using the miRanda software [58] that is based on the local alignment between the query miRNA sequences and the reference transcriptome and on the thermodynamic stability of miRNA/mRNA duplexes (parameters used were score $\geq$ 160, free energy $\leq$ -25kcal/mol).

Results

Trace metals analysis – Differences between individuals collected in both sites are presented in Table 1. First, AB and GE sites differed in salinity and dissolved oxygen concentrations. We observed no difference in Fulton condition factor between the two sites. However, the Hepato-Somatic Index (HSI) of AB eels was higher than GE eels. No difference was detected in OI and with a value below the threshold value of 6.5 [45], all the individuals were indeed at the yellow eels stage. As expected from previous results [41], trace metal concentrations were significantly higher in the liver of eels from GE, with the exceptions of Cr and Zn that showed no difference and Ni that was higher in AB. Cadmium showed the largest difference with a concentration more than 200-times higher in the liver of GE 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

anguilla's transcriptome assembly [34]. The *A. anguilla* transcriptome contains 18,113 contigs with high homology with known sequences (BLASTx, Evalue ≤ 1e-10). Although 3'UTR regions are the most important regulatory sites for post-transcriptional regulation of gene expression by miRNAs, we considered whole length transcripts. 242 miRNAs showed a potential to hybridize within the transcriptome wherein 3,637 contigs were identified as potential targets (additional file 4). As expected, one miRNA could target several transcripts and one transcript could be targeted by several miRNAs. For instance, the novel miRNA aan-scaffold-2762 could hybridize with up to 271 targets and the transcript contig_15454, encoding a homologue of E3 ubiquitin-protein ligase UBR2 (Uniprot accession number Q8IWV8), was potentially the target of 8 different miRNAs. The entire set of predicted mRNA targets was further functionally categorized through GO annotation and KEGG pathway analysis. No GO term, nor KEGG pathway, was significantly enriched in the list of target genes compared to the transcriptome background, suggesting that miRNAs might affect any biological process.

Differentially expressed mature miRNAs between the two sites – One objective of this study was to evaluate the response of miRNAs to differing levels of contamination. A multidimensional scaling of the count data showed that despite inter-individual variability, samples are well separated according to their site of origin (additional file 5). We identified 41 mature miRNAs with differential expression (DE) in GE compared to the reference site (AB). Nineteen (19) miRNAs were up-regulated, while 22 were down-regulated (Table 3). The most up-regulated and down-regulated miRNAs were respectively mir-29a (2.77 times more expressed in GE) and mir-462 (3.97 times less expressed in GE). These results were validated by qRT-PCR ($r^2 = 0.88$, additional file 5). Amongst all the DE miRNAs, 35 were likely to target 490 sequences in *A. Anguilla*'s transcriptome (Table 3). The targets of up-regulated miRNAs were functionally enriched in transcripts encoding proteins involved in ribosome biogenesis. This process is hence expected to decrease in GE. Biological processes affected by down-regulated miRNAs were related to the response to hormones (*e.g.* GO:0016568), response to chemicals (e.g. GO:0071417) and chromatin organisation (*e.g.* GO:0006325) (Figure 3). These processes are then expected to increase in GE.

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

Discussion

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

knowledge, our work is the first *in situ* study using miRNA in an aquatic organism. MicroRNAs are being characterized in an increasing number of teleost fish species where they repress target mRNA abundance or translation in a highly conserved manner across most eukaryotes [68].

In this study, we conducted a comprehensive identification of the microRNA repertoire in the liver of the European eel *A. anguilla* and its response to environmental pollution. This organ was chosen owing to its role in pollutant metabolism, accumulation and toxicity. Only trace metal concentrations were reported here since they were measured in the liver. However, one should note that eels from the Gironde Estuary also showed higher levels of persistent organic pollutants (Poly-Chlorinated Biphenyls, Organo-Chlorine Pesticides, and Poly-Brominated Diphenyl Ethers) in their muscle compared to eels from the Arcachon Bay, as evidenced by previous analyses [34,43]. Owing to panmixia and proximity between the two sites, this contrast is most likely due to water quality rather than population differences.

The size distribution of the small RNA libraries revealed that 22-23 nt microRNAs were dominant, followed by small RNAs of 28-29 nt in length (Figure 1). This distribution is similar to what was found in the sister species *A. marmorata* [20]. It is interesting to note that, in both eel species, the relative abundance of 22-23 nt and 28-29 nt varies depending on environmental conditions, which could represent a new path of investigation in the future. These 28-29 nt sRNA might correspond to piRNA (piwi-interacting RNA that have a length of 24-32 nt [1,69]), another smallRNA silencing system that plays a role in germline specification, gametogenesis, transposon silencing, genome integrity, and stem cell maintenance across the animal phylogeny [70].

We identified a total of 299 mature miRNA in eel liver, of which 158 were known and 141 were novel. This number is comparable to what is currently available in the miRBase database for the model species *Danio rerio* (375 mature sequences). This identification benefited from the availability of a sequenced genome of *A. anguilla* [37]. Only mature miRNAs were considered in our analysis since they eventually regulate gene expression at the post-transcriptional level. In all libraries, mir-122 was the most abundant mature sequence. This was not surprising since it is the most abundant liver 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

expression level of 19 miRNAs increased in the polluted site while the expression of 22 miRNAs was higher in AB. This relatively small number of DE miRNA is similar to what was found in other fish species exposed to environmental stressors [19–21,24] and could be due to the relatively low number of samples per site and inter-individual variability (additional file 5) that should be taken into account in future studies. The most expressed known miRNAs in AB (>3 fold) were miR-462, miR-15e and miR-23a, and the most up-regulated known miRNAs in GE (>2 fold) were miR-29a, miR-122 (discussed above) and miR-203. As previously, the fold change values measured here are in agreement with what can be found for Teleosts in the literature [19–21,24]. The role of these miRNAs could be inferred from previous studies.

Despite the highly conserved mode of action of miRNAs, caution must be taken when comparing fish and mammals as only a small fraction of miRNA-mRNA interactions (~10%) is conserved [79] and identification of conserved targets between species remains challenging [80]. For instance, in fish, miR-462 was shown to be upregulated after hypoxia treatment in liver [81,82] or virus infection [18,83]. MiR-462 is only described in teleost fish and is actually an orthologue of the human miR-191 that plays a role in cell cycle regulation, but not in viral infection [83]. The role of miR-23a instead seems to be conserved. It was identified as an essential regulator of adipocyte differentiation of which increased expression might reduce lipid accumulation and triglyceride content in adipocytes [84]. This role was confirmed in our study by the identification of a transcript encoding a very long-chain acyl-CoA synthetase (Uniprot accession O14975, additional file 5) as a putative target of miR-23a. In human, miR-29a is known to regulate cell proliferation, differentiation and apoptosis as several of its targets are oncogenes or anti-apoptotic genes [85]. Amongst the predicted mRNA targets of miR-29a, we identified a thyroid receptor-interacting protein homologue (Uniprot accession Q15643, additional file 5) of which degradation could reduce gene transcription in response to thyroid hormone while miR-122 predicted targets included transcripts encoding proteins involved in lipid synthesis, energy generation, platelet activation, apoptosis and transcription regulation. The role of miR-203 remains unclear as its overexpression might promote both apoptosis and tumor growth, as well as host-virus interactions [86]. This difference in lipid metabolism is not confirmed by our observation as fish from the two sites

showed no significant difference regarding their muscular lipid content (table 1), contrary to previous studies that showed that eels exposed to Cd [87] and organic pollutants [88] increased their fat consumption and/or reduced their energy reserves. We can here hypothesize that miRNA regulation in the liver contributes to maintain a significant production of triglycerides and their export to the main site of lipid storage in eels, *i.e.* the muscle [87]. At the opposite, the lower HSI values in fish from the estuary could indicate an impairment in the accumulation of energy reserves under the form of carbohydrate such as glycogen in hepatocytes.

More comprehensive information was gained from the functional analysis of miRNA targets in *A. Anguilla*'s transcriptome (Figure 3). Based on gene ontology of these targets, changes in miRNA expression might result in reduced ribosome biogenesis and increased response to hormone and chemical, and chromatin modification. The effect on ribosome biogenesis could be evidenced by a significantly reduced level of total proteins in the liver of GE eels (Table 1). One could expect to find some similar functions in the predicted targets of up-regulated miRNAs and down-regulated transcripts, and conversely. In spite, we found no relation between enriched gene ontology in miRNA targets and mRNA, as well as a small number of miRNA-mRNA pairs with negative correlation (Table 4). This situation was also observed in a comparable study carried out in *Pelteobagrus vachelli* exposed to hypoxia [21] in which the authors identified only 97 negative correlations out of 308 miRNA-mRNA pairs. In the same study, and in the same manner as our results, most of the DE mRNA (43 out of 60) were target by a single DE miRNA. This highlights that the identification of functional miRNA-targets interaction is still challenging and/or false positives might still be frequent with *in silico* analyses [89]. MicroRNAs are obviously not the only mode of regulation of gene expression, however based on the lack of correspondence between miRNA targets and DE transcripts, we could hypothesize that the degradation of messenger RNA was not the major mode of action of miRNAs here and that they act more likely by inhibiting mRNA translation into proteins, instead. This inhibition might be reinforced by the targeting of mRNA involved in ribosome assembly and function. This result is in accordance with those obtained in juvenile zebrafish exposed to the antibacterial agent triclosan where "structural constituent of ribosome" and "ribosome biogenesis" are amongst the 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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Supporting information

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

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

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

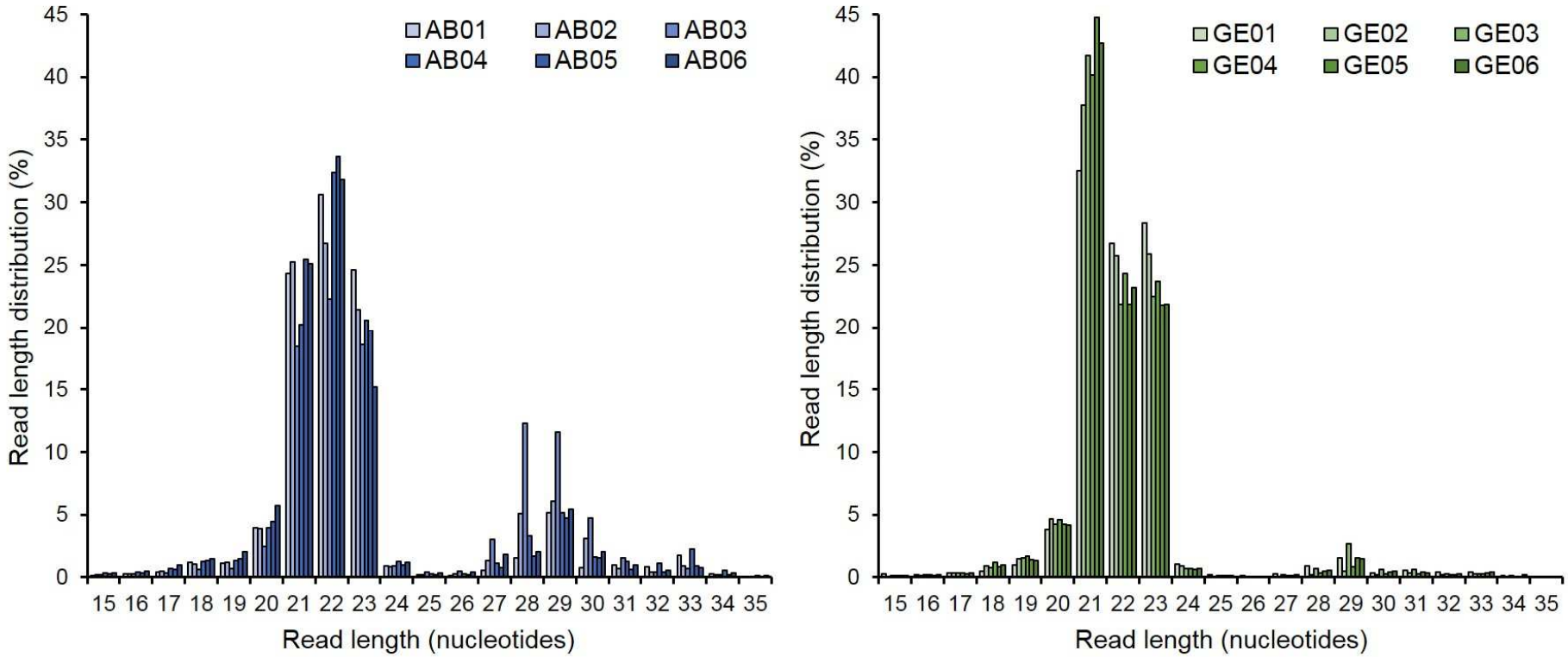
Table 3: Relative expression and number of target transcripts of differentially expressed miRNA (FDR≤0.05) in GE compared to AB groups.

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

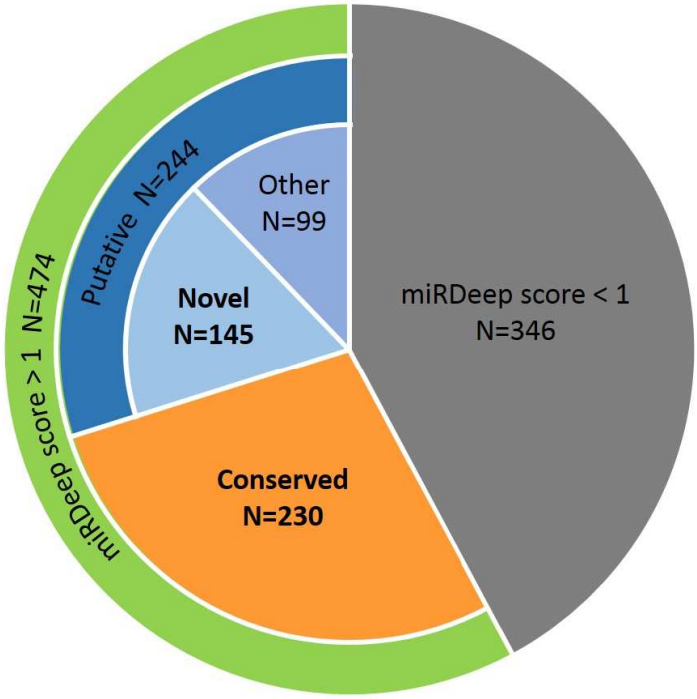
659

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.

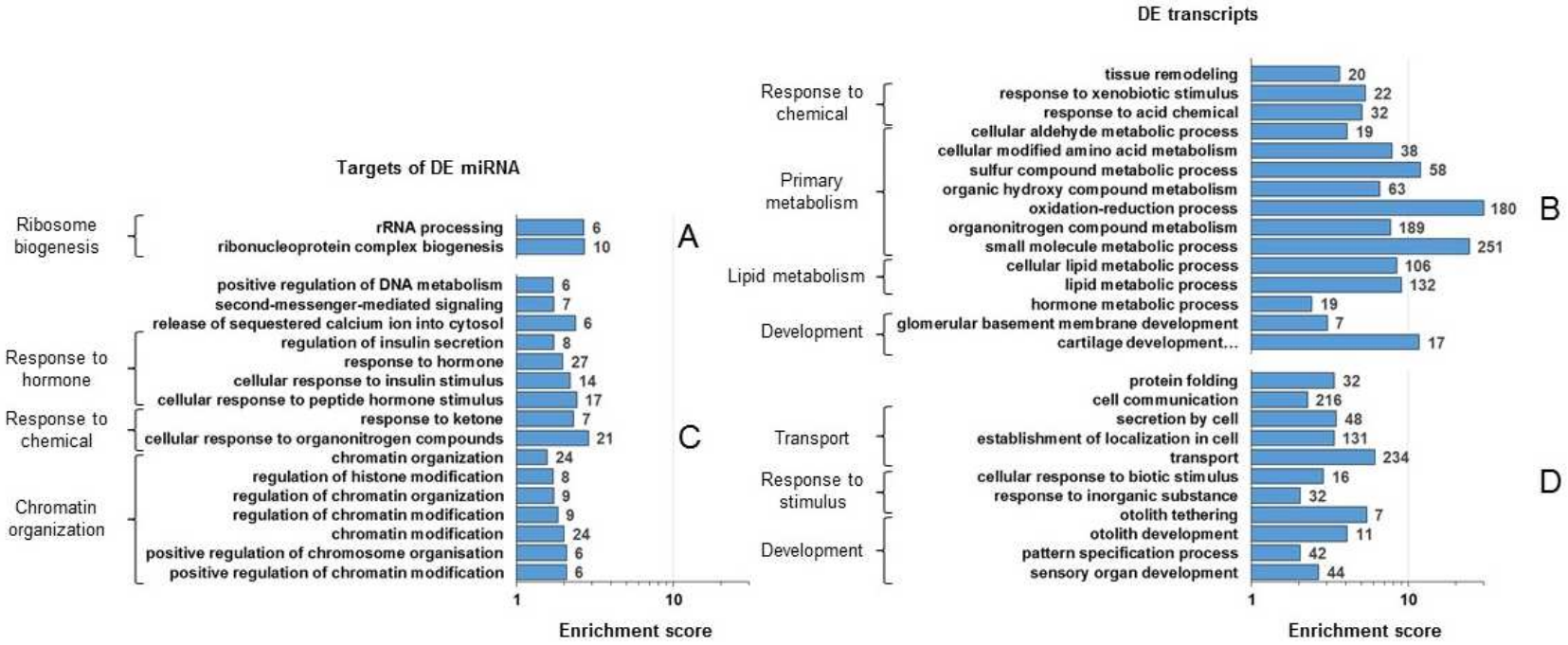
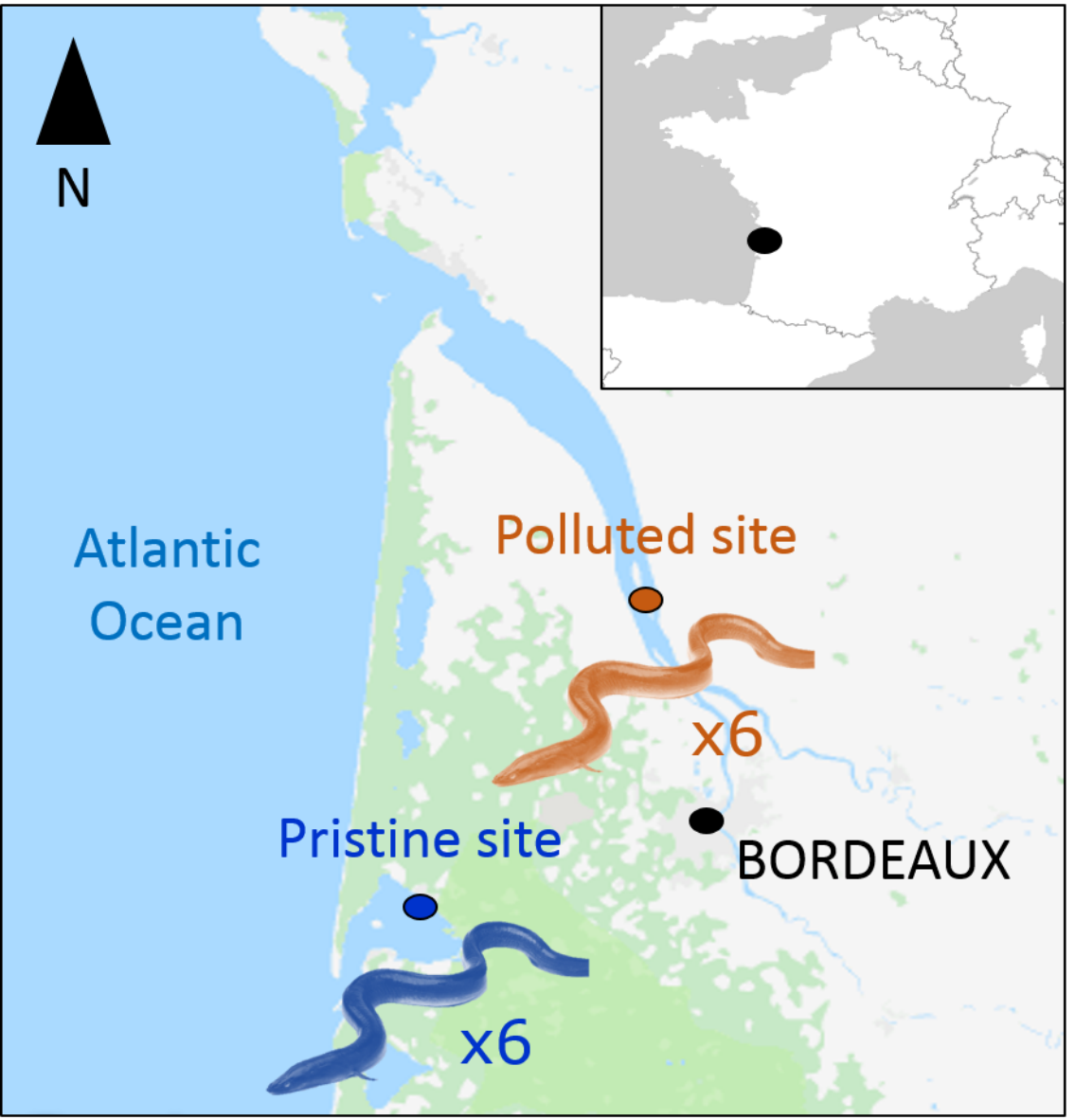
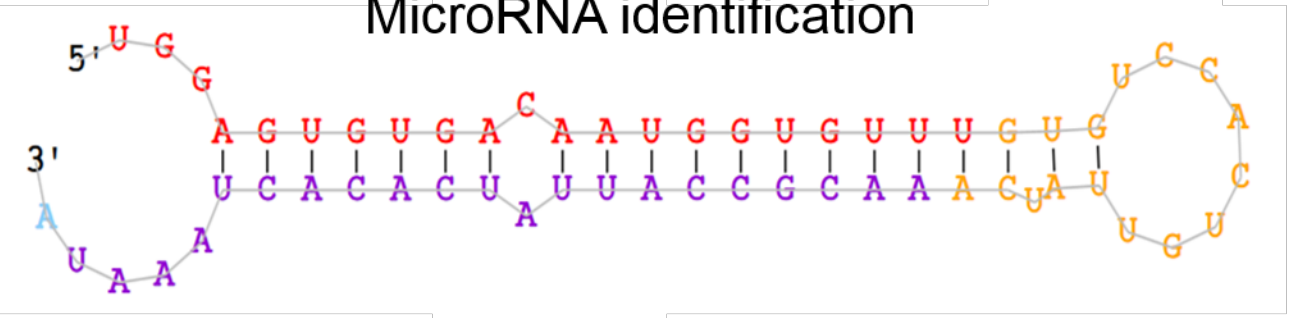


Figure 3. GO enrichment analysis (p-value ≤ 0.01 ; ≥ 5 sequences) of the transcript targets of differentially expressed miRNAs (Left panel) and differentially expressed mRNAs (Right panel), **A:** targets of up-regulated miRNAs. **B:** down-regulated mRNAs, **C:** targets of down-regulated miRNAs, **D:** 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.

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 (-log10 Pvalue); Y-axis, Biological processes. The number of sequences in each category is presented on the right of the corresponding bar.



MicroRNA identification



Expression in polluted site

22 DOWN

19 UP

Targets prediction