Environmental Pollution
June 2023, Volume 326, Pages 121472 (11p.)
https://doi.org/10.1016/j.envpol.2023.121472
https://archimer.ifremer.fr/doc/00829/94090/

Molecular and phenotypic effects of early exposure to an environmentally relevant pesticide mixture in the Pacific oyster, *Crassostrea gigas*.

Sol Dourdin Thomas [1, *], Rivière Guillaume [2], Cormier Alexandre [3], Di Poi Carole [4], Guyomard Killian [5], Rabiller Manuella [4], Akcha Farida [1], Bah Thierno Sadialiou [1], Le Monier Pauline [1], Sussarellu Rossana [6]

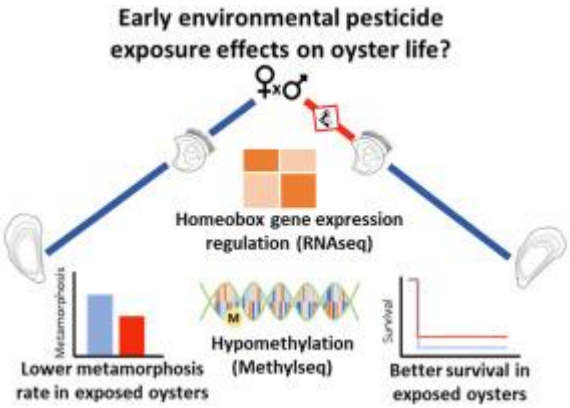
[1] Ifremer, Unité Contamination Chimique des Ecosystèmes Marins, Nantes, France
[2] Biologie des Organismes et Ecosystèmes Aquatiques (BOREA), UMR8067, Muséum National D'Histoire Naturelle (MNHN), Centre National de La Recherche Scientifique (CNRS), Institut de Recherche et Développement (IRD), Sorbonne Université (SU), Université de Caen Normandie (UCN), Université des Antilles (UA), 75231, Paris, CEDEX, France
[3] Ifremer, Service de Bioinformatique de L'Ifremer, Brest, France
[4] Laboratoire des Sciences de L'environnement Marin (LEMAR), France
[5] Ifremer, Plateforme Mollusques Marins Bouin, Bouin, France
[6] Ifremer, Physiologie et Toxines des Microalgues Toxiques, Nantes, France

* Corresponding author : Thomas Sol Dourdin, email address : Thomas.Sol.Dourdin@ifremer.fr

Abstract :

Early life stages are crucial for organism development, especially for those displaying external fertilization, whose gametes and early stages face environmental stressors such as xenobiotics. The pacific oyster, Crassostrea gigas, is considered a model species in ecotoxicology because of its ecological characteristics (benthic, sessile, filter feeding). So far studies have investigated the impact of xenobiotics at embryotoxic, genotoxic and physiological endpoints, sometimes at the multigeneration scale, highlighting the role of epigenetic mechanisms in transmitting alterations induced by exposure to single xenobiotics. However, to date, little is known about the impact of environmentally-mimicking contaminants cocktails. Thus, we examined the impact of an early exposure to environmentally relevant mixture on the Pacific oyster life history. We studied transcriptomic, epigenetic and physiological alterations induced in oysters exposed to 18 pesticides and metals at environmental concentration (nominal sum concentration: 2.85 µg.L−1, measured sum concentration: 3.74 ± 0.013 µg.L−1) during embryo-larval stage (0–48 h post fertilization, hpf). No significant differences in embryo-larval abnormalities at 24 hpf were observed during larval and spat rearing; the swimming behaviour of exposed individuals was disturbed, while they were longer and heavier at specific time points, and exhibited a lower epinephrine-induced metamorphosis rate as well as a higher survival rate in the field. In addition, RNA-seq analyses of gastrula embryos revealed the differential expression of development-related genes (e.g. Hox orthologues and cell cycle regulators) between control and exposed oysters. Whole-genome DNA methylation analyses demonstrated a significant modification of DNA methylation in exposed larvae marked by a demethylation trend. Those findings suggest that early exposure to an environmentally relevant pesticide mixture induces multi-scale latent effects possibly affecting life history traits in the Pacific oyster.

Graphical abstract



Highlights

► *Crassostrea gigas* embryos were exposed to an environmental pesticide mixture for 48 h. ► Carry-over effects were observed along the lifecycle of the oysters. ► The expression of developmental genes was modified during the exposure. ► Exposed oysters exhibit a global DNA demethylation during and after the exposure window. ► Some phenotypic endpoints were impaired: swimming behavior, metamorphosis, field survival.

Keywords : Mixture, Epigenetics, Gene expression, Molluscs, Pesticides, DNA methylation

Introduction

The widespread use of contaminants leads to the presence of complex mixtures of substances in continental and coastal water bodies [1,2,3], with a majority of herbicides [4]. To face the degradation of water quality, the EU Water Framework Directive listed 45 priority substances (annex I) to be monitored, among which 20 are pesticides [5].

Pesticides threat marine environments from the organisms to the ecosystemic scale [6–9] and are especially harmful for non-target organisms like molluscs, whose external early developmental stages constitute critical windows of high susceptibility to environmental stressors [10–15]. Among those species, the Pacific oyster, *Crassostrea gigas* (Thunberg, 1873; e.g. *Magallana gigas*) has emerged as an emblematic bivalve species widely used in ecotoxicology [14,16–18] due to its biological features (wide spread, filter feeding, sessile) [19,20] and its economic importance as a farmed species worldwide [21].

The impact of pesticides in bivalve molluscs has been widely investigated from molecular to physiological scales and from embryo-larval to adult gametogenesis stages and also for a wide variety of herbicides such as atrazine [22,23], diuron [24,25], glyphosate [8,26, 27], isoproturon

[28], or metolachlor [29]. Exposure to diuron during gametogenesis can not only impact the exposed oysters [24,25,30] but also leads to multigenerational effects from the molecular to the phenotypic scale [31,32].

DNA methylation is one of the numerous epigenetic mechanisms [33]. It is essential for the regulation of gene expression [34] and is implicated in phenotypic plasticity, adaptation and non-genetic inheritance [35]. Several studies demonstrated the role of DNA methylation in mediating the response to environmental changes [33] and to environmental chemicals [36,37], making 5-methylcytosine levels one of the most studied epigenetic markers in ecotoxicology [38–41]. In the pacific oyster, DNA methylation is critical for the early development [42,43] and exposure to copper leads to modifications of both the expression and methylation profiles of homeotic genes [44], raising questions about the importance of DNA methylation disruption in the developmental sensitivity window for pollutant-induced long-term impairments [45].

Besides, pollutant effects are mostly assessed in a substance-per-substance manner, thereby neglecting combination effects of contaminant mixtures that are environmentally more relevant [46]. While understanding the effects of pesticide cocktails is one of the most important issues in ecotoxicology nowadays [47], to date they have been little investigated in marine invertebrates [48–51] and the latent effects of early exposure to environmental cocktails remain unknown in the oyster.

Thus, this study investigated the impact of an early exposure to an environmentally relevant pesticide mixture on the Pacific oyster life history, by exploring the effects at different biological levels. For this purpose, *C. gigas* embryos were exposed during 48 h to a mixture of 18 pesticides at environmental concentration (nominal sum concentration: 2.85 µg.L^{-1}), and the effects were investigated from molecular (genotoxicity, gene expression and DNA methylation) to phenotypic (larval development and swimming behaviour, metamorphosis ability, spat growth) scales.

Materials and Methods

1. Experimental design

1.1 Chemical mixture

A complete review of the literature was conducted to identify the most frequent pesticides found in the main aquaculture areas in France during the oyster reproduction period (from June to August). Reported molecules were first sorted according to their presence and concentration. The type of matrix in which they were measured as well as the $LogK_{ow}$ were considered in order to best reflect on the water column contamination that swimming oyster larvae may encounter. This approach allowed the selection of 18 molecules (tab.1) contributing to a putative environmentally relevant pesticide mixture, with a total nominal concentration for the sum of the compounds of 2.85 $\mu g.L^{-1}$ (measured concentrations are in tab.SM1).

Individual stock solutions were prepared from various pesticide powders in their respective solvent (methanol or ultra-pure water). A mixture stock solution (x100000 concentration) was prepared from individual compounds stock solutions.

Table1: Compounds of the mixture and their nominal concentrations.

Compound	Nominal concentration ($ng.L^{-1}$)
Atrazine	8.0
Diuron	15.0
Glyphosate	590.0
AMPA	122.0
Terbuthylazine	0.4
Carbendazime	1.4
DE-Atrazine	55.0
Isoproturon	355.0
Acetochlor ESA	13.7

Acetochlor	73.0
Chlortoluron	57.0
IRgarol	70.0
Imidacloprid	30.0
Azoxystrobine	0.4
Metolachlor	770.0
Simazine	80.0
Copper	600.0
Cadmium	13.3

97

98 **1.2 Broodstock conditioning and fertilization**

99 In January 2021, 3 year old Ifremer-standardized *Crassostrea gigas* adults [52] underwent
100 broodstock conditioning in non-contaminated sea water (NCSW: 1 µm-filtered, UV-treated and
101 activated carbon-filtered, 8 weeks at 18°C, fed *ad libitum* with *Skeletonema costatum* and
102 *Isochrysis lutea*). Spawning of mature broodstock animals was induced by thermal shock,
103 gametes were collected and used for in vitro fertilization as previously described (fig.1) [53].

104

105 **1.3 Embryonic development under chemical contamination**

106 After polar body emission, the pool of embryos was divided equally into 2 experimental
107 conditions: (i) control (C: 25°C, NCSW with solvents (methanol and milliQ water representing
108 $5x10^{-5}$ % and $9.5x10^{-4}$ % of the total volume), 32 psu, air bubbling, n=3 tanks, 50 L tanks,
109 100 embryos.mL^{-1}) and (ii) exposed to the environmental pesticide mixture (E: 1/100000 v/v
110 dilution of the mixture stock solution in NCSW, 25°C, 32 psu, air bubbling, 50 L tanks, n=3
111 tanks, 100 embryos.mL^{-1}). The embryos developed under these conditions for 48 h. Sampling
112 was carried out at gastrula stage (6 hpf, 6 hours of exposure) for RNAseq, Methylseq and
113 genotoxicity analyses, and D larva stage (24 hpf, 24 hours of exposure) for embryotoxicity,
114 genotoxicity and swimming behaviour.

115

116 **1.4 Larval rearing**

117 After the 48 h exposure, D-larvae from both conditions were transferred in a flow-through
118 larval rearing system supplied with NCSW (25.8 ± 0.3°C, 31.4 ± 0,3 psu, 5 $L.h^{-1}$, 50 larvae per
119 mL, n=6 tanks per condition, fig.1) A nutritional supply included *ad libitum Isochrysis lutea*
120 from 2 dpf to 6 dpf and then equal volumes of *Isochrysis lutea* and *Thalassiosira weissflogii*.
121 Nutritional input was kept *ad libitum* by adjusting micro algae to a homogenous outflow of 1.5
122 million $mm^3.L^{-1}$ for each replicate. [54]. No significant differential mortality was observed
123 between tanks from the control and exposed conditions. Finally, 50% of the larvae from each
124 condition reached the metamorphosis competent pediveliger stage (MCP larva, exhibiting eye-
125 spotted shell), needed to start the settlement, at 19 dpf (17 days post-exposure, fig.1).

126

127 **1.5 Spat rearing**

128 MCP larvae from the 6 tanks were pooled according to their original batch in order to
129 homogenise larval concentrations, transferred into a 150 µm sieve containing cultch material
130 (fig.1) and immersed in a flow-through raceway supplied with NCSW (25.0 ± 1.7°C, 33.4 ± 0,9
131 psu, 100 $L.h^{-1}$) for settlement. Phytoplankton intake was composed of a 1:1 volume ratio of
132 *Isochrysis lutea* and *Skeletonema costatum* kept *ad libitum* by adjusting micro algae to a
133 homogenous outflow of 1.5 million $mm^3.L^{-1}$. After metamorphosis and settlement (10 days),
134 cultch was removed and micro-spats were reared on a 300 µm sieve. When spat reached 1mm
135 in length, they were reared in UV-treated, 10 µm-filtered sea water at environmental
136 temperature and fed *ad libitum* with *Skeletonema costatum*. Twelve months old oysters were
137 deployed in March 2022 in the Coupelasse site (47.026571, -2.030872) and their survival was

checked every two weeks until the end of September following the methodology described by Fleury *et al.* (2020) [55].

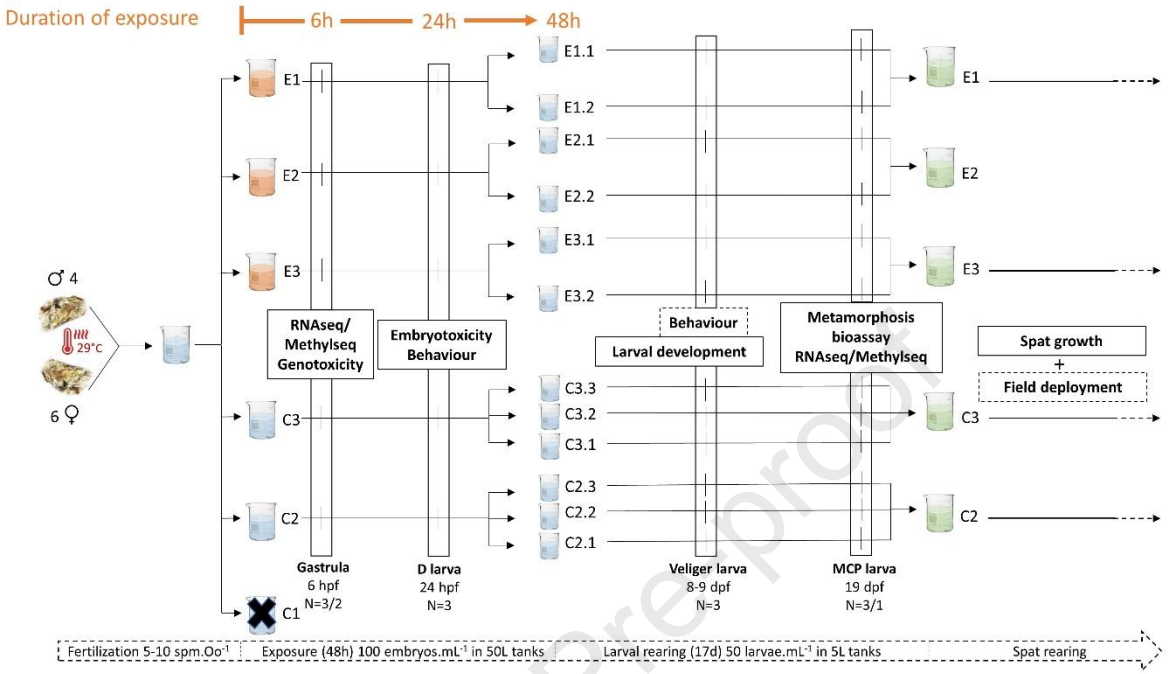


Figure 1: Experimental design. E= Exposed tanks; C= Control tanks; orange= contaminated seawater; blue= non-contaminated seawater (NCSW, 1µm filtered, UV-filtered and active coal-filtered, 25°C), green=pre-treated seawater (UV-treated, 10µm filtered); Oo= fertilized oocytes; spm= spermatozoa. N = number of samples collected per tank for each analysis. One control tank (C1) was not included in the subsequent analyses due to an arrest in larval development.

2. Sampling protocols and analyses

2.1 Seawater chemical analyses

Water chemical analyses were carried out at T0 (before embryo inoculation) and T48 h (seawater was filtered on 20 µm in order to remove oyster larvae) for each tank to assess the effective mixture contamination in exposed tanks and check the absence of target molecules in control ones. For copper and cadmium, 100 mL of seawater were sampled and pre-concentrated with Nobias chelate PA-1 resin (Hitachi High Technology) following a protocol adapted from Sohrin *et al.* [56] and Hatje *et al.* [57] (blank: pre-cleaned seawater, certified material: CASS-6). Copper and Cadmium concentrations were then measured by ICP-MS (I-CAP-TQ, Thermo).

For the organic compounds, water samples were analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS). For 3 pesticides (azoxystrobine, carbendazime, terbutylazine), regarding the low nominal expected concentration, an on-line solid phase extraction was coupled to the LC-MS/MS (SPE-LC-MS/MS) [58] to improve the quantification capacity. For glyphosate and AMPA, samples were analysed according to an adapted AQUAREF procedure [59] performing a derivatization (FMOC-C1) followed by a Liquid/Liquid (LLE) with ethyl-acetate and an SPE extraction before LC-MS/MS. The limit of quantification (LOQ according to S/N > 10) in samples were comprised between 0.06 to 15 ng.L^{-1} (tab.SM1).

2.2 Ecotoxicology tests and morphometric analyses

2.2.1 Comet assay

Three replicates per tank of gastrulae (6 hpf, 6 hours of exposure) (n=50000 per replicate) were sampled and the Comet assay performed as in Sussarellu *et al.* [53].

2.2.2 Embryotoxicity tests and EC_{50} estimation

Fertilized embryos (100.mL^{-1}) were exposed in 10 mL in 6 well microplates, in triplicates, to 8 concentrations of the mixture (X0.1= 0.29 µg.L^{-1}; X1= 2.85 µg.L^{-1}; X5= 14.27 µg.L^{-1}; X10= 28.54 µg.L^{-1}; X20= 57.09 µg.L^{-1}; X30= 85.63 µg.L^{-1}; X50= 142.71 µg.L^{-1}; X100= 285.43 µg.L^{-1}). Copper was used as positive control according to the standardized embryo-larval bioassay (ISO, 2015 [60]; C1= 1 µg.L^{-1}; C2= 5 µg.L^{-1}; C3= 10 µg.L^{-1}; C4= 15 µg.L^{-1}; C5= 20 µg.L^{-1}; C6= 30 µg.L^{-1}). After 24h at 25°C, larvae were stored in 0.1% seawater-formaldehyde solution for visual inspection. Larvae were analysed under an Olympus CK40 inverted microscope (x20 magnification) in order to determine the proportion of abnormal larvae, *i.e*. those presenting shell and/or mantle deformities according to the standardized embryo-larval bioassay (n=100

per sample) [60]. The EC_{50} of the mixture was estimated using the Log-Logistic.3 (LL.3) model from the R DRC package (drc_V3.0-1) [61]. At 24 hpf (24 hours of exposure), 10 mL from each tank were sampled in triplicate and stored in 0.1% seawater-formaldehyde solution. Larvae were visually inspected for developmental abnormalities.

2.2.3 Larval behaviour

D-shaped (2 dpf, 48 hours of exposure) and veliger larvae (8-9 dpf, 6-7 days post-exposure) were transferred alive to a 96-well microplate filled with 100 µL seawater from the respective larval tanks (maximum 16 animals per well). Larvae were filmed for 30 sec under an inverted transmission light microscope connected to a camera (IDS UI-3480LE). Three videos per replicate were recorded. The mean velocity ($mm.s^{-1}$), the mobility (percentage of changed pixels of the detected subject between current sample and the previous one, %) and the proportion of time moving over the duration of the sequence (%) of each animal in each well were quantified with EthoVision XT 13.0 (Noldus Information Technology, The Netherlands) as described in Di Poi *et al.* [62].

2.2.4 Larval growth

Every 2-3 days, 10 mL were sampled in each flow-through tank and stored in 0.1% seawater-formaldehyde solution in microplates. Pictures were taken with an IDS Ueye UI-3480LE Rev.2 camera attached to an Olympus CK40 inverted microscope (x20 magnification). The pictures were analysed for larvae Feret's diameter with a dedicated macro using ImageJ software [63].

2.2.5 Metamorphosis bioassay

Competent eye-spotted pediveliger larvae (19 dpf, 17 days post-exposure) were retrieved on a 150 µm sieve and triplicates of 100 larvae per tank were deposited in a 24-well microplate. Epinephrine (Sigma-Aldrich®) was added at a final concentration of 10^{-4} M and D-larvae were incubated for 24 h at 25°C to induce metamorphosis [64]. Larvae were then stored in 0.1%

seawater-formaldehyde solution and the metamorphosis rate was estimated by counting the larvae exhibiting gills, velum disappearance and irregularly edged dissoconch establishment [65] under an Olympus CK40 inverted microscope (x20 magnification). Metamorphosis rates were calculated by considering both percentages of non-metamorphosed and dead D-larvae versus metamorphosed ones.

2.2.6 Spat growth

From metamorphosis, fifty animals were sampled every month in each tank and their total weight were measured.

2.3 Statistical analysis for ecotoxicology tests and biometric data

Results are presented as the mean +/- 95% confidence interval (CI) of independent replicates. For all the phenotypic analyses, data were processed and analysed using R/BioConductor (R Development Core Team, V4.0.5) [66]. Pairwise comparison between experimental conditions were carried out using two-tailed Student's T test. Normality and homogeneity of variances were tested using Shapiro-Wilk test and F test, respectively. Wilcoxon's test was used as non-parametric alternative when required. The α threshold for significance was fixed at 0.05 for all tests. Percentage data underwent angular transformation before statistical analyses. Survival rates were represented as Kaplan-Meier curves. Significant differences in survival rates between conditions were evaluated using a log-rank test with the Survival r package (v3.2-11) [67].

2.4 Molecular analyses

2.4.1 Sampling protocol, RNA and DNA extraction and sequencing

Gastrula embryos (6 hpf, 6 hours of exposure, 50000 animals per replicate, n=6 replicates per 50 L tank) and MCP larvae (19 dpf, 17 days post-exposure, 1500 animals per 5 L tank) were sampled, sieved on 20 µm and 150 µm respectively, concentrated by centrifugation (250 g, 5 min, 4°C) and immediately frozen in liquid nitrogen.

Total RNA was extracted using 1.5 mL of Trizol (Invitrogen) according to the manufacturer's instructions. RNA purity and concentration were assayed by UV spectrometry (ND-1000 spectrophotometer ThermoScientific, Waltham MA, USA) and fluorometry (Qubit 4 fluorometer, ThermoFisher). RNA integrity was assessed using RNA 6000 Nano kits (Agilent Technologies) on an Agilent bioanalyzer for a mean RNA Integrity Number (RIN) of 8.7 ± 1.2.

Fifteen gastrula and 12 MCP larvae samples (9 and 6 controls and 6 and 6 exposed, respectively) were sent for polyA-enriched RNA-seq library preparation and paired-end 2x150 bp sequencing on an Illumina NovaSeq6000 platform to an external service provider (Genewiz Azenta, Leipzig, Germany). Sequencing led to *ca*. 981.7 million of paired-end reads *i.e*. 32.7 ± 10.35 $x10^6$ reads per sample, with an average quality score of 35.8 ± 0.03, corresponding to a presumptive *ca*. 50x coverage per sample.

Genomic DNA was extracted using the E.Z.N.A Mollusc DNA Kit (Omega Biotek, Norcross, USA) following the manufacturer's instructions. DNA purity and concentration were quantified by UV spectrometry (Nanodrop 2000, Thermo Scientific) and fluorometry (Qubit 4 fluorometer, ThermoFisher). Ten gastrula and 12 MCP larvae samples (4 and 6 controls and 6 and 6 exposed, respectively) were sent for NEBNext Methylseq library preparation and paired-end 2x150 bp DNA sequencing on an Illumina NovaSeq6000 platform to an external service provider (Genome Quebec, Montreal, Canada). Sequencing of gastrula samples led to *ca*. 1.2 billion of paired-end reads *ie*. 119.2 ± 23.7 x 10^6 reads per sample, with an average quality score of 35.4 ± 0.6, corresponding to a presumptive *ca*. 6x coverage per sample. Sequencing of MCP larvae samples led to *ca*. 1.1 billion of paired-end reads *i.e*. 92.3 ± 12.7 x 10^6 reads per

sample, with an average quality score of 36, corresponding to a presumptive *ca*. 6x coverage per sample. Bioinformatic treatment of the data was carried out on the Ifremer's high performance computing cluster (Datarmor)

2.4.2 RNAseq data analysis

Raw read quality was checked using *FastQC* (fastqc_v0.11.9) [68], all bases were above 30 Q_c score. Reads were mapped to the indexed GCA902806645v1 reference genome [69] using *STAR* software (star_v2.7.9a) [70] with the following parameters (--alignIntronMin 20 --alignIntronMax 1000000). The number of reads mapping to mRNA transcripts was counted on a per-gene basis and their differential expression was examined using the R *DESeq2* package (default parameters, except *log*-2 fold-change (*log*2FC) threshold: 0.5 and *P*-adj < 0.05) [71]. Heatmap was built from the normalized counts matrix (DESeq's median of ratios) with Euclidian distances using the R *pheatmap* (pheatmap_1.0.12) [72] package.

2.4.3 Methylseq data analysis

Raw reads quality was checked using *FastQC* (fastqc_v0.11.9) [68]. Illumina adapters were trimmed by *fastp* (fastp_v0.20.1) [73], and reads were filtered by length (≥ 125 bp). The remaining reads were re-submitted to quality check with *fastQC*. The alignment of reads to the indexed GCA902806645v1 reference genome [69] was performed using *BWA-Meth* (bwa-meth_v0.2.3) [74]. *MethylDackel* (methyldackel_v0.6.1) (https://github.com/dpryan79/MethylDackel) was used to perform methylation bias identification and methylated bases (mCpGs) were called using the following parameters : --minDepth 10 --methylKit --nOT 0,0,0,145 --nOB 0,0,7,0. Finally, differential methylation analyses were performed using the R *MethylKit* Package [75] (default parameters : difference threshold: 25%, maximal significant *q*-value: 0.01). Differentially methylated cytosines (DMCs) were then annotated by genomic feature with the R *genomation* package [76] and the

distribution frequency of DMCs within genomic features (promotor: 1000bp up- and -down-stream transcription start sites, exon, intron and intergenic regio) was tested using chi-squared test considering an α threshold of 0.05.

DMCs coordinates were matched with gene coordinates from the GCA902806645v1 genome assembly, using the R *Bedtools intersect* (bedtoolsv_2.30.0) [77], to identify DMC-related genes (DMG). Gene ontology enrichment analyses of DMG subsets was carried out with the R *ClusterProfiler* package [78]. GO terms with Bonferroni-corrected $p < 0.05$ (exact Fisher's test) were considered significantly enriched.

Results

1. Chemical contamination.

Measured concentrations (MC) were stable throughout the exposure period and were consistent with nominal concentrations (NC) of the mixture compounds, except for simazine (NC=80.0 $ng.L^{-1}$; MC=44.1 ± 6.1 $ng.L^{-1}$), glyphosate (NC=590 $ng.L^{-1}$; MC=1026,7 ± 57,4 $ng.L^{-1}$) and cadmium, for which the contamination of 2 tanks was higher than expected (C2, MC_{T0}=210 $ng.L^{-1}$; E3, MC_{T0}=209 $ng.L^{-1}$) and increased during the exposure (C2, MC_{T48}=1197.0 $ng.L^{-1}$; C3, MC_{T48}=1576.0 $ng.L^{-1}$). In control tanks, organic contaminants were under the detection limits, and copper and cadmium were measured at 303.5 ± 72.5 $ng.L^{-1}$ and 244.7 ± 431.8 $ng.L^{-1}$ respectively. (tab.SM1).

2. Embryotoxicity and genotoxicity

The estimated EC_{50} of the pesticide mixture for embryo-larval development was 100.68 ± 9 $\mu g.L^{-1}$ (fig.SM1). The test was validated according to the standardized criteria from the test ISO

298 17244:2015 (larval abnormalities in control seawater 6.13 ± 0.27% and EC_{50} value for copper
299 estimated at 15.16 ± 1.2 µg.L^{-1} [60]). In 50 L tanks, D-larvae normality rates were not
300 significantly different between groups (control: 82.5 4.0%; exposed: 82.6 1.7%, Student's T
301 test, $p > 0.05$). The comet assay did not reveal an increase in DNA strand breaks upon pesticide
302 exposure (control: 16.5 ± 1.7%; exposed: 16.5 ± 1.1%, Student's T test $p > 0.05$).

303

304 **3. Larval behaviour**

305 Following exposure (2 dpf), the pesticide mixture did not induce significant changes in larval
306 mobility (control vs exposed: 9.559 ± 1.373 vs 8.366 ± 1.798%, fig.2B) and velocity (control
307 vs exposed: 0.308 ± 0.038 vs 0.293 ± 0.059%, fig.SM2A), anyway the time spent moving was
308 significantly higher in exposed animals than in controls (control vs exposed: 27.697 ± 4.786 vs
309 38.061 ± 7.356%, $p < 0.01$, fig.SM2B). In contrast, the exposure to pesticides influenced
310 swimming behaviour later in larval development (8 dpf). Exposed larvae were less mobile
311 (control vs exposed: 15.824 ± 1.689 vs 10.098 ± 1.122 %, $p < 0.001$, fig.2B), slower (control
312 vs exposed: 0.447 ± 0.056 vs 0.301 ± 0.033 mm.s^{-1} $p < 0.001$, fig.SM2A) and spent less time
313 moving (control vs exposed: 55.729 ± 7.949 vs 39.198 ± 5.319 %, $p < 0.01$, fig.SM2B).

314 In addition, the velocity, the time moving and the mobility increased between 2 dpf and 8 dpf
315 for control larvae (2 dpf vs 8 dpf: velocity, 0.308 ± 0.019 vs 0.447 ± 0.056 mm.s^{-1}; time moving,
316 27.697 ± 4.785 vs 55.729 ± 7.949 %; mobility, 9.559 ± 1.373 vs 15.825 ± 1.689 %, $p < 0.001$)
317 while they remain stable for exposed larvae (tab.SM2).

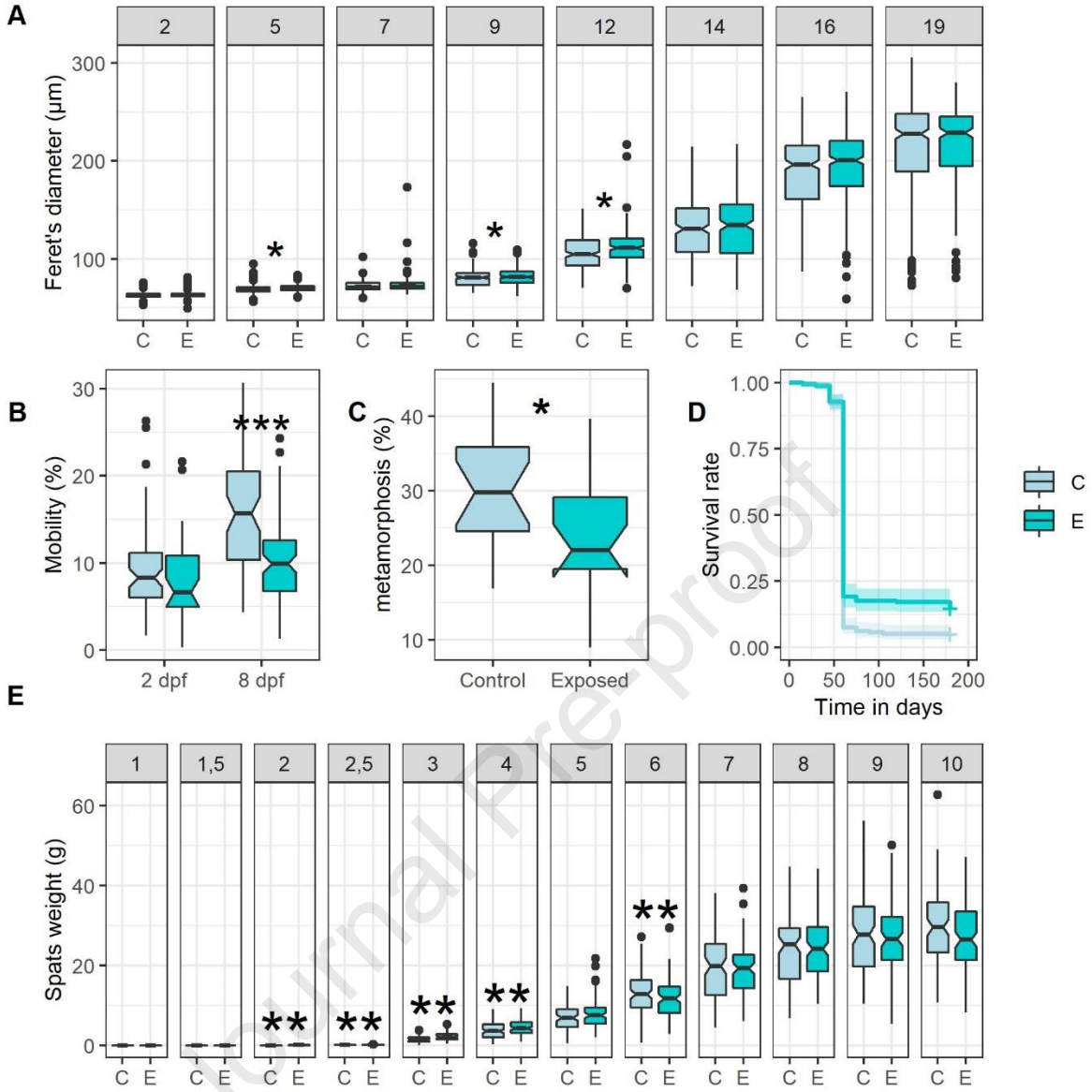


Figure 2: A) Larval length across development (Feret's diameter, µm) from 2 to 19 dpf. B) Larval mobility. dpf: day post-fertilization. C) Metamorphosis bioassay. D) Survival rate of spats in the field. Error interval represent the 95% CI (log-rank test, p < 0.0001). E) Spat total weight (g) from 1 to 10 months post-fertilisation. C: Control, E: Exposed, Notches represent the 95% CI (, p<0.05; **, p<0.01; ***, p<0.001, Student's T test or Wilcoxon's test).*

4. Larval growth and metamorphosis

Pesticide exposure did not induce any overall difference in larval growth, however exposed larvae exhibited a significantly longer Féret's diameter than control larvae at 5 (control: 69.44 ± 0;33 µm; exposed: 70.12 ± 0.28 µm), 9 (control: 80.24 ± 1.15 µm; exposed: 81.86 ± 1.07 µm) and 12 (control: 106.61 ± 2.41 µm; exposed: 111.64 ± 2.61 µm) dpf, respectively (Student's T

329 test, $p < 0.05$, fig.2A). The competence for metamorphosis was reached for both conditions at
330 19 dpf, and no difference in eye-spotted D-larvae percentage was observed (control: 53.4 ±
331 6.2%; exposed: 47.0 ± 3.0%, Student's T test $p > 0.05$). The metamorphosis bioassay showed
332 a significantly lower metamorphosis rate in treated MCP larvae than in controls (control: 30.33
333 ± 3.77 % vs exposed: 23.64 ± 3.69 %, Student's T test, $p = 0.01$, fig.2C).

334

335 **5. Spat growth**

336 Exposed spat were significantly heavier than controls from 2 to 4 months post-fertilization
337 (mpf, control vs exposed: 2 mpf, 0.069 ± 0.020 vs 0.126 ± 0.031 g; 2.5 mpf, control: 0.171 ±
338 0.030 vs 0.233 ± 0.028 g; 3 mpf, 1.549 ± 0.331 vs 2.279 ± 0.440 g; 4 mpf, 3.727 ± 0.401 vs
339 4.591 ± 0.363 g, Student's T test, $p < 0.01$), this trend was then mitigated and eventually
340 switched from 6 mpf (control: 13.295 ± 1.136 g; exposed: 11.861 ± 1.056 g, Student's T test, p
341 < 0.01), then no significant difference occurred between groups from 7 mpf (fig.2E). Exposed
342 oysters placed in the field had a better survival rate than controls (+9.5%, log-rank test, $p =$
343 0.0003, fig.2D).

344 **6. Gene expression**

345 The pesticide exposure was not associated with clear transcriptome-wide signatures among the
346 21350 genes examined in gastrula embryos. However, 14 genes were differentially expressed
347 (DEG) between conditions (fig.3). Among them, 10 genes were down-regulated and 4 genes
348 were up-regulated in exposed embryos. The expression fold change (FC) of this limited number
349 of genes is above 2.5 regardless of those genes being repressed (FC<1) or stimulated (FC>1)
350 (tab.SM3). Six of the DEGs encode transcription factors (6/14), some of which being putatively
351 implicated in developmental mechanisms, such as Pax6, Elf-2, Cell death specification protein

2, Goosecoid and Engrailed-like. There were no significant DEGs in MCP larvae (*log*2FC threshold = 0.5 and *P*-adj threshold = 0.05)

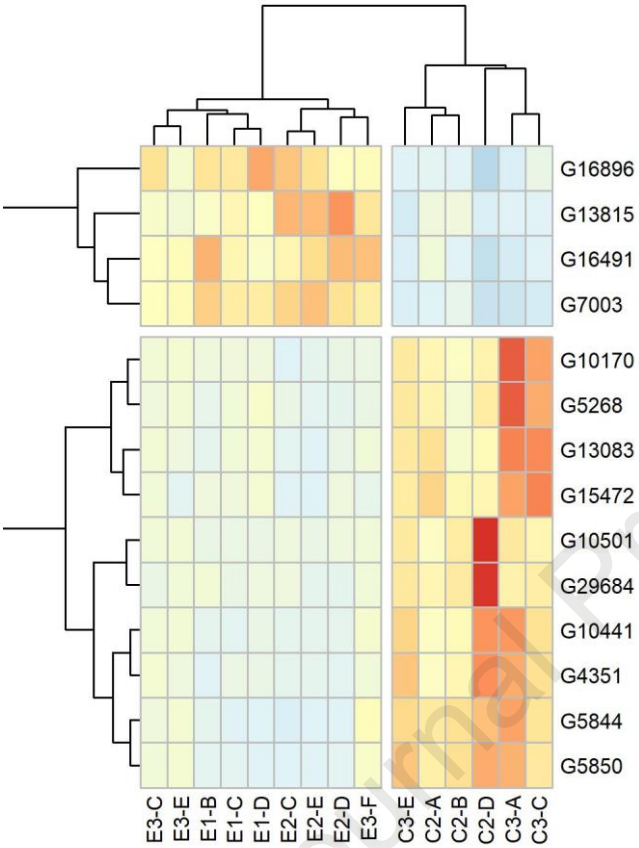


Figure 3: Normalized counts (DESeq²'s median of ratios) of DEGs and their associated protein between control and exposed gastrulae. Cols and rows are clustered based on Euclidean distances. E: batch exposed; C: batch control; letters A to F refer to the replicates.

7. DNA methylation

Almost 70% of methylated CpGs dinucleotides (mCpGs) in control gastrulae and MCP larvae were located in gene bodies. Differential methylation analysis revealed that the pesticide exposure induced the differential methylation of 507 and 220 cytosines in gastrulae and MCP larvae, respectively. Differentially methylated cytosines were mostly located in genes in gastrulae and MCP larvae (75% and 84% in promotors, exons or introns, respectively). The

366 distribution of mCpGs within the genome is different from the distribution of DMCs in both
367 gastrulae and MCP larvae (χ^2, p<0.0001 for gastrulae and MCP larvae), with differential
368 methylation preferentially affecting intergenic regions in gastrulae and both intergenic regions
369 and introns in MCP larvae (fig.SM3).

370 Overlap between DMCs and gene coordinates revealed 386 and 175 genes containing at least
371 one DMC (DMGs; see tab.SM4) in gastrulae and MCP larvae, respectively. In gastrulae, 147
372 contained hypermethylated cytosines (hyper-MC), whereas 227 DMGs contained
373 hypomethylated cytosines (hypo-MC) and, in MCP larvae, 40 contained hyper-MC while 135
374 genes contained hypo-MC. Gene ontology analyses did not highlight any functional term
375 enrichment in either gastrula and MCP larvae.

376 Among the 175 DMGs found in MCP larvae, 18 were already differentially methylated
377 gastrulae. Ten out of those 18 DMGs retained a similar profile being either hyper- or hypo-
378 methylated in both stages, and most of them (15/18) were hypomethylated in MCP larvae
379 (tab.SM5).

380

381 **Discussion**

382 This study aimed to investigate the impact of an early exposure to an environmentally realistic
383 pesticide mixture on the life history of the Pacific oyster, *Crassostrea gigas*. Fertilized oocytes
384 were exposed to contaminated seawater (cocktail of 18 pesticides, NC: 2.85 µg.L^{-1}) from 0 to
385 48 hpf, and thereafter animals were reared in non-contaminated seawater. Numerous analyses
386 were carried out in order to identify potential alterations from the molecular scale (gene
387 expression and DNA methylation in gastrulae and MCP larvae) to the phenotypic scale
388 (development features of larvae and spat). To our knowledge, this study is the first
389 environmentally relevant long-lasting investigation in the Pacific oyster. As such, it may

contribute to incorporate the epigenetic aspect in ecological risk assessment frameworks, in line with what was proposed by Shaw *et al*. [79].

The measured contamination of seawater is mostly consistent with results expected from the nominal concentration used (tab.SM1). In the control seawater, organic contaminants concentrations were below our detection threshold, assessing the efficiency of the active carbon treatment of the water used for the experiments. Cadmium concentrations in some experimental tanks (C2 and E3) were more than 5-fold higher than the nominal concentration and increased throughout the exposure window. Complementary tests suggest that stainless steel taps of the oxygenation system might be the source of this contamination. However, the maximum measured concentration (1.58 $\mu g.L^{-1}$) remains far below the cadmium embryo-larval toxicity thresholds for the Pacific oyster (lowest observed effect concentration (LOEC): 106,0 $\mu g.L^{-1}$ and estimated EC_{50}: 272,2 $\mu g.L^{-1}$) [80]. Consequently, larvae were maintained for subsequent rearing and analyses. Besides, the mean copper concentration measured in control tanks (303.5 ± 72.5 $ng.L^{-1}$) is consistent with the previously measured concentrations in the North-eastern Atlantic near-shore waters in July (300 $ng.L^{-1}$ to 3800 $ng.L^{-1}$) [81] and in the Loire estuary (*c.a.* 600 $ng.L^{-1}$) during summer [82], and is less than half the levels measured in exposed tanks (743.3 ± 57.6 $ng.L^{-1}$). Overall, the total and individual measured concentrations of contaminants are of the same order of magnitude than recently measured in the Arcachon Bay [2] and other French lagoons [83].

Two developmental stages were investigated during the exposure period: gastrula embryos (6 hpf, 6 hours of exposure) and D-larva (24 hpf, 24 hours of exposure). Oyster gastrulae undergo a strong global DNA demethylation associated with the expression regulation of specific developmental genes and fundamental for organogenesis [42], which is almost complete in D-larvae. Therefore, DNA methylation disruption and/or phenotypic abnormalities during those developmental stages caused by contaminants can potentially impair developmental processes

with subsequent impacts in the later life. Here, no embryotoxic effects were identified in exposed D-larvae and the comet assay did not reveal genotoxic effects after the pesticide exposure in gastrulae. Those results were expected as genotoxicity is supposed to be an early marker of morphological embryotoxicity [84], and the mixturenominal experimental concentration used in the present study (sum of compounds 2.85 $\mu g.L^{-1}$) is far under its EC_{50} for embryo-larval abnormality (100.68 $\mu g.L^{-1}$). To date, only a few studies have investigated the embryotoxicity of pesticide mixtures in the oyster. On the one hand, Mai *et al.* [85] found that larval abnormalities increase from 17.5 ± 1.5 % to almost 70% in oysters D-larvae exposed for 24h from fertilization to an environmentally realistic cocktail of 15 pesticides at a nominal concentration of 1.557 $\mu g.L^{-1}$. On the other hand, Kuchovská *et al*. [83] highlighted a moderate but significant increase (from 15% to almost 30%) of larval abnormality in D-larvae exposed for 30h from fertilization to an environmentally-relevant mixture of 5 pesticides at a total concentration of 0.32 $\mu g.L^{-1}$. Moreover, in this study an early exposure to an environmentally-relevant pesticide mixture only affected the swimming behaviour of oyster D-larvae in terms of time moving. Similarly, Kuchovská *et al*. [83] did not find reduction of the swimming speed of *C. gigas* larvae upon pesticide exposure. In contrast, early exposure to single pesticide compound, such as chlortoluron (from 0.015 $\mu g.L^{-1}$), reduced the swimming speed of oyster D-larvae along with growth and developmental issues [28], while other compounds like glyphosate, isoproturon or S-metolachlor did not, even at relatively high concentrations compared to the one used in this study (11.1 $\mu g.L^{-1}$, 7.8 $\mu g.L^{-1}$ and 1$\mu g.L^{-1}$ respectively) [28, 87]. Interestingly, the effects of the herbicide chlortoluron, a pesticide used in our cocktail, occurred at ecologically relevant levels (0.015 $\mu g.L^{-1}$) [28]. Because the temporality of the exposures is the same, we assume the same window of susceptibility are targeted [10]. Therefore, the highly diverse biological effects observed between the different chemical mixtures could be due to differences in their composition, and thereby to different interactions

between the chemicals within, depending on their modes of action [88]. However, the modes of action, understood as a causal-mechanistic description of the toxicity phenomenon [89], are complex to detangle. First, most of the modes of action of the single molecules used herein are unknown to date in molluscs because they are non-target organisms. Second, interaction between compounds are hard to highlight when using many molecules [46]. So far, studies mainly focus on binary or tertiary mixtures and much less on complex cocktails [88], which require specific design lying beyond the scope of the present work.

In addition, the swimming performances of exposed animals were significantly reduced after the 48h exposure. The unexposed larvae were more mobile, active and faster at the veliger stage, whereas the swimming behaviour of the exposed larvae remained constant. Previous studies evidenced that swimming parameters generally increased with larval size in the oyster larvae [90] in order to feed and select a suitable settlement habitat. Because the MCP stage was reached at the same time (19 dpf) and larval growth was similar for both conditions, behavioural differences are likely to be related to energetic metabolism or neuro-muscular system impairment. Indeed, several compounds in the mixture have a neurotoxic mode of action (e.g. imidacloprid, [91]) or present neurotoxic effects in various marine organisms like *Mytilus edulis* (glyphosate, [92]) or *Danio rerio* (atrazine, [15]). Overall, changes in larval behaviour, even transiently, can have direct effects on survival at the organism and ultimately at the population level.

Later on, the capacity of larvae to metamorphose was tested using epinephrine. This hormone induces metamorphosis in *C. gigas* by activating the adrenergic pathway [64, 93]. Here, the epinephrine bioassays resulted in a significantly lower metamorphosis rate in exposed larvae. This suggests that exposed animals could be delayed in acquiring metamorphosis competency despite the presence of the eye-spot [64]. In the field, such delay could be detrimental for the survival of the larvae and potentially limit the recruitment.

At the molecular level, a slight modulation of gene expression in gastrulae was observed, which does not last after the exposure period. The differential expression analysis revealed that only 14 genes were differentially expressed between exposed and control gastrulae. Interestingly, none of those genes are known to be implicated in xenobiotic metabolism or stress response. Consequently, traditional effect biomarkers genes (i.e. GST, CYP1A, AhR), mostly investigated by real-time PCR, might not be the only tools to efficiently detect and warn against adverse biological effects of environmental chemical contamination as previously advised [94]. Indeed, the gastrulation is a critical developmental step during which morphogenetic rearrangements lead to the segregation of embryonic sheets (Stern, 2004 in [95]). Thus, even if the absence of DEGs in MCP larvae fails to explain the hypothetic delay in the acquisition of metamorphosis competency in exposed oysters, the observed transcriptomic modulation in gastrulae could participate to the subsequent long-lasting effects on the life history of the oysters.

Actually, among the 14 DEGs, 10 are down-regulated in exposed embryos. Six out of these genes are homeotic transcription factors that may play a role in development (*pax6*, *elf-2*, *cell death specification protein 2*, *goosecoid*, *engrailed-like*). Especially, *goosecoid* is an early driver of mesoderm specification in molluscs and it is expressed from the setup of the antero-posterior axis to the end of gastrulation [96]. *Goosecoid* and *engrailed* are also shell formation genes which expression delineate the molluscan shell compartment [97,98]. Interestingly, *engrailed* is over-expressed in *C. gigas* in response to a decreasing pH [99]. Previous studies also demonstrated the potential impact of chemical stressors on homeotic transcription factors involved in shell formation in oyster larvae [44]. Indeed, environmental stress might disrupt early developmental processes, having potential impacts later on the life history by decreasing the ability of oyster larvae to face environmental changes.

Because gene expression reflects the organism instantaneous physiological state, it is not surprising that the expression pattern in MCP larvae was no longer impacted by the early exposure. By contrast, DNA methylation changes are long-lasting and may reflect past stimuli. Likewise, we observed DNA methylation changes 17 days after the exposure ended. Such an epigenetic memory was already reported in the literature as a key mechanism of stress response and adaptation [100, 101]. This may constitute a new perspective for biomonitoring by allowing the investigation of not only current but also past-contaminations.

The whole-genome DNA methylation analysis revealed that more than 70% of mCpGs in control embryos and MCP larvae were located in gene bodies, which is consistent with previous studies on oyster development [43]. However, differential methylation preferentially affects cytosines within intergenic regions in both gastrulae and MCP larvae, indicating a non-random mechanism (fig.SM3). Interestingly, intergenic regions host transposable elements (TEs) [102] which silencing is critical for a normal development and which are known to be silenced by DNA methylation from plants to vertebrates [103–105]. The methylation of TEs is highly variable during gastrulation, organogenesis and metamorphosis in the Pacific oyster [43], and, in this study, the enrichment of the differential methylation in intergenic regions in exposed gastrulae and MCP larvae could be related to a potential impairment of developmental mechanisms. Therefore, our results highlight the need for future studies dedicated to the impacts of environmental stressors on TE methylation and to what extent such impacts may have phenotypic outcomes as mentioned in Fallet *et al*. [106].

Pesticide exposure seems therefore associated with a demethylation trend (60.7% and 77.1% of DMCs are hypomethylated in gastrulae and MCP larvae, respectively). DNA hypomethylation was already described in several model organisms [107–111], as well as in humans [112] in response to different stressors, thus becoming a marker of environmental responses. However, a lack of correlation between differential methylation and expression has already been

514 highlighted in previous studies [32,53,113] in line with present results (fig.SM4), suggesting
515 that the functional meaning of differential methylation in response to environmental stressors
516 is more complex than a linear relationship between methylation and expression. Because in
517 adult oysters highly methylated genes are related to housekeeping functions whereas sparsely
518 methylated genes are associated with inducible expression genes [114], the hypomethylation
519 could be associated with more transcriptional opportunities, that may include alternative
520 splicing or transcript variants with functional outcomes, and result in a higher phenotypic
521 plasticity [115–117]. In this context, the implications of the demethylation observed in this
522 study still have to be clarified. Indeed, the limited number of DMGs did not allow robust GO
523 enrichment analyses to decipher whether hypo- or hyper-MC-related genes constitute specific
524 functional pathways. Nevertheless, the coincidence between the DNA hypomethylation trend
525 and the lower metamorphosis rate in exposed larvae, without related gene expression
526 modifications raises the question of the mechanisms that convey and translate information from
527 DNA methylation to the functional phenotypic scale, and by which DNA methylation may
528 enhance phenotypic plasticity of organisms in the context of changing environments [113–115].

529 The hypothesis of an increased phenotypic plasticity in exposed individuals is also supported
530 by the results from spat monitoring. On the one hand, exposed spats were significantly heavier
531 than control ones from months 2 to 4, and this trend was mitigated after 6 mpf. On the other
532 hand, on-field monitoring of spat conducted twice a month from March 2022 to October 2022
533 revealed high mortality after 45 days for both conditions, which likely corresponds to the
534 Pacific oyster mortality syndrome (POMS) [52,55]. Indeed, exposed animals exhibited a better
535 survival rate than control animals, suggesting a decreased susceptibility to pathogens. Those
536 two remarkable phenotypic changes may be an expression of the improved phenotypic plasticity
537 in exposed individuals. Together with behavioural and metamorphosis analyses, these results
538 point out to the influence of the early adverse environment in the expression of various traits in

later life. Such so-called “carry-over” effects are increasingly considered acclimatization mechanisms in bivalves coping with environmental change [116,117] and our results corroborate the hypothesis that DNA methylation could be one of their molecular bases.

8. Conclusion

This study is the first investigation about environmentally relevant pesticide mixtures long-lasting effects in the Pacific oyster. It demonstrates that an early exposure to an environmentally-relevant pesticide mixture affects in a slight but significant manner both the expression of some homeotic genes during embryo development as well as the methylation of DNA at critical developmental steps, during and after the exposure window. Phenotypic modifications were also observed, including the swimming behaviour, response to metamorphosis enhancers and survival rates. This study provides evidences for the threat low pesticide marine environment contamination might represent for organisms exhibiting external fertilization, and which may trigger long term consequences at the population level. Our results highlight the potency of multiscale biological and temporal investigations as an asset for the future analysis of delayed harmful effects of environmentally relevant chemicals in toxicity assessment and ecological risk management. Because the contamination of marine environments is chronical, further investigations are now required to better understand how it impacts exposed organisms across generations.

Abbreviations

DEG: differentially expressed gene

DMC: differentially methylated cytosines

562 DMG: DMC-related gene

563 Dpf: day post-fertilization

564 EC_{50}: 50% effect concentration

565 Hyper/Hypo-MC: hyper/hypo-methylated cytosine

566 LOEC: lowest observed effect concentration

567 MC: measured concentration

568 MCP larva: metamorphosis competent pediveliger larva

569 Mpf: month post-fertilization

570 NC : nominal concentration

571 NCSW : non-contaminated sea water

572 **Acknowledgments**

573 We are grateful to the French National Research Agency (ANR) for funding the PESTO project
574 (Projet-ANR-19-CE34-0004). We also thank the EPOC-LPCT platform for the analyses of
575 organic contaminants in the experimental water and the Ifremer Marine Shellfish Platform of
576 Bouin (PMMB) who hosted the experimentations.

577

578 **Data availibity**

579 RNAseq and Methylseq data have been submitted to the European Nucleotide Archive (projects
580 PRJEB58194 and PRJEB58545) for free access. All non-molecular data are available upon
581 request.

582

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Highlights

- *Crassostrea gigas* embryos were exposed to an environmental pesticide mixture for 48h.
- Carry-over effects were observed along the lifecycle of the oysters.
- The expression of developmental genes was modified during the exposure.
- Exposed oysters exhibit a global DNA demethylation during and after the exposure window.
- Some phenotypic endpoints were impaired: swimming behavior, metamorphosis, field survival.

Author Statement

Thomas Sol Dourdin: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization

Guillaume Rivière: Conceptualization, Methodology, Validation, Investigation, Writing - Original Draft, Writing - Review & Editing, Supervision

Alexandre Cormier: Software, Validation, Writing - Review & Editing

Carole Di Poi: Methodology, Investigation, Resources, Writing - Review & Editing

Killian Guyomard: Investigation, Methodology, Writing - Review & Editing

Manuella Rabiller: Investigation, Methodology, Writing - Review & Editing

Farida Akcha: Methodology, Investigation, Resources, Writing - Review & Editing

Thierno Bah Sadialiou: Investigation, Formal analysis, Writing - Review & Editing

Pauline Le Monier: Methodology, Formal analysis, Investigation, Writing - Review & Editing

Rossana Sussarellu: Conceptualization, Methodology, Validation, Investigation, Resources, Writing - Original Draft, Writing - Review & Editing, Supervision, Project administration, Funding acquisition

XXX On the behalf of all the authors.

Declaration of interests

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

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: