***Supplementary File S1***

***Materials and Methods***

***Oyster and experimental set up***

Adult oysters were produced in February 2020 according to Petton et al. (2015) and from the Ifremer’s nursery (Bouin, France) were transferred to the Ifremer’s experimental facilities (Argenton, France). Oysters were acclimated during one week in a 600 L tank supplied with UV-treated 1-μm filtered seawater. Seawater temperature was gradually increased from 12°C to 17°C by 1°C day-1. Histology analyses confirmed that oyster gonadic development was stage 0 or early stage 1 at the beginning of the experiment (according to Steele and Mulcahy, 1999). After acclimation, oysters were placed in eighteen 50-L experimental PMMA tanks (40 oysters tank-1) supplied continuously with UV-treated 1-µm filtered seawater (12 L h-1; 17.7 ± 0.2 °C; 34.5 ± 0.4 PSU; pH 8.2 ± 0.1) containing a balanced mixture (50/50, v/v) of two microalgae, *Tisochrysis lutea* (T-iso CCAP927/14, cell volume = 40 µm3) and *Chaetoceros* sp. (CCAP 1010/3, cell volume = 80 µm3) at a daily ratio equal to 8% dry weight algae/dry weight oyster. The algal concentration was measured then adjusted every morning using an electronic particle counter (Multisizer 3; Beckman Coulter, USA). This conditioning period lasted 44 days to ensure complete gametogenesis. Photoperiod was 12 h light:12 h dark.

Six treatments were applied: (1) Control (no particle exposure; CTL); (2) Exposure to TP at low concentration of microplastics in ocean (10 TP mL-1; 5.2 µg L-1, namely MR-L micro-rubber low); (3) Exposure to TP at high concentration (100 TP mL-1; 52 µg L-1, namely MR-H micro-rubber High); (4) Exposure to leachate corresponding to a low concentration of TP (5.2 µg L-1; LEA-L); (5) Exposure to leachate corresponding to the high concentration of TP (52 µg L-1; LEA-H); (6) Exposure to natural particles (diatomite; 52 µg L-1; NAT). Due to the technical limitations, there is a general scarcity of information about the environmental concentrations of rubber debris in aquatic environments. The only known concentrations to which we have referred were only obtained from runoff river sediments (179 mg L−1 sediment) and highway runoff sediments (480 mg g−1 sediment) (reviewed in Arias et al., 2022). Moreover, our tested concentrations were chosen to be very low compared to those used for tires and CRG toxicity (*e.g* 0.5-10 g L-1 Wik and Dave, 2006). For instance, our highest concentration 52 µg L-1 is around 20 times lower than the lowest leachate concentration tested in oyster juveniles (equivalent masses: 0, 1, 10, and 100 μg tire mL−1; Tallec et al., 2022b). The system was set up with three replicate tanks per treatment. To ensure a good circulation of particles inside tanks, we used air bubbling and pressurized the water inflow (*sensu* Sussarellu et al., 2016). Stock solution of TP, leachates and natural particles in sterile 0.2-μm filtered seawater were administered using peristaltic pumps from concentrated TP, leachates or natural particle solutions in order to conduct chronic and continuous exposures. Tween-20 was added in stock solutions to avoid MP aggregating giving a final concentration of 0.0002% in the tanks (that is below the non-toxic concentration, 0.0007% v/v; Khosrovyan & Kahru, 2022). Tween-20 was distributed in all tanks to avoid confounding effects. Every 48 hours, tanks were emptied and cleaned; the first wash water was put into the effluent treatment system.

***Leachates preparation***

Leachates were obtained by the incubation of tire particles in 0.2 µm-filtered and sterile seawater for 14 days in dark condition at room temperature with a constant agitation using an orbital shaker (240 rpm). These conditions were chosen based on published data (Capolupo et al. 2020) to avoid the photooxydation of organic compounds under light exposure and to ensure comparability of our data acquired in oyster (Tallec et al. 2022a,b). At the end of the incubation, leachates were filtered on glass-fiber filter (GF/F; porosity 1.2 µm) to remove tire particles. To avoid degradation of chemicals in the leachate during the exposures, a new and fresh leachate solution was used every day.

***Chemical analyses***

Chemical compounds were assayed on the crude materials, i.e. the stock powder supplied by Edge Rubber (Chambersburg, USA). Organic micropollutants were analysed by a multi-residue method allowing the determination of the following compounds: organochlorine pesticides, organophosphorus pesticides, polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), polybrominated diphenyl ethers (PBDEs) and chlorobenzenes (the list is given in Table S1 below). Molecules were extracted using methanol and a targeted search by gas chromatography coupled with tandem mass spectrometry (GC-MSMS, Agilent 7890 GC system linked to an Agilent 7010 triple quadrupole MS) and identification by MRM (Multiple Reaction Monitoring) spectral mode was carried out. This mass spectrometry technique allows the selective and sensitive quantification of compounds in complex matrices. Briefly, powder was immersed in methanol (24h, long leaching) to extract the pollutants adsorbed on tire particles. The extracted micropollutants were recovered in dichloromethane to allow GC/MSMS injection. In-house standard solutions were used for the determination of the micropollutants. The search for additives was carried out by gas chromatography-tandem mass spectrometry (GC-MSMS, Agilent 7890 GC system linked to an Agilent 7010 triple quadrupole MS) in scan mode. Molecules extraction was performed through a brief leaching (30 s) in methanol and pollutants were covered with dichloromethane. The extracts were then concentrated before injection in Scan mode (non-targeted research). Due to the lack of laboratory standards, only identification (without quantification) was performed.

***Ecophysiological parameters***

*Algal consumption and absorption efficiency*

Algal counts measured every morning allowed to estimate the algal consumption (AC; µm3 day-1 oyster-1) as: AC = *fl* × (I – O) ÷ *n* where *fl* is the flow rate (mL min-1), I and O are the algal concentrations (µm3 µL-1) in the water inflow and outflow, respectively, while *n* is the number of oysters per tank. After 12-, 23-, 32- and 40-days of exposure, 24-hours feces were collected from each tank as well as a sample of the diet (500 mL) to estimate the absorption efficiency (AE; %) of organic matter from the diet using the Conover’s method. Samples were centrifugated at 3000 rpm during 10 minutes to eliminate the supernatant. Cell pellets were rinsed three times with ammonium formiate then centrifugated at 3000 rpm during 10 minutes. Finally, cell pellets were retrieved on foil cup and their weights were measured after 24h at 60°C then 4h at 450°C (Pousse et al., 2020). The AE is calculated as: AE = (*f* – *e*)/((1 – *e*) × *f*), where *f* is the organic fraction of the microalgae ration and *e* is the organic fraction of the feces (Conover, 1966).

*Histological analysis*

Histological analysis was performed on G1 oyster reared in the field by sampling 30 oysters per treatment (10 per oyster bag) at the final sampling in September 2022. For each sample, a 3-mm cross-section of the visceral mass was excised in front of the pericardic region and immediately fixed in modified Davidson’s solution (4°C) for 48h. Sections were dehydrated in ascending ethanol solutions, cleared with histosol and embedded in paraffin wax. Sections of 5 µm were cut, mounted on glass slides and stained with Harry's hematoxylin-Eosin Y. Samples were observed under a light microscope to the sex and gametogenic stage according to the reproductive scale of Steele and Mulcahy (1999) and look for tissue damages. The percentage of the surface occupied by the gonad, compared to the total surface of the visceral mass, was estimated on a median section as described in Fabioux et al. (2005).

***Molecular analyses***

*RNA extraction, RNA quality check and library preparation*

Oocytes’ total RNA was extracted by Extract-All kit (Eurobio Scientific; Les Ulis, France) following the manufacture’s protocol with a further DNase treatment (DNase Maxi Kit; MO BIO Laboratories, Berlin, Germany) and addressed to RNA-sequencing for transcriptomic analysis. Furthermore, filtered seawater (250 ml of water per tank per condition filtrated under vacuum using a 47 mm-diameter glass fiber-filter following Mérou et al. (2020), modified) from each aquarium (3 samples per condition) at T0 and T1 and then stored at -80°C until the RNA extraction performed by the RNeasy PowerWater Kit (Qiagen) used for microbiota characterization (16S).Quality and quantity check of RNA from each animal/water sample was performed using the spectrophotometry (Nanodrop; Thermo Scientific, Villebon-sur-Yvette, France), Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA) and Tape Station (Agilent D1000, Waldbronn, Germany).

Library pools for gene expression analyses were prepared using QuantSeq 3’ mRNA-Seq Library Prep Kit and sequenced on Illumina Novaseq 6000 (CRIBI; University of Padova) (75 bp single-end) obtaining an average yield of 6,836,963 reads per sample (sequences available in NCBI SRA; https://www.ncbi.nlm.nih.gov/sra; BioProject PRJNA856813).

For microbiota analyses, RNA (1 μg) of gills, digestive gland (from the same samples used for RNA-seq analysis) and filtered seawater was reverse-transcribed to cDNA using the Superscript IV kit (Invitrogen, Life Technologies, Monza, Italy). Library construction and sequencing (MiSeq Illumina 300 PE) (BMR Genomics, Padova, Italy) were carried out using primers targeting the V3–V4 gene region as described by Milan et al. (2018).

*Mapping and data normalization (RNA-sequencing)*

Quality check of input reads and bionformatic pipeline for mapping and normalization were performed accordingly to Peruzza et al. (2021). Quality of the input reads was checked through FastQC/v0.11.9 and consequently low-quality reads and residual adaptors were trimmed and removed using BBTools suite of BBDuk program. Mapping of remaining high-quality reads was carried out against the reference genome of *Crassostrea gigas* (Ensembl Metazoa database) by using STAR software. The final raw reads count table obtained was imported into R/v4.1.1, filtered and normalized (using RUVSeq/v1.18 library (Gerstner et al. 2016; Verma et al. 2020)).In detail, raw reads were filtered based on Counts per Million (CPM) by choosing CPM=1 in a minimum number of samples (samples threshold) equal to 6 for gills and digestive gland and 4 for oocytes. Filtered reads were normalized using the RUVs function (parameter “k = 6” for gills and digestive gland, “k=7” for oocytes). Normalized counts were used to perform the Principal Component Analysis (PCA) and pairwise comparisons between CTL and each exposed group (NAT, MR-L, MR-H, LEA-L, LEA-H) with edgeR/v3.26.0 (Robinson et al., 2010) (p-value < 0.05 and FC ≥ |2|). Gene Set Enrichment Analysis (GSEA; Subramanian et al., 2005; Mootha et al., 2003) was performed using the clusterProfiler/v4 library. Hallmark categories and other gene sets (Liberzon et al., 2015) used for the Gene Set Enrichment Analysis are reported here below.

|  |
| --- |
| **Pathway Name** |
| HALLMARK\_PEROXISOME |
| HALLMARK\_APICAL\_JUNCTION |
| HALLMARK\_APICAL\_SURFACE |
| HALLMARK\_ADIPOGENESIS |
| HALLMARK\_PANCREAS\_BETA\_CELLS |
| HALLMARK\_MYOGENESIS |
| HALLMARK\_ANGIOGENESIS |
| HALLMARK\_SPERMATOGENESIS |
| HALLMARK\_EPITHELIAL\_MESENCHYMAL\_TRANSITION |
| HALLMARK\_UV\_RESPONSE\_UP |
| HALLMARK\_DNA\_REPAIR |
| HALLMARK\_UV\_RESPONSE\_DN |
| HALLMARK\_COAGULATION |
| HALLMARK\_INTERFERON\_RESPONSE |
| HALLMARK\_INTERFERON\_RESPONSE |
| HALLMARK\_COMPLEMENT |
| HALLMARK\_IL6\_JAK\_STAT3\_SIGNALING |
| HALLMARK\_INFLAMMATORY\_RESPONSE |
| HALLMARK\_BILE\_ACID\_METABOLISM |
| HALLMARK\_CHOLESTEROL\_HOMEOSTASIS |
| HALLMARK\_XENOBIOTIC\_METABOLISM |
| HALLMARK\_FATTY\_ACID\_METABOLISM |
| HALLMARK\_GLYCOLYSIS |
| HALLMARK\_OXIDATIVE\_PHOSPHORYLATION |
| HALLMARK\_PROTEIN\_SECRETION |
| HALLMARK\_HYPOXIA |
| HALLMARK\_REACTIVE\_OXYGEN\_SPECIES\_PATHWAY |
| HALLMARK\_UNFOLDED\_PROTEIN\_RESPONSE |
| HALLMARK\_APOPTOSIS |
| HALLMARK\_MITOTIC\_SPINDLE |
| HALLMARK\_MYC\_TARGETS\_V1 |
| HALLMARK\_MYC\_TARGETS\_V2 |
| HALLMARK\_P53\_PATHWAY |
| HALLMARK\_G2M\_CHECKPOINT |
| HALLMARK\_E2F\_TARGETS |
| HALLMARK\_WNT\_BETA\_CATENIN\_SIGNALING |
| HALLMARK\_TNFA\_SIGNALING\_VIA\_NFKB |
| HALLMARK\_NOTCH\_SIGNALING |
| HALLMARK\_IL2\_STAT5\_SIGNALING |
| HALLMARK\_ESTROGEN\_RESPONSE |
| HALLMARK\_ESTROGEN\_RESPONSE |
| HALLMARK\_TGF\_BETA\_SIGNALING |
| HALLMARK\_HEDGEHOG\_SIGNALING |
| HALLMARK\_ANDROGEN\_RESPONSE |
| HALLMARK\_MTORC1\_SIGNALING |
| HALLMARK\_PI3K\_AKT\_MTOR\_SIGNALING |

Gene Ontology (GO) and KEGG pathways

|  |
| --- |
| GO\_DNA REPAIR |
| GO\_DNA\_DAMAGE\_RESPONSE\_DETECTION\_OF\_DNA\_DAMAGE |
| GO\_CELLULAR\_RESPONSE\_TO\_DNA\_DAMAGE\_STIMULUS |
| KEGG\_NUCLEOTIDE\_EXCISION\_REPAIR |
| GO\_CELL\_CYCLE |
| GO\_REGULATION OF CELL CYCLE |
| GO\_ATP BIOSYNTHETIC PROCESS |
| GO\_ENERGY\_DERIVATION\_BY\_OXIDATION\_OF\_ORGANIC\_COMPOUNDS |
| GO\_OXIDATIVE\_PHOSPHORYLATION |
| GO\_RESPIRATORY\_CHAIN\_COMPLEX |
| KEGG\_CITRATE\_CYCLE\_TCA\_CYCLE |
| KEGG\_FATTY ACID METABOLISM |
| KEGG\_GLYCOLYSIS-GLUCONEOGENESIS |
| KEGG\_PYRUVATE\_METABOLISM |
| HALLMARK\_XENOBIOTIC\_METABOLISM |
| KEGG\_ABC\_TRANSPORTERS |
| KEGG\_DRUG\_METABOLISM\_CYTOCHROME\_P450 |
| KEGG\_DRUG\_METABOLISM\_OTHER\_ENZYMES |
| GO\_SULFOTRANSFERASE\_ACTIVITY |
| GO\_REGULATION\_OF\_REPRODUCTIVE\_PROCESS |
| GO\_RESPONSE\_TO\_ESTROGEN |
| HALLMARK\_SPERMATOGENESIS |
| GO\_ORGAN DEVELOPMENT |
| KEGG\_PATHWAYS\_IN\_CANCER |
| GO\_ENDOSOME |
| GO\_CELLULAR\_RESPONSE\_TO\_EXTERNAL\_STIMULUS |
| GO\_CHROMATIN |
| GO\_ION\_TRANSMEMBRANE\_TRANSPORT |
| GO\_MACROAUTOPHAGY |
| GO\_NUCLEOSOME\_ORGANIZATION |
| GO\_PROTEIN\_FOLDING |
| GO\_REGULATION\_OF\_CELLULAR\_RESPONSE\_TO\_STRESS |
| GO\_RESPONSE\_TO\_ENDOPLASMIC\_RETICULUM\_STRESS |
| GO\_RESPONSE\_TO\_OXIDATIVE\_STRESS |
| GO\_RIBOSOME |
| GO\_TRANSPORT\_VESICLE |
| GO\_GOLGI\_APPARATUS |
| GO\_ION\_HOMEOSTASIS |
| KEGG\_AMINOACYL\_TRNA\_BIOSYNTHESIS |
| KEGG\_ECM\_RECEPTOR\_INTERACTION |
| KEGG\_FOCAL\_ADHESION |
| KEGG\_LYSOSOME |
| KEGG\_PPAR\_SIGNALING\_PATHWAY |
| KEGG\_PROTEASOME |
| KEGG\_UBIQUITIN\_MEDIATED\_PROTEOLYSIS |
| GO\_PROTEOLYSIS |
| GO\_RESPONSE\_TO\_OXIDATIVE\_STRESS |
| GO\_RNA processing |
| GO\_SPLICEOSOME |
| GO\_REGULATION\_OF\_NEUROTRANSMITTER\_LEVELS |
| GO\_CHOLINERGIC\_SYNAPSE |
| GO\_GABA\_ERGIC\_SYNAPSE |
| GO\_EXCITATORY\_SYNAPSE |
| GO\_GLUTAMATERGIC\_SYNAPSE |
| GO\_SYNAPSE |
| GO\_INHIBITORY\_SYNAPSE |
| GO\_AUTOPHAGIC\_CELL\_DEATH |
| GO\_DEATH\_INDUCING\_SIGNALING\_COMPLEX |
| GO\_INTRINSIC\_APOPTOTIC\_SIGNALING\_PATHWAY |
| GO\_REGULATION\_OF\_CELL\_DEATH |
| KEGG\_APOPTOSIS |
| GO\_CANONICAL\_WNT\_SIGNALING\_PATHWAY |
| KEGG\_NOD\_LIKE\_RECEPTOR\_SIGNALING\_PATHWAY |
| KEGG\_NOTCH\_SIGNALING\_PATHWAY |
| KEGG\_P53\_SIGNALING\_PATHWAY |
| KEGG\_WNT\_SIGNALING\_PATHWAY |
| KEGG\_TOLL\_LIKE\_RECEPTOR\_SIGNALING\_PATHWAY |
| GO\_ACTIVATION\_OF\_IMMUNE\_RESPONSE |
| GO\_DEFENSE\_RESPONSE\_TO\_VIRUS |
| GO\_DEFENSE\_RESPONSE\_TO\_VIRUS |
| GO\_INNATE\_IMMUNE\_RESPONSE |
| GO\_IMMUNE\_RESPONSE |
| GO\_IMMUNE\_SYSTEM\_PROCESS |
| KEGG\_ARACHIDONIC\_ACID\_METABOLISM |
| GO\_RESPONSE\_TO\_BACTERIUM |
| GO\_WOUND\_HEALING |

*Microbiota bioinformatic analysis*

Bioinformatic analyses were performed as described in Bernardini et al. (2022). Raw reads from microbiome sequencing were uploaded in QIIME 2 (Quantitative insights into microbial ecology; Bolyen et al., 2019). Primer sequences and low-quality reads were removed and filtered using cutadapt and DADA2 (Callahan et al., 2016) respectively and then reads merging and removal of chimeric fragments were performed. Representative sequence alignment was achieved using MAFFT software (Katoh and Standley, 2013) and then Python library Scikit-Learn was employed for their classification. Taxa assignment was carried out using the SILVA database (132 update release). Microbiome Analyst software was adopted to perform statistical analyses (Chong et al., 2020). Samples were organized through Principal Coordinate Analysis using Bray-Curtis distance at OTU level and Diversity Indexes (Chao1, Simpson’s and Shannon’s Index) were calculated. Pairwise comparisons between treatments (CTL vs NAT, MR-L, MR-H, LEA-L, and LEA-H) and the control group were performed using DESeq2 method to identify over- and under- represented taxa (FDR < 0.05).

***Gametes quality***

In G0 and G1 oysters, gametes from 12 males and 12 females per treatment were collected by stripping and sieving on 100 µm-mesh the gametes which were then separated in four pools (3 males or 3 females per pool). Gamete concentrations were estimated by flow cytometry (EasyCyte Plus cytometer; Guava Merck Millipore) and adjusted with 1-µm filtered seawater (SW) at 108 spermatozoa mL-1 and 105 oocytes mL-1. Spermatozoa and oocyte were incubated separately in SW for 45 minutes before sampling to assess gamete quality.

Mitochondrial function was assessed on G1 oocytes following the analysis of ATP levels and mitochondrial content. Oocytes were sampled (20 µm strain) and transferred to filtered seawater (0.2 µm) at a concentration of 6,600 gametes /ml. During the sampling and transfer processes, samples were stored on ice. The mitochondrial metabolism was assessed on G1 oocytes by the analysis of ATP levels (per oocyte) and mitochondrial content (per larvae). For ATP levels, the samples were gently resuspended, and 330 gametes were transferred to a 96-well solid white plate, followed by the addition of 50 µl of CellTiter GLO 3D kit (PROMEGA G7570). After shaking for 2 minutes to promote cellular lysis, the samples were incubated at room temperature and in the dark for 15 minutes to equilibrate the assay temperature. Luminescence was measured using a PerkinElmer EnSpire plate reader, and ATP levels were determined following the standard curve ranging from 1 to 5000 nM. For G2 D-larvae mitochondrial metabolism assessment, 6,600 G2 D-larvae were gently resuspended and fixed in 200 µl of 4% paraformaldehyde solution to be stored at 4°C. Later, 330 larvae (20 µl) were transferred to 96-well black plates, followed by the addition of 80 µl of filtered seawater and MitoID Green (Enzo Life Sciences) (final dilution of 500x), which stains the mitochondria in live of fixed cells regardless of mitochondrial membrane potential. Larvae were incubated for 1 hour in the dark and analyzed for MitoID green fluorescence at a TECAN plate reader at 460/560nm.

***Fertilization, embryo-larval development and settlement (G1, G2)***

*Settlement behavior of larvae*

At 15 dpf, eyed-larvae were sampled to compare the settlement behavior among treatments. A total of three settlement conditions were tested: (1) Seawater, (2) Seawater + oyster (*C. gigas*) fragrance, (3) Seawater + predator (*Ocenebra erinaceus*) fragrance. These conditions were tested as oyster larvae are sensitive to waterborne chemical cues during the settlement process (Turner et al., 1994; Zimme-Faust and Tamburri, 1994) and allowed to test potential variability in the response to chemical cues following parental exposure to micro-rubber and leachates.

To prepare the fragrance, 450 mL of crushed *C. gigas* or *O. erinaceus* (without the shell for both species) were mixed with 550 mL of UV-treated and filtered SW. Then, glass slides (2.5 cm²) were immerged on their respective solutions for 24h at 4°C. After a quick rinse with filtered SW, the glass slides were further placed in petri dish (12.6 cm²) filled with FSW (25°C; 7 mL) and ≈100 eyed-larvae for 24h under constant light. Three petri dishes were used per chemical cue and per treatment (3 petri dishes × 3 cue conditions x 6 treatments). The percent of crawling larvae, i.e. the percent of larvae at the bottom exploring the substrate to settle and not the swimming larvae was estimated in each petri dish by scan sampling for 30 seconds.

***Results***

***Tables***

***T******able S1.*** *Chemical analyses performed on tire powder. LOQ= Limits of quantification.*

|  |  |  |
| --- | --- | --- |
| **Chemicals** | **LOQ (µg kg-1)** | **Values (µg kg-1)** |
| Acenaphtene | 20 | <20 |
| Acenaphtylene | 20 | 198 |
| Anthracene | 20 | 688 |
| Benzanthracene | 50 | 1024 |
| Benzo(a)pyrene | 60 | 1078 |
| Benzo(b)fluoranthene | 60 | 1269 |
| Benzo(ghi)perylene | 100 | 5582 |
| Benzo(k)fluoranthene | 60 | 446 |
| Chrysene | 50 | 3175 |
| Dibenzanthracene | 100 | 112 |
| Fluoranthene | 40 | 5481 |
| Fluorene | 20 | 311 |
| Indenopyrene | 100 | 740 |
| Naphtalene | 100 | 811 |
| Phenanthrene | 20 | 7025 |
| Pyrene | 40 | 13865 |
| PCB101 | 3 | 4 |
| PCB105 | 3 | <3 |
| PCB118 | 3 | 4 |
| PCB138 | 6 | <6 |
| PCB153 | 6 | <6 |
| PCB156 | 6 | <6 |
| PCB180 | 3 | <3 |
| PCB205 | 6 | <6 |
| PCB207 | 20 | <20 |
| PCB28 | 10 | <10 |
| PCB52 | 2 | 7 |

**Table S2**. Length (mm), dry mass (g) and absorption efficiency (%) of adult oysters after 44 days of exposure to: (1) Control – CTL; (2) Natural particles (52 µg L-1) – NAT; (3) Tire particles low (5.2 µg L-1, 10 particles mL-1) – MR-L; (4) Tire particles high (52 µg L-1, 100 particles mL-1) – MR-H; (5) Leachate low (5.2 µg L-1) – LEA-L; (6) Leachate high (52 µg L-1) – LEA-H. One-way ANOVA was conducted to compare treatments at the 5% level; n = 6 per treatment for length and dry weight; n = 12 for the absorption efficiency.

|  |  |  |  |
| --- | --- | --- | --- |
| **Treatment** | **Length (mm)** | **Dry weight (g)** | **Absorption efficiency (%)** |
| CTL | 62.1 ± 4.5 | 1.26 ± 0.29 | 61 ± 12 |
| NAT | 63.7 ± 4.1 | 1.13 ± 0.30 | 62 ± 19 |
| MR-L | 61.5 ± 3.4 | 1.35 ± 0.10 | 65 ± 15 |
| MR-H | 58.4 ± 2.5 | 1.18 ± 0.21 | 61 ± 18 |
| LEA-L | 59.2 ± 2.6 | 1.21 ± 0.31 | 58 ± 19 |
| LEA-H | 59.5 ± 2.6 | 1.29 ± 0.18 | 59 ± 15 |

**Table S3.** Number of differentially expressed genes obtained in each pairwise comparison between control (CTL) and exposed oysters. Down and over mean down- and over-regulated.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Treated vs CTL** | | | | | | | | |
|  | **Digestive gland** | | | **Gills** | | | **Oocytes** | | |
|  | TOT | Down | Over | TOT | Down | Over | TOT | Down | Over |
| NAT | 0 | 0 | 0 | 16 | 6 | 10 | 580 | 452 | 128 |
| MR-L | 2 | 0 | 2 | 7 | 1 | 6 | 488 | 375 | 113 |
| MR-H | 26 | 9 | 17 | 2 | 2 | 0 | 553 | 436 | 117 |
| LEA-L | 3 | 0 | 3 | 3 | 1 | 2 | 667 | 439 | 228 |
| LEA-H | 21 | 3 | 18 | 19 | 8 | 11 | 401 | 283 | 118 |

**Table S4.** Number of differently represented taxa (species and genus level) in treatments compared to the control.Down and over mean down- and over-represented.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Species** | | | | | | | | |
|  | **Digestive gland** | | | **Gills** | | | **Filtered water** | | |
|  | TOT | Down | Over | TOT | Down | Over | TOT | Down | Over |
| NAT | 1 | 0 | 1 | 4 | 3 | 1 | 0 | 0 | 0 |
| MR-L | 1 | 0 | 1 | 1 | 1 | 0 | 14 | 9 | 5 |
| MR-H | 2 | 1 | 1 | 4 | 2 | 2 | 1 | 0 | 1 |
| LEA-L | 3 | 1 | 2 | 3 | 0 | 3 | 0 | 0 | 0 |
| LEA-H | 5 | 0 | 5 | 2 | 1 | 1 | 1 | 0 | 1 |
|  | **Genus** | | | | | | | | |
|  | **Digestive gland** | | | **Gills** | | | **Filtered water** | | |
|  | TOT | Down | Over | TOT | Down | Over | TOT | Down | Over |
| NAT | 3 | 1 | 2 | 3 | 1 | 2 | 0 | 0 | 0 |
| MR-L | 2 | 1 | 1 | 5 | 2 | 3 | 23 | 10 | 13 |
| MR-H | 4 | 4 | 0 | 2 | 1 | 1 | 4 | 3 | 1 |
| LEA-L | 5 | 3 | 2 | 5 | 4 | 1 | 4 | 1 | 3 |
| LEA-H | 3 | 0 | 3 | 1 | 1 | 0 | 1 | 1 | 0 |

**Table S5.** Behavior of larvae (G1) (2 dpf) issued from control genitors (CTL), genitors exposed to diatomite (52 µg L-1; NAT), tire particles at low dose (5.2 µg L-1; MR-L), tire particles at high dose (52 µg L-1; TP-H), leachate at low dose (5.2 µg L-1; LEA-L) or leachate at high dose (52 µg L-1; LEA-H). One-way ANOVA was conducted to compare treatments at the 5% level; n = 3.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Treatments** | **Activity (%)** | **Active duration (sec)** | **Distance traveled (mm)** | **Velocity (mm sec-1)** |
| CTL | 96.1 ± 2.3 | 34.8 ± 9.8 | 16.0 ± 5.1 | 0.39 ± 0.10 |
| NAT | 97.0 ± 1.7 | 38.9 ± 7.4 | 17.5 ± 3.3 | 0.34 ± 0.12 |
| MR-L | 97.0 ± 3.3 | 43.0 ± 7.2 | 19.4 ± 3.1 | 0.28 ± 0.11 |
| MR-H | 93.0 ± 3.8 | 36.6 ± 10.2 | 16.1 ± 4.8 | 0.28 ± 0.06 |
| LEA-L | 97.3 ± 2.5 | 42.3 ± 16.2 | 18.7 ± 7.7 | 0.30 ± 0.07 |
| LEA-H | 96.3 ± 4.9 | 38.9 ± 18.2 | 20.1 ± 10.7 | 0.35 ± 0.12 |

**Table S6.** Behavior of larvae (G1) (15 dpf) issued from control genitors (CTL), genitors exposed to diatomite (52 µg L-1; NAT), tire particles at low dose (5.2 µg L-1; MR-L), tire particles at high dose (52 µg L-1; MR-H), leachate at low dose (5.2 µg L-1; LEA-L) or leachate at high dose (52 µg L-1; LEA-H). Three type of substrate were tested: (1) Without fragrance, (2) Predator’ fragrance, (3) Oyster fragrance. One-way ANOVA was conducted to compare treatments at the 5% level; n = 3.

|  |  |  |  |
| --- | --- | --- | --- |
| **Treatments** | **Without fragrance** | **Predator + fragrance** | **Oyster fragrance** |
| CTL | 38.8 ± 8.7 | 76.2 ± 12.4 | 65.9 ± 2.0 |
| NAT | 33.8 ± 9.0 | 67.8 ± 19.7 | 58.1 ± 31.4 |
| MR-L | 38.6 ± 10.6 | 67.7 ± 13.8 | 74.7 ± 18.4 |
| MR-H | 43.3 ± 6.9 | 76.1 ± 16.2 | 61.7 ± 10.8 |
| LEA-L | 40.1 ± 7.2 | 60.1 ± 5.8 | 64.7 ± 13.8 |
| LEA-H | 43.2 ± 17.2 | 80.1 ± 9.2 | 61.5 ± 16.0 |

**Table S7**. Cumulative mortality (%), individual growth (total weight, drained meat weight, shell weight, shell length) and reproductive effort (i.e. the area occupied by the gonad = mean percentage surface occupied by the gonad / total area of the visceral mass ± standard deviation) estimated after 10 months in situ rearing of the first G1 generation obtained after experimental reproduction of G0 parental oysters exposed to (1) No treatment – CTL; (2) Natural particles (52 µg L-1) – NAT; (3) tire particles low (5.2 µg L-1, 10 particles mL-1) – MR-L; (4) tire particles high (52 µg L-1, 100 particles mL-1) – MR-H; (5) Leachate low (5.2 µg L-1) – LEA-L; (6) Leachate high (52 µg L-1) – LEA-H. \* = p-value < 0.05; n = 3.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Treatment** | **Cumulative mortality (%)** | **Total weight (g)** | **Drained meat weight (g)** | **Shell weight (g)** | **Shell length (mm)** | **Reproductive effort** |
| CTL | 25.7 ± 6.3 | 47.9 ± 12.9 | 11.58 ± 3.46 | 28.9 ± 8.1 | 77.2 ± 11.1 | 50.6 ± 16.0 |
| NAT | 25.3 ± 13.6 | 46.2 ± 11.6 | 11.81 ± 3.13 | 27.1 ± 7.2 | 72.1 ± 8.6 | 52.5 ± 12.3 |
| MR-L | 36.3 ± 10.2 | 44.4 ± 11.9 | 9.87 ± 3.62 | 26.7 ± 7.0 | 73.5 ± 6.4 | 52.4 ± 8.1 |
| MR-H | 17.3 ± 12.1 | 43.6 ± 8.5 | 10.25 ± 2.01 | 25.1 ± 5.0 | 75.8 ± 8.6 | 53.6 ± 7.2 |
| LEA-L | 28.0 ± 6.6 | 42.6 ± 17.7 | 10.41 ± 4.74 | 25.5 ± 10.3 | 72.9 ± 15.1 | 58.4 ± 9.8 |
| LEA-H | 20.3 ± 16.0 | 41.5 ± 9.6 | 9.29 ± 2.23 | 25.9 ± 6.5 | 72.3 ± 7.2 | 53.0 ± 9.7 |

**Table S8**. Distribution of sex after 10 months in situ rearing of the first G1 generation obtained after experimental reproduction of G0 parental oysters exposed to (1) No treatment – CTL; (2) Natural particles (52 µg L-1) – NAT; (3) tire particles low (5.2 µg L-1, 10 particles mL-1) – MR-L; (4) tire particles high (52 µg L-1, 100 particles mL-1) – MR-H; (5) Leachate low (5.2 µg L-1) – LEA-L; (6) Leachate high (52 µg L-1) – LEA-H.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  |  | Sex | | |
|  | n | female  % | male  % | hermaphrodite  % |
| CTL | 15 | 66.7 | 33.3 | 0 |
| NAT | 14 | 85.7 | 7.1 | 7.1 |
| MR-H | 15 | 53.3 | 456.7 | 0 |
| MR-L | 15 | 66.7 | 33.3 | 0 |
| LEA-H | 15 | 86.7 | 13.3 | 0 |
| LEA-L | 15 | 86.7 | 13.3 | 0 |

n = number of individuals analyzed.

**Table S9**. *Matrix of P values over all the 6 conditions for distribution of sex using Fisher exact tests (above diagonal), with associated statistical significance (below diagonal) after 10 months in situ rearing of the first G1 generation obtained after experimental reproduction of G0 parental oysters exposed to (1) No treatment – CTL; (2) Natural particles (52 µg L-1) – NAT; (3)* tire particles *low (5.2 µg L-1, 10 particles mL-1) – MR-L; (4)* tire particles *high (52 µg L-1, 100 particles mL-1) – MR-H; (5) Leachate low (5.2 µg L-1) – LEA-L; (6) Leachate high (52 µg L-1) – LEA-H.*

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | CTL | NAT | MR-H | MR-L | LEA-H | LEA-L |
| CTL |  | 0.1727 | 0.4621 | 1.0 | 0.3898 | 0.3898 |
| NAT |  |  | 0.0157 | 0.1727 | 1.0 | 1.0 |
| MR-H |  | \* |  | 0.4621 | 0.0502 | 0.0502 |
| MR-L |  |  |  |  | 0.3898 | 0.3898 |
| LEA-H |  |  |  |  |  | 1.0 |
| LEA-L |  |  |  |  |  |  |

\* Values significant at the P < 0.05 level.

**Table S10**. Percentage G1 motile spermatozoa (%), sperm velocity (VAP; µm sec-1), oocyte diameter (µm) when circularity>0.8, fertilization yield (%), and D-larval yield (%) of the second G2 generation obtained after experimental reproduction of the G1 generation obtained from the G0 parental oysters exposed to (1) No treatment – CTL; (2) Natural particles (52 µg L-1) – NAT; (3) tire particles low (5.2 µg L-1, 10 particles mL-1) – MR-L; (4) tire particles high (52 µg L-1, 100 particles mL-1) – MR-H; (5) Leachate low (5.2 µg L-1) – LEA-L; (6) Leachate high (52 µg L-1) – LEA-H. \* = p-value < 0.05; n = 3.

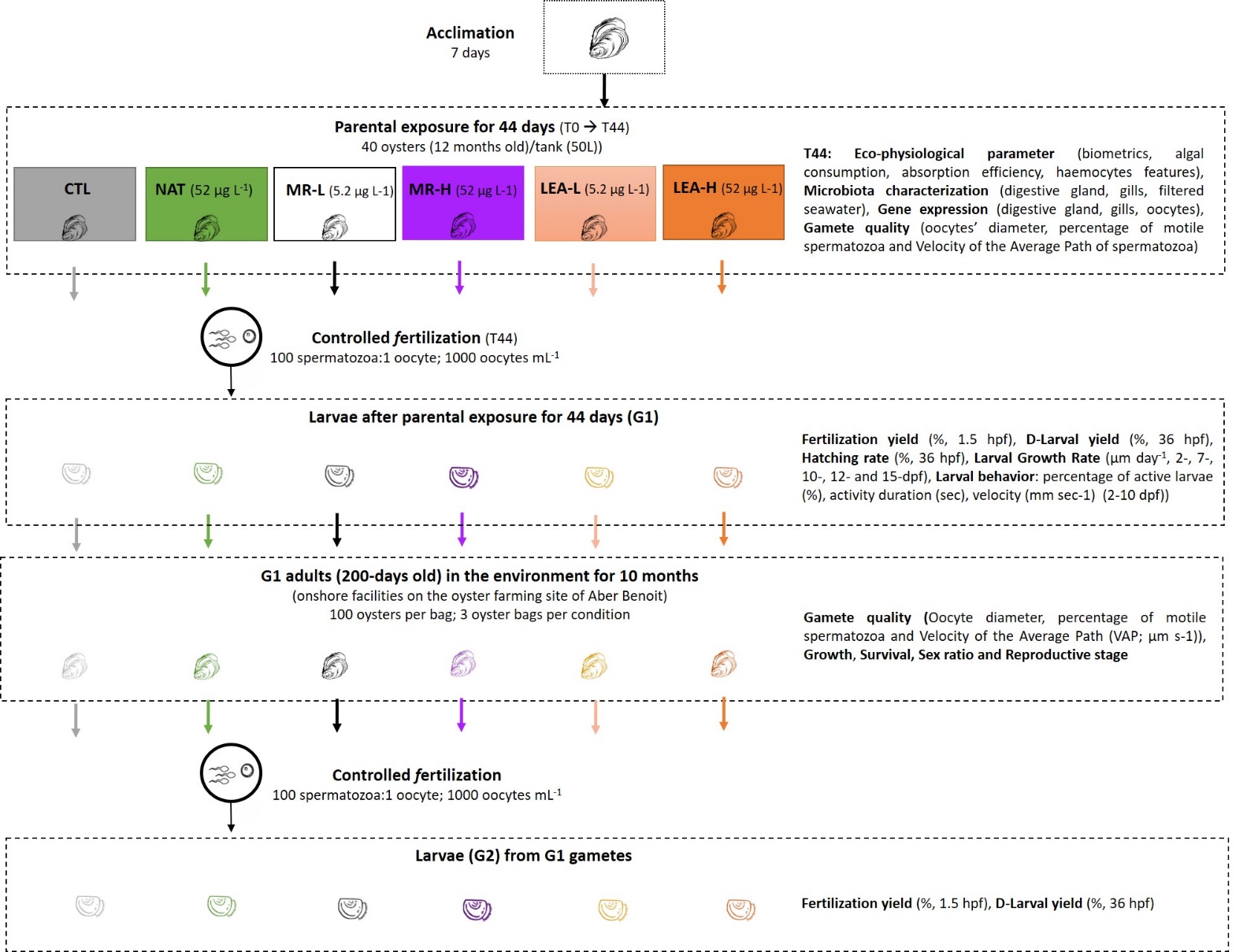
|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Treatment** | **Sperm motility (%)** | **VAP**  **(µm sec-1)** | **Oocyte Diameter (µm)** | **Fertilization Yield (%)** | **D-Larval Yield (%)** | **D-larval size (µm)** |
| CTL | 21.1 ± 14.14 | 79.5 ± 15.06 | 58.1 ± 3.56 | 96.4 ± 0.8 | 74.8 ± 11.9 | 78.7 ± 2.92 |
| NAT | 16.2 ± 10.82 | 66.9 ± 2.37 | 60.7 ± 3.76 | 86.2 ± 3.0 | 65.2 ± 3.0 | 76.1 ± 3.8 |
| MR-L | 22.6 ± 10.34 | 71.7 ± 1.61 | 60.2 ± 3.68 | 94.8 ± 3.6 | 71.9 ± 15.7 | 76.8 ± 3.7 |
| MR-H | 17.6 ± 10.41 | 72.0 ± 14.95 | 61.3 ± 4.01 | 96.3 ± 1.4 | 67.5 ± 12.6 | 76.7 ± 3.3 |
| LEA-L | 15.3 ± 6.93 | 69.3 ± 12.57 | 64.9 ± 13.09 | 93.6 ± 3.4 | 70.6 ± 14.5 | 77.8 ± 3.7 |
| LEA-H | 16.1 ± 6.74 | 70.9 ± 11.35 | 63.5 ± 12.56 | 97.2 ± 1.6 | 80.9 ± 15.6 | 76.8 ± 3.7 |

**Table S11**. ATP content (fmol 10−12 oocyte) of G1 oocytes obtained after experimental reproduction of G0 parental oysters exposed to (1) No treatment – CTL; (2) Natural particles (52 µg L-1) – NAT; (3) TP low (5.2 µg L-1, 10 particles mL-1) – TP-L; (4) TP high (52 µg L-1, 100 particles mL-1) – TP-H; (5) Leachate low (5.2 µg L-1) – LEA-L; (6) Leachate high (52 µg L-1) – LEA-H, and ATP content (fmol 10−12 D-larvae) of G2 D-larvae after experimental reproduction of G1 oysters. \* = p-value < 0.05; n = 3.

|  |  |  |
| --- | --- | --- |
| **Treatment** | **ATP content (fmol 10−12 G1 oocyte)** | **ATP content (fmol 10−12 G2 D-larvae)** |
| CTL | 378.0 ± 28.8 | 35.3 ± 18.2 |
| NAT | 382.0 ± 64.5 | 41.9 ± 11.3 |
| TP-L | 316.0 ± 10.8 | 27.1 ± 17.6 |
| TP-H | 338.7 ± 69.7 | 43.3 ± 7.6 |
| LEA-L | 446.2 ± 115.2 | 25.8 ± 13.9 |
| LEA-H | 333.9 ± 40.0 | 21.8 ± 13.9 |

***Figures***

***Figure S1. Experimental plan***



***Figure S2.*** *Live cells (%; A), ROS production (A.U.; B) and MXR activity (A.U.; C) of oyster hemocytes after 44 days of exposure to: (1) No treatment – CTL; (2) Natural particles (52 µg L-1) – NAT; (3) Tire particles low (5.2 µg L-1, 10 particles mL-1) – MR-L; (4) Tire particles high (52 µg L-1, 100 particles mL-1) – MR-H; (5) Leachate low (5.2 µg L-1) – LEA-L; (6) Leachate high (52 µg L-1) – LEA-H. One-way ANOVA was conducted to compare treatments at the 5% level; n = 5-6 oysters par treatment.*



***Figure S3.*** *Principal Coordinates Analysis (PCoA) of digestive gland (A), gills (B) and filtered seawater (C). For each tissue PCoA were performed indicating i) samples of digestive gland of oysters collected before the treatments (T0) and after 44 days of exposure; ii) samples of control group (CTRL), treated with natural particles (NAT), low and high concentration of micro-rubber (MR-L, MR-H), low and high concentration of leachates (LEA-L, LEA-H) collected after 44 days of exposure.*

**A) Principal Coordinates Analysis - Digestive gland**

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**Day 44**

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F:\Dottorato\Esperimento TOYOTA\paper\immagini\PCoA_T0_microbiota.tif**B) Principal Coordinates Analysis – Gills**

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**Day 44**

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C) **Principal Coordinates Analysis – Filtered seawater**

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**ii)**

**Day 44**

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***Figure S4.*** *Graphical representation of Chao1, Shannon and Simpson’s Index for microbial diversity in digestive gland (A), gills (B) and filtered seawater (C).*

**A) Chao1, Shannon and Simpson Indices - Digestive gland**

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**B) Chao1, Shannon and Simpson Indices - Gills**

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**C) Chao1, Shannon and Simpson Indices – Filtered seawater**

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