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Seasonal dynamics of the microbiome-host response to pharmaceuticals and pesticides in *Mytilus galloprovincialis* farmed in the Northwestern Adriatic Sea

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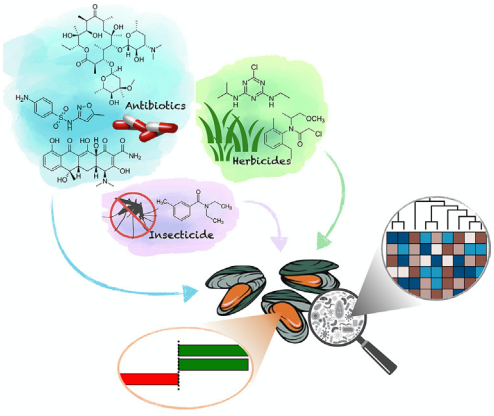
HIGHLIGHTS

- Farmed mussels are exposed to a complex and dynamic pool of emerging contaminants.
- Summer is associated with the highest level of exposure to contaminants.
- *M. galloprovincialis* transcriptional response is affected by pollutant exposure.
- Mussel digestive gland microbiome is key in pollutants degradation and resistance.

GRAPHICAL ABSTRACT



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ABSTRACT

Marine mussels, especially *Mytilus galloprovincialis*, are well-established sentinel species, being naturally resistant to the exposure to multiple xenobiotics of natural and anthropogenic origin. Even if the response to multiple xenobiotic exposure is well known at the host level, the role of the mussel-associated microbiome in the animal response to environmental pollution is poorly explored, despite its potential in xenobiotic detoxification and its important role in host development, protection, and adaptation. Here, we characterized the microbiome-host integrative response of *M. galloprovincialis* in a real-world setting, involving exposure to a complex pattern of emerging pollutants, as occurs in the Northwestern Adriatic Sea. A total of 387 mussel individuals from 3 commercial farms, spanning about 200 km along the Northwestern Adriatic coast, and in 3 different seasons, were collected. Multiresidue analysis (for quantitative xenobiotic determination), transcriptomics (for host physiological response), and metagenomics (for host-associated microbial taxonomical and functional features) analyses were performed on the digestive glands. According to our findings, *M. galloprovincialis* responds to the presence of the complex pattern of multiple emerging pollutants – including the antibiotics sulfamethoxazole, erythromycin, and tetracycline, the herbicides atrazine and

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metolachlor, and the insecticide N,N-diethyl-m-toluamide – integrating host defense mechanisms, *e.g.*, through upregulation of transcripts involved in animal metabolic activity, and microbiome-mediated detoxification functions, including microbial functionalities involved in multidrug or tetracycline resistance. Overall, our data highlight the importance of the mussel-associated microbiome as a strategic player for the orchestration of resistance to the multixenobiotic exposure at the holobiont level, providing strategic functionalities for the detoxification of multiple xenobiotic substances, as occurring in real world exposure settings. Complementing the host with microbiome-dependent xenobiotic degradative and resistance genes, the *M. galloprovincialis* digestive gland associated microbiome can have an important role in the detoxification of emerging pollutants in a context of high anthropogenic pressure, supporting the relevance of mussel systems as potential animal-based bioremediation tool.

1. Introduction

Due to a plethora of human activities, even exacerbated under the climate change scenario, coastal marine ecosystems are currently suffering from multiple and pressing threats, compromising the provision of important ecosystem services for human and planet health (Crain et al., 2009; Frapiccini et al., 2020). Levels of aquatic pollution in coastal areas are increasing worldwide, including metals (Fazio et al., 2014; Aliko et al., 2018), radioactive compounds (Faggio et al., 2018), oil pollution (Frapiccini et al., 2018; de Giovanni et al., 2022), fertilizers and pesticides (Grilli et al., 2020; Cabral et al., 2019) and emerging pollutants, such as micro and nano plastics (Peng et al., 2017; Barboza et al., 2018), pharmaceuticals for human and veterinary use, cosmetics, and personal care products deriving from farms and municipal wastewaters (Fabbri and Franzellitti, 2016; Noguera-Oviedo and Aga, 2016).,

Marine mussels are highly homeostatic systems, naturally resistant to the exposure of multiple xenobiotic substances of natural and anthropogenic origin and, thus, colonizing habitats suffering extreme anthropogenic threats (Silva Dos Santos et al., 2022; Martínez-Escauriaza et al., 2021). This makes mussels as the ideal sentinel species for the biomonitoring the quality of costal marine environments (Viarengo et al., 2007; Provenza et al., 2022). The *M. galloprovincialis* response to the multiple xenobiotic exposure is well known at the host level, for what concerns the molecular, cellular, and even behavioral response (Silvestre, 2020; Franzellitti et al., 2020), however, to the best of our knowledge, little is known about the possible functional tole of the mussel-associated microbiome. The importance of considering marine organisms as holobionts, and thus as metaorganisms sharing a close symbiosis with an associated microbial community, has been stressed (Trevathan-Tackett et al., 2019; Wilkins et al., 2019; Dittami et al., 2021). In this framework, several research has recently been conducted on the mussel microbiome, demonstrating its relevance for different aspects inherent in host biology, such as development, protection, and acclimatization to multiple stressors (Lokmer and Mathias Wegner, 2015; Meisterhans et al., 2016; Musella et al., 2020; Wathsala et al., 2021). In particular, it has been suggested that the digestive gland (DG)-associated microbiota may affect host responses to environmental pollution, even if the mechanisms still need to be elucidated (Milan et al., 2018). For instance, as observed for pesticides resistant bean bugs (Kikuchi et al., 2012), the mussels may leverage on associated microbes to acquire new traits that are adaptive in the local environment (Henry et al., 2021). In this context, we believe that, for a full understanding of the mussel responses to environmental pollution, as well as other abiotic or biotic stressors, its associated microbiome cannot be ignored. On the other hand, through an integrative microbiome-host assessment at the holobiont level, it is possible to provide a holistic overview of chemical effects on animal biology and health, also in the prospective to integrate microbiome factors into the environmental risk assessment.

In the present study, we integrate multiresidue chemical analysis, transcriptomics and metagenomics to assess the level of real-world exposure to pharmaceuticals and pesticides, and the consequent microbiome-host integrative response in the Mediterranean mussel (*Mytilus galloprovincialis*) collected from commercial farms in the Northwestern Adriatic Sea. The Northwestern Adriatic Sea is characterized by shallow waters and the influence of several river inputs, mainly the Po, Adige and Brenta (Rampazzo et al., 2013). Riverine freshwater supplies contribute to the high inflow of inorganic nutrients and organic matter along the Northwestern Adriatic coast (Mangoni et al., 2008; Marini et al., 2015), thus making this area particularly suitable for mussel farming, which accounts for 50 % of the Italian mussel production (Prioli, 2006). The peculiar geochemical and hydrodynamic features make the Northwestern Adriatic a remarkable model of a marine system dynamically influenced by complex anthropogenic pressures, including the massive release of chemical pollutants from the industrial and highly urbanized inlands of the North Italy (Danovaro, 2003; Zuccato et al., 2005; Zuccato et al., 2006), resulting in a widespread contamination by metals, polyaromatic hydrocarbon (PAHs), pesticides, and, more recently, microplastics and pharmaceuticals (Bajt et al., 2019; Combi et al., 2016; Frapiccini et al., 2018; Strafella et al., 2019; Mezzelani et al., 2020). The subclasses of chemicals analyzed in this study belong to contaminants as antibiotics (sulfonamides, macrolides, tetracyclines), a psychotropic drug (carbamazepine), insect repellants (N,N-Diethyl-meta-toluamide, DEET) and herbicides (chloroacetanilide, atrazine), and were selected based on their extensive worldwide use, legal status and/or known persistence (Aminot et al., 2019; Baralla et al., 2021; Chiţescu et al., 2021). The occurrence of some these chemicals in the North Adriatic Sea has been documented. For example, the antibiotics erythromycin and sulfamethoxazole are reported at concentration as high as 4.1 ng/L and 5.8 ng/L, respectively (Nödler et al., 2014), whereas different tetracycline compounds were measured in several bivalve species up to 125 ng/g d.w. (Chiesa et al., 2018). DEET, which is the most used pesticide and insect repellant for topical use and the active ingredient of about 505 commercial products, was detected in marine water samples 16 km offshore from Venice (Italy), at concentrations ranging from 0.3 to about 5 ng/L (Loos et al., 2013). In the same samples, metalochlor was detected at 0.3–2.8 ng/L concentrations range (Readman et al., 1993; Loos et al., 2013). Despite its EU-wide ban in 2004 (EC, 2004) and the consequent reduction of its environmental burden (Nödler et al., 2013), atrazine may be still detected in NW Adriatic coastal waters at concentrations up to 1.5 ng/L (Nödler et al., 2014).

Generally, in the highly anthropized Adriatic coast, the large consumption of human and veterinary medicines and the inadequate presence and typology of wastewater treatment plants can explain their relevant release in the coast waters, trough the riverine inputs and runoff, resulting in the observed contamination with multiple pharmaceuticals (Mezzelani et al., 2020). On the other hands, herbicides of the chloroacetanilide and triazine groups are widely used in agriculture (Gutiérrez-Martín et al., 2023), being transported to the sea through surface (Boithias et al., 2011) and groundwaters (Businelli et al., 2000). It has been estimated that 1 % of the applied herbicides can reaches watercourses, however during heavy rain events this percentage can increase up to 60 % (Boithias et al., 2011).

In this study mussels were collected from 3 different commercial farms spanning about 200 km along the Northwestern Adriatic coast from the Po Delta southward and in 3 different seasons, autumn, spring, and summer and mussel digestive glands (DGs) were evaluated in parallel for the presence of the selected pharmaceuticals and pesticides and the corresponding microbiome-host responses, implementing transcriptomics and metagenomics. The digestive gland has been described as an ideal target for estimating the impact of xenobiotics on mussel's health because of its sensitivity to environmental perturbations, due to its involvement not

only in digestion, absorption, and storage of nutrients, but also in the detoxification of a wide range of chemicals through their sequestration and accumulation (Moschino et al., 2016).

Overall, the data collected in this study attempt to provide a holistic vision of the mussel response to combined xenobiotic exposure across sites and seasons in the Northwestern Adriatic Sea, showing the importance of the DG associated microbiome in providing the impressive degree of resilience of these animals at the holobiont level, and the possibility of implementing a new panel of integrative biomarkers for environmental risk assessment of emerging pollutants in marine ecosystems.

2. Materials and methods

2.1. Mussels collection

A total of 387 Mediterranean mussels – 54 for multiresidue analysis, 108 for transcriptomics and 225 for metagenomics – were collected from November 2018 to August 2019 from three government-certified farms located in Goro, Cattolica, and Senigallia (Fig. 1). The influence of the Po River plume on the western Adriatic is known and peculiar for each season. This river runoff decreases from north to south and from inshore to offshore (Marini et al., 2008). For this reason, we have chosen the arrangement of the measuring stations along a decreasing gradient with respect to the plume of the Po. At each location, three sampling campaigns were performed, corresponding to autumn, spring, and summer. The selected sampling sites are regularly monitored by the Regional Agency for Prevention, Environment and Energy (ARPA) of the Emilia-Romagna and Marche regions, to evaluate seawater parameters (Supplementary Table S1).

At each timepoint, 2 L of seawater and 43 randomly selected mussels of commercial size (5–7 cm long) were collected directly in the field and stored in cooled containers (+4 °C) for transfer to the laboratory. Mussel biometric parameters and condition factors are reported in Supplementary Table S2. Water samples were processed for microbiota determination, while mussels were immediately processed for DG dissection under sterile conditions. Tissues (digestive glands) were individually dissected from mussels, snap-frozen in liquid nitrogen, and stored at −80 °C until further processing.

2.2. Liquid chromatography-mass spectrometry analysis

Instrument information and liquid chromatography-mass spectrometry (LC-MS) experimental conditions are reported in Supplementary Methods. Briefly, LC-MS was performed using a 2690 Alliance system (Waters, Milford, MA, USA) coupled to an ESI source and a triple quadruple mass spectrometer (Quattro-LC, Micromass) in the multiple reaction monitoring (MRM) acquisition mode. Analyte separation was obtained by using an analytical column Atlantis T3 (5 μm, 2.1 mm × 150 mm, Waters) in gradient elution. Quantitative xenobiotic determination was obtained in MRM mode on the MS/MS most abundant fragments by an internal standard (IS) calibration method with isotopically labeled IS. The analyte and IS selected MRM transitions are reported in Supplementary Table S3.

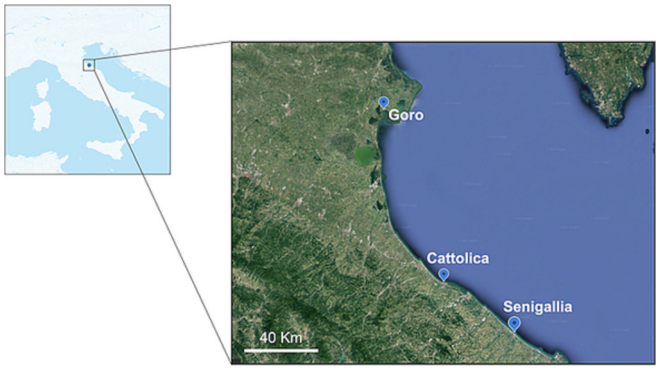

Fig. 1. Sampling sites. Map of mussel farms for the collection of *Mytilus galloprovincialis* and seawater samples. The three farms are located about 3 nautical miles offshore in Goro, near the Po delta, Cattolica, about 160 km southern to the Po delta, and Senigallia, about 200 km southern to the Po delta - in the Northwestern Adriatic Sea (source: Google Earth, earth.google.com/web/; map data: SIO, NOAA, U.S. Navy, NGA, GEBCO, IBCAO).

Analyte standard stock solutions were prepared in methanol and stored until use at −80 °C, standard solutions used for calibration were obtained diluting stock solutions in mobile phase. Sample treatment, under refrigerated conditions, involved a double solid-liquid extraction with two solvent mixtures of increasing polarity, addition of CHAPS as emulsifier and methanol.

Pollutant accumulation data in the mussel DG were evaluated based on the mean value of each pollutant in all samples and represented as a heatmap. In particular, accumulation values within one standard error of the mean were considered as within the mean range, whereas pollutant concentrations below or above one standard error of the mean were considered to be lower and higher than the mean, respectively. The Total Index Pressure (TIP) (Aylagas et al., 2017) for each sample was calculated by computing the number of pollutants detected with values above the mean, plus one standard error. TIP ranged from 0 to 4, corresponding to the concomitant presence of 4 different pollutants showing above-average values. Samples between 0 and 2 TIP values were indicated as low TIP, whereas samples between 3 and 4 TIP values as high TIP.

2.3. Mussel RNA extraction, cDNA preparation, and qPCR analysis

For each animal, 200 mg of DG were independently homogenized in a suitable volume of TRI Reagent (Sigma Aldrich, Milan, Italy) and total RNA was extracted using the DirectZol kit (Zymo Research, Freiburg, Germany) following the manufacturer's instructions. RNA concentration and quality were confirmed using the Qubit system (Thermo Scientific, Milan, Italy) and electrophoresis with a 1.2 % agarose gel under denaturing conditions. First strand cDNA for each sample was synthesized from 1 μg of total RNA using the iScript supermix (BioRad Laboratories, Milan, Italy) following the manufacturer's instructions.

Expression profiles of selected transcripts were assessed by qPCR using primer pairs and protocols reported previously (Wathsala et al., 2021). 18S and 28S rRNA were selected as reference gene products for qPCR data normalization by a preliminary stability analysis of 6 established candidate transcripts (Balbi et al., 2016). Relative expression values of target mRNAs were calculated by a comparative C_T method (Schmittgen and Livak, 2008) using the StepOne and DataAssist software (Thermo Fisher, Milan, Italy).

2.4. Microbial DNA extraction, 16S rRNA gene amplification and sequencing

Total microbial DNA was extracted from approximately 20–30 mg of DG using the DNeasy PowerSoil kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with only minor adjustments in the homogenization step (Musella et al., 2020). Specifically, all samples were homogenized using a FastPrep instrument (MP Biomedicals, Irvine, CA, USA). The elution step was repeated twice in 50 μL, incubating the columns for 5 min at 4 °C before centrifugation. DNA samples were stored at −20 °C for subsequent processing.

Seawater samples were filtered on 0.45-μm pore size MF-Millipore membrane filters (Merck, Darmstadt, Germany) using a vacuum pump (Campbell et al., 2015; Sadik et al., 2017; Su et al., 2018). Total microbial DNA was extracted from membrane filters using the DNeasy PowerWater kit (Qiagen) according to the manufacturer's protocol.

Library preparation was performed following the Illumina 16S Metagenomic Sequencing Library Preparation (Illumina, San Diego, CA, USA). Briefly, the V3–V4 hypervariable region of the 16S rRNA gene was PCR amplified in a final volume of 5 μL with 25 ng microbial DNA, 2× KAPA HiFi HotStart ReadyMix (Roche, Basel, Switzerland), and

200 nmol/L of 341F and 785R primers with added Illumina adapter overhang sequences (Klindworth et al., 2013). The PCR thermocycle was as follows: 3 min at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C, and a final 5-min step at 72 °C (as modified by Musella et al., 2020). PCR products were purified with Agencourt AMPure XP magnetic beads (Beckman Coulter, Brea, CA, USA). Indexed libraries were prepared by limited-cycle PCR, using Nextera technology (Illumina), and cleaned-up as described above. Libraries were then quantified using the Qubit 3.0 fluorimeter (Invitrogen, Waltham, MA, USA), normalized to 4 nM and pooled. The sample pool was denatured with 0.2 N NaOH and diluted to a final concentration of 4.5 pM with a 20 % PhiX control. Sequencing was performed on an Illumina MiSeq platform using a 2 × 250 bp paired-end protocol, according to the manufacturer's instructions (Illumina).

2.5. Shotgun sequencing

A subset of 9 mussel DG samples (one per site and season) were further processed for shotgun sequencing. DNA libraries were prepared according to the QIAseq FX DNA library kit (Qiagen) manufacturer's instructions. Shortly, 100 ng of each DNA sample was fragmented to a 450-bp size, end-repaired, and A-tailed using FX enzyme mix with the following thermal cycle: 4 °C for 1 min, 32 °C for 8 min, and 65 °C for 30 min. Adapter ligation was performed by incubating DNA samples at 20 °C for 15 min in the presence of DNA ligase and Illumina adapter barcodes. A first purification step with Agencourt AMPure XP magnetic beads (Beckman Coulter) was performed, followed by library amplification with a 10-cycle PCR amplification and a further purification step. Samples were pooled at an equimolar concentration of 4 nM to obtain the final library. Sequencing was performed on an Illumina NextSeq platform using a 2 × 150-bp paired-end protocol, following the manufacturer's instructions (Illumina).

2.6. Bioinformatics and statistics

qPCR data were analyzed using a 2-way permutation multivariate analysis of variance (PERMANOVA) using PRIMER v6 (Anderson, 2008) to test for variations of transcriptional profiles among sampling sites and seasons. Permutation pairwise comparisons through PRIMER v6 were carried out among the different level of each significant factor. Data visualization, and graphics were obtained with the ggplot2 R package in R (R Development Core Team, 2017). In any case, statistical differences were accepted when $P < 0.05$.

Linear discriminant analysis (LDA) effect size (LEfSe; Segata et al., 2011) was performed to identify discriminating host transcriptional responses between low and high TIP samples. The model was adjusted for seasonality, which might influence the tested parameters. Only genes with an LDA score threshold of ±2 (on a log10 scale) and a *p*-value ≤0.05 were retained. The online Galaxy Version interface (https://huttenhower.sph.harvard.edu/galaxy/, last access February 2023) was used.

For 16S rRNA gene analysis, raw sequences for a total of 234 samples (225 mussel DG samples and 9 seawater samples) were processed using a pipeline combining PANDAseq (Masella et al., 2012) and QIIME 2 (Bolyen et al., 2019). High-quality reads were retained using the "fastq filter" function of the Usearch11 algorithm (Edgar, 2010), then clustered into amplicon sequence variants (ASVs) using DADA2 (Callahan et al., 2016). Taxonomy was assigned using the VSEARCH classifier (Rognes et al., 2016) and the SILVA database (December 2017 release) as a reference (Quast et al., 2013). Alpha diversity was calculated using the number of observed ASVs, the Chao1 index, and Faith's phylogenetic diversity. Beta diversity was estimated by computing weighted UniFrac distances. All sequences assigned to eukaryotes or unassigned were discarded. Overall, an average sequencing depth of 4.4 ± 3.2 thousand high-quality reads per sample was obtained, resulting in a total of 9022 ASVs.

For shotgun sequencing analysis, raw reads for a total of 9 samples (one representative for each condition) were filtered for the eukaryotic host *M. galloprovincialis* (NCBI accession GCA_900618805.1) using BMTagger (Rotmistrovsky and Agarwala, 2011) and quality filtered (NIH Human Microbiome Project website, last access February 2023; The Human Microbiome Project Consortium, 2012). A total of 64 million high-quality microbial reads were retained, averaging 7.2 ± 3.6 million reads per sample. The taxonomic classification, at family, genus, and species level, was obtained with Kaiju (v1.8.2) (Menzel et al., 2016), using the "marDB" database with greedy mode and considering paired reads for each sample. A customized dataset of enzymes for xenobiotic degradation and resistance was produced (Supplementary File and Supplementary Table S4) by retrieving antibiotic degradation and resistance genes from the CARD database (Alcock et al., 2020), based on the results of multiresidue analysis. Moreover, the degradation enzymes for atrazine, and metolachlor and DEET (N,N-diethyl-meta-toluamide) were retrieved from UniProt (UniProt: the universal protein knowledgebase in 2021; UniProt Consortium, 2021) and NCBI (Liu et al., 2021; Rivera-Cancel et al., 2012), respectively. All enzymes were grouped into 8 different functional categories as follows: (i) atrazine (ATRA)-degrading and resistance genes; (ii) DEET-degrading genes; (iii) macrolide (MAC)-degrading genes, which include genes encoding erythromycin and tetracycline degradation and resistance enzymes; (iv) metolachlor (METOL)-degrading and resistance genes; (v) macrolide-lincosamide-streptogramin (MLS) resistance genes, including genes encoding erythromycin-degrading and resistance enzymes; (vi) sulfonamide, macrolide and tetracycline resistance genes, including genes encoding sulfamethoxazole, erythromycin and tetracycline degradation and resistance enzymes (SULF); (vii) sulfonamide resistance genes, including only sulfamethoxazole (SULFO) degradation and resistance genes; and (viii) tetracycline (TET)-degrading and resistance genes. Diamond (v2.0.8.146) (Buchfink et al., 2021) was applied for sequence alignment of high-quality microbial reads to the customized dataset. Output tables of counts for degrading enzymes matching microbial reads were converted to reads per kilobase (RPKs), which accounted for the enzyme length of each reference, and then normalized to copies per million (CPM) units, as follows:

$$\frac{\left[\frac{reads\ counts\ for\ enzyme\ in\ a\ given\ sample}{\left(\frac{enzyme\ length\ (aa)}{1000\ (aa)}\right)}\right]}{\left[\frac{n^{\circ}reads\ per\ sample}{10^{6}}\right]}$$

All statistical analyses were performed using R software (R Core Team; www.r-project.org), v4.1.2, with the packages "made4" (Culhane et al., 2005), "vegan" (Oksanen et al., 2013; https://CRAN.R-project.org/package=vegan), "vegan3d" (https://CRAN.R-project.org/package=vegan3d), "scatterplot3d" (Ligges and Mächler, 2002), "plot3D" (https://CRAN.R-project.org/package=plot3D), "rgl" (https://CRAN.R-project.org/package=rgl), "ggplot2" (Wickham, 2016), "Maaslin2" (Mallick et al., 2021), "reshape2" (Wickham, 2007), "RColorBrewer" (https://CRAN.R-project.org/package=RColorBrewer), "factoextra" (https://CRAN.R-project.org/package=factoextra), and "mixOmics" (Rohart et al., 2017). Data separation in the Principal Coordinates Analysis (PCoA) of beta diversity was tested using a permutation test with pseudo-F ratio (function "adonis" in the vegan package). The Wilcoxon rank-sum test was used to assess significant differences in alpha diversity among couples of seasons. MaAsLin2 (Multivariate Association with Linear Models 2, v1.8.0), a modified general linear model for feature-wise multivariate modeling, was used to identify differentially abundant taxa in relation to seasonal/environmental data. Sparse partial least square discriminant analysis (sPLS-DA) as implemented in the mixOmics package was adopted to find associations, *via* multiple regressions, among the xenobiotic degradation genes represented in DG metagenomic profiles, seawater environmental conditions, and accumulation data. Associations were visualized in a correlation plot, projecting the variables inside a correlation circle plot, with associated variables projected in the same direction. *P*-values were corrected for multiple testing using the Benjamini–Hochberg method, with a false discovery rate (FDR) ≤ 0.05 considered statistically significant.

3. Results

3.1. Accumulation of pharmaceuticals and pesticides in mussel digestive glands

For each site (Goro, Cattolica, and Senigallia) and season (autumn, spring, and summer), levels of pharmaceuticals and pesticides in mussel DGs were measured by LC-MS-based multiresidue analysis. Seven of the 11 compounds included in this study were found at concentrations above their respective method detection limit (MDL). Overall, the most abundant contaminants among pesticides and herbicides were atrazine (7 times higher than its method quantitation limit – MQL), metolachlor and DEET (found at about the MQL concentration); among pharmaceuticals, the concentrations of sulfamethoxazole, tetracycline and erythromycin were much higher than their MQLs, whereas carbamazepine was close to the MQL but in only 2 samples. In particular, tetracycline and atrazine were the most abundant and prevalent compounds, with concentrations between 24.3 and 140.8 ng/g d.w. and 11.8 and 34.7 ng/g d.w., respectively, across sites and seasonality. Erythromycin and DEET were less prevalent, with concentrations ranging from 4.8 to 15.9 ng/g d.w. and 0.004 to 0.009 ng/g d.w., respectively. Atrazine desethyl-desisopropil, alachor, amoxicillin and doxycycline hyclate (pharmaceuticals) were under the MDLs (Supplementary Table S5).

To find out a specific accumulation profile of the compounds in mussel DGs across site and seasons, a heatmap of their abundance across sites and seasons was generated (Fig. 2). For this analysis, the 6 compounds detected at or above the MQL (*i.e.*, atrazine, metolachlor, DEET, sulfamethoxazole, tetracycline and erythromycin) were considered. Our data overall showed a site-specific accumulation profile, which fluctuated with seasonality. Regardless of site, summer samples were generally characterized by a higher abundance and diversity of compounds. In particular, the summer samples from Senigallia and Cattolica showed a higher abundance of sulfamethoxazole, tetracycline, and DEET, with the former also showing metolachlor accumulation. Finally, summer samples from Goro showed higher abundances of erythromycin, DEET, and atrazine. We also scored the different DG samples according to the TIP index, computing the total exposure level to the targeted compounds. All summer DG samples were at high TIP, regardless of site, while all spring DG samples were at low TIP, with a site-dependent behavior observed for autumn samples (Fig. 2).

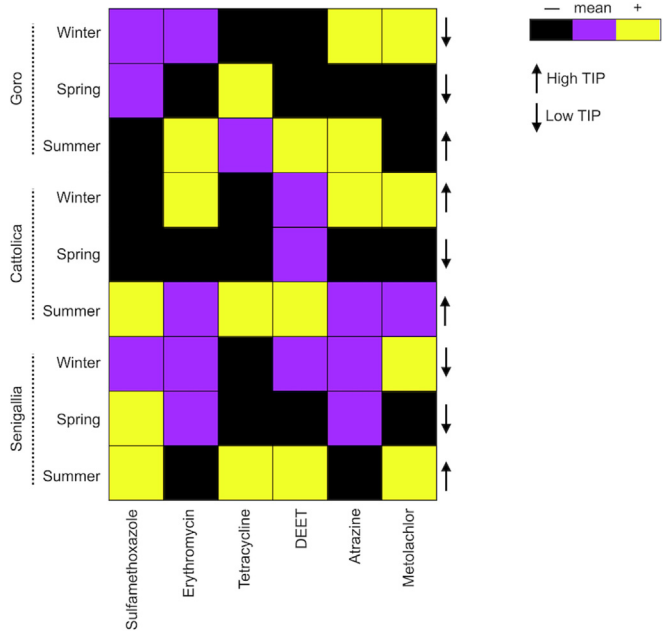

Fig. 2. Pollutant accumulation in mussel digestive glands. Accumulation was evaluated based on the mean value of 6 pollutants found in all samples, in concentrations above the detection limits (see Materials and Methods). Pollutant concentrations within one standard error of the mean are considered as within the mean range (purple), whereas values below or above one standard error of the mean are colored in black and yellow, respectively. High and low values of Total Index Pressure (TIP) for each site (Goro, Cattolica, Senigallia) and season (autumn, spring, summer) are indicated with an arrow next to each row. Abbreviations: DEET = N,N-diethyl-meta-toluamide.

3.2. Host gene transcriptional profiles

Variations of gene transcriptional profiles related to several physiological functions of mussels across sites and seasons are reported in detail in Supplementary Fig. S1A). PERMANOVA analyses demonstrated that the single factors "Season" and "Location" had a significant effect on the whole dataset, and a significant interaction between the factors was observed (p-value = 0.001) (Supplementary Fig. S1B).

To assess the host transcriptional response to total xenobiotic pressure, samples at high and low TIP were compared through LEfSe analysis (Segata et al., 2011) (Fig. 3). High TIP differs from low TIP samples for the relative increase of transcription levels for genes involved in DG metabolic activity namely (*AMYL, PK and LYS)*, as well as for the relative decrease of transcription levels for genes involved in detoxification (*MT10*) and lysosome (*CTSL*) functions.

3.3. Dynamics of the mussel digestive gland-associated microbiome across sites and seasons

First, we demonstrated that mussel DG-associated microbiomes segregated from those of the surrounding seawater in the weighted UniFrac-based PCoA (permutation test with pseudo-F ratio, p-value = 0.001) (Supplementary Fig. S2), thus constituting separate communities. Both sampling season and site contributed to this segregation, with 15 % and 12 % of variability explained by season and site, respectively. To further elucidate their contribution to the mussel DG microbial composition, samples were stratified either by site or by season, and separate PCoA plots were generated. In all cases, significant segregation was found between the microbial profiles (p-values ≤ 0.001) (Fig. 4). Regarding alpha diversity (Supplementary Fig. S3), while some significant variation was observed, a consistent trend across seasons or sites could not be identified.

As for the compositional structure of the mussel DG microbiome, the phylum-level relative abundance profiles were quite similar among all samples (Supplementary Fig. S4). In particular, Firmicutes was the dominant phylum in the Cattolica and Senigallia samples regardless the season, with a relative abundance between 40 and 70 %. In contrast, Goro samples were dominated by Planctomycetes (mean relative abundance ± standard deviation, 31 ± 18 %) in autumn, with Firmicutes representing the second most abundant phylum (17 ± 18 %), by Proteobacteria (46 ± 14 %) in spring and by Firmicutes (45 ± 24 %) in summer. At the family level, the microbial composition of mussel DGs became much more heterogeneous, with significant variations in the proportions of taxa across different sites and seasons (data not shown).

For each site, the MaAsLin2 correlation model was used to detect DG microbiome components showing significant variation across seasonality (Fig. 5). In particular, Goro samples were characterized by a higher relative abundance of *Prevotellaceae* and *Pseudomonadaceae* in autumn, while of DEV007 (Verrucomicrobia), *Flavobacteriaceae*, *Hyphomicrobiaceae*, JTB255 marine benthic group (gammaproteobacterial *Woeseiaceae*), and *Rhodobacteriaceae* in spring. Clostridiales vadinBB60 group, *Ruminococcaceae*, and *Sphingomonadaceae* were characteristic of summer. For Cattolica, *Bifidobacteriaceae*, *Clostridiaceae 1*, *Coriobacteriaceae*, *Lachnospiraceae*, *Peptostreptococcaceae*, *Planctomycetaceae*, *Prevotellaceae* and *Pseudomonadaceae* were more abundant in autumn samples. Spring samples were characterized by higher proportions of *Enterobacteriaceae*, *Lactobacillaceae* and *Sphingomonadaceae*, whereas summer samples showed an overrepresentation of *Christensenellaceae*, *Clostridiales* vadin BB60 group and *Ruminococcaceae*. Finally, the autumn profile of Senigallia resembled that of Cattolica, while *Enterobacteriaceae* and *Ruminococcaceae* were overabundant in spring, and Family I of Cyanobacteria, *Lactobacillaceae* and *Sphingomonadaceae* in summer.

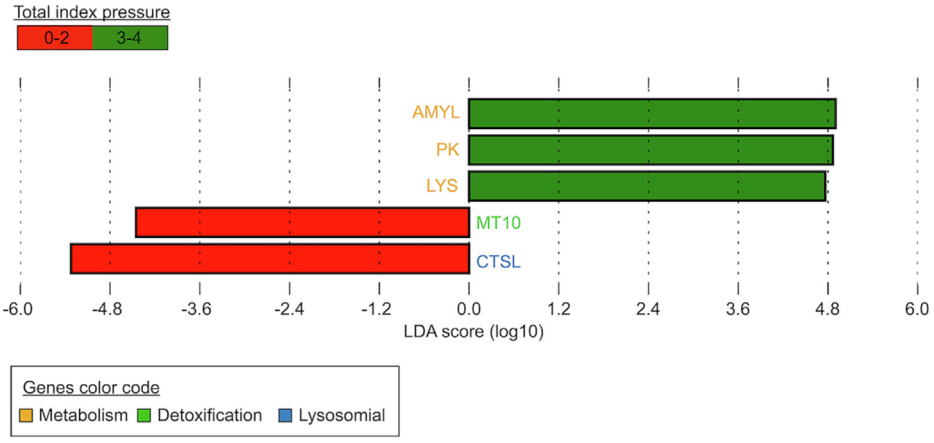

Fig. 3. Transcriptional signatures of the mussel digestive gland related to pollutant exposure. Linear discriminant analysis (LDA) effect size (LEfSe) revealed differentially transcribed host genes between samples with high (3–4) and low (0–2) Total Index Pressure (TIP). Only genes with LDA score threshold of ± 2 (on a log10 scale) and a *p*-value ≤ 0.05 were retained.

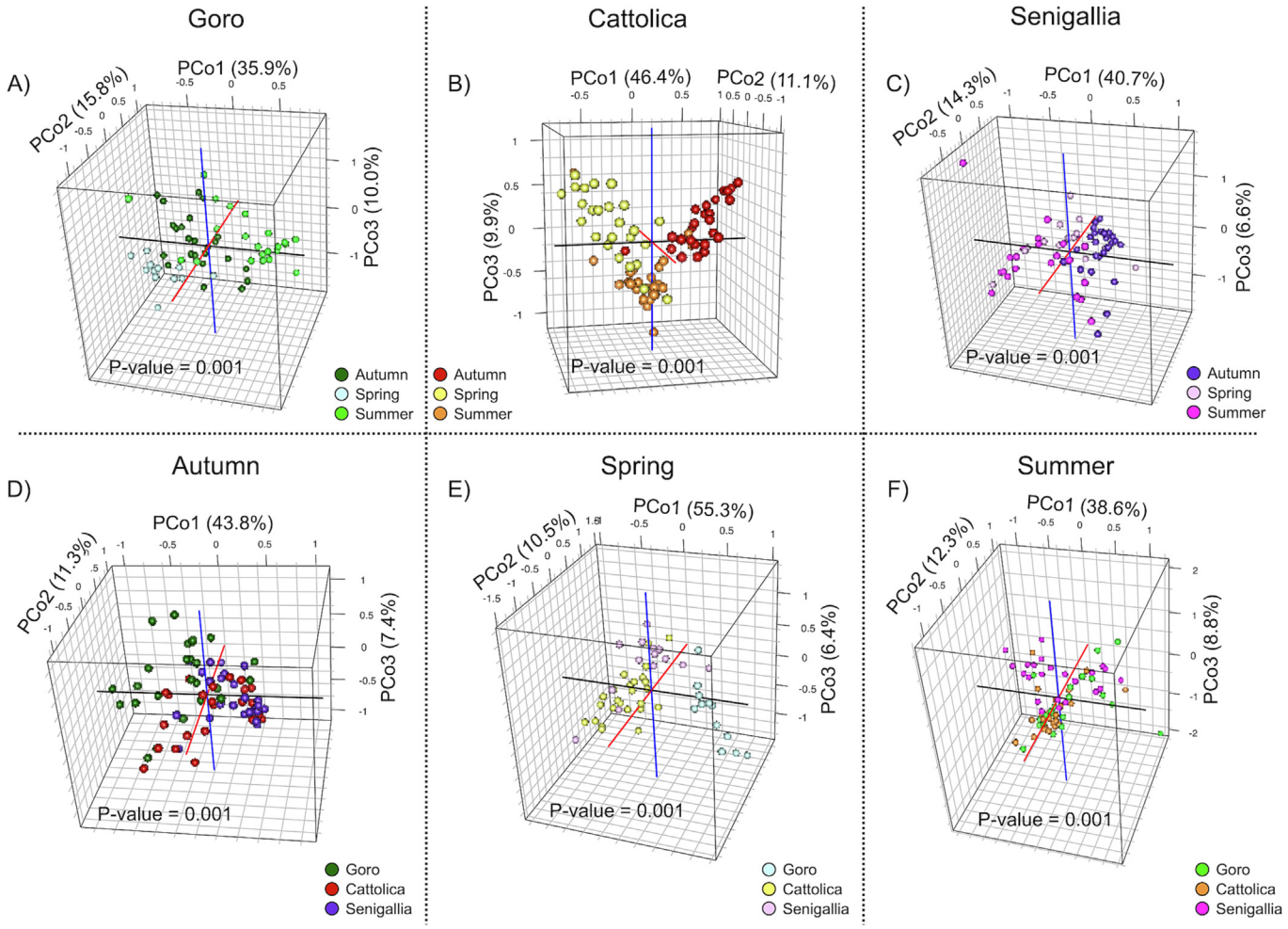

Fig. 4. Compositional structure of the mussel digestive gland-associated microbiome by sampling site and season. 3D Principal Coordinates Analyses (PCoAs) based on weighted UniFrac distances between microbial profiles of mussel digestive glands from different sampling sites (Goro, Cattolica, and Senigallia; A-C) and seasons (autumn, spring, and summer; D—F). The first, second and third principal components (PCo1, PCo2 and PCo3) are plotted in all graphs, and the percentage of variance in the dataset explained by each axis is reported. Permutation test with pseudo-F ratio, p-value = 0.001 for all comparisons.

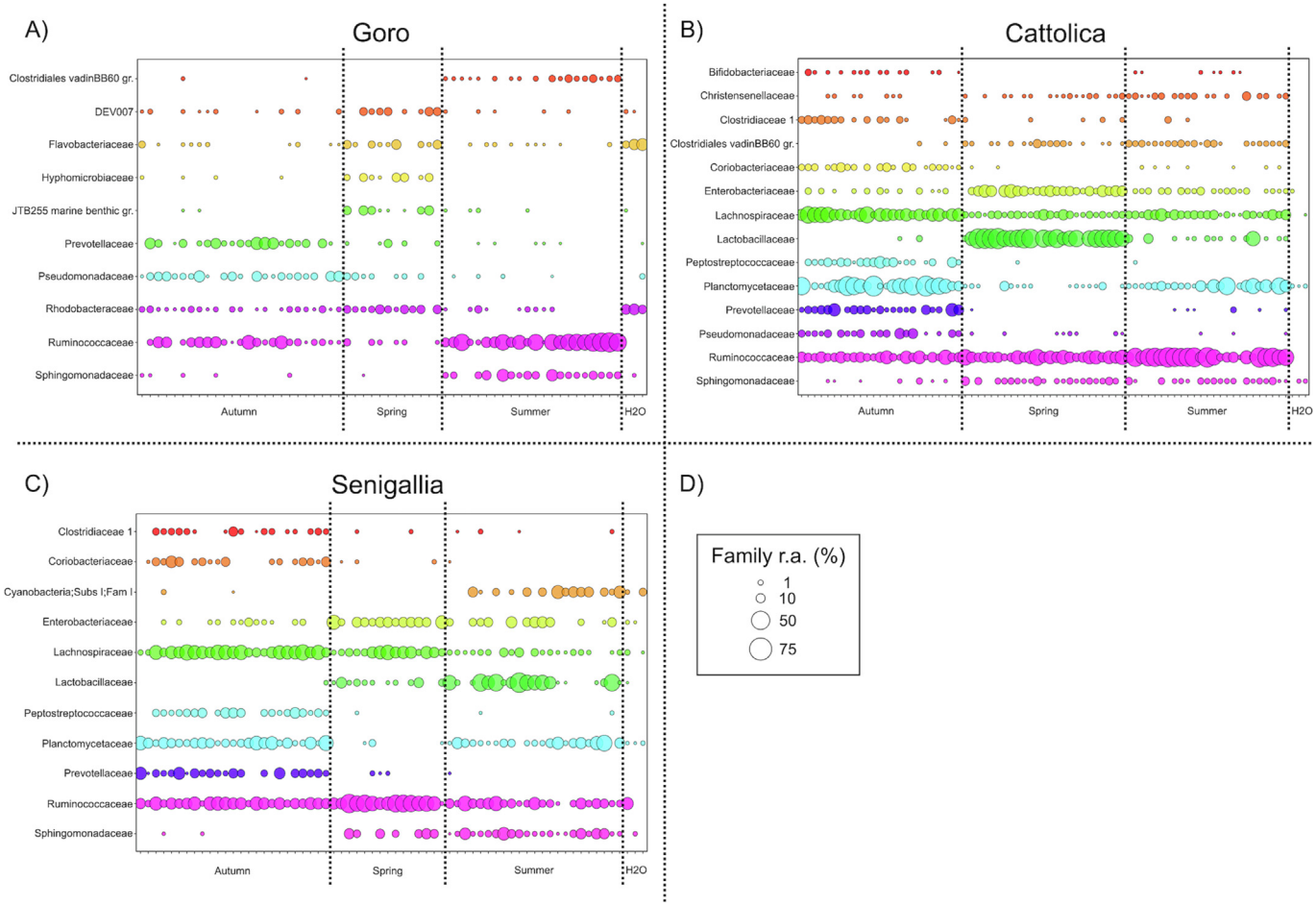

Fig. 5. Taxonomic signatures of the mussel digestive gland-associated microbiome related to sampling site and season. Bubble plots of the family-level microbiome compositional structure in mussel digestive glands for all sampling sites (Goro, Cattolica, and Senigallia) across different seasons (autumn, spring, and summer), including surrounding seawater (H_2O). Bubble size is proportional to the relative taxon abundance (r.a.) according to the legend. Abbreviations: gr = group; Subs = Subsection; Fam = Family.

3.4. Xenobiotics-degrading genes in the mussel digestive gland-associated microbiome

In order to assess the functional response of the DG microbiome to the 6 detected xenobiotics (*i.e.*, atrazine, metolachlor, DEET, sulfamethoxazole, tetracycline and erythromycin), shotgun metagenomics microbial reads were aligned to a customized internal dataset of enzymes for the degradation and resistance mechanisms of the corresponding chemical compounds, resulting in the identification of 254 bacterial enzymes (see Materials and Methods, and Supplementary Table S4). Enzymes were grouped into 8 functional categories for the degradation of: (i) atrazine, (ii) DEET; (iii) macrolides (*i.e.*, erythromycin and tetracycline); (iv) metolachlor; (v) erythromycin; (vi) sulfamethoxazole, erythromycin and tetracycline; (vii) sulfamethoxazole; and (viii) tetracycline. Samples were clustered according to the abundance profiles of xenobiotic degradation genes, and then represented as a heatmap (Fig. 6; see corresponding values and gene names in Supplementary Table S6), with genes belonging to the same functional category grouped together. Although in the context of impressive variation in gene abundance profiles between samples, it was interesting to note that all summer samples clustered together, suggesting that combined exposure to multiple pollutants, *i.e.*, high TIP, selected for a similar (and highly diverse) xenobiotic-degrading gene pattern in the DG metagenome. For each of the 8 functional categories of xenobiotic degradation genes, the associated microbiome families are shown in Supplementary Fig. S5. The results from this taxonomic assignment showed a considerable amount of xenobiotic degradation genes with an unassigned taxonomy, further highlighting the still limited representation of marine microbial taxonomic diversity in public databases. When focusing on genes with an assigned taxonomy, we found that each functional category was quite heterogenous, *i.e.*, accounted for different taxa in different sites and seasons. Nevertheless, *Pirellulaceae*, *Planctomycetaceae* and *Rhodobacteraceae* were overall the most represented families among xenobiotic-degrading bacteria, with a cumulative relative abundance ranging from 1.5 % to 20 % depending on site and season, being present in at least one season per site, and generally carrying the greatest diversity potential and the highest counts for xenobiotic degradation (Fig. 7).

Possible associations between xenobiotic degradation genes of the DG microbiome, the abundance of xenobiotic compounds in DGs, and seawater parameters at the time of mussel collection were investigated through sPLS-DA (Fig. 8). The variance explained by the three factors was 57 %, 46 % and 39 %, respectively, on the first component and 5 %, 37 % and 28 % on the second component, respectively. Most of the genes for antibiotic degradation (*i.e.*, those for macrolide, erythromycin, sulfamethoxazole, and tetracycline degradation), all herbicide degradation genes (for atrazine and metolachlor degradation) and the DEET degradation genes correlated with each other and with atrazine concentrations. Furthermore, we found a correlation among turbidity and chlorophyll *a* parameters, erythromycin concentration, two antibiotic resistance genes (*emtA* and *tet(44)*) and one herbicide degradation gene (*atzA*). Tetracycline concentration and seawater surface temperature were also correlated. Finally, five antibiotic degradation genes (*tet(K)*, *tet(S)*, *erm(47)*, *ermX* and *ykkC*) correlated with seawater salinity and sulfamethoxazole accumulation.

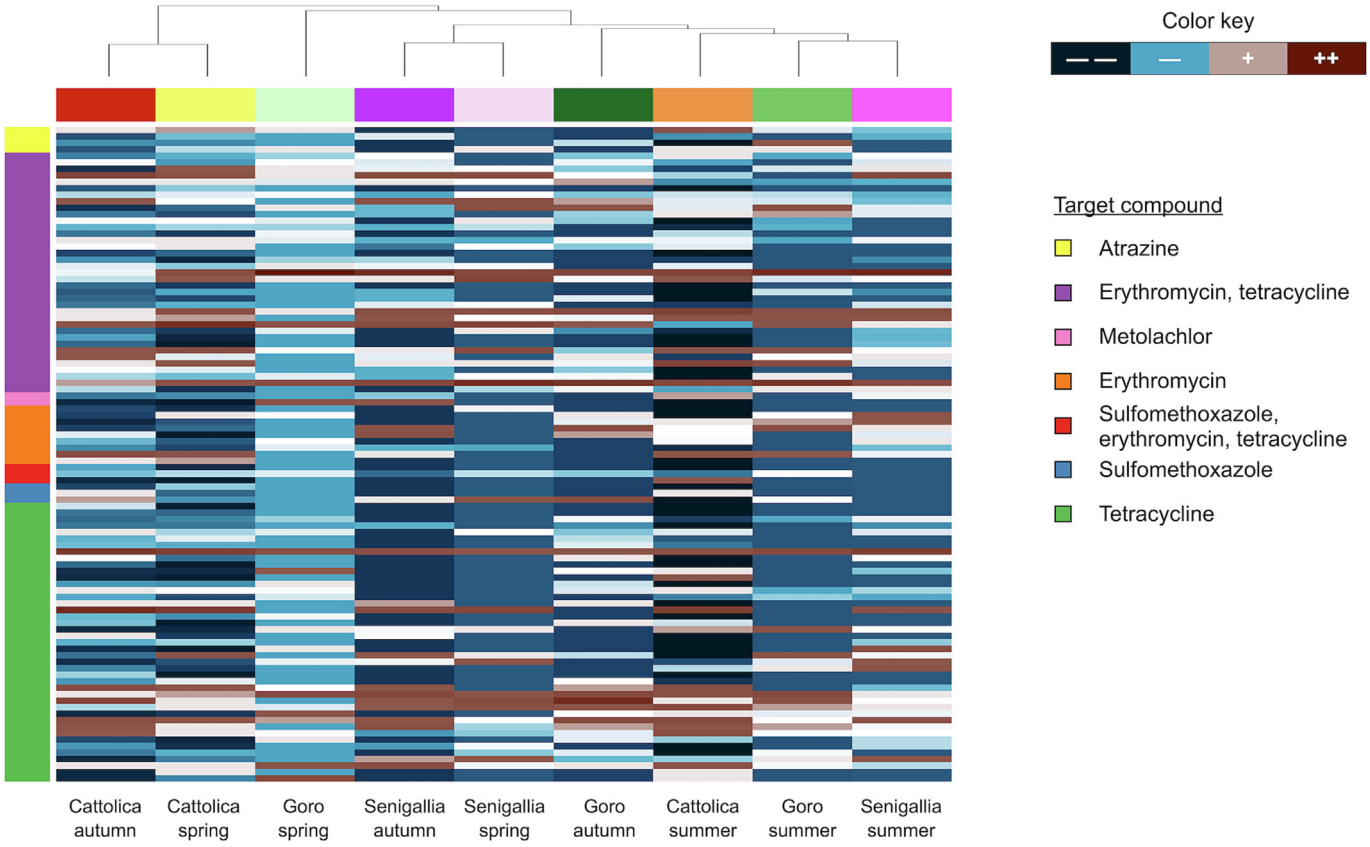

Fig. 6. Abundance profiles of xenobiotic degradation genes in the mussel digestive gland metagenome by sampling site and season. Hierarchical Ward linkage clustering based on the Spearman correlation coefficients of copies per million reads (CPM) of xenobiotic degradation genes in the mussel digestive gland metagenomes from different sampling sites (Goro, Cattolica, and Senigallia) and seasons (autumn, spring, and summer), filtered for gene presence in at least one sample. Genes involved in the degradation of xenobiotics detected by multiresidue analysis were considered (see Materials and Methods for further details).

4. Discussion

In our work we provide some glimpses on the role of DG-associated microbiome for the mussel response to exposure to multiple pollutants in real world settings, demonstrating the strategic importance of the host-associated microbiome for the extraordinary natural multi xenobiotic resistance observed in this marine animal.

According to our findings, out of the 11 targeted pollutants, 7 among pharmaceuticals, herbicides, and pesticides, were detected in the DG of mussels collected from 3 different commercial farms, spanning for about 200 km along the Northwestern Adriatic coast, and in 3 different seasons. Among pharmaceuticals, tetracycline and the herbicide atrazine were the most abundant and prevalent compounds, while erythromycin among pharmaceuticals and DEET among pesticides were less prevalent. More specifically, we found a site-specific and complex pattern of the detected chemicals, which fluctuated with seasonality. Summer samples were overall characterized by relatively higher concentrations and diversity of detected contaminants, as suggested by TIP scoring of the total exposure levels. In contrast, all samples collected in the spring were at low TIP, while the autumn samples showed site-dependent TIP scores. Previous seasonal and interannual analyses of pharmaceutical accumulation in mussels from the same area of the Adriatic Sea showed an overall heterogeneous distribution of the compounds analyzed thus far, with no clear seasonal accumulation trends (Mezzelani et al., 2020). However, in line with our data, accumulation peaks of anti-inflammatories, anxiolytics, and antidepressants were reported in summer, suggesting the impact of the increasing anthropogenic pressure from touristic activities occurring in the area (Mezzelani et al., 2020).

Despite this summer increase for some pollutants, and since we observed complex patterns of mussel chemical accumulations across conditions, mussel holobiont responses in terms of transcriptomic (host) and metagenomic (microbiome) changes were evaluated comparing sample groups according to their respective TIP (Total Index Pressure; Aylagas et al., 2017). From the mussel host side, samples from high TIP (which mainly comprise summer sampling time points, irrespective from the sampling site) and low TIP conditions mainly differ for differential regulation of transcripts involved in metabolic (AMYL, PK, LYS), antioxidant and lysosomal responses. Amylase (AMYL) and pyruvate kinase (PK) are key enzymes in glycolitic metabolism (Canesi et al., 2007; Liu et al., 2017) and considered robust biomarkers of mussel digestive functions (Connor et al., 2016). Although the principal function attributed to lysozymes (LYS) in bivalves is host defense in circulating hemolymph, in the digestive gland a digestive function for some LYS isoforms has been suggested in many studies (Xue et al., 2010, and reference therein). The relative expression patterns of these gene products may suggest an increased utilization of metabolic resources as a requirement for mussel acclimatization at high TIP conditions, or a generalized metabolic activation from chemical biotransformation processes. Relative decrease of lysosomal (CTSL) and antioxidant (MT10) responses in high TIP conditions compared to low TIP conditions may be related stimulation of DG metabolic activity, ad both lysosomes and the antioxidant system are tightly involved in degradative processes (Shaw et al., 2019). In particular, either CTSL up- or down- regulation may be a signature for alteration of the lysosomal system due to accumulation of complex contaminant mixtures (Khoma et al., 2022).

Focusing on the microbiome counterpart, a core DG microbiome was recognizable at the high taxonomic level (Musella et al., 2020; Wathsala et al., 2021), showing Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, and Planctomycetes among the main components, always present regardless of site and seasonality, although in different proportions. Not surprisingly, at lower taxonomic levels, a recognizable seasonal

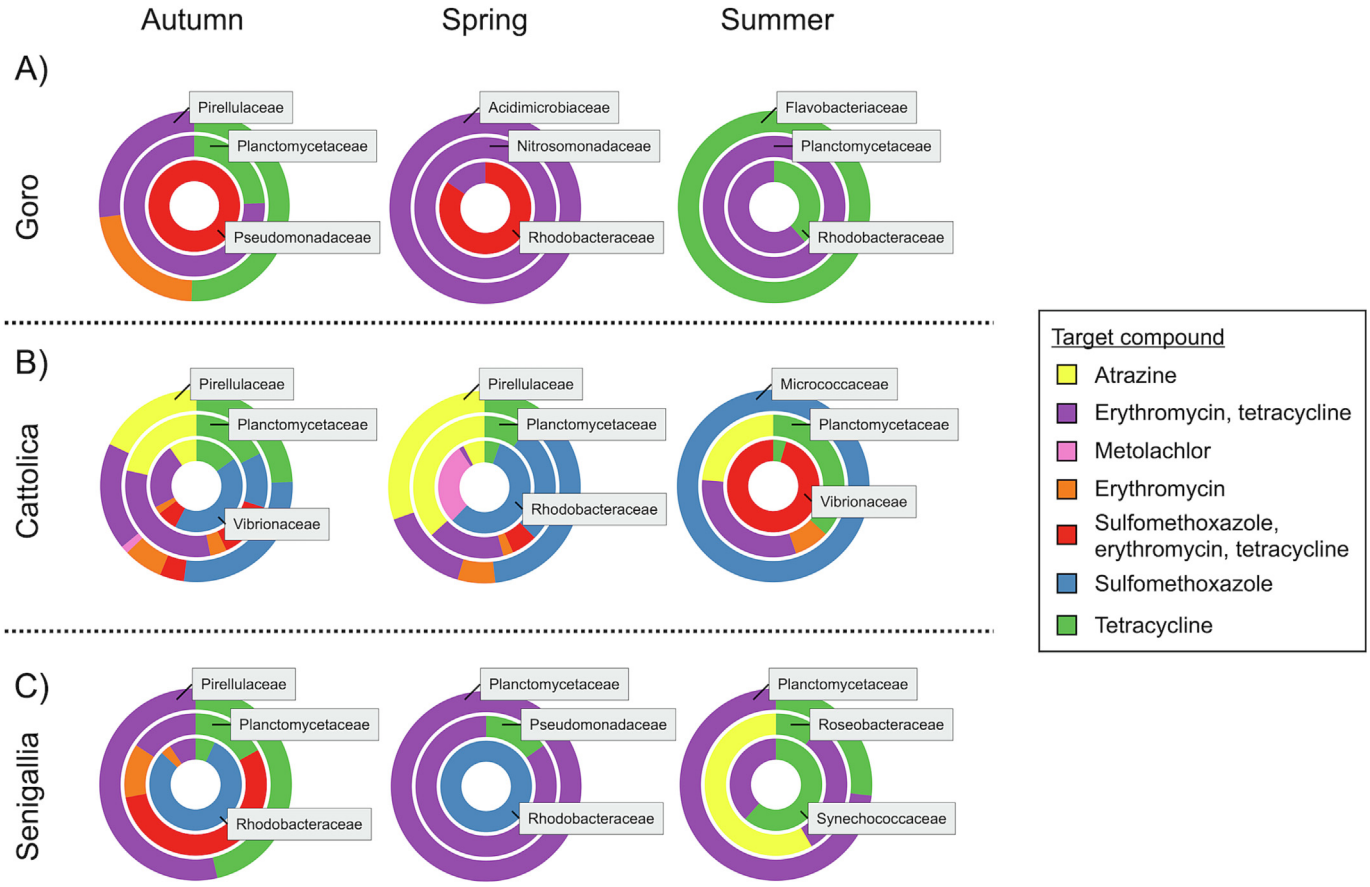

Fig. 7. Mussel digestive gland microbial families with the highest counts of xenobiotic degradation genes and their respective functional potential. Donut plots representing, for each sampling site (Goro, Cattolica, and Senigallia) and season (autumn, spring, and summer), the 3 bacterial families showing the highest potential (in terms of copies per million reads - CPM) toward the degradation of xenobiotics and the corresponding functional distribution. For functional categories, see also Materials and Methods.

dynamic was observed, with specific declinations depending on the site. Overall, the autumn samples were characterized by higher proportions of *Prevotellaceae* and *Pseudomonadaceae*, with Cattolica and Senigallia sites showing a very similar microbial configuration. The spring and summer samples were instead characterized by a more site- and season-specific profile, with *Enterobacteriaceae* being overabundant in the spring samples from Cattolica and Senigallia, while Clostridiales, *Ruminococcaceae* and *Sphingomonadaceae* sharing higher relative abundances in the summer samples from Goro, Cattolica or Senigallia. Taken together, these data confirmed the relevant degree of fluidity of the mussel DG-associated microbiome, whose composition can fluctuate in response to site and seasonality.

In order to explore the functional response of the mussel DG microbiome to exposure to the detected emerging contaminants, genes for the degradation of these xenobiotics were specifically searched across sites and seasons. Our data highlighted a relevant degree of functional complexity and plasticity of xenobiotic-degrading gene patterns in the DG microbiome. Indeed, we detected a total of 254 bacterial genes corresponding to 8 different functional categories of contaminant degradation, hence supporting the potential of the DG microbiome as an integral player in affecting animal ability to tolerate a high pollutant burden, or even detoxify environmental xenobiotics. This finding may also explain the apparent lack of a clear and consistent detoxification response in the mussel host. Overall, the degradation profile was site- and season-specific, with only a few genes conserved across all samples, such as *vgaE* (for macrolide resistance) (Feßler et al., 2018) and *adeR* (for tetracycline resistance) (Grossman, 2016). It was also possible to identify a cluster of tetracycline degradation and resistance genes (such as *tetA* and *tetB*) (Sreejith et al., 2022) shared among the majority of the samples, although in different amounts. Moreover, only a few gene clusters were shared in sequential seasons at the same site, such as *evgA* (a transcriptional regulator controlling the expression of multiple genes conferring antibiotic resistance) (Nishino et al., 2003) – present in autumn and spring in Cattolica and Senigallia – and *salA* (ATP-binding cassette protein conferring resistance to macrolide-lincosamide-streptogramin group of antibiotics) (Hot et al., 2014) – shared between autumn and spring in Goro. Nonetheless, all DG samples collected in the summer, scored as high TIP, showed a higher overall load of degradation genes, regardless of site, especially genes involved in the degradation of macrolides, such as the multidrug resistance gene poxtA (Antonelli et al., 2018), and tetracycline (*tetA* and *tetB*) (Sreejith et al., 2022). These data are consistent with the presence, in the summer samples, of a higher average abundance of tetracycline (for Cattolica and Senigallia) and erythromycin (for Goro). *poxtA* is a member of the protein family ARE ABC-F, the ATP-binding cassette proteins associated with antibiotic resistance belonging to the F lineage (Antonelli et al., 2018), with a resistance mechanism mediated by ribosomal protection (Sharkey et al., 2016). As for tetracycline, its resistance mechanisms are governed by *tet* genes, which are involved in active drug efflux, ribosomal protection, or enzymatic drug modification (Hedayatianfard et al., 2014).

When seeking for associations between the detected contaminants and the profile of xenobiotic-degrading genes in the DG-associated microbiome, we found that the herbicides metolachlor and atrazine, and the antibiotic erythromycin, were associated with the highest diversity of xenobiotic-degrading genes, whereas tetracycline and sulfamethoxazole showed the opposite behavior. Particularly, sPLS-DA analysis showed that metolachlor, atrazine and erythromycin combined into a large and diverse pool of functional features, including genes for degradation of antibiotics, herbicides, and DEET. In contrast, tetracycline and sulfamethoxazole correlated with

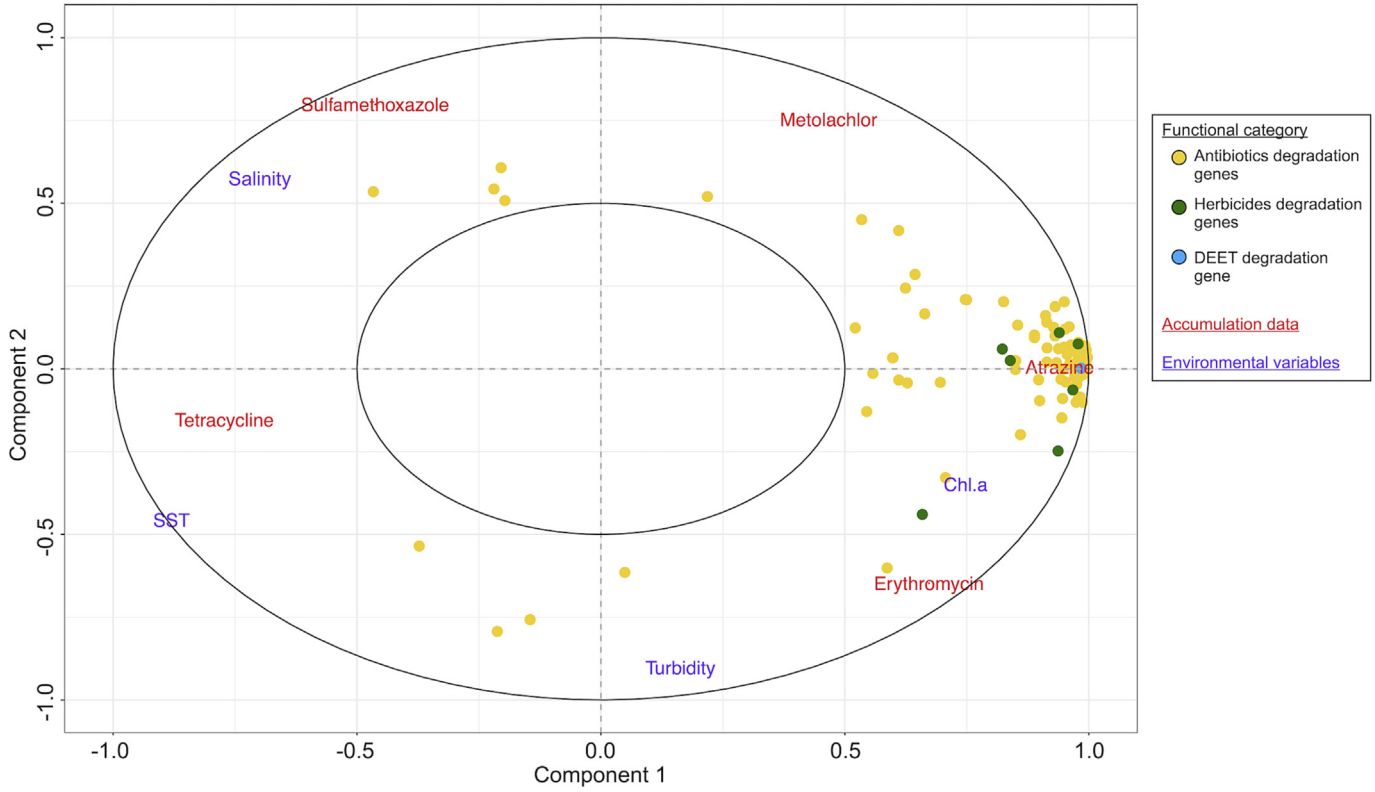

Fig. 8. Associations between microbial genes of xenobiotic degradation, accumulation data and seawater parameters. Correlation circle plot for the first two sPLS components, with correlations depicted for < -0.5 and > 0.5. The two circumferences show correlation coefficient radii at 0.5 and 1.0. The farther from the center a variable is, the greater the association with the component. Variables projected in the same direction of the plot are positively correlated, while variables in diametrically opposite position are negatively correlated. Variables located perpendicular to each other are not correlated. Xenobiotic degradation genes are indicated as circles of different colors based on the functional category, according to the legend on the right (see also Materials and Methods). Accumulation data, namely the abundance of xenobiotic compounds, are shown in red. Seawater parameters are shown in purple. Abbreviations: Chl.a = chlorophyll *a*; DEET = N,N-diethyl-meta-toluamide; SST = seawater surface temperature.

a smaller pool of antibiotic-degrading genes. Overall, these findings provide evidence in support of the importance of the mussel DG-associated microbiome for the animal adaptation to the local ecological context, particularly in term of resistance – and even degradation – to harmful xenobiotics compounds of anthropogenic origin. This suggests the importance of microbiome-dependent functionalities for the rapid adaptation and persistence of mussels in a complex polluted environment, complementing the limited plasticity of the host genomic traits. An extremely dynamic pattern of microbiome taxa carrying the observed degrading functions was observed, where the same functionalities were provided by different microbial groups, depending on site and season. For instance, sulfamethoxazole, erythromycin and tetracycline degradation and resistance functions in Goro samples were mainly contributed by *Pseudomonadaceae* in autumn and *Rhodobacteraceae* in spring. In summer, *Rhodobacteraceae* provided functions involved in degradation pathways of macrolides and tetracycline. In Cattolica samples, the *Vibrionaceae* family provided a panel of function across all degrading categories in autumn, whereas its role was limited to sulfamethoxazole, erythromycin and tetracycline degradation and resistance pathways in summer. Finally, in Senigallia samples, macrolide and tetracycline degradation functions were provided in a similar fashion by *Pirellulaceae* and *Planctomycetaceae* in autumn and summer, respectively. Since several of the above-mentioned taxa incudes well known benthonic free-living marine microorganism population the North Western Adriatic Sea (Scicchitano et al., 2022), we hypothesize that mussels can select and retain locally adapted microbioes, whose pangenomes may evolve novel function firster that the host, for persisting and adapting to a complex and fluctuating pattern of pollutants, as occurring in the North Western Adriatic sea.

5. Conclusions

Our results demonstrate that farmed mussels in the Northwestern Adriatic Sea are exposed to a complex and dynamic pool of emerging contaminants, with a site- and season-specific variation pattern, with summer showing the highest level of exposure. The peculiar geochemical and hydrodynamic conditions characterizing the Northwestern Adriatic make it an ideal real-world model for marine ecosystems dynamically influenced by complex anthropogenic pressures (Capolupo et al., 2017). In this scenario, we were able to dissect the mussel response to this complex exposome at the holobiont level, elucidating the synergy between host- and microbiome-dependent detoxification and resistance mechanisms, which enable the mussel to resist and, ultimately, adapt to combined xenobiotic exposure. Thus, our findings support possible important implications of using mussels for animal-based bioremediation applications, in line with previous results. For example, mussels have already been shown to be potentially effective as bioremediation tools for eutrophication (Filippini et al., 2023), microplastics pollution (Masiá et al., 2020) or to reduce potential pathogen load (Bianchi et al., 2014). Our results thus constitute a further step in the implementation of animal-based bioremediation strategies by exploiting the mussel capacity to resist and, even, eventually detoxifying emerging pollutants in complex and combined patterns as occurring in real world conditions.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.163948.

CRediT authorship contribution statement

Giorgia Palladino: Conceptualization, Data curation, Formal analysis, Investigation, Project administration, Supervision, Visualization, Writing

– original draft. **Simone Rampelli:** Formal analysis, Visualization, Writing – review & editing. **Daniel Scicchitano:** Data curation, Formal analysis, Visualization. **Enrico Nanetti:** Writing – review & editing. **Letizia Iuffrida:** Data curation, Visualization. **Rajapaksha Haddokara Gedara Rasika Wathsala:** Investigation. **Nicolò Interino:** Investigation. **Mauro Marini:** Writing – review & editing. **Emanuele Porru:** Writing – review & editing. **Silvia Turroni:** Writing – review & editing. **Jessica Fiori:** Data curation, Resources, Writing – original draft. **Silvia Franzellitti:** Conceptualization, Formal analysis, Project administration, Supervision, Writing – original draft. **Marco Candela:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft.

Data availability

Processed reads for 16S rRNA gene sequencing and for metagenomic sequencing are openly available in European Nucleotide Archive (ENA), reference number PRJEB60787.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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