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You shall not pass! (unless you're stress resistant): Selection-driven restructuring and transient invasion in freshwater mussel microbiomes under antimicrobial exposure

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ABSTRACT

Global declines of freshwater mussel populations and of the ecosystem services they provide have been linked to anthropogenic stressors including pollution, but the impacts of antibiotic spillover, e.g. through aquaculture or pharmaceutical wastewater effluents, on mussel health are unclear. Mussel-associated microbiomes contribute to key benthic processes such as nutrient cycling and organic matter transformation. While adverse effects on fish gut microbiomes are increasingly recognized, ecological impacts of antibiotic discharge on microbiomes of non-target organisms like mussels are poorly understood, with unknown consequences for the functioning of aquatic habitats. Using 16S rRNA gene sequencing, we investigated how exposure to florfenicol and peracetic acid via rearing water effluents affected the microbiome of *Anodonta cygnea*. Under baseline conditions, the microbiome was distinct from water and dominated by taxa like *Ferruginibacter* aligning with its suspension-feeding lifestyle. Both compounds significantly altered microbial community structure, with temporal convergence of the host microbiome with external water microbiota. Observed shifts were characterized by the enrichment of detoxifying *Dechloromonas* and *Sphingomonas* as well as modulatory taxa like *Aureispira* and *Nannocystis*, with core commensals persisting across treatments. Co-application of peracetic acid and florfenicol increased florfenicol concentrations in water, highlighting potential compound interactions that may alter florfenicol degradation dynamics and could increase antibiotic pressure in aquatic habitats. Although the stability of core taxa indicates partial resilience rather than complete dysbiosis after single exposure, this study highlights potential ecological implications of antibiotic spillover in freshwater environments, as exemplified here by aquaculture but relevant for risk assessments of other pollution sources.

1. Introduction

Freshwater mussels are keystone species that modulate freshwater ecosystems by coupling benthic and pelagic zones and sustaining nutrient fluxes through filtration and biodeposition (Vaughn, 2018; Zieritz et al., 2025). Supplied services include water clearance, nutrient transformation, and habitat provision through burrowing activities (Zieritz et al., 2025; Geist, 2011; Boeker et al., 2016; Lummer et al., 2016). Of particular relevance is the role of mussel assemblages in regulating nitrogen (N) levels, assimilating excess phosphorus (P) and mitigating eutrophication, a substantial threat for the health of natural freshwater systems (Kellogg et al., 2014). By supplying carbon (C)- and N-rich biodeposits, mussels actively fuel microbial nitrogen cycling in sediments. Host-associated microhabitats (e.g. gut and shells) further contribute to microbial nitrogen transformations (Atkinson and Vaughn, 2015; Hoellein et al., 2015; Caffrey et al., 2016; Humphries et al., 2016). This provision of bioavailable N to primary producers and macroinvertebrates, mediated by microbial remineralization of biodeposits

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Abbreviations: FF, Florfenicol; PAA, Peracetic Acid; pT, post-treatment; N, nitrogen; P, phosphorus; OM, organic matter; RAS, Recirculating aquaculture system; ARG, Antibiotic resistance gene; WWTP, wastewater treatment plant.

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and excretion of dissolved N (primarily NH_3 and NH_4^+) by mussels, sustains aquatic food web productivity, while removal of excess N from the system alleviates nutrient pollution (Vaughn, 2018).

Symbiotic microbial communities contribute to the provision of these ecosystem services (Arfken et al., 2017; Murphy et al., 2019) and support host health and disease resilience (Wegner et al., 2013; Lokmer et al., 2016; King et al., 2019). In addition, host-associated microbial communities themselves are suspected to play an important role in the feeding of freshwater mussels (Geist et al., 2005). Current knowledge is largely limited to taxonomic surveys using amplicon sequencing (Aceves et al., 2018; Weingarten et al., 2019; Mccauley et al., 2021), while the full functional potential of mussel-associated microbiomes remains poorly understood. Nevertheless, existing studies on marine blue mussel (*Mytilus sp.*) and estuarine eastern oyster (*Crassostrea* sp.) have shown substantial variation in microbiome composition across host species (Weingarten et al., 2019; Akter et al., 2023; Gignoux-Wolfsohn et al., 2024), tissue type (Dubé et al., 2019; Musella et al., 2020), geographical location (Chiarello et al., 2022) and season (Lokmer et al., 2016; Mccauley et al., 2021; Akter et al., 2023; Chiarello et al., 2022). Given the permanent exposure to exogenous bacteria during suspension feeding, bivalve microbiomes are particularly susceptible to local fluctuations in environmental conditions, including temperature, salinity, pollution and acidification (Unzueta-Martínez et al., 2021; Green et al., 2019; Stevick et al., 2021; Alma et al., 2020; Gill et al., 2022). How microbial assemblages are formed and maintained depends on an interplay between stochastic (e.g. random dispersal, ecological drift) and deterministic processes like environmental and host filtering (Nemergut et al., 2013; Burns et al., 2017; Wang et al., 2025). The relative contribution of each process is highly context-dependent and may shift under stress conditions with consequences for how microbial communities respond to and reorganize following disturbance (Zhang et al., 2016; Ning et al., 2024). In other aquatic models, divergence between host-associated and environmental microbial communities point towards a stronger impact of deterministic forces in driving phenotypic variation in natural bacterial populations (Schmidt et al., 2015; Weigel, 2020; Chung et al., 2024). In this context, the environment acts both as a source of potential colonizers and as a selective filter, whereby abiotic conditions such as pH, salinity, temperature and resource availability constrain which species can persist. The host further imposes selective filtering through co-evolutionary pressures, actively retaining microbes that support host fitness from the existing pool of external and internal microorganisms. Host traits such as pH gradients within tissue niches, mucosal structure, and the secretion of immune effectors bias microbial colonization toward taxa carrying traits that benefit the host, in combination with diet-dependent nutrient availability and stabilization through microbial interactions within the network (Youngblut et al., 2019; Woodhams et al., 2020; Ortiz et al., 2021). Clear separation between host and external communities has been shown for several bivalves, suggesting that selective retention and colonization resistance maintain a functional barrier despite continuous influx. While direct studies on host-environment interactions in mussels are lacking, experimental evidence supports the hypothesis of functional adaption through selective microbe acquisition, for instance through the enrichment of denitrifying taxa (especially *nosZII, nirK* and *nirS* carriers) in bivalve tissue and sediments associated with mussel assemblages that provide microaerobic and anoxic conditions (Arfken et al., 2017; Murphy et al., 2019; Black and Just, 2018). Rapid adaptation to environmental changes through functional diversification and selective recruitment is essential for freshwater mussels, whose populations are under growing pressure due to climate change, mass mortality events, and anthropogenic pollution (Lopes-Lima et al., 2018; Stoeckl et al., 2020; Cope et al., 2021; Cushway et al., 2025). Given the dependency of mussel-mediated ecosystem services on stable host-microbe interactions, understanding how such stressors impact these communities is critical, particularly as the close host-environment coupling creates a potential point of vulnerability under stress. Currently, there is a lack of knowledge regarding the specific microbiome responses towards antibiotics and disinfectants which can enter aquatic ecosystems through both point and diffuse sources (e.g. municipal wastewater discharge or agricultural runoff). Aquaculture can be a major source of environmental pollution, as farming practices have increasingly shifted toward intensive production systems to meet rising demand for aquatic products. Frequent disease outbreaks in aquaculture necessitate the routine use of antimicrobials for disease control, often with limited regulatory oversight. As a consequence, antimicrobial consumption in aquaculture far exceeds that of terrestrial livestock or even human medicine when compared on an equivalent biomass basis (Schar et al., 2020). As most aquaculture facilities operate as open systems without effective wastewater treatment (incl. land-based systems like raceways and ponds), chemical residues and resistant pathogens are directly discharged into surrounding freshwater habitats, extending adverse side effects to a wide range of non-target organisms. Antibiotics primarily enter the environment via release through feces, uneaten feed residues, and farm effluents. Chronic exposure to sublethal concentrations of widely used broad-spectrum antimicrobials like florfenicol (FF) or oxytetracycline has been shown to compromise mussel health through cellular stress, immunotoxicity (Gust et al., 2012; Nicolussi et al., 2022) and microbiome dysbiosis, as indicated by reduced abundance of symbiotic bacteria like *Spirochaeota* and *Mycoplasma* and diminished carbon utilization (Griffin et al., 2021; Ramírez et al., 2022). Disruption of microbial networks and erosion of the commensal barrier may facilitate pathogen invasion, increase disease susceptibility and hinder the ability of mussels to mitigate stressors, comparable to responses observed in vertebrate models (Payne et al., 2022; Streb et al., 2025; Wang et al., 2019). Peracetic Acid (PAA), a commonly used disinfectant in salmonid aquaculture, is widely accepted as a safe alternative to conventional treatments due to its low environmental toxicity (Lazado and Good, 2021; Liu et al., 2024). Potent antimicrobial effects of PAA have predominantly been demonstrated at the single-strain level against fish pathogens like *Aeromonas salmonicida* and *Yersinia ruckeri* (Good et al., 2022; Meinelt et al., 2015). However, evidence from a recent study in brown trout showed that PAA can affect host-associated microbiomes at community level, including the loss of commensal taxa and reorganization of microbial networks, with effects comparable in magnitude to those reported for traditional antibiotics (Streb et al., 2025). Although the enrichment of antimicrobial resistance genes (ARGs) close to fish farms (Xiong et al., 2015; Muziasari et al., 2017; Zheng et al., 2021; Jiang et al., 2024) and mussels (Ramírez et al., 2022; Grevskott et al., 2017; Albini et al., 2022) as well as the detection of trace concentrations of commonly applied antibiotics (Jiang et al., 2024; Sargenti et al., 2020; Thiang et al., 2021) clearly highlights the spillover risk to adjacent environments and non-target organisms, ecological risk assessments remain largely centered on in-farm effects and on the farmed fish themselves. While long-term accumulation of PAA in water and sediments is unlikely due to its short environmental half-life, short-term exposure through continuous release via farm effluents represents a largely unexplored risk.

Considering that freshwater ecosystem stability relies on bottom-up food web support by bivalves, their exclusion from antibiotic risk assessment creates a critical gap. To address this gap, we selected the swan mussel (*Anodonta cygnea*), a common inhabitant of European freshwater systems, as model organism. While globally considered less threatened than other unionid species, regional population declines, including in Germany, indicating growing vulnerability to anthropogenic stressors such as habitat degradation and chemical pollution (Lopes-Lima et al., 2017; Bundesamt für Naturschutz, 2020). Nevertheless, the host microbiome of *A. cygnea* remains entirely unexplored despite being a key component of host resilience. Using 16S rRNA gene amplicon sequencing, we investigated the effects of indirect exposure to two commonly applied antimicrobials in European aquaculture, FF and PAA, on the *A. cygnea* microbiome. Given the strong interaction between filter feeders and the surrounding water column, we specifically focused on host-environment dynamics under antimicrobial stress over an extended post-treatment period. Drawing on evidence from other study systems, we hypothesized that exposure to antimicrobial residues would disrupt selective microbial recruitment and barrier stability, leading to microbiome destabilization and progressive convergence with the surrounding water community through invasion of opportunistic R-strategists. By examining this hypothesis in a non-target species of high ecological relevance under controlled conditions, our study expands the understanding of the ecological consequences of veterinary florfenicol and, for the first time, PAA use in aquaculture.

2. Material and methods

2.1. Experimental set-up and treatment

Mimicking natural spillover pathways, the effects of indirect exposure via rearing water effluents to florfenicol and peracetic acid on the composition of the host-associated microbiome of swan mussels (*Anodonta cygnea*) were investigated in a closed, recirculating aquaculture setup (RAS). Mature swan musselswere introduced into the existing rearing system for juvenile brown trout (*Salmo trutta*) as the main cultured species over an eleven-week period, including a three-week acclimation phase without intervention. Both species originated from the internal broodstock of the Aquatic Systems Biology Unit (Technical University Munich, Freising, Germany) and had no prior exposure to antimicrobials. No mortality was observed during the experiment. Species identity as *Anodonta cygnea* was additionally confirmed via PCR amplification using the primer set ITS-1-F (5‘-AGACTGGGTTGCGGAGGT-3‘) and ITS-1-R (5‘-CGAGTGATCCACCGCTTAGA-3‘) following the protocol developed by Zieritz et al. (2012). Sanger sequencing of PCR products from three individuals was performed at the Sequencing Facility of the Ludwig Maximilian University (Munich, Germany) on an ABI 3730 capillary sequencer, taxonomic identity was validated via BLASTn (99.31 % identity).

Each RAS unit consisted of a 1.7 m^3 fish tank (stocking density ~80 fish) connected to a sediment-filled trench above the tanks, stocked with 25–30 *A. cygnea* individuals (mean shell length 113.66 ± 20 mm). Rearing water was continuously pumped through the mussel compartment and recirculated back into the tank, ensuring sustained exposure to the pharmaceuticals used (see Supplementary Fig. S1).

The setup included four independent experimental units: (i) untreated control, (ii) florfenicol (FF), (iii) peracetic acid (PAA) and (iv) combined FF + PAA. Treatments were applied directly to the fish; mussels received no additional input. Florfenicol (Cayman Chemical, USA) was dispersed in rapeseed oil prior to top-coating the fish feed. The medicated feed was administered to the fish at a dosage of 10 mg/kg bw for ten consecutive days. The control group received oil-coated feed lacking florfenicol to ensure comparability across groups. 0.005% v/v Peracetic acid (Wolfasteril Classic©, Kesla Hygiene AG, Germany) was applied directly to the water surface on days one, three, six and nine during the treatment phase. Consequently, mussels were exposed only to residual concentrations of FF and PAA in the circulating rearing water, originating from fish excretion, feed-derived losses and partial degradation, rather than to the full dosage applied. Details on maintenance procedures of the animal experiment can be found in the Supplementary Material, Section 1.1.

2.2. Sample collection

Tissue swabs and water samples were collected at seven defined time points, each at least three hours post feeding: day 0 (pre-treatment), day 10 (end of treatment), and 4, 11-, 18-, 32- and 40-days post-treatment. Three individuals per treatment group were sampled non-invasively by carefully opening the shells with sterilized forceps and swabbing the internal tissue (foot, gill, and mantle) using sterile swabs (Carl Roth, Germany), targeting primary filtration and exposure sites. After the specimen collection, mussels were individually marked to avoid resampling and returned to the trenches. Swabs were aseptically transferred into sterile 2 ml Eppendorf tubes and snap-frozen on dry ice. In parallel, 2x 50 ml of tank water per treatment group was collected with sterile 50 ml Falcon tubes at each sampling point. Samples were stored at – 20 °C until downstream DNA extraction.

2.3. Determination of florfenicol and florfenicol amine by two-dimensional liquid chromatography coupled to tandem mass spectrometry (LC-LC-MS/MS)

Quantification of florfenicol and its major metabolite, florfenicol amine (FFA) in water samples was performed using LC-LC-MS/MS with a limit of quantification (LOQ) of 0.5 $\mu g\ L^{-1}$ and a detection limit of 0.2 $\mu g\ L^{-1}$.

The analytical technique was previously validated for florfenicol in fish fillet, liver and kidney samples (de Queiroga et al., 2024) and water. Chromatographic separation was performed using a mobile phase composed of water and methanol, both acidified with 0.01% (v/v) formic acid, following a gradient elution program: 0–1.3 min, 85:15 (v/v) water:methanol; 1.3–3.0 min, 0:100 methanol; 3.0–5.0 min, 0:100 methanol; and 5.0–6.0 min, 85:15 water:methanol. A total of 150 μL of sample was injected using a water:methanol (85:15, v/v) mixture as the loading solvent at a flow rate of 1.0 $mL\ min^{-1}$. Quantification was performed in selected reaction monitoring (SRM) mode, targeting transitions for both analytes and the isotopically labeled internal standard FF-d_3). Internal matrix-matched calibration curves were constructed using aqueous standards of FF and FFA at concentrations of 0.5, 1.0, 5.0, 10.0, 20.0, 30.0, and 50.0 $\mu g\ L^{-1}$, each containing a fixed concentration of the isotopically labeled internal standard (FF-d_3) at a concentration of 20.0 $ng\ mL^{-1}$. Procedural blanks were included in every analytical batch and processed under identical analytical conditions as the study samples. The absence of detectable FF or FFA residues in these blanks confirmed that there was no background contamination or analytical carryover. Data acquisition and instrument control were carried out using MassLynx™ v4.1 software, and chromatographic data were processed with TargetLynx™ (Waters Laboratory Informatics).

A complete description of the MS parameters is provided in de Queiroga et al. (2024) andQueiroga et al. (2025), monitored transitions used for quantification and confirmation are listed in the Supplementary Material (Table S3). Further information on sample processing can be found in the Supplementary Material (Section S1.4.).

2.4. 16S rRNA amplicon sequencing

Genomic DNA from 84 mussel tissue swabs was extracted using the NucleoSpin Forensic DNA Kit (Macherey Nagel, Germany) following the standard protocol. For water samples, each 50 mL sample was split into three pseudo-technical replicates per tank (15 ml each) and concentrated by centrifugation for 20 min at 14,000 rpm. After removal of the supernatant, pellets were resuspended in 1 ml of DEPC-treated H_2O and DNA extraction was performed with the NucleoSpin Soil Kit (Macherey Nagel, Germany), following the “soil and sediment” protocol. Negative extraction controls were included (Kit content, sterile swab, DEPC-H_2O used for resuspension) to monitor potential contamination during sampling and DNA extraction. Resulting DNA of 168 samples was quantified using Quant-iT Picogreen dsDNA Assay Kit (Thermo Fisher Scientific, USA), purity was assessed spectrophotometrically with a NanoDrop 1000 device (ThermoFisher Scientific, USA). Amplicon sequencing targeting the hypervariable V4 region of the 16S rRNA gene was performed using the primer pair 515F/806R (Apprill et al., 2015; Parada et al., 2016) including non-template PCR and extraction controls. Details on sequencing library preparation can be found in the Supplementary Material (Section S1.2.). High-Quality DNA was pooled in an equimolar ratio of 4 nM, sequencing was carried out on an Illumina MiSeq (2 x 300 bp paired-end) with Reagent Kit v3 (600 cycles) in two independent runs per sample type. Libraries were spiked with 7 pM 20 % phiX.

Raw reads and metadata are deposited in the Sequence Read Archive under Bioproject PRJNA1330032.

2.5. Raw data processing and ASV filtering

Pre-Processing of raw sequencing data and downstream statistical analyses were performed in RStudio (R version 4.4.2; (R Core Team, 2020). Paired-end sequencing yielded 4,709,653 reads from mussel samples and 7,797,700 reads from water samples. Sequencing adapters were trimmed using cutadapt v4.5 (Martin, 2011) and initial sequence quality was assessed with fastQC v0.12.1 (Andrews, 2012). Subsequent read processing including trimming of low-quality bases, estimation of error rates and inference of Amplicon Sequencing Variants (ASVs) was done with DADA2 v1.30.0 (Callahan et al., 2016) separately for the two sequencing runs. Trimming parameters were set to n-terminal trimming of 20 bp, truncation at position 240 (forward) and 210 (reverse) for water samples, and 250 and 210 for mussel samples with an expected error rate of 2 and 4. The resulting ASV tables were merged using the *mergeSequenceTables* option and taxonomic classification was performed using the SILVA v138 database (Quast et al., 2013). Putative contaminants were removed based on prevalence in extraction and PCR controls. ASVs were classified as contaminants if present in $\geq$25 % of controls, corresponding to a presence in $\geq$2 of 9 controls (water) or $\geq$4 of 16 controls (mussels), resulting in the removal of 90 and 98 ASVs, respectively. In addition, non-target sequences including unassigned/eukaryotic ($n = 43/53$), chloroplast ($n = 55/83$), mitochondrial ($n = 71/148$) and singleton ASVs ($n = 327$ in water, none detected in mussels) were removed from the dataset. The final dataset contained 9,327,713 high-quality reads, corresponding to an average sequencing depth per sample of 73,623 ± 21,250 reads (water) and 37,872 ± 21,044 reads (mussels). These reads represented a total of 17,721 unique ASVs with 9,917 ASVs detected in water samples and 10,948 ASVs in mussel samples. Adequate sequencing depth was confirmed via rarefaction analysis using the vegan R package (v 2.6.10; (Oksanen et al., 2016), as shown in Supplementary Fig. S2.

2.6. Microbial community analysis and statistical analysis

To account for varying sequencing depths, amplicon counts were normalized using cumulative sum scaling (CSS). Unless stated otherwise, downstream analyses were conducted using phyloseq v1.46, microbiome v1.24 and dplyr v1.1.4. All plots were generated using ggplot2 v3.5.1, ggpubr v0.6 and finalized in Inkscape.

Compositional differences between treatment groups based on Bray-Curtis dissimilarity were calculated with a two-way PERMANOVA (9999 permutations) and spatial ordination of samples was inferred using principal coordinate analysis (PCoA). SourceTracker2 (v2.0.1, Knights et al., 2011) was used to estimate the contribution of water ASVs to the mussel microbiome. Water samples were defined as source, mussel samples served as sink with CSS-normalized data as input.

To quantify whether antimicrobial exposure increased community similarity between water and mussel samples, we calculated the relative change in shared ASVs per treatment and timepoint. ASVs were considered as shared if they appeared in at least two out of three replicates in both sample types within a treatment-timepoint combination. This threshold ($\geq$60 %) was chosen to account for high inter-individual variation. Observed changes in the treatment groups were expressed relative to natural fluctuations in the control group.

To further resolve underlying dynamics of community structuring, we partitioned compositional dissimilarity based on Jaccard distances between microhabitats (mussel associated vs. ambient rearing water) into species turnover and nestedness components (i.e. selective species loss or gain leading to the less diverse assemblage becoming a subset of the more diverse community) using the betapart R package (v1.6; Betapart, 2012). This distinction provides insights into whether treatment-induced shifts may reflect either a restructuring of community membership (ASV replacement) or a selective loss of sensitive taxa without replacement. Mean values and standard deviations were summarized per treatment and timepoint group. To account for observed baseline variation between groups and avoid artefacts from near-zero denominators at the pre-treatment timepoint, we calculated adjusted delta values (Δ) as a descriptive measure of temporal change. For each treatment, the absolute change in β-jtu and β-jne relative to its own pre-treatment baseline was computed and then normalized by subtracting corresponding fluctuations in the control group at the same timepoint.

Differentially abundant ASVs in treatment groups of mussel samples compared to the un-exposed control group were identified using a linear model approach implemented in the microViz R package (v.0.12.1, Barnett et al., 2021). P-values were adjusted using the Benjamini-Hochberg method, only ASVs with an FDR-adjusted p-value > 0.05 and occurring in at least two out of six timepoints were considered significant. ASV-level heterogeneity and potential niche-specific recruitment patterns of *Flavobacterium*, *Dechloromonas* and *Aureispira* were assessed by extracting all respective ASVs from the full dataset and calculating their mean rel. abundance per sample type, treatment and experimental phase. Log_{10} transformed abundance values ($>$0.1) were visualized using pheatmap v1.0.12 (Kolde, 2025). Finally, statistical differences in genus-level abundances between groups were tested with Wilcoxon rank sum tests.

Detailed descriptions of supplementary analyses (e.g. alpha diversity, phylogenetic tree construction, and venn diagrams of shared ASVs counts between sample types), as well as additional statistical details, are provided in the Supplementary Material (Section S1.3.).

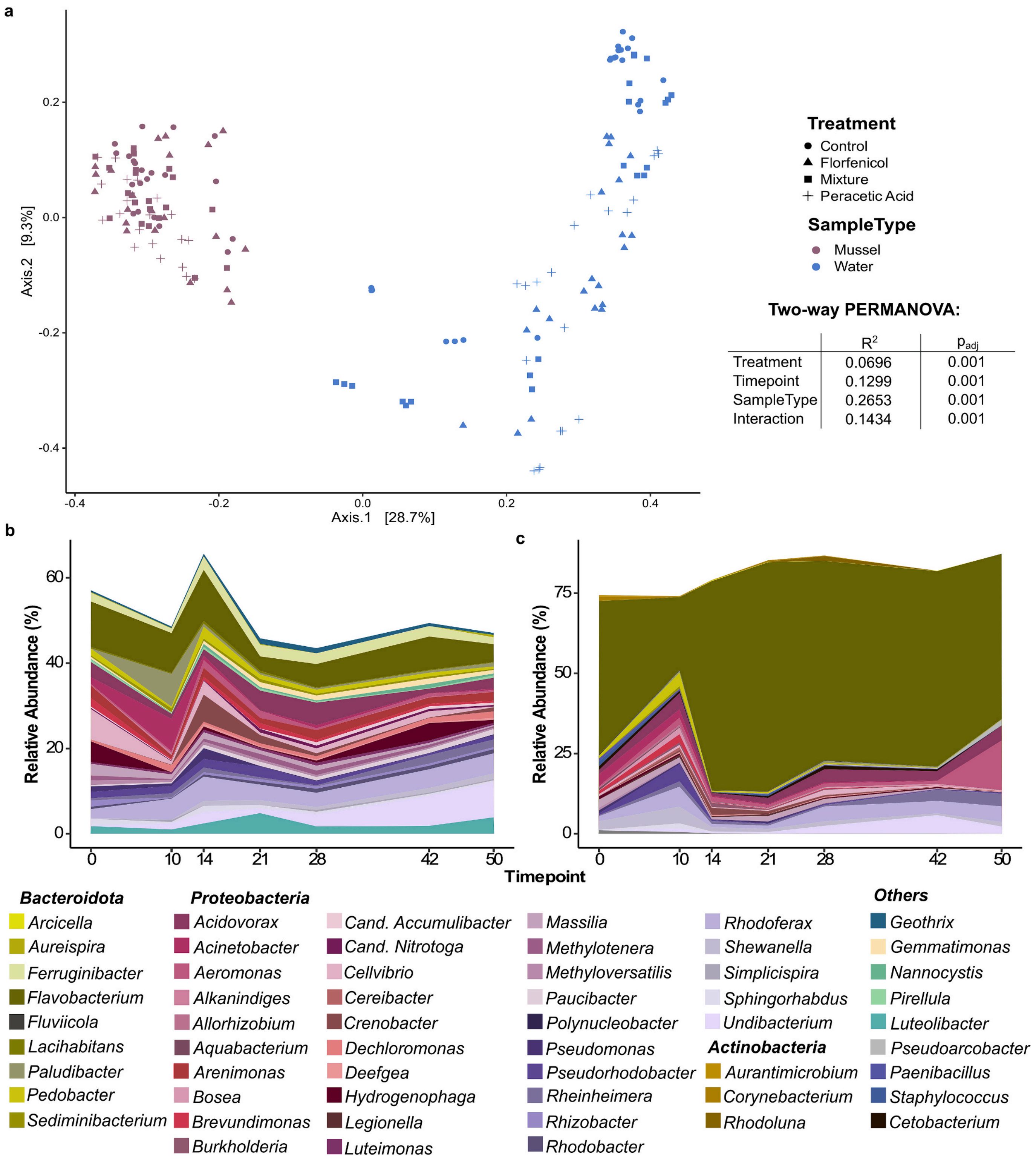
3. Results

3.1. *Anodonta cygnea* harbors a specialized microbiome distinct from rearing water

To establish a baseline for accurately assessing microbiome dynamics under stress, we first examined the surface-associated microbial composition of *A.cygnea, from the internal mantle and gill region,* and its interaction with the surrounding water in the untreated control group.

Microbial community composition differed significantly between mussel and water samples, with sample type explaining the largest proportion of variation (PERMANOVA; $R^2 = 0.2653$, $p_{adj} = 0.001$; see Fig. 1a). Despite stable tank conditions, temporal shifts were evident, with sampling timepoint alone accounting for 13 % of observed variation between groups. This pattern was also reflected in alpha diversity: *A. cygnea* maintained an overall more balanced microbiome than the bacterial community in the water, while ASV richness fluctuated during the early phase of the experiment and was higher in mussels at later sampling timepoints (ASV richness: F-value = 23.973, pval = 0.0001; Pielou evenness: F-value = 834.864, pval = 0.0001, Supplementary Fig. S3). No significant correlation of ASV Richness or Evenness with physicochemical water parameters (e.g. temperature, pH, NH_4^+-N) was detected in the mussels (Supplementary Table S2).

Consistent with observed ß-diversity patterns, only 40 % of detected genera (aggregated across timepoints) were shared between mussel and water samples (Supplementary Fig. S4). Mussels harbored a substantially higher proportion of unique genera (44 %) compared to the surrounding water column (16 %) despite constant exchange. Across sample types, the most abundant genera were affiliated with two main phyla, *Proteobacteria* and *Bacteroidota*: *Flavobacterium, Aeromonas, Acidovorax, Rhodoferax, Undibacterium, Pedobacter, Rheinheimera* and *Shewanella.* Mussel-associated communities consistently showed higher relative abundances of *Luteolibacter, Geothrix, Ferruginibacter, Dechloromonas* and *Hydrogenophaga* (Wilcoxon, $p < 0.05$). In contrast, water samples were enriched *in Rhodoluna, Aurantimicrobium*, and *Cetobacterium* (Wilcoxon, $p < 0.05$), likely reflecting input from fish feces and



Two-way PERMANOVA:

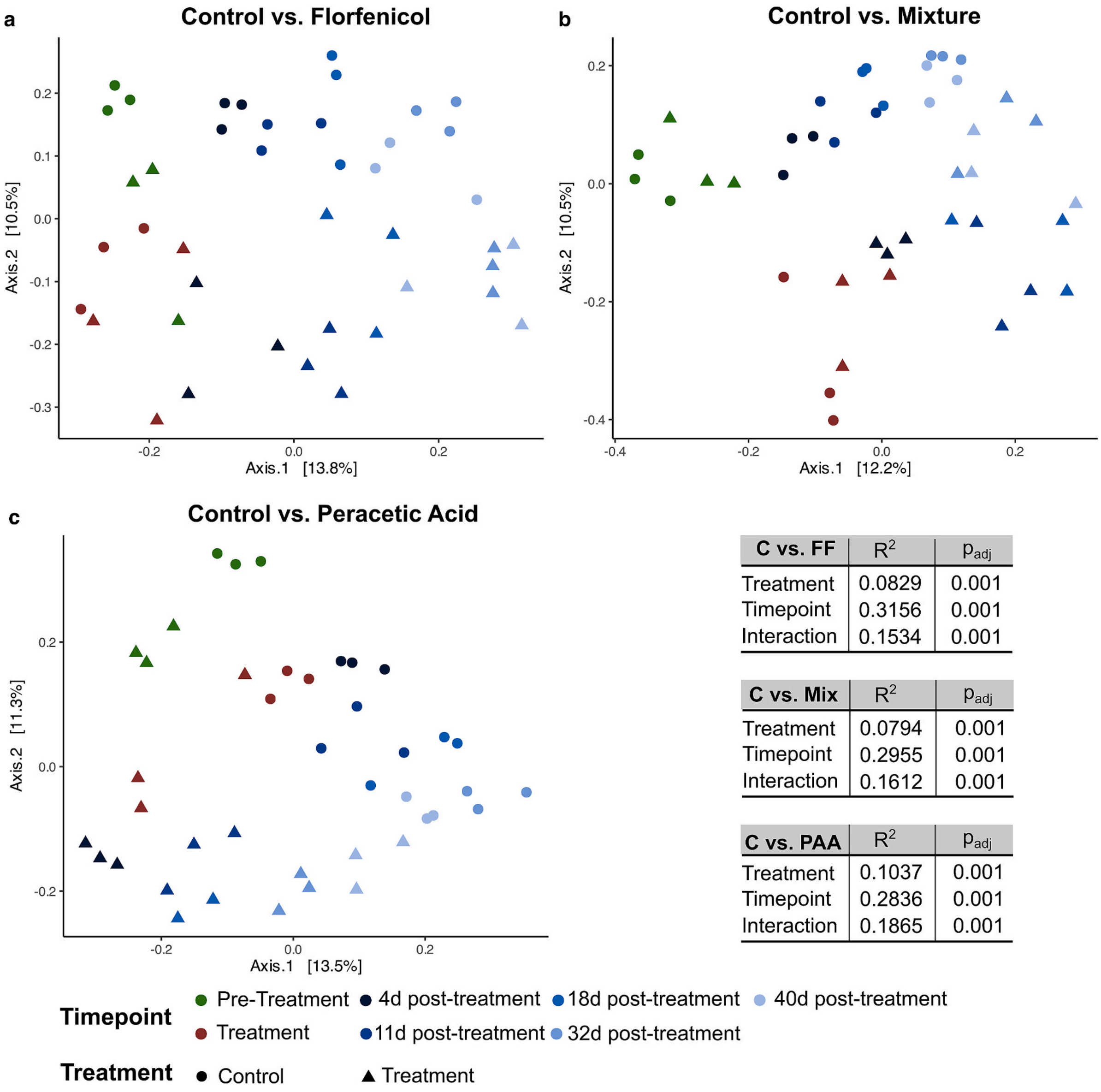
	R^2	p_{adj}
Treatment	0.0696	0.001
Timepoint	0.1299	0.001
SampleType	0.2653	0.001
Interaction	0.1434	0.001

Fig. 1. Differences in microbiome composition of *A. cygnea* and rearing water. (a) PCoA plot based on Bray Curtis dissimilarities between samples from freshwater mussels (pink) and water samples (blue) over 50 days. PERMANOVA results are included. Temporal dynamics of top 40 bacterial genera present in control samples from mussel (b) and water (c), sorted by phylum.

suspended detrital material.

3.2. *Environmental exposure to florfenicol and peracetic acid persistently alters the A. cygnea microbiome*

Indirect exposure to pharmaceutical residues of florfenicol and peracetic acid via the rearing water led to persistent shifts of the microbial community of *A. cygnea*. The strongest treatment effect was observed in mussels co-reared with fish treated with PAA (PERMANOVA; $R^2 = 0.10$, $p_{adj} < 0.0001$; Fig. 2). Based on visual clustering, the strongest separation between control and exposed mussels occurred in the early and mid post-treatment phase (between 4 and 32 days after



C vs. FF	R^2	p_{adj}
Treatment	0.0829	0.001
Timepoint	0.3156	0.001
Interaction	0.1534	0.001

C vs. Mix	R^2	p_{adj}
Treatment	0.0794	0.001
Timepoint	0.2955	0.001
Interaction	0.1612	0.001

C vs. PAA	R^2	p_{adj}
Treatment	0.1037	0.001
Timepoint	0.2836	0.001
Interaction	0.1865	0.001

Fig. 2. Effect of treatment on bacterial ß-diversity. PCoA plots based on Bray-Curtis distances comparing temporal microbiome shifts of control mussels to (a) florfenicol, (b) mixture, and (c) peracetic acid treated swan mussels. Color represents the different sampling timepoints, shape distinguishes control and treatment groups. PERMANOVA results are shown for each comparison.

treatment termination). Despite the absence of further antimicrobial intervention, no clear return to pre-treatment composition was detected by the end of the 40 day recovery period. Temporal dynamics remained a dominant driver of community variation across treatments ($R^2 = 0.31$, 0.29 and 0.28, respectively), however significant treatment × timepoint interaction indicates that microbiome responses were modulated over time rather than by exposure or time alone.

Quantitative LC-LC-MS/MS confirmed that florfenicol levels in tank water dropped below the detection limit of 0.2 μg/L 7 days after the final treatment application (see Supplementary Table S4). However, observed microbiome shifts in *A. cygnea* extended far beyond the quantifiable presence of the drug in the rearing water. Notably, tank water from the mixture group (PAA + FF) showed higher residual florfenicol concentrations compared to the florfenicol-only group. Florfenicol amine, the main metabolite of FF, was not detected at any timepoint.

3.3. Antibiotic and oxidative stressors drive temporal shifts in species turnover and environmental recruitment

To better understand natural microbial dynamics in a filter-feeding host, we first examined the temporal overlap between the mussel-associated microbiome and the surrounding water as the main source, focusing on spatial recruitment and species turnover rates under controlled baseline conditions. On average, 55 % of ASVs in the mussel microbiome were attributable to the water microhabitat over the experimental period, with SourceTracker2 estimates ranging between 43.18 and 71.71 % (Fig. 3a). Gradual convergence of both communities through selective recruitment of a defined subset of waterborne ASVs was further mirrored in a steady decrease in species turnover and increasing nestedness over time (Fig. 3c,d).

As shown in Fig. 3a, indirect exposure to the different antimicrobials altered environmental recruitment dynamics in a treatment- and time-dependent manner, aligning with the main response window visible in the PCoA Plots (Fig. 2). Under FF treatment, the mean contribution of waterborne ASVs increased consistently during the mid- to late post-

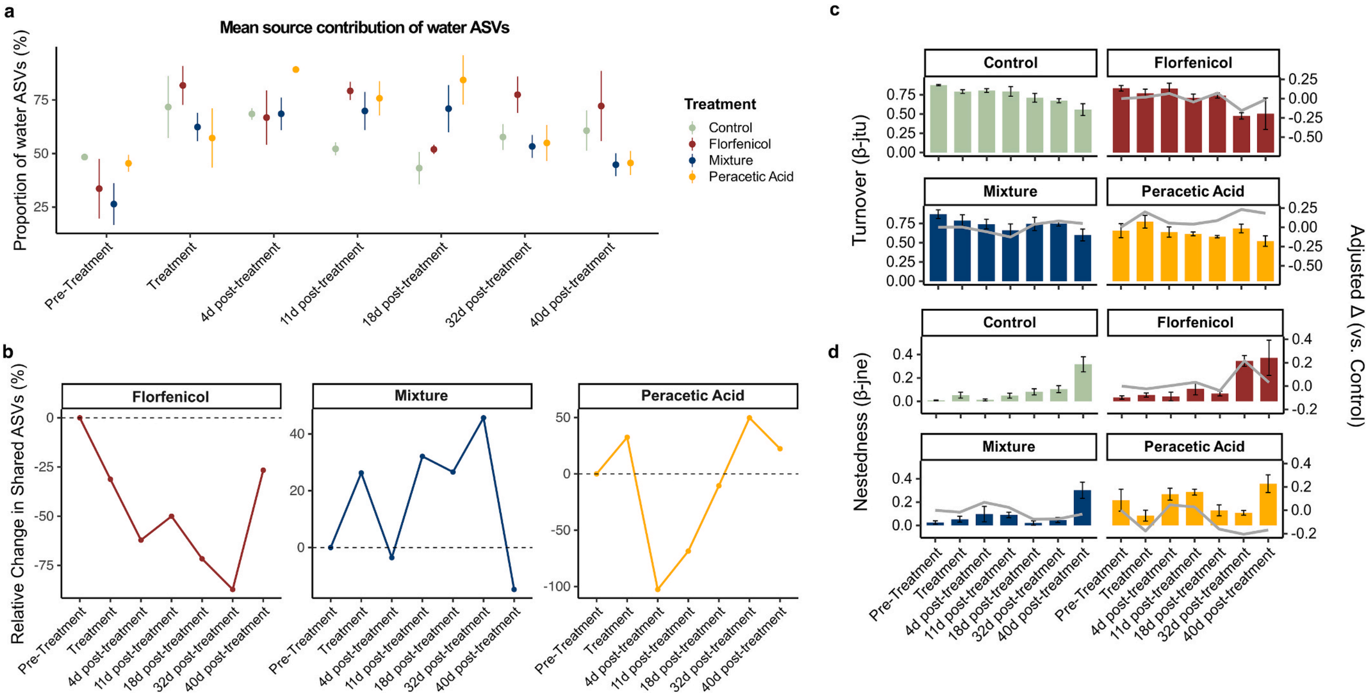


Fig. 3. Environmental recruitment dynamics between water and mussel samples following treatment. (a) SourceTracker2 estimates showing the contribution of water-borne ASVs over the experimental period. Shown are mean values of three replicates with error bars representing standard deviation. (b) Relative change in the number of shared core ASVs (present in 2/3 replicates per SampleType – Treatment – Timepoint combination) compared to natural fluctuations observed in the control group. (c and d) Species turnover values (ß-jtu) and nestedness (ß-jne) between mussel and water samples within each sampling group. Bars indicate mean turnover/nestedness values ± standard deviation (left axis). Temporal change relative to each group's pre-treatment baseline normalized by natural changes in the control is shown as adjusted Δ values (line and right axis).

treatment phase (18 to 40 days pT), averaging up to 15 % above control levels. In PAA-treated mussels, source contribution of water ASVs temporarily exceeded baseline levels, with values ranging from 75 to 89 %. However, by the final sampling timepoint, SourceTracker2 estimates had dropped significantly below the control (45 vs. 60 %). In the mixture group, observed changes were largely driven by the PAA component, with proportions of water ASVs remaining largely comparable to control levels for most of the experiment. Temporal increases occurred between 11 and 18 days pT but declined below control values in the late phase (44 vs. 60 %).

Interestingly, enhanced environmental influx did not necessarily translate into a consistently greater taxonomic overlap between mussels and water (Fig. 3b). In the FF group, the number of shared core ASVs persistently declined throughout the experiment compared to natural fluctuations observed in the control. In line with the observed time-dependent trajectory in environmental recruitment, shared ASV counts in the PAA group dropped by up to 102 % relative to the control until 18 days pT, following an initial increase during the active treatment phase. Values recovered above control levels by the end of the experiment. Although no synergistic effect of the joint application of FF and PAA was detected in the SourceTracker analysis, distinct interaction dynamics based on shared ASV counts were evident compared to the singular treatments. Here, the number of core ASVs shared between *A. cygnea* and rearing water increased sharply during the treatment and intermediate pT period by up to 40 %.

Treatment-specific community-level dissimilarity metrics further supported these observations (Fig. 3c). In FF-treated mussels, turnover values (ß-jtu) varied only moderately over the experimental period (adj. Δ +0.019 during treatment to −0.16 at 32 days pT), and nestedness (ß-jne) remained largely comparable to natural dynamics in the control. The temporal instability seen under PAA exposure was reflected by consistently elevated species turnover between microhabitats relative to the control (adj. Δ +0.04 to +0.23). Water communities represented a smaller subset of the mussel microbiome, as indicated by a decline in baseline-corrected nestedness change during the application phase and adj. Δ values remaining below control levels by the end of the experiment (−0.20). Interestingly, the strong increase in shared ASVs in mussels exposed to both compounds could not be correlated to corresponding shifts in species turnover or nestedness, with both metrics showing no clear deviation from control values over time.

3.4. A microbial survival strategy: selection-driven restructuring without diversity loss

Microbial key responders of the *A.cygnea* microbiome to the different treatments were identified using a linear modelling approach. Fig. 4 summarizes responding ASVs with a consistent response pattern across at least two out of six sampling timepoints. Despite the distinct chemical properties of the different antimicrobial agents, the number of differentially abundant ASVs was similar across treatments (FF: 28; PAA: 29; Mix: 26). General responders enriched in all treatment groups included *Cellvibrio* sp. (ASV135), *Aureispira maritima* (ASV12), *Hydrogenophaga* sp. (ASV584/437); *Sphingomonas koreensis* (ASV507) and among others. Only two ASVs, *Ferruginibacter* sp. (ASV1756) and *Gemmatimonas* sp. (ASV2526), showed strongly reduced abundances in the late pT timepoints of all treatments. *Dechloromonas* sp. (ASV393/884), *Brevundimonas bacteroides* (ASV236) and *Sulfuritalea* sp. (ASV23000) were strongly enriched in mussels exposed to PAA and Mixture, but not FF. Overall, FF shared most of its responding ASVs with PAA, including *Alkanindiges* sp. (ASV107), *Marinagarivorans* sp. (ASV180) and *Pseudomonas* sp. (ASV813). Indirect exposure to both florfenicol and PAA strongly diminished the abundance of *Bdellovibrio* sp. (ASV2000) and *Flavobacterium* sp. (ASV16) among others. F*lavobacterium (ASV48), Novosphingobium* sp. (ASV533), Sph*ingorhabdus* sp. (ASV93) and *Rhodoferax* sp. (ASV29) were significantly depleted in mussels collected from fish tanks receiving either FF or PAA treatment. Notably, most

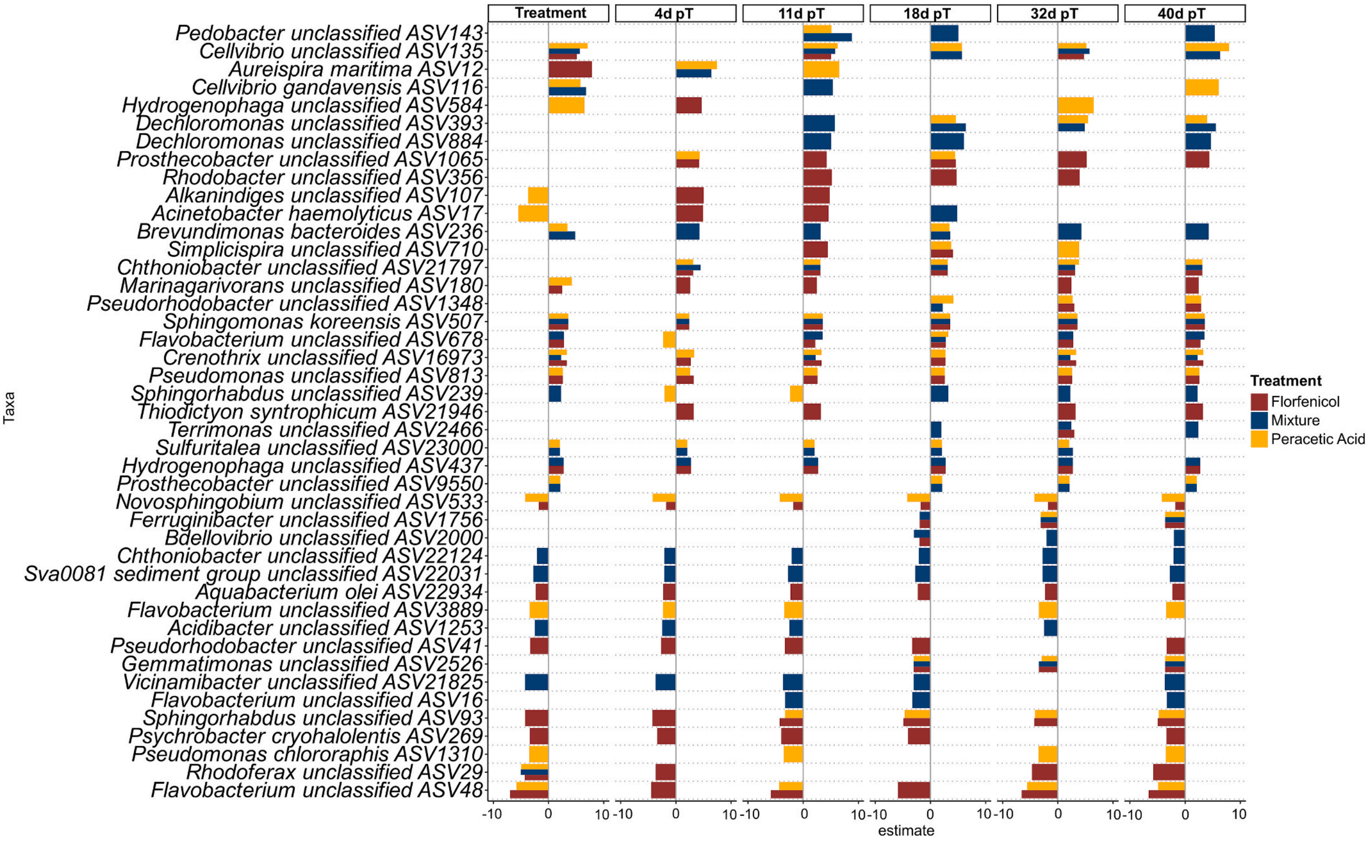


Fig. 4. Differentially abundant ASVs identified by a GLM. Bars represent model estimates, indicating direction and magnitude of change in abundance relative to the control group. Only ASVs detected in at least two sampling timepoints and with padj < 0.05 are shown.

observed shifts persisted throughout experiment and did not recover their initial abundance by the end of the experiment. No consistent enrichment of ASVs belonging to genera with known opportunistic pathogens was observed in any treatment group, except for *Acinetobacter haemolyticus (ASV17)*.

Comparative analysis of pre- and late post-treatment taxonomic profiles between compartments showed that the enrichment of certain taxa in the mussel microbiome in response to FF or PAA exposure was not directly attributable to an increased abundance in the ambient water (Fig. 5). Despite the observed compositional restructuring following chemical exposure, relative abundance of several core constituents of the microbiome remained stable over the course of the experiment, including *Luteolibacter* and *Rhodoferax*. Compared to the initial pre-treatment composition, *Nannocystis* and *Sediminibacterium*, which were initially present at very low abundance, accounted for a large share of the endpoint composition across all treatments (Fig. 5). In PAA- and mixture-exposed mussels, *Dechloromonas* established as a key member in the post-treatment community (0.39 % ± 0.12 to 6.56 % ± 6.25).

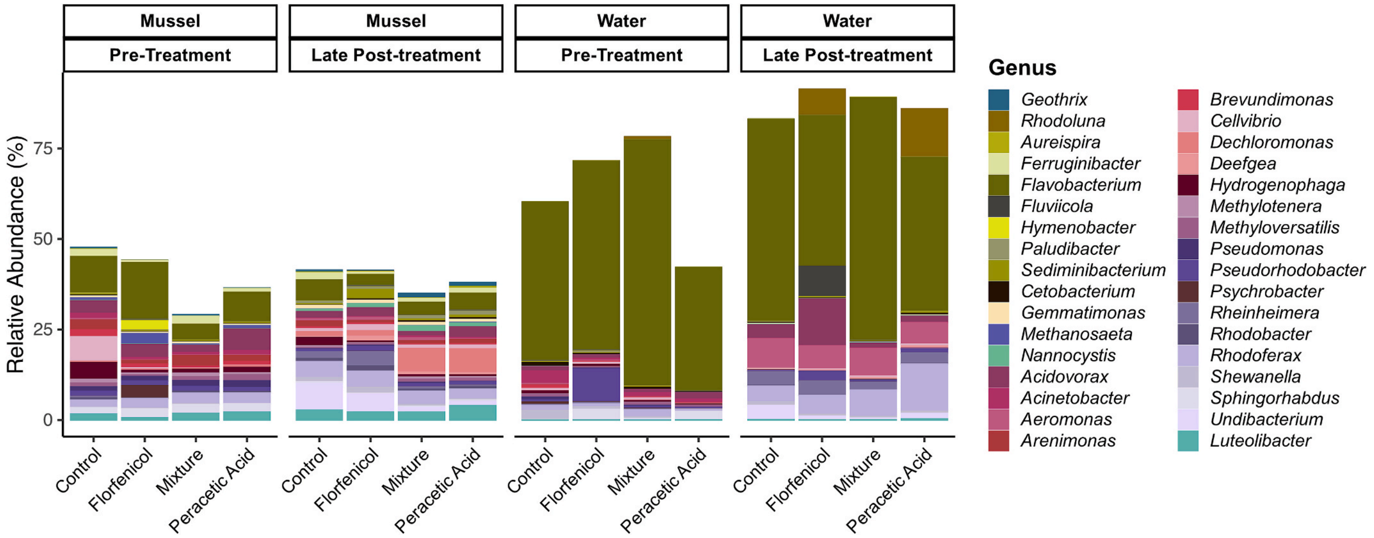


Fig. 5. Top 35 genus-level composition of pre- and post-treatment communities of mussel and water samples. “Late post-treatment” summarizes rel. abundance values from 32 and 40 days pT samples.

Similarly, *Geothrix* and *Methyloversatilis* increased in relative abundance towards the end of the experiment. An interesting transient pattern was observed for *Aureispira*, present at very low levels in both habitats pre-treatment and post-exposure (0.04 % ± 0.09). However, a strong temporal increase up to 11 days pT was evident in both sample types (see Supplementary Fig. S5) and all treatments. *Acidovorax*, *Aeromonas* and *Flavobacterium* did not constitute a larger share of the endpoint composition in the mussel microbiome proportional to their strong enrichment in water.

Contrary to the initial hypothesis of progressive homogenization under antimicrobial stress, no treatment group displayed a reduction of community diversity or a complete shift towards a community structure fully resembling those of the surrounding rearing water. ASV richness and Evenness remained stable, indicating the stress-induced restructuring was not accompanied by a loss of diversity (see Supplementary Fig. S3, Table S2).

3.5. Strain-level partitioning of Flavobacterium reveals habitat-specific subpopulations

To further explore the strain-level heterogeneity underlying the observed selective enrichment, we examined all ASVs assigned to *Flavobacterium*, *Dechloromonas* and *Aureispira* across treatments. Distinct habitat-specific subpopulations were evident within each genus (Fig. 6). In particular, *Flavobacterium* displayed high intra-genus variation and ecological divergence, with certain ASVs representing commensal strains adapted to the host environment, while others were predominantly associated with the water column. Interestingly, despite *Flavobacterium* dominating the rearing water microbiome, species-level heterogeneity was significantly lower compared to the more diverse subpopulations in mussels. Similarly, notable species-level variation in response to the different treatments were observed (see Fig. 3). Phylogenetic placement against curated reference genomes showed that water-associated *Flavobacterium* ASVs were closely related to environmental generalists such as *F. terrigena* and *F. saccharophilum*, whereas mussel-associated ASVs clustered with both host- and environmental species, e.g. *F. columnare*, *F. johnsoniae* and *F. crassostrea* (see Supplementary Fig. S6). Notably, responding ASVs to the treatments were not among the dominant *Flavobacterium* ASVs in the microbial community of *A. cygnea*, suggesting that the disturbance primarily affected less abundant, water-associated strains rather than the stable core community. *Dechloromonas* was largely restricted to mussels and clustered with *D. dentrificans* and *D. agitata.*

4. Discussion

4.1. Host-environment filtering shapes microbiome assembly in A. cygena

Understanding how chemical spillover from aquaculture shapes microbiomes of non-target organisms in adjacent environments is critical for developing antimicrobial stewardship strategies that limit both resistance dissemination and ecological disruption. To our best knowledge, this study is the first to investigate the singular and combined effects of florfenicol and peracetic acid on the microbiome of swan mussels (*A. cygnea*) in a controlled setting, focusing on how antimicrobial exposure alters interactions between the host microbiome and exogenous bacteria as a potential source for invasion. Under baseline conditions, a selected subset of taxa originating from rearing water was

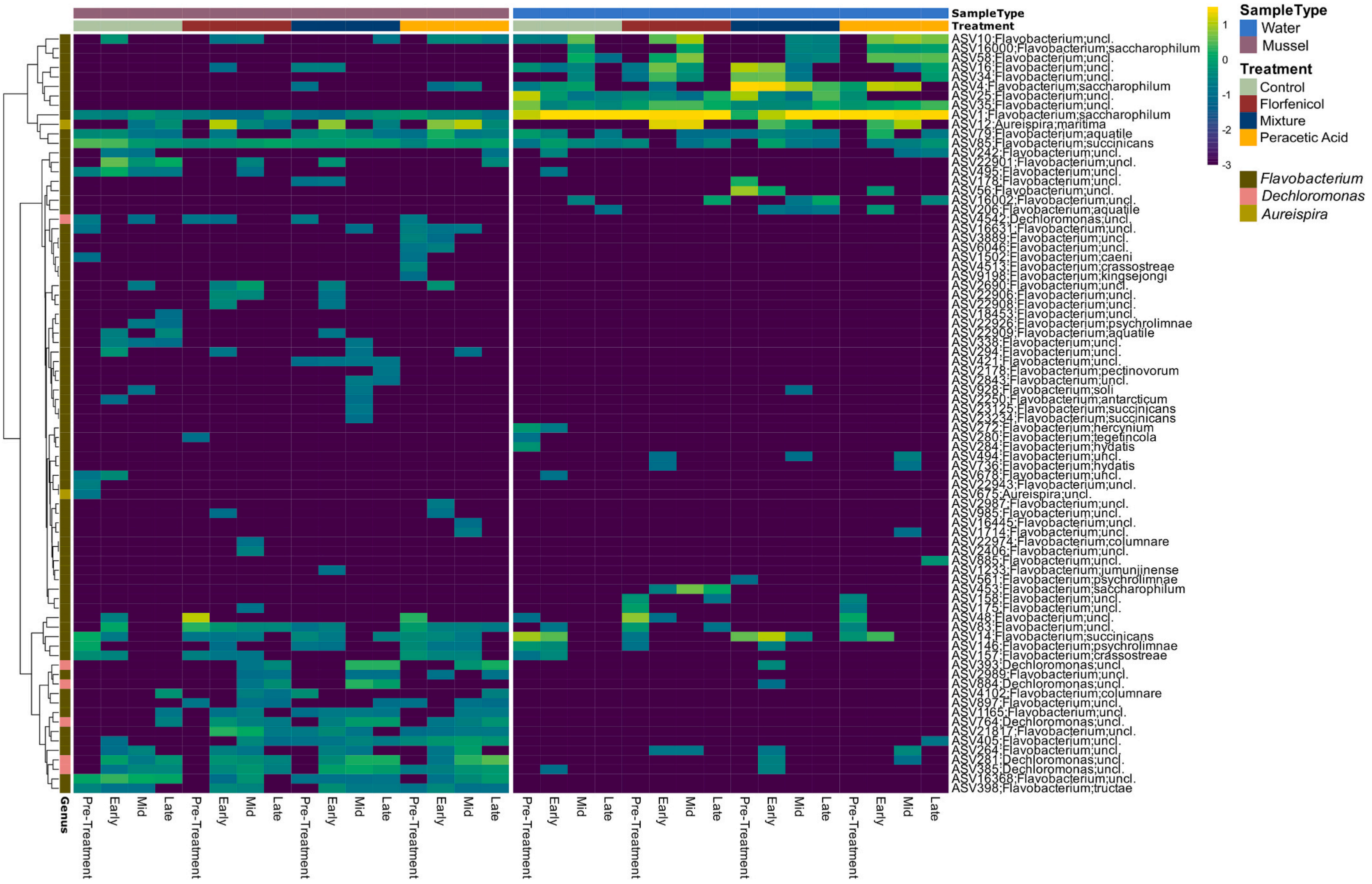


Fig. 6. Strain-specific abundance patterns of ASVs assigned to *Flavobacterium*, *Dechloromonas* and *Aureispira*. Heatmap showing log-transformed abundances of all ASVs assigned to the three genera across treatments, sampling phases and sample types. pre-treatment (day 0), early (day 10, 4 days post-treatment), mid (11 & 18 days post-treatment), late phase (32 & 40 days post-treatment). ASVs were hierarchically clustered using Ward.D2 linkage.

retained within the microbiome of *A. cygnea* with community turnover between host and environment stabilizing over time. This suggests the establishment of a host-specific equilibrium, in which community composition is not passively determined by ingested environmental bacteria but actively structured through environmental influx and host-mediated filtering. Similar differentiation between host and environmental microbiomes have been reported elsewhere for marine and freshwater bivalves (Weingarten et al., 2019; Akter et al., 2023; Chiarello et al., 2022). The dominance of *Proteobacteria* and *Bacteroidota* was consistent with previous findings in mollusks and freshwater systems. While genus-level composition differed from other studies, such variation is expected given the strong influence of host species (Weingarten et al., 2019; Akter et al., 2023; Gignoux-Wolfsohn et al., 2024), habitat (Chiarello et al., 2022), and methodological differences such as the investigated tissue type (Musella et al., 2020). Nevertheless, functional convergence across studies indicates that bivalve-associated microbiomes are selectively structured around lineages that directly support host physiology and ecosystem processes orchestrated by their host (e.g. presence of N-fixing bacteria (Arfken et al., 2017; Murphy et al., 2019; Pfister et al., 2010), sulfur-oxidizers in deep sea mussels (Ücker et al., 2021). Mussel-specific ASVs like *Luteolibacter* and *Ferruginibacter* detected in our study further support this observation. By degrading algal-derived organic matter (OM) and other detrital polysaccharides – a major component of ingested seston during filter feeding – into bioavailable forms, these bacteria may increase nutrient availability to the host and mediate nutrient fluxes within aquatic food webs (Ohshiro et al., 2012; Hu et al., 2023; Ren et al., 2025; Zhu et al., 2025). Anthropogenic nutrient pollution has become a major stressor for freshwater ecosystems and benthic fauna worldwide, as elevated N and P inputs from wastewater, agricultural runoff and aquaculture effluents accelerate eutrophication (Camargo and Alonso, 2006; Gillis et al., 2017; Luo et al., 2018; Wurtsbaugh et al., 2019; Dalbem Barbosa et al., 2024). Iron-reducing *Ferruginibacter* plays a pivotal role in these settings. By catalyzing the reductive dissolution of bound Fe(III) oxides into soluble Fe(II) serving as electron donors for denitrification and Fe-anammox processes, *Ferruginibacter* strongly contributes to nitrogen removal under aerobic and anoxic conditions (Yao et al., 2019; Yang et al., 2021; Pang and Wang, 2021; Hao et al., 2024). A comparable involvement in balancing nitrogen levels can be attributed to *Hydrogenophaga*, a methylotrophic denitrifier frequently found in nitrogen-rich hotspots such as activated sludge (Choi et al., 2020), pacific oysters (Baek et al., 2017), zebra mussels (Marzocchi et al., 2021) and the intestinal tract of fish (Narrowe et al., 2015; Streb et al., 2025) and amphibians (Walke et al., 2014). Furthermore, functional plasticity under high N + P loads in estuarine environments has been recently reported in oysters (Stevick et al., 2021). Although further verification through assessment of denitrification rates and metagenomics is needed, it is plausible that the accumulation of these taxa within the *A. cygnea* microbiome supports host health by mitigating nitrogen pollution and maintaining habitat conditions favorable for mussel survival in nitrogen-enriched freshwater systems affected by aquaculture.

ASV-level partitioning of *Flavobacterium* between microhabitats provides further evidence for the role of intrinsic host filtering in shaping microbiome composition. Together with *Acidovorax* and *Aeromonas*, *Flavobacterium* accounted for a major fraction of overlapping taxa between mussels and water. These genera are environmental generalists with broad metabolic versatility, commonly detected across aquatic habitats (Chaix et al., 2017; Ciric et al., 2023; Vettorazzo et al., 2024), soil (Singleton et al., 2018; Seo et al., 2024), fish (Li et al., 2017; Yajima et al., 2023; Bell et al., 2024) and different bivalve species (Maravić et al., 2013; Choi et al., 2017; Rao et al., 2024). Their concurrent occurrence may reflect ingestion of local microbial consortia, either particle-bound or planktonic, and could also be influenced by input from fish faeces, as both *Aeromonas* and *Flavobacterium* were dominant taxa in the intestinal tract of brown trout, the main cultured species used in this study (Streb et al., 2025). Within *Flavobacterium*, mussel-associated ASVs aligned most closely with strains (e.g. *F. johnsoniae*, *F. crassostraea*) linked to aquatic hosts like pacific oysters (Choi et al., 2017) and the utilization of detrital substrates such as laminarin, a key component of filter-feeding diets (Sack et al., 2011). In contrast, water-associated ASVs predominantly clustered with terrestrial strains, whose genetic repertoires are on average documented to be more suited to plant-derived polysaccharides, consistent with genomic studies showing terrestrial *Flavobacterium* harbor broader sets of carbohydrate-degrading enzymes for compounds such as xylose, arabinose and pectin (Kolton et al., 2013; Kim et al., 2023). This divergence likely reflects need-driven specialization for their feeding ecology rather than random stochastic uptake from the environment based on external availability. Although amplicon sequencing cannot resolve strain-level heterogeneity, the observed partitioning aligns with the well-documented niche differentiation based on metabolic capability of *Flavobacterium* (Kim et al., 2023) and highlights this genus as a valuable model for studying host-driven microbiome assembly.

Moreover, the highly selective strain-level partitioning observed may represent a key element of ecological stability through functional redundancy, potentially buffering the host against antibiotic-induced stress.

4.2. Selective restructuring maintains microbiome integrity under antimicrobial perturbation

Consistent with recent findings from field studies of clams and mussels collected from chemically polluted habitats (Ramírez et al., 2022; Milan et al., 2018), our study showed that continuous exposure to residual FF and PAA over a 10 day period had a persistent influence on the microbiome of *A. cygnea*. Despite its reputation as a low-risk disinfectant, PAA exerted the strongest shifts in our experiment. This outcome may partly reflect differences in exposure routes, since PAA was applied directly to the water column whereas FF exposure occurred through medicated feed, meaning that mussels were only exposed to partially metabolized residues compared to the full dosage. However, this mode of exposure closely mirrors real-word conditions, where non-target organisms are typically affected through effluents rather than direct application. The transient increase in taxonomic overlap and source contribution from water following PAA withdrawal indicates that the barrier function of the mussel microbiome was temporarily compromised. This response likely reflects oxidative stress inflicted by PAA, which induces niche disruption through rapid cellular damage, in contrast to the slower bacteriostatic mechanism of FF through inhibition of bacterial protein biosynthesis. While the antimicrobial activity of PAA at the single-strain level is well established (Good et al., 2022; Meinelt et al., 2015), evidence regarding its effects on complex communities remains conflicting. Some studies report only minor disruption in water communities (Liu et al., 2018; Suurnäkki et al., 2020), whereas host-associated microbiomes appear considerably more susceptible (Galgano et al., 2023, 2024; Streb et al., 2025).

However, unlike in many vertebrate models, where antibiotic exposure often leads to the enrichment of opportunistic pathogens at the expense of host-beneficial commensals (Wang et al., 2019; Payne et al., 2022; Narrowe et al., 2015), microbiomes of treated mussels largely preserved their core composition. Bacterial richness remained stable and community structure following antibiotic withdrawal indicated that the observed restructuring was driven by the selective enrichment of stress-resilient taxa, either emerging from the resident microbiome or recruited from the environment. The consistently higher abundance of *Dechloromonas, Sphingomonas* and *Hydrogenophaga* throughout the experiment reflects microbial signatures typical of polluted environments and can be explained by their ability to degrade a broad range of xenobiotic pollutants, including polycyclic aromatic hydrocarbons (PAHs), chlorophenols and pesticides along with documented tolerance to disinfectants such as triclosan (Gabriel et al., 2005; Coates et al., 2001; Salinero et al., 2009; Narrowe et al., 2015; Xu et al., 2015; Mulla et al., 2016; Takeo et al., 2020; Zhang et al., 2021; Semenova et al., 2024). In addition, stress-mitigation traits such as biofilm formation and ROS tolerance systems like chlorite and perchlorate dismutases that can convert PAA into hydrogen peroxide in *Sphingomonas* and *Dechloromonas* plausibly explain their persistence under PAA exposure. Furthermore, diverse multidrug resistance determinants, including those against phenicols (e.g. acetyltransferases and novel *aphA* genes) are frequently detected in *Sphingomonades*. Similar microbial adaptations have been reported in manila clams (Milan et al., 2018) and in bivalve-associated communities isolated from oil spills (Bayat et al., 2015), where enrichment of PAH-degrading bacteria likely mitigated pollutant loads. These findings highlight that the ability to detoxify harmful xenobiotics, either through host metabolism or via the synergistic activity of the microbiome, is vital for sessile organisms like *A. cygnea*, which are not very mobile and thus fully exposed to local habitat conditions and must rapidly adapt to persist under stress. The enrichment of *Nannocystis* and *Aureispira* suggests a complementary layer of resilience through the modulation of microbial interactions. Within microbial consortia, community dynamics are not only determined by intrinsic traits or resource availability, but also by interspecific mechanisms regulating colonization such as chemical interference and predation. Both processes can stabilize disturbed microbial communities by suppressing opportunistic invaders and preserving resident taxa after niche disruption, thereby contributing to a more resilient microbial community (Machado et al., 2021; Geesink et al., 2024; Woelfel et al., 2024). *Nannocystis* belongs to the class of predatory *Myxobacteria* capable of producing diverse bioactive metabolites, including nannozinones with reported antimicrobial properties, which may confer a competitive advantage and potentially support the observed post-exposure restructuring of the microbiome (Jansen et al., 2014; Bhat et al., 2021). Although its specific role in bivalve microbiomes has not been characterized yet, evidence for community modulation by *Nannocystis* and other predatory myxobacteria exists in soils and wastewater treatment plants (Petters et al., 2021; Zhang et al., 2023). *Aureispira* may exert top-down control through ixotrophic predation, as recently demonstrated against *Vibrio*, a genus containing several highly relevant fish pathogens (Lien et al., 2024). Comparable predator–prey dynamics have been reported in coral microbiomes, where commensal *Halobacteriovorax* populations modulate *Vibrio* abundance and contribute to microbiome stability under infection pressure (Welsh et al., 2016; Welsh et al., 2017; Ottaviani et al., 2020). Internal stabilization through antagonistic and syntrophic interactions within the microbiome may have reinforced the external defensive layer after temporary barrier erosion during singular and combined PAA treatment. Despite the strong increase of *Aeromonas* and *Flavobacterium* in water samples, they did not expand beyond their initial abundance in treated mussels. As both taxa are characterized by high stress tolerance and broad metabolic versatility, allowing them to effectively occupy vacant niches, their failure to establish in mussels implies that the selective exclusion was not driven by physiological constraints but by active host filtering processes, that restricted overgrowth of these taxa. Strain-specific responses of *Flavobacterium* to the different treatments further supported this pattern. The observed heterogeneity likely reflects resistant and susceptible lineages, consistent with the selective, function-driven assembly discussed in 4.1. It should be noted that the methodology applied in this study does not allow discrimination between the specific host filtering mechanisms predominately driving the observed restructuring, such as stress-induced changes in immune mediation, increased selection for detoxifying or modulatory bacteria or suppression of overgrowth through niche occupation. Nevertheless, the stable abundance of *Aeromonas* and *Flavobacterium* within mussels across all groups, despite the strong proliferation in water samples under constant physicochemical tank conditions, indicates that passive, environment-mediated filtering alone is unlikely to account for the observed patterns.

Considering the lack of pathogen proliferation and overall community structure in *A. cygnea*, the mussel microbiome likely retained its structural integrity throughout the exposure period. While some low-abundant taxa involved in OM degradation and nutrient transformation declined under antimicrobial exposure (e.g. *Psychrobacter, Pseudomonas, Gemmatimonas*) (Deng et al., 2021; Hou et al., 2022; Gong et al., 2024; Orlando et al., 2025), their functions are broadly shared among microbial lineages and are likely to be compensated by other members of the microbiome after disturbance. In the context of the holobiont framework, dysbiosis is commonly characterized by a loss of microbial homeostasis, indicated by diversity loss, depletion of beneficial taxa and pathogen overgrowth (Hooks and O'Malley, 2017; Berg et al., 2020). Given the persistence of central core members and the observed redundancy within the microbiome, no signs of dysbiosis occurred under the tested conditions, at least not based on the taxonomic data presented in this study. Nevertheless, further verification through denitrification assays or metagenomic profiling of host-beneficial functions are required to confirm whether the observed taxonomic stability also translates into functional resilience. However, our findings also highlight a critical knowledge gap regarding the assessment of resistance dissemination patterns. *Aeromonas* is frequently used as a proxy species for AMR surveillance in aquaculture settings and, similar to *Flavobacterium*, represents a major group of fish pathogens responsible for considerable economic losses in recent years, particularly in temperate aquaculture systems (e.g. trout farming) (Derome et al., 2016; Lamy et al., 2022; Roh and Kannimuthu, 2023; Lee et al., 2023). While some studies have highlighted the potential of bivalves as reservoirs for ARG dissemination, we could not verify the presence of frequently reported AMR carriers detected in mollusks in our study, such as *Vibrio* sp.*, E. coli* sp. and *Pseudomonas* sp. (Grevskott et al., 2017; Albini et al., 2022; Ramírez et al., 2022). In this context, mussels may therefore not represent major ARG hotspots, although they could act as transient reservoirs under chronic exposure in a more complex, real-world setting. As the experiment was performed under controlled conditions and represents a single exposure event in a naïve host, the observed responses likely underestimate the magnitude of effects occurring under natural environmental conditions. Especially in industrialized regions, where freshwater ecosystems are under severe pressure from continuous pollution load and habitat destruction (Reid et al., 2019; Glibert et al., 2022), both abiotic and biotic stressors (e.g. hydrodynamics, temperature fluctuations, fertilizers or elevated nutrient levels) may drastically influence outcomes. The extrapolation of controlled laboratory experiments to complex natural environments is a key consideration when interpreting the adaptive responses observed here. Host-environmental filtering should be understood as a dynamic mechanism whose effectiveness depends on both host regulatory capacity and the integrity of the resident microbial community. The conditions tested in this study represent a comparatively mild exposure scenario, under which microbiome stability was maintained through selective recruitment and community reorganization. However, this balance is likely sensitive to increasing pressure. When antimicrobial stress strongly reduces microbial biomass and erodes key functional groups mediating colonization resistance, host filtering may no longer suffice to maintain microbiome homeostasis. Repeated exposure during the vulnerable post-treatment recovery window, higher dosages and combined application of antimicrobials may amplify this by inducing physiological stress responses in the host that alter immune mediation and tissue-specific microenvironments. Moreover, species-specific variation should be considered, as *A. cygnea* is relatively resilient to altered habitat conditions, and thus may not fully reflect responses of more sensitive mussel species (Lopes-Lima et al., 2017). Co-selection of antibiotic and heavy metal resistance genes represents an additional, often underestimated risk in such settings (Pavón et al., 2022; Yi et al., 2022; Di Cesare et al., 2023; Gillieatt and Coleman, 2024). Over time, these pressures may gradually erode the stress mitigation capacity observed here. Recurrent stress events could promote the accumulation of highly prevalent, water borne AMR carriers like *Aeromonas* in non-target organisms with a tangible risk of ARG spread within and beyond aquatic environments, including human exposure through recreational activities. Moreover, seasonal variation in bivalve and water microbial communities may affect both initial composition and microbial recruitment dynamics, as higher water temperatures are associated with increased pathogen loads and could therefore intensify infection pressure under natural conditions (Karvonen et al., 2010; Tout et al., 2015; Milan et al., 2018).

However, some methodological constraints should also be acknowledged. Community-profiling approaches like 16S rRNA gene sequencing have inherent limitations regarding the interpretability of the results, as they provide reliable information on taxonomic changes, but do not allow direct inference of shifts in metabolic function or mechanistic links between individual taxa and host health. As such, conclusions derived from taxonomic patterns should be viewed as provisional rather than direct evidence of functional change. Nevertheless, such approaches are essential for establishing baseline knowledge, especially in understudied models like freshwater mussels, with results presented in this study providing a foundation for targeted, follow-up investigations. Further studies should include metatranscriptomic analysis to e.g. assess active regulation of detoxification and stress response pathways to establish a link between taxonomic and functional data. Integrating this data with physiological host-response parameters allows for comprehensive assessment of microbiome-mediated resilience, that are currently not available for freshwater mussels. Finally, although all analyses were expressed relative to natural fluctuations and the respective pre-treatment baseline in the control group, a certain degree of technical bias cannot be fully excluded due to the lack of replication at tank level.

4.3. Compound dynamics shape ecological risk of antibiotic usage in aquaculture

Antimicrobial residue monitoring in aquaculture is primarily used to ensure food safety, rather than to control environmental contamination resulting from wastewater discharge of fish tanks. Regulatory frameworks establish a maximum residue limit of 1000 μg/kg in fish fillet (muscle plus skin in natural proportions) for the sum of FF and all its metabolites, measured as FFA, to ensure safe human consumption. The withdrawal period is temperature dependent and has been reported to range from 48 degree-days (water temperature × days) to 162 degree days (European Union, 2009; Queiroga et al., 2025). In contrast, no comparable limits exist for effluent discharge into marine or freshwater environments, and no comparable global monitoring system is currently in place, despite aquaculture being a major source of antimicrobial contamination (Choi et al., 2020; Rodriguez-Mozaz et al., 2020; Thiang et al., 2021; Hanna et al., 2023). In our experiment, FF residues were no longer quantifiable in water samples three days after withdrawal and measured concentrations were well below reported values in effluents from WWTPs or commercial aquaculture facilities. Residual concentrations are, however, strongly influenced by temperature, drug-specific bioavailability, and environmental context, which may explain these differences (Björklund et al., 1991; Ueno et al., 2004; Rairat et al., 2019; Kim et al., 2023). However, the ecological risk of antimicrobial discharge stems not only from the presence of residual compounds, but mostly from long-term biological effects on exposed organisms. Microbiome dysbiosis and broad-spectrum ARGs and MGEs persist long after the legally defined withdrawal period (Huang et al., 2025; Rosado et al., 2023; Streb et al., 2025). Although FF induced overall milder shifts than PAA, our results demonstrate that biological effects extend far beyond the active lifetime of the compound. While this relationship is well established for fish, our study provides one of the first experimental evidence that even sublethal, indirect exposure via waterborne residues can elicit distinct microbiome alterations in a non-target organism. Current risk assessments based solely on residue monitoring therefore underestimate the extended ecological consequences of antimicrobial use in aquaculture. Consistently higher FF levels in tanks receiving both antimicrobials highlight that chemical interaction can alter compound kinetics and should be more closely examined in the future. While the underlying mechanisms cannot be resolved in the frame of this work, several plausible explanations exist: (i) PAA may suppress microbial pathways involved in the biotransformation of FF; (ii) oxidative stress induced by PAA can transiently affect fish metabolism, immune activity, and mucosal integrity, thereby modulating antibiotic uptake and excretion (Soleng et al., 2019; Gamal et al., 2024); and (iii) reduced feed intake or altered digestion through depletion of key bacterial members in the gut of brown trout could have modified the release of unmetabolized residues into the water. On the other hand, oxidative degradation of antibiotics by PAA, including chloramphenicol, a structural analogue of FF, is frequently discussed as a promising approach for the effective removal of organic contaminants from wastewater (Rizzo et al., 2018; Chen et al., 2022). However, PAA stability and reactivity is strongly influenced by water parameters like salinity and organic matter (DOC) content. High DOC, which is characteristic for intensive rearing systems with high stocking densities, significantly accelerates PAA decay, thereby reducing its potency. (Pedersen et al., 2013; Liu et al., 2014) Due to the short half-life of PAA and the persistence of elevated FF concentrations two days after the last PAA application, it is unlikely that chemical interactions between the two compounds alone explain the observed pattern, pointing instead to a more dominant role of system-level processes. Given the rising incidence of parasitic and bacterial co-infections, the combined application of disinfectants and antibiotics is expected to become more frequent. Although widely regarded as a safe and effective agent against ectoparasites such as *Ichthyophthirius multifiliis* (Abu-Elala et al., 2021)*;* its impact on host-associated microbiomes and the chemical interaction with antibiotics warrants re-evaluation of its risk profile for use in aquaculture. Effective risk assessment must account for compound interactions, exposure routes, and delayed post-treatment effects rather than relying on generalized safety profiles. Only such system-specific evaluations will allow the design of treatment strategies that minimize unintended ecological disruption and safeguard both cultured fish and surrounding aquatic ecosystems.

5. Conclusion

Our study has shown that exposure to peracetic acid and florfenicol through rearing water effluents persistently alters the microbiome of the freshwater mussel *Anodonta cygnea*. While key commensals involved in host and ecosystem support like *Luteolibacter* remained stable, the enrichment of stress-resilient taxa such as *Dechloromonas* und *Sphingomonas* points towards a selection-driven change that enables short-term adaptation under pollution stress. These mechanisms may serve as a safeguard at the host-environment interface. However, this resilience could diminish under repeated or complex pollution scenarios, where erosion of the microbial barrier function as observed in the post-treatment phase, may facilitate pathogen invasion into the host microbiome, and consequently increase the risk of resistance spread within the One Health context, potentially including the transmission back into fish farms or to humans through handling or direct water contact. Understanding how their microbiomes respond to chemical disturbance provides essential guidance for evaluating long-term ecological risk of antimicrobial use in aquaculture and aquatic resistome dynamics. Despite the rapid degradation, our results highlight that the antimicrobial effect of PAA is strongly context-dependent and requires careful reconsideration. Future strategies toward sustainable and ecologically responsible aquaculture should aim to reduce antibiotic and biocidal input at the source – for instance through improved husbandry practices, optimized dosing regimens, and implementation of effective wastewater treatment regiments to remove residual pharmaceuticals and ARGs from effluents. As aquaculture accounts for only part of the overall antibiotic input into waterbodies, future integrative risk assessments should also extend to additional sources such as pharmaceutical and municipal wastewater effluents to fully capture cumulative exposure risks for non-target species. It should be noted, however, that the type and intensity of antimicrobial application vary considerably between regions and farming systems, which may influence the antimicrobial load discharged into the environment and the resulting ecological risks. Caution is therefore warranted when extrapolating these findings beyond the specific experimental context. Further studies involving more sensitive mussel species and varying aquaculture regimens are needed to provide a more comprehensive understanding of potential ecosystem implications.

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CRediT authorship contribution statement

Lisa-Marie Streb: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Michelle Kligman:** Methodology, Data curation. **Juergen Geist:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Gabriela Freitas Pereira de Souza:** Writing – review & editing, Methodology, Formal analysis. **Susanne Rath:** Writing – review & editing, Funding acquisition, Conceptualization. **Susanne Walch:** Methodology. **Michael Schloter:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Ethics approval

Sampling of swan mussels did not require specific ethical approval, as invertebrates are exempted from animal welfare regulations under German law. The experimental setup was evaluated and approved by the TUM Animal Welfare Office in accordance with existing regulations and following best veterinary practices.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2026.110138.

Data availability

Raw sequencing data was deposited on NCBI SRA under Bioproject PRJNA1330032.

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