



Effects of aquaculture waste feeds and antibiotics on marine benthic ecosystems in the Mediterranean Sea

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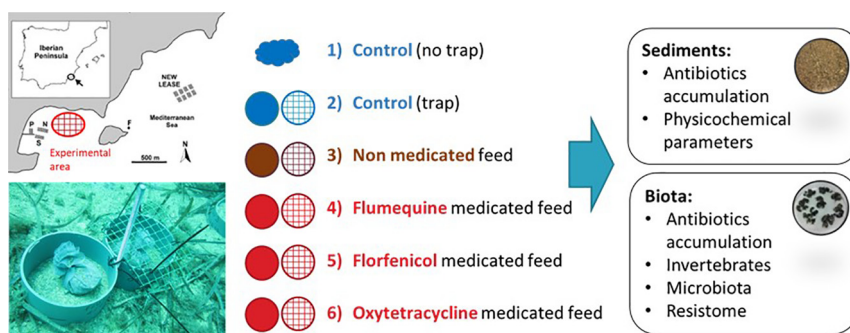
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HIGHLIGHTS

- Effects of aquaculture feed waste and antibiotics were assessed on benthic ecosystems.
- Oxytetracycline and flumequine accumulated in sediments and wild invertebrates.
- Feed waste altered habitat conditions and decreased macroinvertebrate diversity.
- Feed waste significantly influenced the structure of microbial communities.
- Florfenicol and oxytetracycline correlated with resistance to several antibiotics.

GRAPHICAL ABSTRACT



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ABSTRACT

Intensive aquaculture is an important source of organic waste and antibiotics into the marine environment. Yet, their impacts on benthic marine ecosystems are poorly understood. Here, we investigated the ecological impacts of fish feed waste alone and in combination with three different antibiotics (i.e., oxytetracycline, florfenicol and flumequine) in benthic ecosystems of the Mediterranean Sea by performing a field experiment. We assessed the fate of the antibiotics in the sediment and their accumulation in wild fauna after two weeks of exposure. Moreover, we investigated the impact of the feed waste alone and in combination with the antibiotics on sediment physico-chemical properties, on benthic invertebrates, as well as on the microbiota and resistome of the sampled sediments. One week after the last antibiotic application, average oxytetracycline and flumequine concentrations in the sediment were <1% and 15% of the applied dose, respectively, while florfenicol was not detected. Flumequine concentrations in wild invertebrates reached $3 \mu\text{g g}^{-1}$, while concentrations of oxytetracycline were about an order of magnitude lower, and florfenicol was not detected. Feed waste, with and without antibiotics, increased the concentration of fine particulate matter, affected the pH and redox conditions, and significantly reduced the biodiversity and abundance of benthic invertebrates. Feed waste also had a significant influence on the structure of sediment microbial communities, while specific effects related to the different antibiotics ranged from insignificant to mild. The presence of antibiotics significantly influenced the normalized abundance of the measured antibiotic resistance genes. Florfenicol and oxytetracycline contributed to an increase

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of genes conferring resistance to macrolides, tetracyclines, aminoglycosides and chloramphenicol, while flumequine had a less clear impact on the sediment resistome. This study demonstrates that feed waste from aquaculture farms can rapidly alter the habitat and biodiversity of Mediterranean benthic ecosystems, while antibiotic residual concentrations can contribute to the enrichment of bacterial genes resistant to antibiotic classes that are of high relevance for human medicine.

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1. Introduction

The aquaculture industry is growing worldwide as a consequence of the increasing seafood demands and the decline in catchable wild fisheries (FAO, 2018). One of the main environmental impacts related to the expansion of intensive aquaculture is the release of organic waste, including uneaten feeds and feces (Mente et al., 2006; Wang et al., 2012). Excess organic waste release can lead to eutrophication and anoxic conditions in sediments, which can contribute to a biodiversity loss and may have long-term effects on ecosystem functioning (Hyland et al., 2005; Kalantzi and Karakassis, 2006).

The aquaculture industry is also characterized by the periodic use of antibiotics to treat and prevent bacterial disease outbreaks (Burrige et al., 2010; Miranda et al., 2018; Lulijwa et al., 2020). Antibiotics are usually administered via formulated feeds that contain therapeutic doses. A small fraction of these feeds is not eaten by the cultured fish and passes through the net pens directly into the open environment. Moreover, antibiotics are only partly metabolized by fish, so a significant amount is also released by feces, which accumulate in the marine benthic environment (Leal et al., 2019).

To date, the impact that aquaculture antibiotics may have on marine ecosystems, in combination with organic waste, is rather unknown. On the one hand, antibiotics are expected to modify benthic microbial communities and can be taken up by wildlife, possibly affecting their microbiota and their physiological status (Björklund et al., 1990; Liu et al., 2017). On the other hand, antibiotics can contribute to the selection of antibiotic resistance genes in areas surrounding aquaculture farms, thus threatening the effectivity of future antibiotic administrations (Chen et al., 2018; Higuera-Llantén et al., 2018). Moreover, the environmental release of aquaculture antibiotics can result in the co-selection of resistance genes associated with different antibiotic classes, such as tetracyclines, fluoroquinolones or beta-lactams (Gao et al., 2012; Jiang et al., 2012; Verner-Jeffreys et al., 2009), which are of critical importance for human medicine (World Health Organization, 2019). In this regard, antibiotic pollution from aquaculture farms can contribute to the whole antibiotic resistance burden and may have direct health implications for farmers and swimmers in areas nearby aquaculture facilities (Cabello et al., 2013; Tomova et al., 2015; Amarasiri et al., 2020).

Here, we provide the first investigation on the ecological impacts of fish feed waste alone and in combination with antibiotics in marine benthic ecosystems of the Mediterranean Sea. Particularly, we assessed: 1) the fate of antibiotics in marine benthic ecosystems, including their accumulation in wild fauna (invertebrates); 2) the impact of feed waste and antibiotics on sediment physico-chemical properties; 3) their impacts on benthic invertebrates, by assessing their community composition, abundance, and species richness; and 4) their impacts on sediment microorganisms and the prevalence of antibiotic resistance genes. A field experiment was performed in the western part of the Mediterranean Sea (Murcia, Spain) by applying controlled amounts of commercial feeds used in seabream and seabass production, and similar feeds containing antibiotics, which are sold as therapeutic treatments for the same aquaculture species. Three different therapeutic treatments were used, which contained oxytetracycline, florfenicol and flumequine, respectively. These three antibiotics are among the most used compounds in aquaculture production in the Mediterranean region (Rico et al., 2019), and are therefore expected to be found in marine

sediments near to aquaculture farms (Kalantzi et al., 2021). The main objective of this study was to describe how feed waste release from aquaculture farms of the Mediterranean Sea can affect habitat characteristics and biodiversity of benthic ecosystems, and to assess whether the presence of different antibiotics influences the environmental effects of organic waste contamination.

2. Materials and methods

2.1. Experimental set-up and sampling

A field experiment was performed in July of 2017 in an un-impacted bay in the southeast coast of Spain (Águilas, Murcia Spain; 37° 24'33" N - 1° 33'27" W), where previous aquaculture activities had taken place a decade ago (Sanz-Lázaro and Marin, 2006). The average water depth in the study area was 10 m, and the water temperature during the time of the experiment oscillated between 24 and 26 °C. Twenty-five benthic traps made of polyvinyl chloride (PVC) round cylinders (sediment surface: 63 cm²) were placed by scuba diving in a muddy-sand bottom with dead rhizomes of *Posidonia oceanica*. Each trap was formed by two PVC cylinders. One of them was open and the other was covered with a net (2.5 cm mesh size) to avoid fish bioturbation and fish impacts on the colonization by benthic invertebrates. The traps were fixed to the seafloor with a metal spike and pulled down to an approximate height of 8 cm within the sediment. The traps were filled with approximately 1500 g of sandy sediment from a nearby area, which raised the sediment bottom 4 cm inside the cylinder. Traps were left for stabilization for one week prior to the application of the treatments.

The experiment included the following treatments: 1) no-trap or control (natural seabed); 2) control with trap (no addition of feed); 3) addition of non-medicated fish feed; 4) addition of oxytetracycline medicated fish feed; 5) addition of flumequine medicated fish feed; and 6) addition of florfenicol medicated fish feed. Each treatment consisted of five replicates (i.e., 5 benthic traps), except for the no-trap or control treatment, which implied direct sampling of the natural seabed in triplicate. Feed addition consisted on the introduction of net bags (1 mm mesh size) filled with 75 g of commercial fish feed into the benthic traps (one bag per cylinder). The amount of feed contained in each bag was calculated as the amount of uneaten feed deposited (5%) from an average weekly application of medicated fish feed on a seabream and seabass farm in the Mediterranean Sea, following the sedimentation rates calculated in a previous study (Sanz-Lázaro et al., 2011). Approximately one third of the surface of the net bags was in direct contact with the sediment and allowed a slow release of the feeds into the sediment bed, simulating feed waste deposition on sediments underneath fish farms. The first feed addition to the traps was done one week after the placement of the benthic traps in the field. After one week, the bags had released almost all feed content into the sediment and were replaced by new ones with fresh fish feeds by scuba diving. In such way, sediments were exposed to simulated feed waste deposition for a period of two weeks. The feeds used in the experiment were commercial feeds typically used in seabass and seabream production. The antibiotic concentrations in the three different medicated feeds were 15,000 mg kg⁻¹ oxytetracycline, 2500 mg kg⁻¹ flumequine and 2000 mg kg⁻¹ florfenicol. The commercial names and composition of the tested feeds are provided in Table S1.

One week after the second feed administration, sediment and invertebrate samples were collected by scuba diving. The sediment samples collected from the seabed and the benthic traps were used for the analysis of antibiotics, physicochemical parameters, and for the analysis of the microbiota and antibiotic resistance genes. These were collected in falcon tubes (50 mL) and frozen at -20°C until further processing. The invertebrate samples were collected by sieving (mesh size: 0.5 mm) the benthic trap content onsite. The collected invertebrate material was kept on 1 L plastic flasks and transported to the laboratory. Once in the laboratory, 3 of the trap replicates were fixed with 10% buffered formaldehyde for species identification and counting, while the other 2 were kept frozen at -20°C for assessing antibiotic accumulation in the sampled organisms.

2.2. Antibiotic analysis

Sediments were analyzed following the analytical method described by González-Gaya et al. (2018). Briefly, 3 g of freeze dried and grinded sediment were spiked with isotopically labeled flumequine (flumequine-(1,2-carboxy- $^{13}\text{C}_3$), which was used as internal standard (IS) for the three antibiotics. The chemical extraction was done with 30 mL of acidified methanol (0.01 M oxalic acid, 0.01% formic acid and 12 mg EDTA). The sample was vortexed and homogenized on an ultrasound and centrifuged (10 min, 4000 rpm). The supernatant was evaporated (*speedvac* Savant SPD 131DDA, by Thermo Scientific) and reconstituted in 10 mL MilliQ water (0.1% formic acid). Then, the samples were subjected to a solid phase extraction process with HLB cartridges (SPE-HLB Oasis 60 mg, 3 cc, Waters). The eluted extracts were evaporated and reconstituted in 3 mL of methanol:water (10:90 v/v, 0.1% formic acid) and filtered over a PVDF 0.22 μm syringe.

The analysis of antibiotics in marine invertebrates was performed as described previously (González-Gaya et al., 2018). The pool of invertebrates (0.8–280 mg, pooling 2 replicates of each treatment) was freeze dried and spiked with the IS at 20 ng mL $^{-1}$, as done with the sediment samples. Then, 30 mL of acidified methanol (oxalic acid 0.01 M, 0.1% formic acid) were added. Like the sediment samples, extraction was assisted by vortexing, sonication and centrifugation. After centrifugation, the supernatant was transferred into vials and evaporated on a *speedvac*. After dryness, the sample was reconstituted with 100 mL MilliQ water (0.1% formic acid), and then 10 mL of diluted extracts were purified over a SPE tandem consisting on a top MAX cartridge 150 mg and a bottom HLB 60 mg cartridge (Oasis, Waters). Cartridges were conditioned with 6 mL methanol and 6 mL deionized water. After the sample loading, a washing step with 9 mL deionized water and 6 mL of methanol: water (20:80 v/v) was performed. Next, the cartridges were dried under vacuum and eluted with 8 mL of acetonitrile: methanol (70:30 v/v, formic acid 0.1%). The eluted extract was evaporated on a *speedvac* (using the same conditions as for the sediment samples), reconstituted on 1 mL of methanol:water (10:90 v/v, 0.1% formic acid), and filtered over a 0.22 μm PVDF syringe filter.

The antibiotic analysis was done by high-pressure liquid chromatography coupled with a time-of-flight mass spectrometer (HPLC-TOF-MS) with a LC Agilent MSD TOF from Agilent Technologies 6230 (Palo Alto, CA, USA). The chromatographic separation was carried out with a Luna Omega Polar C18 column (100 mm $\times$ 2.1 mm $\times$ 5 μm , Phenomenex) using 0.1% formic acid in water (A) and methanol (B) as mobile phases (0.4 mL min $^{-1}$). Mass spectrometry with an electrospray ionization mode (ESI) was performed in positive mode. Quantification limits for the sediment matrix were 0.1 $\mu\text{g L}^{-1}$ for oxytetracycline and flumequine, and 0.5 $\mu\text{g L}^{-1}$ for florfenicol, with satisfactory precision (relative standard deviation, RSD, <10%). Quantification limits for the biological samples were 100 $\mu\text{g kg}^{-1}$ for oxytetracycline and flumequine, and 500 $\mu\text{g kg}^{-1}$ for florfenicol with satisfactory precision (RSD <10%). For further information on the QA/QC please refer to González-Gaya et al. (2018).

2.3. Physico-chemical parameter analysis

We assessed the grain and elemental composition of the sediments as well as the organic matter (OM) content, and the pH and redox potential. For the grain and elemental composition analysis, unfiltered sediments were dried overnight (70 $^{\circ}\text{C}$ in the oven) and sieved with an electronic Ika-vibrax-VXR shaker using 2, 1, 0.5, 0.25 and 0.063 mm mesh size sieves. Fractions were weighted separately. Between 20 and 50 mg of the 0.063 mm and lower fractions were used for elemental characterization of C, H, N and S, and 200 mg were used to assess the P content after a digestion process. Inductively coupled plasma mass spectrometry (ICP-MS) was performed with a Leco CHNS-932 analyzer for C, H and S (with independent detectors of IR, no dispersive solid state) and N (with thermal conductivity). For this, sub-samples of 2 mg were combusted at 1000 $^{\circ}\text{C}$ and sequentially analyzed for the relative mass concentration of the elements. As for P analysis, samples were digested with 3 mL HNO $_3$ and 1 mL of MilliQ water and an ultra-wave program from 850 to 1150 W (100–200 $^{\circ}\text{C}$) for 15 min. Prior to ICP-MS analysis, each sample was diluted in MilliQ water to maintain nitric acid below 2% as necessary for the equipment proper preservation. The OM content was measured on unfiltered sediment (10–25 g), which was first dried overnight at 70 $^{\circ}\text{C}$ and weighted. Then it was burned at 450 $^{\circ}\text{C}$ overnight and weighted again to calculate the loss of weight. The pH, redox and ammonia reduction potential were measured in the fresh sediment samples with a Thermo Orion potentiometer coupled to a Thermo Scientific 9512BNWP ISE ammonia/ammonium and an Orion basic pH electrode.

2.4. Benthic macroinvertebrate analysis

The fixed biological material was washed over 1 mm mesh and elutriated to extract the fauna under laboratory conditions. The specimens were then transferred to a 70% methanol solution. Organisms were identified to the lowest possible taxonomic level using a stereo microscope and their abundances were recorded according to their density per m 2 of sediment. Finally, biological indices, such as species richness (expressed as total number of taxa), abundance (individuals m $^{-2}$) and the Shannon Wiener diversity index [H'] (log $_e$ base) were calculated.

2.5. Microbial analysis

DNA was extracted using the DNeasy power water kit from Qiagen. Next, 16S rRNA sequencing and analysis was prepared and performed as described previously (Buelow et al., 2020). Extracted DNA samples for 16S rRNA sequencing were prepared following a dual barcoded two-step PCR procedure for amplicon sequencing for Illumina. Primers of the first PCR step included universal CS1 and CS2 tags targeting the V4 region of the hypervariable region of the 16S rRNA gene, using the 16SrRNA primer sequences of the Earth Microbiome Project (Gilbert et al., 2010). Samples were sequenced following the Illumina protocol for a 2 $\times$ 301 MiSeq run (Illumina, Inc., San Diego, CA). Sequence reads from the Illumina MiSeq were demultiplexed and classified as follows. First, the Python application dbcAmplicons (<https://github.com/msettles/dbcAmplicons>) was used to identify and assign reads to the appropriate sample by both expected barcode and primer sequences. Barcodes were allowed to have at most 1 mismatch (hamming distance) and primers were allowed to have at most 4 mismatches (Levenshtein distance), as long as the final 4 bases of the primer matched the target sequence perfectly. Reads were then trimmed of their primer sequence and merged into a single amplicon sequence using the application FLASH (Magoč and Salzberg, 2011). Illumina MiSeq forward and reverse reads were processed using the MASQUE pipeline (<https://github.com/aghazlane/masque>). Briefly, raw reads were filtered and combined, followed by dereplication. Chimera removal and clustering were followed by taxonomic annotation of the

resulting OTUs by comparison to the SILVA database. In this way, a BIOM file was generated that combines both OTU taxonomic assignment and the number of matching reads for each sample. Relative abundance levels for microbial taxa at phylum and class levels were obtained and analyzed. The sequencing data obtained as part of this study are included in the European Nucleotide Archive (ENA), with the following code: PRJEB46785.

2.6. Resistome analysis

Nanolitre-scale quantitative PCRs were performed as described previously (Buelow et al., 2018, 2017, 2020) to quantify levels of genes that confer resistance to antimicrobials and heavy metals. The primer sequences are provided in Table S2. In total we targeted 78 individual resistance genes conferring resistance to antibiotics, quaternary ammonium compounds or heavy metals, which were grouped into 16 resistance gene classes. The targeted genes include those commonly detected in the gut microbiota of healthy humans, clinically relevant ones (including genes encoding extended spectrum β -lactamases (ESBLs), carbapenemases, and vancomycin resistance), and heavy metal and quaternary ammonium compound resistance genes suggested to favor cross and co-selection for antibiotic resistance in the environment. We also targeted a total of 8 genetic elements as important transposase gene families (Zhu et al., 2013) and class 1, 2 and 3 integrons by primers described by Barraud et al. (2010), which are important vectors for antibiotic resistance genes and often used as proxies for anthropogenic pollution (Gillings et al., 2015). Real-Time PCR analysis was performed using the 96.96 BioMark™ Dynamic Array for Real-Time PCR (Fluidigm Corporation, San Francisco, CA, U.S.A) as described previously (Buelow et al., 2018, 2017). Thermal cycling and real-time imaging were performed at the Plateforme Génomique GeT – INRA Transfert (France), and Ct values were extracted using the BioMark Real-Time PCR analysis software. Calculations for normalized and cumulative abundance of individual genes and allocated gene classes was done as described previously (Buelow et al., 2018, 2017, 2020). The normalized abundance of resistance genes was calculated relative to the abundance of the 16S rRNA gene ($2^{-(CT_{\text{gene}} - CT_{16S \text{ rRNA}})}$). Finally, the cumulative abundance of each resistance gene class was calculated based on the sum of the normalized abundances of all individual genes detected within a sample.

2.7. Statistical analyses

Statistical analyses were performed to assess the influence of the different treatments on the sediment physico-chemical parameters, on the macroinvertebrates, and on the microbial and resistome data. The analyses for the microbiota were performed at the phylum and class levels, while the analysis of the resistome was done with all identified resistance genes and with the resistance genes grouped by classes according to their function. A permutational multivariate analysis of variance (PERMANOVAs) was performed with the physico-chemical and the macroinvertebrate dataset including two factors: net covering (net or no net) and feed treatment (including the five treatments: control, feed, feed with oxytetracycline, feed with flumequine, and feed with florfenicol). As for the microbial and resistome datasets, only the samples with no net were included as the covered ones were not evaluated for these endpoints. The PERMANOVA analyses were performed using log ($x + 1$) transformed data, and were based on Euclidean distances and 999 Monte Carlo permutations (Anderson et al., 2008). Pair-wise comparisons were also calculated when significant differences were observed for at least one of the evaluated factors. Finally, a Principal Coordinate Analysis (PCoA) was done to display the relationship between the evaluated ecosystem parameters and the samples. All statistical analyses were performed with the PRIMER-v6™ software, using the PERMANOVA+ add-on package (Anderson et al., 2008).

3. Results and discussion

3.1. Environmental fate of antibiotics

Oxytetracycline was detected in all sediment samples, including the control traps with an average concentration of 30 ng g^{-1} (Table S3). To test whether this contamination came from previous aquaculture activities developed in the area or was related to field cross contamination, 6 additional sediment samples were taken in March 2019 (20 months after the experiment). No oxytetracycline was found in any of the samples, indicating that oxytetracycline was not ubiquitous in the study area. Despite the traps were placed randomly in the sea floor and few meters away from each other, some cross contamination could have happened since fish was actively feeding in some traps with no cover, thus distributing feed residues and/or feces into other areas. In addition, partial resuspension of organic matter and deposition could have happened due to natural currents and during scuba diving and sampling. However, in the traps treated with feeds containing oxytetracycline, the concentration of oxytetracycline was hundred times higher, with an average concentration of 2700 ng g^{-1} (Relative Standard Deviation, RSD: 18%) in the traps without net cover and nearly 8000 ng g^{-1} (RSD: 123%) in the net-covered traps (Fig. 1). It is noteworthy that there was a relatively large variation in the concentrations within replicates of the same treatment, particularly for the net-covered traps, which could be attributed to the aforementioned factors. The overall trend towards a larger average concentration in the net-covered traps can be attributed to several reasons. First, the feed in the open traps was partly eaten by wild fish, leading to lower concentrations of antibiotics deposited and adsorbed into the sediment. In addition, sediment in the open traps was bioturbated by fish and burrowed deeper into the sediment compartment. Bioturbation enhances aerobic conditions, which may have favored microbial degradation (Martinez-Garcia et al., 2015; Nickell et al., 2003).

Flumequine was also detected at low concentrations in some of the samples that were not treated with flumequine feeds (particularly in the net-covered traps treated with uncontaminated feeds), probably due to the same reasons as described above. However, the concentrations in the traps treated with flumequine-containing feeds were one to four orders of magnitude higher, except for one sample that showed

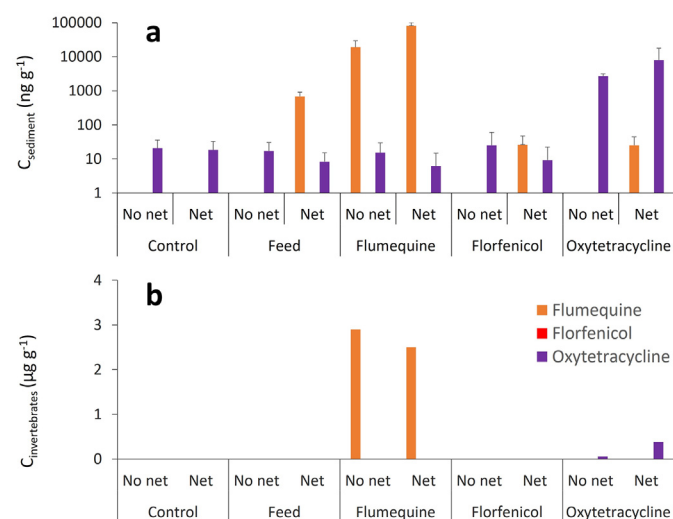


Fig. 1. Measured concentration of antibiotics in a) sediment samples (average, ng g^{-1}) and b) in benthic macroinvertebrates (average, $\mu\text{g g}^{-1}$). In (a) error bars represent one SD. The replicate of the flumequine treated traps with net that was deviating was not included in the calculations shown in the graph (see Table S3). Control: traps without feed; Feed: traps treated with regular aquaculture feeds; Flumequine: traps treated with feeds containing flumequine; Florfenicol: traps treated with feeds containing florfenicol; Oxytetracycline: traps treated with feeds containing oxytetracycline.

a very low concentration (102 ng g^{-1} ; Table S3). Probably this sample contained a mixture of the sediment inside the trap and the sediment in the surroundings of the trap, as there was quite some resuspension of it during the sampling by scuba diving. However, we verified that the feed bag was inside the trap, so the sediment had received the appropriate dosing. The average flumequine concentrations were $19,000 \text{ ng g}^{-1}$ (RSD: 55%) and $81,000 \text{ ng g}^{-1}$ (RSD: 24%) in the traps that were not covered and covered with the net, respectively, when the deviating sample is not included (Fig. 1).

Florfenicol was not detected in any sediment sample, including those that contained its medicated feeds (Fig. 1), indicating a rapid leaching of this antibiotic from fish feeds and dissipation from the sediment compartment. This is in line with previous investigations, which show a very weak adsorption capacity of this antibiotic to soil organic matter and a high dissipation capacity from marine sediments due to its high solubility in water (Hektoen et al., 1995; Hu et al., 2007; Zong et al., 2010).

Based on the different therapeutic doses (6 times higher for oxytetracycline), we expected to find a higher concentration of oxytetracycline in the sediments as compared to flumequine. However, we estimated that the accumulation of the supplemented antibiotic in the sediment was lower than 0.5% of the applied dose for oxytetracycline, and between 8% and 22% for flumequine by the end of the experiment (Table 1). This indicates that flumequine has a higher capacity to accumulate in marine sediments as compared to oxytetracycline, which is in line with the hydrophobic characteristics of these substances (Log K_{ow} were 2.56 and -1.22 for flumequine and oxytetracycline, respectively; González-Gaya et al., 2018). Flumequine half-life in sediments has been reported to be 15 days under laboratory conditions, although it could have a longer persistence under dark (buried) or anoxic conditions (Lai and Lin, 2009). On the other hand, the half-life of oxytetracycline in marine sediments has been reported to be of several months (Hansen et al., 1992; Hektoen et al., 1995; Nepejchalová et al., 2008; Norambuena et al., 2013), and may explain the differences in antibiotic dispersal and occurrence in the non-treated samples between both substances.

We detected the presence of flumequine and oxytetracycline in benthic macroinvertebrates collected from the sediment traps, while florfenicol was not detected (Fig. 1, Table S4). Concentrations of flumequine in the invertebrates collected from the traps treated with flumequine-containing feeds reached $2.5\text{--}3.0 \mu\text{g g}^{-1}$ in the covered and uncovered traps. Oxytetracycline was also found to accumulate in the invertebrates collected from the oxytetracycline-treated traps, with concentrations up to $0.05 \mu\text{g g}^{-1}$ in the uncovered traps and $0.38 \mu\text{g g}^{-1}$ in the net-covered traps. Thus, the concentrations of flumequine in wild fauna were an order of magnitude higher than those for oxytetracycline. The mean calculated biota-sediment accumulation factor (BSAF) based on sediment exposure without organic carbon correction (Van der Oost et al., 2003) were 1.0×10^{-4} and 3.4×10^{-5} for flumequine and oxytetracycline, respectively. However, these figures should be taken with caution as they are based on the pool of collected organisms and some uncontrolled factors (i.e., interspecies differences in uptake/depuration, species mobility, residual antibiotic concentrations in the gut content) may have influenced these values (Arnot and Gobas, 2006). BSAFs lower than one generally indicate low bioaccumulation potential for benthic organisms. Similarly, other studies have reported mid-to-low bioconcentration factors for

oxytetracycline in mussels (Le Bris and Pouliquen, 2004), oysters and crabs (Capone et al., 1996), and shrimps (Schmidt et al., 2007; Thuy et al., 2011). While quinolones have also shown a low accumulation potential in laboratory experiments performed with bryozoans (Delépée et al., 2004) and in benthic species under natural conditions (Xie et al., 2017; Zhang et al., 2020). However, through this experiment we have demonstrated that, despite having low bioaccumulation potential, antibiotics are prone to be taken up and transported by wild invertebrates in the surroundings of aquaculture farms. Further research should be dedicated to assess their long-term effects on microorganisms associated with wild fauna and their potential side effects on marine food webs.

3.2. Effects on physicochemical conditions

We did not identify clear differences in physico-chemical conditions (i.e., sediment grain size, elemental composition, chemical conditions) between the sediment samples taken from the study area and the sediment samples taken from the control traps (Tables S5, S6, S7; Fig. S1). As shown by the PERMANOVA analysis, the feed treatments ($p = 0.001$) as well as the fact of covering the traps with net or not ($p = 0.006$) had a significant influence on the physicochemical conditions of the sediments (Fig. 2; Table S8). As expected, the addition of feeds resulted in a higher relative percentage of organic matter and fine material ($<0.063 \text{ mm}$) compared to the controls, particularly in the traps that were covered with the net (Table S5). In line with this, we found that the relative concentration of P was generally higher in the traps treated with feeds and with net cover (Table S6). Notable differences also appeared between treatments, with controls exhibiting a lower relative concentration of S and a higher relative concentration of N. The increase of S in benthic ecosystems impacted by aquaculture has been reported by other authors, especially as sulfides, since the sulfate reduction rate increases significantly under situations of organic matter enrichment and anoxic conditions (Holmer et al., 2005; Piedcausa et al., 2012).

Feed waste also altered the chemical conditions of the sediment, and resulted in a slight pH increase and a decrease of the redox and ammonia reduction potential (Table S7), contributing to (partial) anoxia, with sediments turning dark gray. The pair-wise comparisons did not show significant differences among the medicated and non-medicated feed treatments, suggesting that the main driver for these physico-chemical changes was the addition of feeds, and to a minor extent the net cover, but not the presence of antibiotics (Fig. 2; Table S8). As observed while sampling, large wild fish contributed to the depletion of feed waste

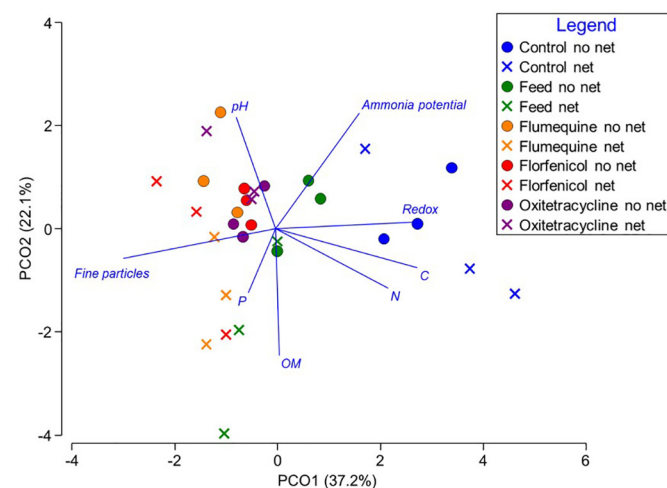


Fig. 2. PCoA biplot of physicochemical parameters in the sediment samples. The grain size was included as synthetic predictor by taking the first axis of the PCA of the dataset, which pointed towards the samples with finer particles. S was not included as there were too many values below the limit of quantification (Table S6).

Table 1

Percentage of the dose of applied antibiotics found in the sediment samples at the end of the experimental period (i.e., one week after the last application). n.d.: not detected.

Treatment	Oxytetracycline (%)	Florfenicol (%)	Flumequine (%)
Traps without net	0.18	n.d.	7.65
Traps with net	0.53	n.d.	21.7
Average	0.36	n.d.	14.7

and bioturbation of the sediments in the traps without net cover, thus reducing the organic matter content of these sediments. Therefore, we conclude that the net effects of aquatic feeds on Mediterranean benthic ecosystems depend on the presence and bioturbation caused by megafauna, as also suggested in other studies (Nickell et al., 2003; Callier et al., 2018), and confirms the need to include this process in monitoring and modelling studies aimed at assessing the environmental impacts of aquaculture in the Mediterranean Sea (Cromeu et al., 2012).

3.3. Effects on benthic invertebrates

A total number of 134 invertebrates were identified in the traps, belonging mainly to the Polychaeta (70% of individuals, 25 different families) and Mollusca (14% of individuals, 5 species) groups, and to a lower extent to the Crustacea group (2 taxa) and Echinodermata larvae (1 species) (Table S9). No clear differences were observed between the species found in the control traps and those sampled from the sediment samples randomly taken in the study area (i.e., no trap or control). The total number of individuals was notably higher in the control traps as compared to the traps treated with feeds (Fig. 3). The species richness and biodiversity were also notably different. The control traps had between 6 and 8 species, while the traps treated with feeds contained between 2 and 4 species. The results of the PERMANOVA analysis showed that the treatment ($p = 0.001$) and the presence or absence of net ($p = 0.022$) significantly influenced the macroinvertebrate community composition (Table S10). The control traps were dominated by Pisionidae and Oweniidae polychaetes, the bivalves *Donacilla cornea* and *Acanthocardia tuberculata*, and contained a significantly larger amount of crustacea (Amphipoda and *Anapagurus laevis*) (Fig. 4, Table S9). In the traps treated with feeds, there was a sporadic occurrence of some worm taxa (e.g. Onuphidae and Dorvilleidae) (Table S9). Generally, there were no clear differences between the traps treated with regular feeds and those treated with medicated feeds, with the exception of the traps medicated with florfenicol (Table S10), the latter having a larger number of crustaceans (*A. laevis*) and Echinodermata (Fig. 3, Table S9). Interestingly, we found that the biodiversity and the number of individuals in the control traps was slightly higher in those covered with net as compared to the open ones, due to an increase of crustacea (*A. laevis*). Probably, these individuals found a suitable habitat where they could escape from predators. However, in the traps treated with feeds (medicated or not) we observed an opposite trend (Fig. 3).

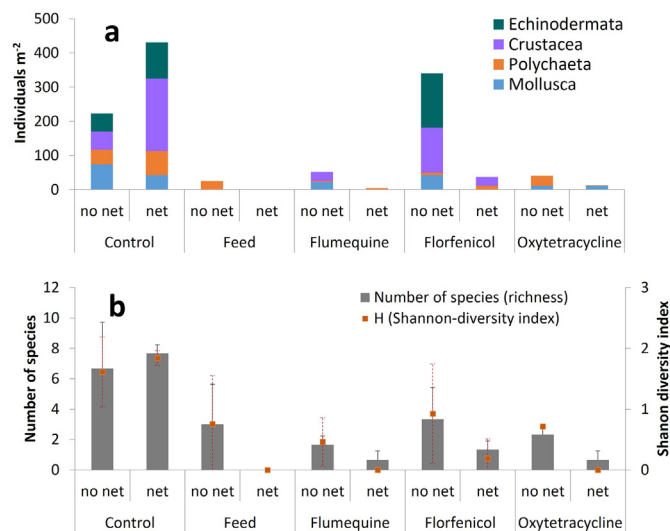


Fig. 3. a) Abundance of invertebrates in the sediment samples. b) Number of invertebrate species (bars) and calculated Shannon diversity index (dots) in the different samples. Data show mean values and calculated standard deviations for the replicates ($n = 3$).

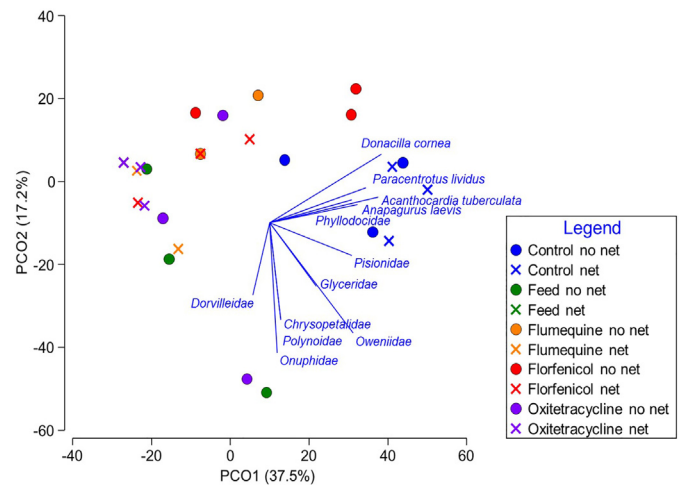


Fig. 4. PCoA biplot for macroinvertebrates in the sediment samples. Only taxa with vector overlay of Spearman rank correlations >0.09 with the PCO axes were included.

A reduction of invertebrate's abundance and species richness has been described in benthic ecosystems impacted by aquaculture waste feeds in other regions of the globe (Borja et al., 2009; Mirto et al., 2002; Tomassetti et al., 2016; Wang et al., 2017). It is generally accepted that if the organic matter turnover rate is fast (i.e., organic enrichment under aerobic conditions) there is a stimulation of the benthic invertebrate biomass due to an increase of available food resources. However, when there is an excessive accumulation of feed waste (i.e., leading to anoxia, sulfide accretion), the community structure changes notably and is dominated by few tolerant species with high abundances (Wilding and Nickell, 2013; Tomassetti et al., 2016). Our experiment shows that feed waste accumulation from seabream and seabass farms can contribute to an abrupt macroinvertebrate community shift in a relatively short time span (two weeks after the start of feed administration), and suggests that conditions may be worsened if feed waste is accumulated for prolonged periods. Also, we have identified some taxa with low tolerance to the habitat alteration caused by feed waste deposition (primarily Crustacea) that could be used as bioindicators to assess the ecological status of marine benthic sediments impacted by aquaculture in the Mediterranean Sea.

3.4. Effects on sediment microorganisms

The PERMANOVA analyses performed with the phylum ($p = 0.004$) and class ($p = 0.001$) OTU datasets revealed significant differences between the sediment microorganisms in the controls and in the traps containing feed waste (Table S11). At the phylum level, the microorganism community of the control trap samples was dominated by Proteobacteria, while in the traps treated with feeds, an increase in the relative abundance of Bacteroidetes, Firmicutes and Synergistetes was observed (Fig. 5, Table S12). In line with this, the analysis performed on the class level indicated a relative increase of the classes Bacteroidia, Synergistia, Clostridia, Cloacamonas (candidatus) and Negativicutes in the sediments treated with feed waste as compared to the controls (Fig. S2, Table S13). Although the PCoA analysis shows a relatively high dispersion in the trap samples treated with regular feeds and feeds containing different antibiotics (Fig. 5), we could not identify statistically significant differences on the structure of the microorganism community among these sample groups (Table S11). A significant impact of fish farming on the prokaryotic microbiota of marine sediments was described by Kawahara et al. (2009) and Quero et al. (2020), highlighting an increase in Bacteroidetes and Firmicutes in fish farm sediments over time. Our study shows that the increase in Bacteroidetes and Firmicutes in the flumequine traps was more pronounced (Fig. 5)

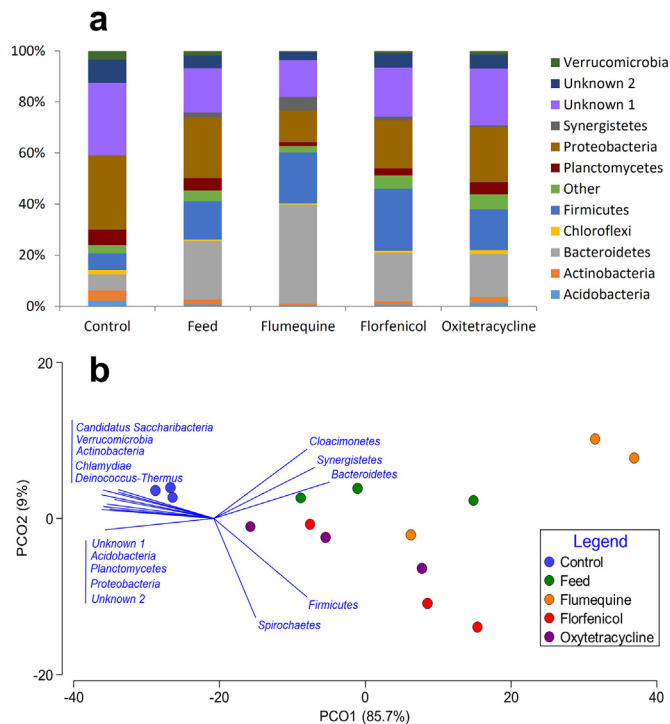


Fig. 5. a) Relative abundance of OTUs (phylum level) in the different samples. b) PCoA biplot of microorganism OTU data (phylum level) in the sediment samples. Only phyla with vector overlay of Spearman rank correlations (> 0.09) with the PCO axes were included.

compared to the control traps and the other treatments containing other antibiotics (with some pair-wise p values < 0.1), indicating that in addition to organic waste, antibiotics present in fish feeds may slightly influence the development of marine sediment microbiota. Due to its toxicological mode of action, flumequine is expected to mainly affect gram negative bacteria. Thus, the notable increase in Firmicutes observed in this sediment compared to the other treatments may be due to the (partial) elimination of certain Proteobacteria sensitive to flumequine. Longer term experiments should be conducted to evaluate whether the observed shifts are fostered by the presence of antibiotics and remain for long-term periods in the benthic environment, also after the cessation of fish feed waste and antibiotic deposition.

3.5. Effects on the sediment resistome

The PERMANOVA analysis indicated a significant influence of the different treatments on the resistome, both at the individual gene level ($p = 0.001$) and on the gene classes ($p = 0.001$) (Table S14). The pair-wise analysis performed with the gene class dataset shows significant differences between the control samples and the rest of the treatments. Moreover, we identified significant differences between the samples treated with regular feeds and feeds medicated with flumequine, respect to those medicated with florfenicol and oxytetracycline (Table 2). The analysis performed on the individual gene level shows similar results (Table S14). Overall, we found a relative increase in the prevalence of tetracycline (*tetM*, *tetB*) and macrolide (*ermB*, *mefA_10*) resistance genes in the samples treated with feeds containing florfenicol and oxytetracycline (Fig. 6), and a higher prevalence of genes conferring resistance to chloramphenicol (*cat*) and aminoglycosides (*aph(3')-III*). Moreover, we observed a higher prevalence of MGEs such as *ISS1N* in these samples (Table S15). On the other hand, our analysis shows that the prevalence of genes conferring resistance to heavy metals, beta-lactams or polymyxins, as well as genes encoding efflux pumps was higher in the control samples (Fig. 6).

Table 2

Results of the PERMANOVA pair-wise comparisons between different treatments and the resistance gene classes dataset. The results indicate the Monte Carlo p values. Significant values ($p < 0.05$) are marked in bold.

	Control	Feed	Flumequine	Florfenicol
Feed	0.008			
Flumequine	0.005	0.101		
Florfenicol	0.023	0.039	0.122	
Oxytetracycline	0.006	0.015	0.021	0.502

The influence of the different treatments on the resistome is a consequence of phenotypic and genotypic changes on the microbial community (Aminov, 2009). The change in the microbial community structure implies a change in hosts that are likely to carry certain resistance genes. Thus, differences in the resistome between the control and the sediments that received fish feeds may be partly explained by the increase of Bacteroidetes and Firmicutes, while the increase of clostridia (firmicutes) and gram-positive bacteria was correlated to the observed increase in resistance genes in the florfenicol and oxytetracycline treatments (Portillo et al., 2000; Soge et al., 2009). The increase of the prevalence of *tet* determinants in environmental bacteria in the surroundings of aquaculture facilities as well as chloramphenicol acetyltransferases (*cat*) has been previously reported, and is expected to be directly related to the contamination with oxytetracycline and florfenicol (see Miranda et al., 2013 for a review). However, it is expected that at the antibiotic exposure concentrations and the time windows evaluated in this experiment, co-selection has also occurred, explaining the enrichment of resistance genes to other antibiotic classes such as macrolides and aminoglycosides (Verner-Jeffreys et al., 2009; Pal et al., 2015; Chen et al., 2018).

The spread of antibiotic resistance genes is often facilitated by their location on mobile genetic elements, such as plasmids and transposons. In our study, we found the transposase gene *ISS1N*, which is common in gram positive lactic acid bacteria (Haandrikman et al., 1990), to correlate with the different antibiotic resistance genes in the oxytetracycline and florfenicol treatments. *ISS1N* has been previously identified as a maker of highly contaminated waters (Buelow et al., 2020) and can be considered as a putative vector of antibiotic resistance to aquaculture fish or humans in areas impacted by aquaculture waste feeds and antibiotics (Cabello et al., 2013).

4. Conclusions

Our study shows how the addition of aquaculture waste feeds to benthic ecosystems of the Mediterranean Sea can alter physico-

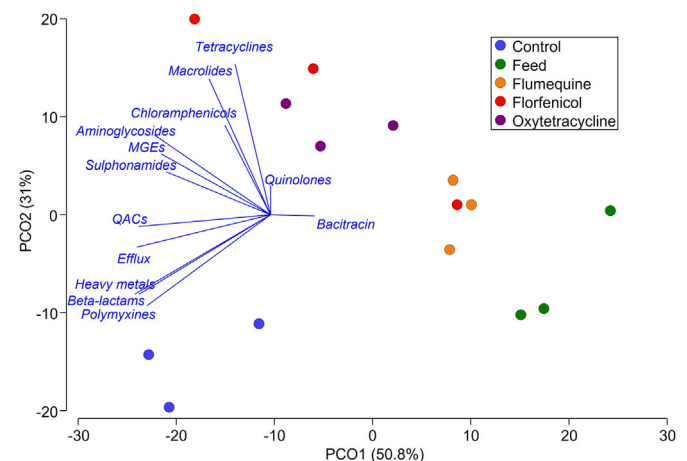


Fig. 6. Principal coordinates analysis (PCoA) of resistance gene classes in the different treatments. Vector overlay of Spearman rank correlations (> 0.07) with the PCO axes.

chemical conditions of the sediment and affect marine biodiversity. The addition of waste feeds for a period of 14 days changed the chemical composition and the redox potential of the ecosystem, and contributed to a local decline of the macroinvertebrates' biomass, species richness and biodiversity. Moreover, it affected the structure of benthic microbial communities. The additional contamination with antibiotics commonly used as therapeutants in aquaculture did not influence the sediment physico-chemical conditions, nor the macroinvertebrate community structure. The influence of the antibiotics on the structure of the microbial community ranged from insignificant to mild, with the flumequine treatment showing the clearest effects in comparison to the treatment that contained non-medicated feed. On the other hand, our study indicates that some antibiotics (flumequine, oxytetracycline) have a moderate persistence in marine sediments and can be taken up by wild fauna, thus having the potential to be incorporated into marine food webs. Our study also shows that the contamination of benthic environments with antibiotic-containing aquaculture feeds alters the resistome of wild bacterial populations. We demonstrate that the environmental release of residual concentrations of oxytetracycline and florfenicol contributes to the selection of resistant genes that have important therapeutic implications for human medicine, such as macrolides, tetracyclines and aminoglycosides. Further studies are recommended to assess the persistence of the resistome alterations after antibiotic exposure, and the transmission potential of antibiotic resistance genes to non-target organisms and people in the surroundings of aquaculture production areas (including farm operators, fishermen, and swimmers).

CRedit authorship contribution statement

Belén González-Gaya: Conceptualization, Investigation, Methodology, Writing – original draft. **Nuria García-Bueno:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Elena Buelow:** Investigation, Methodology, Writing – review & editing. **Arnaldo Marin:** Conceptualization, Investigation, Methodology, Project administration, Supervision, Writing – review & editing. **Andreu Rico:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing.

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.scitotenv.2021.151190>.

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