The impact of antibiotic exposure in water and zebrafish gut microbiomes: a 16S rRNA gene-based metagenomic analysis

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Abstract

In order to supply human demand for food, the aquaculture industry has been growing fast in the last years, being fish usually cultivated in overcrowded conditions. Hence, to prevent disease spreading, antibiotics may be applied to both sick and healthy animals. Due to its broad spectrum, oxytetracycline (OTC) is one of the most used antibiotics in food-production. Yet, although useful to prevent infections, antibiotics residues may persist in the environment and may reshape non-target aquatic animals' microbiome, disturbing hosts' welfare. However, the impact of this exposure to the organism microbiome and its surrounding environment is poorly understood. Then, the objective of this study was to analyze in detail the long-term effect of OTC in both zebrafish gut and water microbiomes. Zebrafish adults were exposed, via water, for two months to three concentrations of OTC (0, 10 and 10000 µg/L). Total DNA was extracted from gut and water samples and the V3-V4 region of the bacterial 16S rRNA gene was sequenced using Illumina technology. Results of alpha and beta-diversity analyses revealed that long-term exposure to OTC impacted both zebrafish gut and water microbiomes. In water samples, effects were observed even at the lowest (10 µg/L) OTC concentration tested resulting in an increase in Deltaproteobacteria, namely the Myxococcales and Bdellovibrionales orders. On the other hand, effects on zebrafish gut were mainly observed at the highest concentration with the selection of Alphaproteobacteria and Actinobacteria classes. Although these classes are common in fish gut, the increase of Actinobacteria may represent a health problem since some genera like *Gordonia* include human pathogens. Nevertheless, in both gut and water, it was observed a decrease in Gamaproteobacteria. On the other hand, the selection of bacterial groups intrinsically resistant to OTC was observed, including emergent fish pathogens. *In silico* functional metagenomic analysis revealed that OTC exposure selected general detoxification mechanisms. Selection of functional genes involved in Quorum Sensing (QS) suggests that QS may help bacteria to survive OTC stress. Future studies should consider post-exposure scenarios for a deeper analysis of the water and zebrafish gut resistome, since bacteria may react differently after exposure ceased.

Key words: tetracycline; microbiome; OTU; Piphillin; *Danio rerio*

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1. Introduction

In the last decades, due to society pressure and overpopulation, aquaculture industry has been rising in order to supply human necessities (Føre et al., 2018). Consequently, organisms are usually cultivated in highly stressed conditions (e.g. overcrowded) in intensive and semi-intensive production, facilitating dissemination of infectious diseases. Hence, to prevent animal death and economic losses, antibiotics may be used as a prophylactic therapy meaning that they are applied to both healthy and sick organisms (Cabello, 2006; Santos and Ramos, 2018). Oxytetracycline (OTC) is one of the most used antibiotics in food-production in European countries (European Medicines Agency, 2017) due to its broad spectrum and effectiveness against Gram-positive and Gram-negative bacteria. In aquaculture, antibiotics are mainly administrated through feed incorporation and thus, uneaten food constitutes an important source of OTC residues in the in the environment. In addition, it is known that organisms such as fish, do not absorb all the OTC ingested and more than 90% of this chemical may be excreted in feces and urine resulting in another source of environmental contamination (Cravedi et al., 1987). Consequently, OTC was already detected at concentrations of 287 ng/L in Dou river in China (Zou et al., 2011), 19.2 ng/L in Po river in Italy (Calamari et al., 2003) and 340 ng/L in USA surface waters (Kolpin et al., 2002). Furthermore, values of 7993 and 15163 ng/L of OTC were also found in aquaculture systems in Brazil and China respectively (Chen et al., 2015; Monteiro et al., 2016). As these compounds remain bioactive in the environment, non-target organisms might be unavoidably exposed. Then, effects in the organisms themselves and in their associated and surrounding bacterial communities can be expected.

The balance of the interactions between the microbiome and its host is of high importance to the host health and welfare (Miller et al., 2018). It is known that this is not a static relationship and organism's microbiome change along its life due to several factors like diet and nutritional status (Xia et al., 2014), immunological conditions (Maynard et al., 2012), environmental stress (Boutin et al., 2013) and chemical exposure (Zhou et al., 2018). Studying the changes in the microbiome is of high importance to understand the impact that these factors may have in host health.

Due to its several advantages, zebrafish has been indicated as a suitable organism to study host-microbiome interaction (Roeselers et al., 2011). In fact, zebrafish have a high

fecundity, allowing to derive and cultivate embryos in germ-free or gnotobiotic conditions (Rawls et al., 2004) while its transparency and rapid development allows the monitoring of all the embryonic development. In addition, its genome sequencing (http://www.sanger.ac.uk/Projects/D_rerio/) instigated the development of several tools for forward and reverse genetic analysis (e.g infection and disease studies) (Lieschke and Currie, 2007). Moreover, numerous works relating zebrafish microbiome changes and host health have been published. For instance, bacterial communities may influence zebrafish intestinal growth and differentiation (Wallace et al., 2005), affect metabolism of fatty acid absorption (Semova et al., 2012) and modulate anxiety-related behavior (Davis et al., 2016). For this reason, studying the changes of organism's microbiome is of high importance to understand the impact of a chemical exposure may have in host health.

Water microbiome plays a role in important environmental processes such as decomposition of organic matter, nutrient and carbon cycle (Moriarty, 1997; Widenfalk et al., 2008) and thus, changes in bacterial communities may lead to alterations in water parameters like pH, ammonia or oxygen content compromising water quality (Moriarty, 1997). Since water microbiome is highly dynamic, it can be easily affected by external factors like salinity, temperature and chemicals (Hollister et al., 2010; Mark Ibekwe et al., 2012; Maul et al., 2006). Consequently, changes in water microbiome may compromise not only biogeochemical processes but also organism's health, which in an aquaculture context may result in economic loss. In addition, aquatic organisms, like fish, are always surrounded by water and in intimal relationship with their environment. Through water ingestion, bacterial communities of water shape fish gut microbiome. However, through feces and urine, the organism itself may also impact water communities. Therefore, changes in water microbiome may not only have a direct, but also an indirect impact at ecological level and thus, an integrated analysis of the antibiotic impact is needed.

For this reason, the objective of this work is to study how the long-term exposure of OTC may impact the bacterial communities of both zebrafish and water. To do that, zebrafish adults were exposed via water to three concentrations of OTC for two months, under the scope of a previously conducted study (Almeida et al., 2019). In that work, effects of OTC in both zebrafish and water microbiome were studied through DGGE analysis. The approach assessed only the effects on the structure of the communities in

terms of their dominant members. No information was obtained on the phylogenetic affiliation of the affected microorganisms. Consequently, a deeper understand of which bacterial phylotypes were affected and possible effects at functional level were needed. Hence, in this work, changes in gut and water microbiomes were studied through Illumina next generation sequencing. This approach allows the structural analysis of bacterial communities of a sample in a fast and cost-effective way (Caporaso et al., 2012; Rud et al., 2017). Moreover, microbiomes functional diversity was predicted *in silico* based on the Illumina sequences using Piphillin software (Iwai et al., 2016).

2. Materials and Methods

2.1 **Zebrafish experiment**

Zebrafish adults used in the experiment were obtained from zebrafish facility stablished at the Biology department in the University of Aveiro, Portugal. The fish were selected following the OECD guideline 230 (OECD, 2009) recommendations. Organisms were exposed to three concentrations of oxytetracycline hydrochloride (0, 10 and 10000 µg/L) for two months under a semi-static condition as described by Almeida et al. (2019). In the end of the experiment (two months of exposure), fish were euthanized with tricaine overdose (tricaine methane sulfonate, Metacain, MS-222; CAS number: 886-86-2) followed by spinal cord severing. Fish guts (5 guts per replicate (5 replicates) in a total of 25 guts per treatment) were sampled individually in aseptic conditions, snap frozen and stored at -80ºC until DNA extraction. Water bacterial community's DNA was collected by filtering 100 ml of each water condition in triplicate (300 ml per treatment) using a 0.22 µm hydrophilic PVDF durapore membrane filter (Merck Millipore; Massachusetts, EUA).

2.2 **Molecular analysis of microbiome**

2.2.1 DNA extraction

For fish gut DNA analysis, the total DNA was extracted individually (1gut = 1 extraction) using the commercial kit PowerSoil® DNA isolation kit (MOBIO laboratories, CA, USA), following manufactures instructions. Later, pools of 5 guts DNA per replicate

were prepared. For water microbiome analysis, total DNA extraction was performed using the commercial kit Genomic DNA Purification kit (Thermo Fisher Scientific; Massachusetts, EUA) as described by Henriques et al. (2004).

2.2.2 Illumina high-throughput sequencing

Fish guts and water samples were prepared for Illumina Sequencing by 16S rRNA gene amplification of the bacterial community. The hypervariable V3-V4 region was amplified with specific primers (Bakt_341F and Bakt_805R) and further reamplified in a limited-cycle PCR reaction to add sequencing adapters and dual indexes. First PCR reactions were performed for each sample using KAPA HiFi HotStart PCR Kit (Table S1 and S2). In the second PCR indexes and sequencing adapters were added to both ends of the amplified target region according to manufacturer's recommendations (Illumina Inc., 2013). Using a SequalPrep 96-well plate kit (ThermoFisher Scientific, Waltham, USA) (Comeau et al., 2017) PCR products were one-step purified and normalized, pooled and paired-end sequenced in the Illumina MiSeq® sequencer, according to manufacturer's instructions (Illumina, San Diego, CA, USA) at Genoinseq (Cantanhede, Portugal). Sequences were then processed at Genoinseq: raw reads were quality-filtered with PRINSEQ version 0.20.4 (Schmieder and Edwards, 2011) to remove sequencing adapters, reads with less than 150 bases and trim bases with an average quality lower than Q25. Furthermore, the forward and reverse reads were merged by overlapping paired-end reads with Adapter Removal version 2.1.5 using default parameters (Schubert et al., 2016).

Sequences were processed using VSEARCH (Rognes et al., 2016) and QIIME pipeline (Caporaso et al., 2012) as described in Alves et al (2016). Briefly, a VSEARCH script was used to identify and discard dereplicated sequences (*derep_fulllength*); to identify Operational Taxonomic Units (OTUs) defined at 97% similarity (*cluster_fast*), and to identify and discard chimeras (*uchime_ref*). Taxonomy assignment was achieved through QUIIME (*assign_taxonomy.py*) using SILVA reference database (132 release; 97% sequence identity) (Quast et al., 2012). Due to new proposed rearrange of classification, the Betaproteobacteria were considered as Betaproteobacteriales, a new order within the class Gammaproteobacteria (Parks et al., 2018).

2.2.3 *In silico* metagenome analysis

Piphillin software (Iwai et al., 2016). was used for functional profile inference based on the OTU sequences and OTU abundance table obtained. These were matched against the Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/) database of phylogenetically referenced prokaryotic genomes using an identity cut-off of 97% to obtain a list KEGG orthologs (KO) and their abundance for each sample.

2.3 **Statistical analysis**

PRIMER v6 software (Primer-E Ltd., Plymouth, UK) (Clarke and Gorley, 2006) was used to perform cluster and principal coordinate analysis (PCoA) using a Bray-Curtis distance matrix constructed by a rarefied and transformed (Log x+1) OTU abundance table. Alpha-diversity, namely species richness (number of OTU; S), diversity (Shannon-Wiener index; H') (Shannon and Weaver, 1964) and evenness (Pielou's evenness index; J) indexes were calculated based on OTU abundance table. Rarefaction curve were obtained through the function *rarecurve* of *vegan* package (Oksanen, J. et al., 2016) from the R software (R Core Team, 2016).

Differences significance among indexes, bacterial abundance at classes, genus and OTU levels, and KO abundance were discriminate using a one-way analysis of variance (ANOVA) followed by the Dunnett's method with the Sigma plot V.12.5 (SysStat software Inc., CA, USA) software. A significant level of 0.05 was considered.

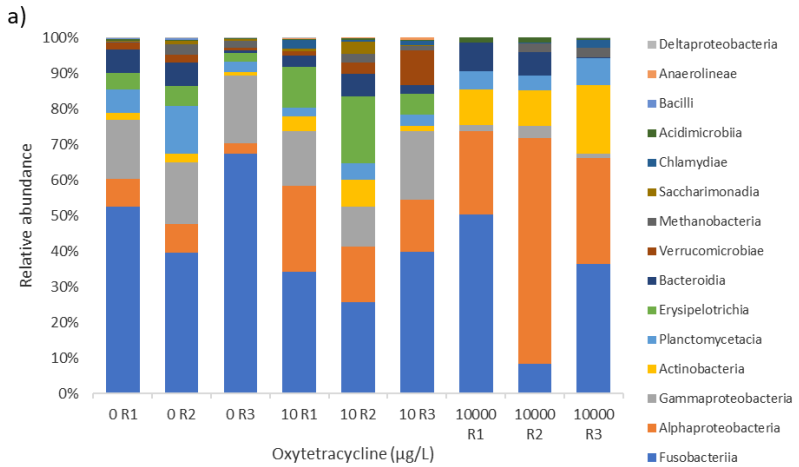
3 **Results**

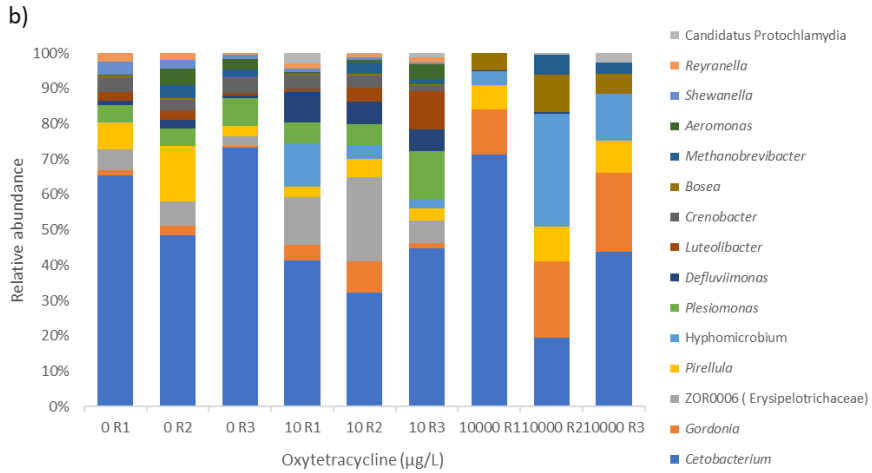
In our study, the impact of OTC in the microbiomes of zebrafish gut and exposure water was assessed through Illumina sequencing analysis. After quality-filtering, a total of 752940 sequence reads from zebrafish gut and 669018 reads from water bacterial communities were achieved. Unassigned reads, singletons and chloroplast-affiliated reads were removed from analysis (Table S3). The obtained rarefaction curves tended to saturation, suggesting for each sample that the OTUs detected were a good estimated of the community richness (Fig. S1).

3.1 Zebrafish gut microbiome

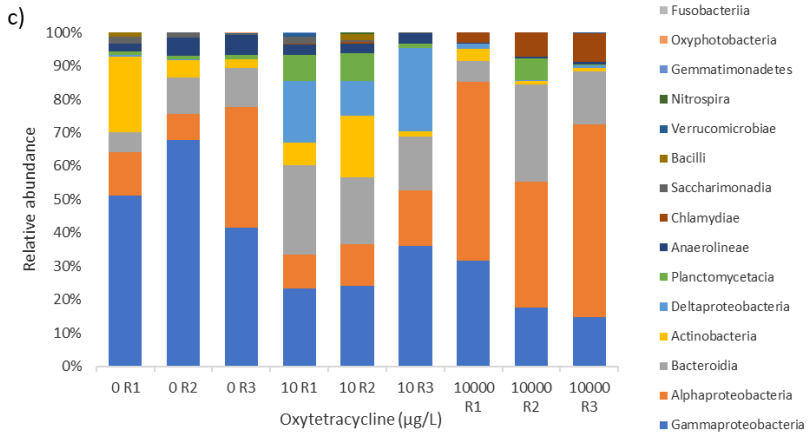
After long-term exposure to OTC, the composition of zebrafish gut microbiome at class level has changed (Fig. 1-a)). In control organisms, most bacterial sequences affiliated with classes Fusobacteria (53.2 ± 13.5%), Gammaproteobacteria (17.7 ± 1.3%), Planctomycetia (7.5 ± 5.3%) and Alphaproteobacteria (6.2 ± 3.0%). On the other hand, in organisms exposed to 10 µg/L of OTC, the classes Fusobacteriia (33.2 ± 7.1%), Alphaproteobacteria (18.2 ± 5.2%), Gammaproteobacteria (15.3 ± 4.0%) and Erysipelotrichia (11.96 ± 6.5%) were the most abundant; in organisms exposed to 10000 µg/L of OTC the most abundant classes were Fusobacteria (31.6 ± 21.4%), Alphaproteobacteria (38.9 ± 21.5%) Actinobacteria (13.1 ± 5.4%) and Planctomycetia (5.6 ± 1.8%). Changes in abundance of specific bacterial classes due to OTC exposure were only statistically significant at the highest concentration (10000 µg/L) for Gammaproteobacteria ($H= 5.60$, $p= 0.05$; abundance decrease) and Actinobacteria ($F= 8.08$, $p= 0.02$; abundance increase). Moreover, at genus level (Fig. 1-b)) the genus *Defluviimonas* ($F= 52.01$, $p< 0.001$; abundance increase) was affected significantly only in the lowest OTC concentration (10 µg/L) while the genera *Gordonia* ($F= 7.91$, $p= 0.021$; abundance increase), *Crenobacter* ($F= 21.12$, $p< 0.002$; abundance increase), *Bosea* ($F= 85.08$, $p< 0.001$; abundance increase) and *Shewanella* ($H= 7.45$, $p= 0.004$; abundance decrease) were affected significantly only in the highest concentration (10000 µg/L) (Fig. 1-b)).

Zebrafish Gut microbiome





Water microbiome



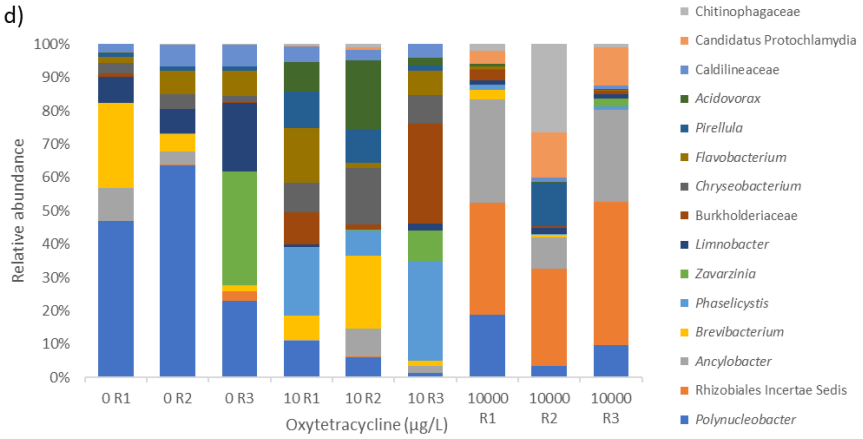


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Fig. 1: Relative abundance of top 15 most abundant classes [a) and c)] and top 15 most abundant genera [b) and d)] of zebrafish gut and water microbiomes exposed to OTC. Genera classified as "uncultured" were indicated with the respective family name. Results of three replicates (R1, R2 and R3) are presented for control (0 µg/L) and for each OTC concentration (10 and 10000 µg/L).

Regarding to OTUs structure and abundance, through cluster and PCoA analysis an impact in the structure due to OTC exposure was observed. Samples of organisms exposed to OTC highest concentration, clustered in a different branch from the other treatments (Fig. 2- a)). The same spatial separation is possible to observe in PCoA (Fig. 2- b)). Although there is some variability among samples, two groups with 50% of similarity were formed.

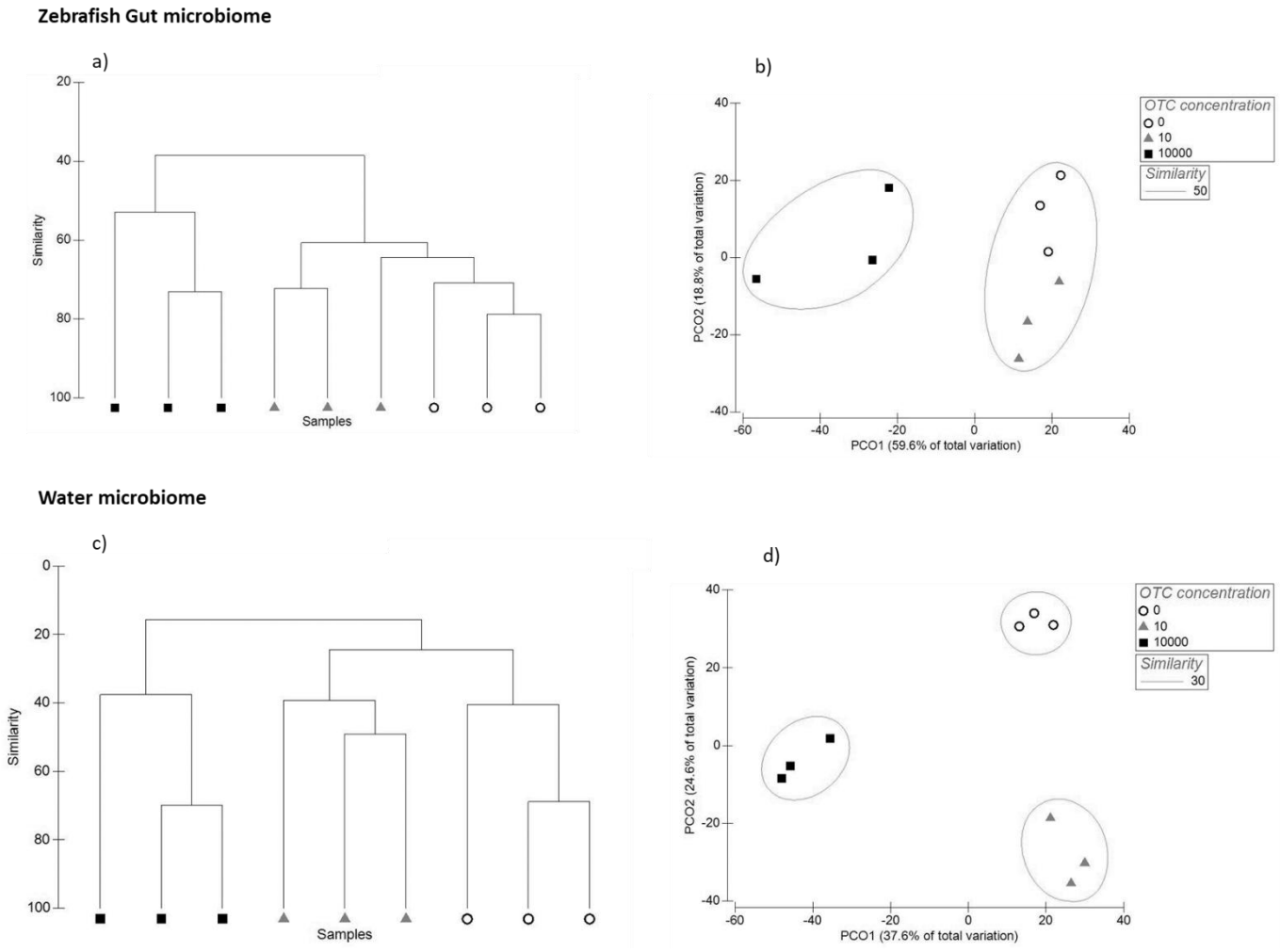

Fig. 2: Changes in zebrafish gut and water microbiomes exposed to oxytetracycline: a) and c) Dendrograms; b) and d) PCoA based on Bray-Curtis similarity matrices. Concentrations are in µg/L.

The top 30 most abundant OTUs, in zebrafish gut, for each condition are represented in the Heatmap presented in Table 1. OTC exposure significantly affected the abundance of 20 OTUs. The OTUs affiliated with Alphaproteobacteria class (Rhodobacteraceae: OTU_25054, Rhodobacteraceae: OTU_24710; *Rhodobacter*: OTU_25507; *Bosea*: OTU_32606; Rhizobiaceae: OTU_31612; Rhizobiaceae: OTU_33018; *Aminobacter*; OTU_24656 and Xanthobacteraceae: OTU_33056), Actinobacteria (*Gordonia*: OTU_7849, OTU_41647, OTU_26247, OTU_26531) and Acidimicrobiia (Microtrichales: OTU_32536) significantly increased in abundance in the highest concentration of OTC (10000 µg/L) (Table S4). OTUs affiliated with Alphaproteobacteria, namely *Defluviimonas* (OTU_35219 and OTU_34457), significantly increased in abundance only in 10 µg/L of OTC. On contrary, the OTUs affiliated with Planctomycetia (*Pirellula*: OTU_7240) and Alphaproteobacteria (*Reyranella*: OTU_9167) significantly decreased in abundance in both OTC concentrations (Table S4). Gammaproteobacteria (*Crenobacter*: OTU_7574 and *Shewanella*: OTU_7855) and Planctomycetia (*Pirellula*: OTU_7123) were only significantly affected (abundance decrease) in the highest concentration of OTC. In addition, some OTUs not present in the control group, emerged in exposed communities: in the highest concentration of OTC (10000 µg/L), a significant abundance increase was observed for the OTUs affiliated with Alphaproteobacteria class (Rhizobiaceae: OTU_31612; Xanthobactereaceae: OTU_33056 and *Rhodobacter*: OTU_25507) (Table S4).

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Table 1: Abundance of the most represented OTUs (30 most abundant per treatment) in zebrafish gut. The color code represents the relative OTU abundance in each sample. Asterisks (*) indicate significant differences in relative abundances towards the respective control ($p \leq 0.05$; Dunnett's test).

#OTU ID	OTC conc. (µg/L)									Phylogenetic affiliation
	0	0	0	10	10	10	10000	10000	10000	
OTU_7409	28.673	21.926	36.135	20.900	12.277	16.171	27.477	4.584	21.864	*Cetobacterium*
OTU_8309	14.172	11.296	19.283	7.959	7.909	13.613	13.419	2.295	9.001	*Cetobacterium*
OTU_20004	8.682	6.844	11.919	4.696	5.578	9.962	8.350	1.480	5.673	*Cetobacterium*
OTU_7123*	5.145	12.094	1.702	2.279	3.679	3.089	3.528	3.477	8.875	*Pirellula*
OTU_8498	2.714	2.904	5.292	3.528	3.477	8.875	0.001	0.001	0.000	*Plesiomonas*
OTU_7574*	3.470	2.478	4.040	3.256	2.800	1.711	0.001	0.000	0.000	*Crenobacter*
OTU_8095	2.957	3.589	1.543	7.211	12.140	3.622	0.000	0.004	0.029	Erysipelotrichaceae
OTU_7854	0.159	3.934	2.824	0.428	0.534	3.807	0.000	0.000	0.002	*Aeromonadaceae*
OTU_7855*	2.568	1.751	1.061	0.700	0.523	0.321	0.000	0.000	0.000	*Shewanella*
OTU_6819	1.853	2.217	0.995	4.188	7.123	2.207	0.000	0.007	0.017	Erysipelotrichaceae
OTU_8449	2.766	1.848	0.358	0.431	0.954	0.054	3.734	3.099	0.000	Chitinophagaceae
OTU_8072	1.732	2.171	0.669	0.664	3.035	9.297	0.024	0.000	0.033	*Luteolibacter*
OTU_8242	2.375	1.729	0.338	0.366	0.918	0.063	3.603	3.096	0.002	Chitinophagaceae
OTU_8202	1.030	1.066	2.009	1.341	1.248	3.351	0.001	0.000	0.000	*Plesiomonas*
OTU_9167*	2.055	1.629	0.368	1.047	0.771	1.319	0.000	0.003	0.000	*Reyranella*
OTU_8415	2.244	1.183	0.527	2.604	0.573	0.171	0.021	0.000	0.017	Burkholderiaceae
OTU_16890	1.193	1.113	1.339	0.862	0.849	0.129	0.639	0.895	0.152	Burkholderiaceae
OTU_25054*	0.989	1.646	0.649	2.542	2.770	3.326	10.247	15.395	7.640	Rhodobacteraceae
OTU_7240*	1.004	1.220	1.050	0.058	0.625	0.038	0.006	0.013	0.000	*Pirellula*
OTU_7849*	0.954	1.763	0.239	3.321	6.542	1.172	6.666	7.161	14.045	*Gordonia*
OTU_35219*	0.769	1.471	0.498	5.418	3.943	4.193	0.034	0.157	0.015	*Defluviimonas*
OTU_24583	1.627	0.477	0.056	0.200	1.450	0.004	0.435	13.357	0.644	Rhizobiales Incertae Sedis
OTU_6111	0.643	0.699	0.815	0.599	0.585	0.081	0.310	0.685	0.141	Burkholderiaceae

Relative abundance (%)	Color code
50 - 10	
10. - 05	
5 - 0.2	
< 0.2	

OTU_29001	0.663	0.532	0.947	0.477	0.314	0.385	0.655	0.120	0.513	Cetobacterium
OTU_7118	0.252	0.735	0.438	0.431	1.985	0.161	0.001	0.000	0.000	Saccharimonadales
OTU_19964	0.000	1.388	0.000	0.325	2.171	1.287	0.004	0.000	0.035	*Chryseobacterium*
OTU_20811	1.041	0.252	0.000	0.010	0.234	0.007	0.000	0.000	0.000	*Fluviicola*
OTU_24710*	0.361	0.610	0.275	0.893	1.350	1.564	4.003	7.011	3.372	Rhodobacteraceae
OTU_9062	0.767	0.275	0.132	0.190	0.075	0.153	0.000	0.000	0.000	*Chitinibacter*
OTU_10920	0.372	0.334	0.373	0.286	0.229	0.041	0.165	0.279	0.050	Burkholderiaceae
OTU_8932	0.137	0.164	0.020	7.909	3.078	2.198	0.123	0.282	5.177	*Hyphomicrobium*
OTU_7515	0.000	0.013	0.000	2.532	0.279	1.128	0.021	0.169	2.273	*Candidatus Protochlamydia*
OTU_7990	0.230	0.533	0.091	0.597	1.769	0.381	0.000	0.000	0.000	*Dinghuibacter*
OTU_34457*	0.189	0.295	0.093	1.049	0.716	0.766	0.004	0.031	0.006	*Defluviimonas*
OTU_19538	0.039	0.010	0.000	2.344	0.069	0.001	2.670	13.765	5.948	*Hyphomicrobium*
OTU_19398	0.000	0.959	0.054	1.336	0.348	0.720	0.303	0.067	0.048	Barnesiellaceae
OTU_7846	0.033	0.020	0.079	0.000	0.000	0.000	4.874	4.223	7.815	*Pirellula*
OTU_32606*	0.276	0.493	0.270	0.173	0.417	0.193	3.366	4.514	4.728	*Bosea*
OTU_41647*	0.128	0.199	0.035	0.238	0.447	0.087	1.365	1.476	2.942	*Gordonia*
OTU_31612*	0.000	0.001	0.004	0.000	0.000	0.000	0.529	3.308	0.305	Rhizobiaceae
OTU_26247*	0.063	0.066	0.011	0.079	0.159	0.020	0.542	0.483	1.074	*Gordonia*
OTU_26531*	0.046	0.063	0.011	0.063	0.133	0.023	0.466	0.445	0.845	*Gordonia*
OTU_8487	0.211	0.072	0.006	0.031	0.174	0.004	0.071	1.436	0.085	Rhizobiales Incertae Sedis
OTU_27070	0.152	0.066	0.014	0.048	0.099	0.048	0.796	0.535	0.245	Microtrichaceae
OTU_25507*	0.000	0.000	0.000	0.048	0.044	0.041	0.031	0.980	0.334	*Rhodobacter*
OTU_33018*	0.002	0.040	0.128	0.019	0.019	0.146	0.463	0.661	0.162	Rhizobiaceae
OTU_33056*	0.000	0.000	0.000	0.000	0.000	0.001	0.145	0.577	0.378	Xanthobacteraceae
OTU_24656*	0.000	0.008	0.000	0.000	0.000	0.000	0.286	0.500	0.164	*Aminobacter*
OTU_24832	0.450	0.263	0.139	0.039	0.033	0.001	0.222	0.177	0.457	*Ancylobacter*
OTU_32536*	0.037	0.040	0.006	0.077	0.072	0.061	0.316	0.204	0.322	Microtrichales
OTU_9185	0.000	0.000	0.000	0.000	0.000	0.000	0.240	0.272	0.177	*Pseudomonas*

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280 Regarding alpha-diversity, no statistically significant effects were observed on the
281 indexes of gut samples (Table 2).

282 The function inference analysis revealed that overall the bacterial communities'
283 metabolism may be affected by OTC. A significant decrease in glycolysis/gluconeogenesis
284 and oxidative phosphorylation was predicted. Also, the biosynthesis of amino acids seems
285 to be affected and a decrease in sequences of tRNA was predicted (Leu; Met; Arg; Val; Ser;
286 Gly and Ala) was predicted (Table 3 and Table S5). From our results, it was also predicted
287 an increase in the detoxification mechanisms, due to a predicted increase in the metabolism
288 of xenobiotics by cytochrome P450; drug metabolism- cytochrome P450 and GST. On the
289 other hand, a decrease in tetracycline resistance mechanisms, namely in the prevalence of
290 determinants such as *tetB*, *tet35*; *tetV*; *tetM* and *tetO*, was predicted for samples exposed to
291 OTC. Our results suggest that Quorum Sensing (QS) cellular communication may also be
292 promoted upon antibiotic exposure (Table 3 and Table S5).

293

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Table 2: Species richness (number of OTU; S), diversity (Shannon-Wiener index; H') and evenness (Pielou's evenness index; J) index of the zebrafish gut and water bacterial communities exposed to oxytetracycline (OTU based profile). Values presented per mean ± standard deviation. Asterisks (*) indicates differences towards the respective control ($p < 0.05$; Dunnett's test).

Samples	Oxytetracycline (µg/L)	Species richness (S)	Shannon - Wiener diversity index (H')	Pielou's evenness index (J)
Zebrafish gut	0	287 ± 56.93	2.83 ± 0.39	0.50 ± 0.07
	10	294 ± 34.04	3.19 ± 0.17	0.56 ± 0.04
	10 000	248 ± 26.35	2.99 ± 0.21	0.51 ± 0.03
Water	0	279 ± 17.06	2.49 ± 0.17	0.44 ± 0.03
	10	358 ± 60.58	**3.31 ± 0.37***	**0.56 ± 0.05***
	10 000	346 ± 21.83	2.89 ± 0.35	0.49 ± 0.06

Table 3: Predicted functional pathway changes of zebrafish gut and water microbiomes exposed to OTC. Differences in relative abundance towards the control (↓ decrease; ↑ increase) are indicated ($p \leq 0.05$; Dunnett's test).

Sample type	KEGG Pathway	KEGG ID	Tendency	F	p
GUT	Metabolic pathways	ko01100	↓	20.805	0.002
	Biosynthesis of secondary metabolites	ko01110	↓	15.752	0.004
	2-Oxocarboxylic acid metabolism	ko01210	↑	6.770	0.029
	Aminoacyl-tRNA biosynthesis	ko00970	↓	6.512	0.031
	Quorum sensing	ko02024	↑	20.536	0.002
	Purine metabolism	ko00230	↓	11.066	0.010
	Ribosome	ko03010	↓	8.251	0.019
	Pyrimidine metabolism	ko00240	↓	6.010	0.037
	Oxidative phosphorylation	ko00190	↓	6.947	0.027
	Glycolysis / Gluconeogenesis	ko00010	↓	18.267	0.003
	Glycine, serine and threonine metabolism	ko00260	↑	6.550	0.031
	Alanine, aspartate and glutamate metabolism	ko00250	↓	11.255	0.009
	Fatty acid biosynthesis	ko00061	↑	5.756	0.040
	Metabolism of xenobiotics by cytochrome P45	ko00980	↑	11.770	0.008
	Drug metabolism - cytochrome P450	ko00982	↑	10.929	0.010
WATER	Two-component system	ko02020	↑	H= 5.600	0.050
	Quorum sensing	ko02024	↑	H= 5.600	0.050
	Glyoxylate and dicarboxylate metabolism	ko00630	↓	182.266	<0.001
	Sulfur metabolism	ko00920	↓	17.525	0.003

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296

297 3.2 Water microbiome

298 Bacterial communities of exposure water were also affected by OTC presence at class
299 level (Fig. 1-c)). In control samples, the classes Gammaproteobacteria (53.5 ± 13.3%),
300 Alphaproteobacteria (18.9 ± 15.0%), Actinobacteria (10.1 ± 10.9%) and Bacteroidia (9.6 ±
301 3.1%), were the most abundant. In the samples exposed to the lowest concentration of OTC

(10 µg/L), the classes Gammaproteobacteria (27.7 ± 7.1%), Bacteroidia (21.0 ± 5.4%), Deltaproteobacteria (17.9 ± 7.3%), Alphaproteobacteria (13.2 ± 3.4%), Actinobacteria (8.9 ± 8.8%) and Planctomycetia (5.8 ± 3.9%) were observed as the most abundant. On the other hand, in the samples exposed to the highest concentration of OTC (10000 µg/L), the classes Alphaproteobacteria (49.7 ± 10.5%), Gammaproteobacteria (21.3 ± 9.0%), Bacteroidia (17.1 ± 11.6%) and Chlamydiae (6.3 ± 2.9%) were the most abundant. Statistically significant differences in the abundance of specific classes due to OTC exposure were observed in the highest concentration (10000 µg/L), namely for Gammaproteobacteria (F= 8.45; p=0.02) and Anaerolineae (F= 9.77; p= 0.01), which decreased in abundance while Alphaproteobacteria (F= 10.00; p=0.01) and Chlamydiae (H= 5.60; p= 0.05) abundance increased. For Deltaproteobacteria a significant abundance increase (H= 6.48; p=0.01) occurred in the lowest concentration (10 µg/L). At genus level, OTC lowest concentration (10 µg/L) significantly affected the genus *Phaselicystis* (H= 6.49; p= 0.011; abundance increase), an uncultured genus from Burkholderiaceae (H= 5.69; p= 0.029; abundance increase), *Chryseobacterium* (H= 6.49; p= 0.011; abundance increase) and *Acidovorax* (H= 7.20; p=0.004; abundance increase). In the highest concentration, the genera uncultured from Caldilineaceae family (F= 9.60; p= 0.013; abundance decrease), uncultured from Rhizobiales Incertae Sedis (F= 21.66; p= 0.002; abundance increase) and the Candidatus Protochlamydia (H= 5.60; p= 0.050; abundance increase) were affected. The genera *Polynucleobacter* (H= 5.60; p= 0.050; abundance decrease) and *Limnobacter* (H= 5.60; p= 0.050; abundance decrease) were affected by both OTC concentrations (Fig. 1-d)).

The analysis of OTUs structure and abundance in water samples is represented in the cluster and PCoA analysis (Fig. 2). In the dendrogram, the highest concentration of OTC impacted the OTU structure and abundance, clustering in a different branch than the control and lowest concentration (10 µg/L) (Fig. 2-c)). Also, the spatial distribution shows that similarity among treatments is less than 30% (Fig. 2-d)).

In water samples, 32 abundant OTUs were significantly affected by the presence of OTC (Table 4 and S6). The OTUs affiliated with the class Chlamydiae (Candidatus Protochlamydia: OTU_7515) presented an increase in the abundance while in the Anaerolineae class (Caldilineaceae: OTU_8489; OTU_24365) was observed an abundance

decrease due to OTC highest concentration exposure. The OTUs affiliated with Deltaproteobacteria (*Phaselicystis*: OTU_6634, OTU_4880, OTU_7538) and Bacteroidia (*Sediminibacterium*: OTU_7428) presented a significant abundance increase in the 10 µg/L OTC concentration (Table S6). Also, due to OTC exposure, some OTUs significantly decreased their abundance, not being detected in exposed samples: OTU_8650 (*Flavobacterium*), OTU_20619 (*Fluviicola*), OTU_33311 (*Prosthecomicrobium*) and OTU_8611 (*Lysobacter*). On the other hand, some OTUs not detected in the control treatments raised their abundance with OTC exposure, namely: OTU_9362 and OTU_7969 (*Runella*); OTU_24165 (*Flectobacillus*); OTU_8242 and OTU_8449 (uncultured Chitinophagaceae); OTU_7691 (*Fluviicola*); OTU_38765 (Rhizobiales Incertae Sedis); OTU_19538 (*Hyphomicrobium*); OTU_7846 (*Pirellula*); OTU_7687 (Burkholderiaceae) and OTU_6796 (*Acidovorax*) (Table S6).

Significant differences in alpha-diversity were observed in the Shannon-Wiener (H: $F= 5.235$; $p= 0.048$) and Pielou's evenness (J: $F= 5.484$; $p= 0.044$) in the lowest concentration tested (10 µg/L OTC) (Table 2).

Regarding to function inference, similar to the gut samples, our results suggest that an increase in Two-Component system and QS (Table 3).

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Table 4: Abundance of the most represented OTUs (30 most abundant per treatment) in water samples. The color code represents the relative OTU abundance in each sample. Asterisks (*) indicate significant differences in relative abundance towards the respective control ($p \leq 0.05$; Dunnett's test).

#OTU ID	OTC conc. (µg/L)									Phylogenetic affiliation
	0	0	0	10	10	10	10000	10000	10000	
OTU_8190*	38.603	53.220	18.869	7.164	4.833	1.070	14.205	1.580	6.938	*Polynucleobacter*
OTU_8401	0.107	0.028	30.455	0.188	0.088	7.486	0.025	0.006	1.828	*Zavarzinia*
OTU_9020	15.203	3.133	1.087	3.334	12.565	0.841	1.294	0.192	0.064	*Brevibacterium*
OTU_7923*	3.777	3.527	10.289	0.257	0.066	0.467	0.557	0.480	0.338	*Limnobacter*
OTU_6849*	2.902	2.681	7.899	0.213	0.050	0.396	0.470	0.389	0.259	*Limnobacter*
OTU_24832	8.781	3.563	0.071	0.063	6.879	1.601	23.984	4.694	20.410	*Ancylobacter*
OTU_24809	7.171	1.429	0.652	1.789	5.457	0.370	0.766	0.195	0.026	*Brevibacterium*
OTU_8339*	2.637	3.586	1.594	5.750	13.246	6.644	0.283	0.034	0.034	*Chryseobacterium*
OTU_9248	0.845	4.642	0.695	0.044	0.008	2.463	0.015	0.000	0.281	*Rhizobacter*
OTU_8489*	0.945	2.500	2.440	1.092	1.175	1.191	0.043	0.225	0.320	Caldilineaceae
OTU_8650*	0.047	0.032	5.743	0.002	0.000	0.001	0.001	0.000	0.000	*Flavobacterium*
OTU_24365	0.849	2.262	2.248	1.040	1.014	1.071	0.043	0.274	0.255	Caldilineaceae
OTU_32698	2.429	1.412	0.002	0.010	0.000	0.020	0.001	0.000	0.000	*Phenylobacterium*
OTU_6535*	1.309	1.559	0.660	0.235	0.123	0.028	0.476	0.069	0.225	*Polynucleobacter*
OTU_7118	1.630	1.291	0.244	1.384	0.346	0.165	0.000	0.001	0.000	Saccharimonadales
OTU_7123	0.725	0.594	1.189	7.600	7.812	1.186	0.015	0.006	0.001	*Pirellula*
OTU_22567	0.449	1.799	0.231	3.328	0.381	1.670	0.131	0.017	0.041	*Flavobacterium*
OTU_31612	0.806	1.295	0.353	0.124	0.235	0.005	0.000	2.099	0.004	Rhizobiaceae
OTU_21385	0.434	1.738	0.221	2.872	0.358	1.620	0.095	0.011	0.041	*Flavobacterium*
OTU_24583*	0.004	0.004	2.186	0.078	0.011	0.040	22.361	13.038	27.658	Rhizobiales Incertae Sedis
OTU_21358	0.411	1.427	0.197	2.709	0.340	1.322	0.103	0.014	0.048	*Flavobacterium*
OTU_25440	0.347	0.328	0.758	0.861	0.358	0.700	0.012	0.064	0.079	Caldilineaceae
OTU_33311*	0.139	0.245	0.977	0.000	0.001	0.002	0.000	0.000	0.000	*Prosthecomicrobium*

Relative abundance (%)	**Color code**
60 - 10	
10 - 5	
5 - 0.2	
< 0.2	

OTU_7688	1.337	0.006	0.000	0.014	0.049	0.000	0.004	0.000	0.000	*Staphylococcus*
OTU_20626*	0.120	0.094	1.120	0.231	0.016	0.097	0.001	0.000	0.000	Chitinophagales
OTU_8611*	0.402	0.639	0.131	0.000	0.003	0.000	0.000	0.000	0.000	*Lysobacter*
OTU_9008	0.147	0.049	0.934	0.300	0.811	0.019	0.028	0.011	0.000	Sphingobacteriales
OTU_20619*	1.016	0.019	0.008	0.000	0.000	0.000	0.000	0.000	0.001	*Fluviicola*
OTU_8999	0.691	0.100	0.168	0.180	0.260	0.008	0.017	0.040	0.187	Burkholderiaceae
OTU_7240*	0.316	0.443	0.096	0.023	0.142	0.009	0.000	0.000	0.005	*Pirellula*
OTU_7687*	0.002	0.002	0.000	6.439	0.811	23.922	2.231	0.130	0.380	Burkholderiaceae
OTU_6796*	0.019	0.032	0.010	6.043	16.994	2.003	0.415	0.133	0.054	*Acidovorax*
OTU_6634*	0.015	0.053	0.010	6.336	2.802	11.030	0.608	0.025	0.305	*Phaselicystis*
OTU_4880*	0.013	0.021	0.010	4.404	2.013	7.873	0.363	0.030	0.217	*Phaselicystis*
OTU_7538	0.011	0.009	0.007	3.123	1.370	5.028	0.308	0.021	0.193	*Phaselicystis*
OTU_8407*	0.000	0.217	0.015	3.124	0.818	1.331	0.190	0.001	0.048	*Sediminibacterium*
OTU_24730	0.066	0.000	0.002	4.088	0.373	0.108	0.209	0.003	0.004	*Bdellovibrio*
OTU_32665	0.006	0.000	0.050	0.026	0.005	3.910	0.014	0.027	0.069	*Phenylobacterium*
OTU_27302	0.083	0.000	0.017	0.000	2.909	0.864	0.000	0.000	0.000	*Bdellovibrio*
OTU_25153	0.002	0.028	0.003	3.368	0.090	0.066	0.048	0.000	0.003	*Brevundimonas*
OTU_32724	0.004	0.045	0.003	2.317	0.534	0.547	0.026	0.003	0.006	*Azospirillum*
OTU_8345	0.008	0.085	0.008	0.372	0.080	2.919	6.604	0.016	1.771	*Ideonella*
OTU_9167	0.011	0.026	0.272	0.611	1.789	0.092	0.000	0.000	0.003	*Reyranella*
OTU_7428*	0.000	0.045	0.002	1.381	0.364	0.636	0.066	0.001	0.024	*Sediminibacterium*
OTU_8844	0.000	0.002	0.000	0.018	1.853	0.002	0.000	0.001	0.000	*Staphylococcus*
OTU_7515*	0.006	0.004	0.035	0.098	0.521	0.004	2.979	6.705	8.489	*Candidatus Protochlamydia*
OTU_7969*	0.036	0.045	0.068	0.213	1.046	0.042	1.295	3.360	10.686	*Runella*
OTU_19538*	0.002	0.000	0.000	0.001	0.002	0.012	1.683	7.715	1.559	*Hyphomicrobium*
OTU_8548	0.006	0.019	0.002	0.001	0.003	0.001	0.085	9.211	0.098	Burkholderiaceae
OTU_7691*	0.002	0.013	0.000	0.001	0.000	0.000	1.195	6.282	0.310	*Fluviicola*
OTU_8487	0.002	0.000	0.257	0.014	0.000	0.006	2.613	1.096	3.297	Rhizobiales Incertae Sedis
OTU_7846*	0.002	0.000	0.000	0.000	0.000	0.000	0.158	6.535	0.265	*Pirellula*
OTU_8242*	0.002	0.000	0.060	0.003	0.000	0.000	0.138	6.603	0.134	Chitinophagaceae

OTU_8449*	0.002	0.002	0.078	0.000	0.003	0.000	0.144	6.579	0.121	Chitinophagaceae
OTU_22727	0.000	0.000	0.819	0.006	0.060	0.000	0.428	4.240	0.108	*Flectobacillus*
OTU_9362*	0.013	0.019	0.023	0.067	0.324	0.005	0.430	0.654	3.426	*Runella*
OTU_25054	0.009	0.196	0.126	0.184	0.089	0.011	0.039	2.835	0.060	Rhodobacteraceae
OTU_9319	0.013	0.049	0.529	0.010	0.000	0.028	2.268	0.235	0.241	*Solimonas*
OTU_38765*	0.000	0.000	0.008	0.000	0.000	0.000	1.353	0.293	0.960	Rhizobiales Incertae Sedis
OTU_6695	0.008	0.168	0.008	0.075	0.216	0.134	0.524	1.348	0.362	*Variovorax*
OTU_24738	0.008	0.002	0.003	1.554	0.036	0.004	1.433	0.000	0.497	*Leifsonia*
OTU_24710	0.002	0.072	0.068	0.068	0.033	0.008	0.025	1.682	0.015	Rhodobacteraceae
OTU_15427	0.002	0.006	0.000	0.000	0.000	0.000	0.011	1.501	0.018	*Caenimonas*
OTU_25509*	0.015	0.081	0.002	0.004	0.000	0.001	0.140	0.564	0.670	*Stella*
OTU_16551	0.000	0.002	0.000	0.228	0.049	0.019	0.399	0.103	0.856	Burkholderiaceae
OTU_24165*	0.000	0.000	0.000	0.001	0.007	0.000	0.076	1.067	0.024	*Flectobacillus*

4 Discussion

The present study assessed the effects of OTC on the bacterial community of both water and fish gut after a long-term exposure to this antibiotic through a metagenomic approach. In a previous work we reported long-term effects of OTC in fish gut and exposure water microbiome structure through a DGGE analysis (Almeida et al 2019). Based on that, this work intended to identify the changes observed, indicating the bacterial phylogenetic groups affected and possible changes in the microbiomes function.

In the literature, teleost fish microbiome is usually characterized by dominance of Gamaproteobacteria, Alphaproteobacteria and Fusobacteria, which constitute the core microbiome (Llewellyn et al., 2014; Roeselers et al., 2011). Since the fish microbiome is defined not only by intrinsic factors (e.g. fish species) but also by the environmental factors (e.g. water, food and geological characteristics) (Parlapani et al., 2018; Pimentel et al., 2017), differences observed in this study, in the control group, were expected. Moreover, the variability observed among samples, natural of interindividual variation of gut microbiome, agrees with literature (Lan and Love, 2012; Stephens et al., 2015).

Considering that OTC exposure was performed via water, it was expected a higher impact in the water bacterial communities. Nevertheless, in our previous work changes in structure of water bacterial communities were mainly observed in the highest OTC concentration tested (Almeida et al. 2019). On the other hand, in this work, changes in the class abundance were observed even in the lowest OTC concentration tested, with the abundance increase of Deltaproteobacteria class. This increase was related with the increase of Myxococcales (Phaselicystidaceae family) and Bdellovibrionales (Bdellovibrionaceae family). Since *Bdellovibrio* and like organisms (BALOs) are a group of major obligate predators of bacteria, some authors indicate that this group may be highly responsive to bacterial community structure changes (Chauhan et al., 2009; Chen et al., 2011). In addition, *Bdellovibrio* have a specific preference for some bacterial species, namely fish pathogens, indicating that they may play a role in keeping organisms healthy (Rogosky et al., 2006). For instance, a previous work has shown that BALOs may reduce the disease occurrence caused by *Aeromonas hydrophila* in fish ponds (Chu and Zhu, 2010). Hence,

382 the exposure to the lowest concentration of OTC (10 µg/L) may favor the increase of some
383 bacterial groups with potential biocontrol in fish diseases.
384 Nevertheless, at the highest OTC concentration, other classes were affected in water
385 samples, namely the Alphaproteobacteria and Chlamydiae, being observed an abundance
386 increase. Chlamydiae is a known pathogen of both mammalians and fish organisms.
387 Members of this group are agents of epitheliocystis disease, responsible for economic loss
388 in fish's aquacultures (Blandford et al., 2018; Stride et al., 2014). Also, the increase of this
389 group may be of high concern since it was reported that Chlamydiae may acquire
390 tetracycline resistance genes (*tet*) when exposed to selective pressure (Sandoz and Rockey,
391 2010). Regarding Alphaproteobacteria, Rhizobiales was the most affected order by OTC
392 exposure. This bacterial group, specially the genus *Hyphomicrobium*, is particularly
393 abundant in biofilms (Chee and Liu, 2007). The biofilm formation is usually a mechanism
394 of stress response. For instance, in the presence of antibiotics, biofilms can provide
395 bacterial cells a higher capacity to resist to antibiotic exposure (Olsen, 2015).
396 Bacterial communities of fish larval stages tend to reflect more the surrounding
397 environment, being more influenced by external factors, such as feed regime and water
398 quality. On the opposite, adult fish tend to have a more specific and stable microbiome
399 (Burgos et al., 2018; Stephens et al., 2015). Yet, in our work, the long-term exposure to
400 OTC induced a significant change in bacterial communities of zebrafish gut, namely, the
401 increase of Actinobacteria and Alphaproteobacteria abundance. Both classes are usually
402 found in the gut microbiome of freshwater organisms like shrimp and fish (Liu et al., 2019;
403 Romero et al., 2014; Rosado et al., 2019). In particular, the Rhodobacteraceae family
404 (Alphaproteobacteria class) was pointed as playing a role in organisms health being usually
405 more abundant in healthy fish (Xue et al., 2017). Indeed, some tropodithietic acid-
406 producing clades were indicated as being a potential probiotic agent due to its effects
407 against pathogens (Sonnenschein et al., 2017). Regarding Actinobacteria, this class presents
408 a global interest in biotechnological application due to its capacity to synthetize secondary
409 metabolites capable of removing xenobiotics (Alvarez et al., 2017; Jami et al., 2015). For
410 instance, *Gordonia* genus has been indicated has a recalcitrant pollutant degrader (Abraham
411 et al., 2013; Alvarez et al., 2017). Thus, these microbiome changes may have provided the
412 host an adaptative advantage to survive in an OTC contaminated environment. On the other

hand, *Gordonia* genus includes human pathogens causing serious infections on soft tissues and bones (Sowani et al., 2017). Therefore, although there may be a positive effect of *Gordonia* abundance to the host and the ecosystem, this abundance increase may represent a public health risk.

On the other hand, in both gut and water samples an abundance decrease in Gammaproteobacteria, a class to which belong many fish pathogens, like the *Shewanella* genus, was observed. Fish microbiome are naturally colonized by potential pathogenic bacteria (Rosado et al., 2019). Nevertheless, the pathogenic effect is only observed when the host undergoes a stress (e.g. chemical). OTC is an antibiotic broadly used in aquaculture to control fish diseases and so, the observed effect in *Shewanella* and other Gammaproteobacteria was expected since these bacteria are included in OTC spectrum of activity.

Differences observed in bacterial communities among the water (environment) and fish gut may be also influenced by host internal regulation mechanisms like immunological system. Usually, healthy animals are associated to a high diversity of bacterial communities, reflecting greater metabolic capacity and well-being (De Schryver and Vadstein, 2014). In our work, a slight impact of OTC in Shannon index was only observed in the water samples exposed to 10 µg/L of OTC, which reflected an increase in diversity. Nevertheless, the impact observed in the relative abundance (increase or decrease) of some bacterial classes and OTUs, due to OTC exposure, may however, had affected the overall bacterial function. Indeed, changes in bacterial diversity have been linked to dysbiosis with consequent metabolic disorders (Jin et al., 2017). In this study, the predicted decrease in cellular activity like the inhibition of protein biosynthesis may be related with OTC mode of action. In particular, OTC molecule prevents aminoacyl-tRNA to bind the ribosome preventing the translation (Chopra and Roberts, 2001). Also, the cellular mechanisms of stress may have been triggered in both water and gut samples resulting in an increase in cell communication mechanisms like Quorum sensing (QS). This mechanism is related with biofilm formation, which, as mentioned before, can provide protection against antibiotic exposure. In addition, the general detoxification mechanisms like the metabolism of xenobiotics by cytochrome-P450 and glutathione S-transferase (GST) activities were likely enhanced. However, an opposite effect was observed in the specific mechanisms of

defense, with the decrease of tetracycline (*tet*) resistance genes. This result may indicate that non-specific mechanisms may be firstly activated, probably due to less energy requirements. On the other hand, since the QS selection occurred, this may be a more advantageous strategy of bacterial communities to fight against the OTC stress.

Worth to note that in our work, in both gut and water samples, OTC exposure has selected intrinsically resistant genera, like *Rhodobacter, Acidovorax* and *Defluviimonas* which may lead to a replacement of the susceptible genera (e.g. *Pirellula*, *Reyranella* and *Fluviicola*). It was also observed the increase of some potential health risk groups like the Chlamydiae (discussed above) and *Chryseobacterium* genus. *Chryseobacterium* is considered as an emerging pathogen, responsible for Bacterial gill disease (BGD) and systemic hemorrhagic septicemia in fish, with a worldwide economic impact (Loch and Faisal, 2015; Shahi et al., 2018). Besides, *Chryseobacterium* was not only reported in literature as a tetracycline resistant taxon but also indicated as a multi-drug resistant bacterium (Harnisz et al., 2015; Zhao et al., 2019). Hence, although OTC exposure lead to a decrease of some pathogenic agents included in Gammaproteobacteria class (also reported in this work) the increase of other taxa may represent a health and economic problem. Therefore, even though in our work a decrease in some *tet* resistance genes was detected, the selection of resistant bacteria may indicate that a different behavior possibly occurs when the exposure ceased.

In this work, a general selection of some groups that play a role in nitrogen cycle (e.g. Rhizobiaceae; *Hyphomicrobium* and *Defluviimonas*) was also observed. Since fish were kept under a semi-static system, ammonia released by the organisms in the water may have influenced bacterial communities. In addition, some of the selected genera were indicated in literature as tetracycline resistant groups explaining our results (Gerzova et al., 2014; Huang et al., 2014). Notwithstanding, the selection of some bacteria genera due to OTC may induce changes in the nitrogen cycle, like nitrification, ammonia oxidation (anammox) and denitrification processes with potential ecological impact in the environment since the accumulation of nitrate ($NO3^{-}$), nitric oxide (NO), and nitrous oxide (N_2O) may be toxic to aquatic organisms (Camargo et al., 2005; Roose-Amsaleg and Laverman, 2016). Thus, in future works, post-exposure scenarios should be considered, since bacterial communities may react differently after exposure ceased. For instance, bacterial selection by OTC may

induce function changes with unknown impact in the environment. Also, fish gut and water bacterial resistome may play a role in pathogen dissemination.

5 Conclusion

In our work, the long-term effect of OTC exposure in water and zebrafish gut bacterial communities was assessed. Overall, the OTC revealed to impact both water and gut bacterial communities' structure. Indeed, in water samples, effects were observed even in the lowest concentration tested with the increase of Deltaproteobacteria class, while in the zebrafish gut, effects were mainly observed in the highest concentration. Changes in zebrafish gut due to OTC exposure revealed to be selective for some taxa like Alphaproteobacteria and Actinobacteria. Although some Actinobacteria genera, like *Gordonia*, may play a role in chemical degradation, this genus may also represent a health problem since it is linked to human infection diseases. However, in both gut and water samples, the decrease in Gammaproteobacteria was observed. Also, the exposure to OTC resulted in the selection of bacterial groups intrinsically resistant to this antibiotic, some of which are considered emergent fish pathogens (e.g. *Chryseobacterium*). Function inference revealed that general mechanisms of defense as the QS were selected, while specific mechanisms of tetracycline resistance (e.g. *tet* genes) decreased. This result may indicate that under stress, general mechanisms may be preferable since the cells do not require as much energy as in the specific ones. In addition, effects in some bacterial groups that have a role in nitrogen cycle may lead to an increase in toxic nitrogen products with a possible ecological effect. Future studies should analyze in detail post-exposure water and zebrafish gut resistome to understand the long-term impact of the observed alterations.

6 Conflict of interest

The authors declare no conflict of interest.

7 Ethics statement

All procedures were conducted under personal and project licenses for this study agrees with European Directive on the protection of animals used for scientific purposes (2010/63/EU).

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References

Abraham, J., Shanker, A., Silambarasan, S., 2013. Role of Gordonia sp JAAS1 in biodegradation of chlorpyrifos and its hydrolysing metabolite 3,5,6-trichloro-2-pyridinol. Lett. Appl. Microbiol. 57, 510–516. https://doi.org/10.1111/lam.12141

Almeida, A.R., Tacão, M., Machado, A.L., Golovko, O., Zlabek, V., Domingues, I., Henriques, I., 2019. Long-term effects of oxytetracycline exposure in zebrafish: A multi-level perspective. Chemosphere 222, 333–344. https://doi.org/10.1016/j.chemosphere.2019.01.147

Alvarez, A., Saez, J.M., Davila Costa, J.S., Colin, V.L., Fuentes, M.S., Cuozzo, S.A., Benimeli, C.S., Polti, M.A., Amoroso, M.J., 2017. Actinobacteria: Current research and perspectives for bioremediation of pesticides and heavy metals. Chemosphere 166, 41–62. https://doi.org/10.1016/j.chemosphere.2016.09.070

Alves, M., Pereira, A., Matos, P., Henriques, J., Vicente, C., Aikawa, T., Hasegawa, K., Nascimento, F., Mota, M., Correia, A., Henriques, I., 2016. Bacterial community associated to the pine wilt disease insect vectors *Monochamus galloprovincialis* and *Monochamus alternatus*. Sci. Rep. 6, 1–12. https://doi.org/10.1038/srep23908

Blandford, M.I., Taylor-Brown, A., Schlacher, T.A., Nowak, B., Polkinghorne, A., 2018. Epitheliocystis in fish: An emerging aquaculture disease with a global impact. Transbound. Emerg. Dis. 65, 1436–1446. https://doi.org/10.1111/tbed.12908

Boutin, S., Bernatchez, L., Audet, C., Derom̂e, N., 2013. Network analysis highlights complex

541 interactions between pathogen, host and commensal microbiota. PLoS One 8, 1–16.
542 https://doi.org/10.1371/journal.pone.0084772

543 Burgos, F.A., Ray, C.L., Arias, C.R., 2018. Bacterial diversity and community structure of the
544 intestinal microbiome of Channel Catfish (*Ictalurus punctatus*) during ontogenesis. Syst. Appl.
545 Microbiol. 41, 494–505. https://doi.org/10.1016/j.syapm.2018.04.006

546 Cabello, F.C., 2006. Heavy use of prophylactic antibiotics in aquaculture: A growing problem for
547 human and animal health and for the environment. Environ. Microbiol. 8, 1137–1144.
548 https://doi.org/10.1111/j.1462-2920.2006.01054.x

549 Calamari, D., Zuccato, E., Castiglioni, S., Bagnati, R., Fanelli, R., 2003. Strategic survey of
550 therapeutic drugs in the rivers Po and Lambro in Northern Italy. Environ. Sci. Technol. 37,
551 1241–1248. https://doi.org/10.1021/es020158e

552 Camargo, J.A., Alonso, A., Salamanca, A., 2005. Nitrate toxicity to aquatic animals: a review with
553 new data for freshwater invertebrates. Chemosphere 58, 1255–1267.
554 https://doi.org/10.1016/j.chemosphere.2004.10.044

555 Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S.M.,
556 Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J.A., Smith, G., Knight, R., 2012. Ultra-
557 high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms.
558 ISME J. 6, 1621–1624. https://doi.org/10.1038/ismej.2012.8

559 Chauhan, A., Cherrier, J., Williams, H.N., 2009. Impact of sideways and bottom-up control factors
560 on bacterial community succession over a tidal cycle. Proc. Natl. Acad. Sci. 106, 4301–4306.
561 https://doi.org/10.1073/pnas.0809671106

562 Chee, M.P., Liu, W.T., 2007. Community structure analysis of reverse osmosis membrane biofilms
563 and the significance of Rhizobiales bacteria in biofouling. Environ. Sci. Technol. 41, 4728–
564 4734. https://doi.org/10.1021/es0701614

565 Chen, H., Athar, R., Zheng, G., Williams, H.N., 2011. Prey bacteria shape the community structure
566 of their predators. ISME J. 5, 1314–1322. https://doi.org/10.1038/ismej.2011.4

567 Chen, H., Liu, S., Xu, X.R., Liu, S.S., Zhou, G.J., Sun, K.F., Zhao, J.L., Ying, G.G., 2015. Antibiotics in
568 typical marine aquaculture farms surrounding Hailing Island, South China: Occurrence,
569 bioaccumulation and human dietary exposure. Mar. Pollut. Bull. 90, 181–187.
570 https://doi.org/10.1016/j.marpolbul.2014.10.053

571 Chopra, I., Roberts, M., 2001. Tetracycline Antibiotics : Mode of Action , Applications , Molecular
572 Biology , and Epidemiology of Bacterial Resistance Tetracycline Antibiotics : Mode of Action ,
573 Applications , Molecular Biology , and Epidemiology of Bacterial Resistance. Microbiol. Mol.
574 Biol. Rev. 65, 232–260. https://doi.org/10.1128/MMBR.65.2.232

575 Chu, W.H., Zhu, W., 2010. Isolation of bdellovibrio as biological therapeutic agents used for the
576 treatment of aeromonas hydrophila infection in fish. Zoonoses Public Health 57, 258–264.
577 https://doi.org/10.1111/j.1863-2378.2008.01224.x

578 Clarke, K.R., Gorley, R.N., 2006. PRIMER v6: User Manual/Tutorial.

579 Comeau, A.M., Douglas, G.M., Langille, M.G.I., 2017. Microbiome Helper: a Custom and
580 Streamlined Workflow for Microbiome Research. mSystems 2, e00127-16.

https://doi.org/10.3109/00016489.2014.952774

Cravedi, J.-P., Choubert, G., Delous, G., 1987. Digestibility of chloramphenicol, oxolinic acid and oxytetracycline in rainbow trout and influence of these antibiotics on lipid digestibility. Aquaculture 60, 133–141. https://doi.org/10.1016/0044-8486(87)90305-X

Davis, D.J., Bryda, E.C., Gillespie, C.H., Ericsson, A.C., 2016. Microbial modulation of behavior and stress responses in zebrafish larvae. Behav. Brain Res. 311, 219–227. https://doi.org/10.1016/j.bbr.2016.05.040

De Schryver, P., Vadstein, O., 2014. Ecological theory as a foundation to control pathogenic invasion in aquaculture. ISME J. 8, 2360–2368. https://doi.org/10.1038/ismej.2014.84

European Medicines Agency, 2017. Sales of veterinary antimicrobial agents in 30 European countries in 2015. Trends from 2010 to 2015. https://doi.org/10.2809/676974

Føre, M., Frank, K., Norton, T., Svendsen, E., Arve, J., Dempster, T., Eguiraun, H., Watson, W., Stahl, A., Magne, L., Schellewald, C., Skøien, K.R., Alver, M.O., Berckmans, D., 2018. Precision fish farming: A new framework to improve production in aquaculture. Biosyst. Eng. 173, 176–193. https://doi.org/10.1016/j.biosystemseng.2017.10.014

Gerzova, L., Videnska, P., Faldynova, M., Sedlar, K., Provaznik, I., Cizek, A., Rychlik, I., 2014. Characterization of microbiota composition and presence of selected antibiotic resistance genes in carriage water of ornamental fish. PLoS One 9. https://doi.org/10.1371/journal.pone.0103865

Harnisz, M., Korzeniewska, E., Gołaś, I., 2015. The impact of a freshwater fish farm on the community of tetracycline-resistant bacteria and the structure of tetracycline resistance genes in river water. Chemosphere 128, 134–141. https://doi.org/10.1016/j.chemosphere.2015.01.035

Henriques, I.S., Almeida, A., Cunha, A., Correia, A., 2004. Molecular sequence analysis of prokaryotic diversity in the middle and outer sections of the Portuguese estuary Ria de Aveiro. FEMS Microbiol. Ecol. 49, 269–79. https://doi.org/10.1016/j.femsec.2004.04.003

Hollister, E.B., Engledow, A.S., Hammett, A.J.M., Provin, T.L., Wilkinson, H.H., Gentry, T.J., 2010. Shifts in microbial community structure along an ecological gradient of hypersaline soils and sediments. ISME J. 4, 829–838. https://doi.org/10.1038/ismej.2010.3

Huang, K., Tang, J., Zhang, X.X., Xu, K., Ren, H., 2014. A comprehensive insight into tetracycline resistant bacteria and antibiotic resistance genes in activated sludge using next-generation sequencing. Int. J. Mol. Sci. 15, 10083–10100. https://doi.org/10.3390/ijms150610083

Illumina Inc., 2013. 16S Metagenomic Sequencing Library. Illumina.com 1–28.

Iwai, S., Weinmaier, T., Schmidt, B.L., Albertson, D.G., Poloso, N.J., Dabbagh, K., DeSantis, T.Z., 2016. Piphillin: Improved prediction of metagenomic content by direct inference from human microbiomes. PLoS One 11, 1–18. https://doi.org/10.1371/journal.pone.0166104

Jami, M., Ghanbari, M., Kneifel, W., Domig, K.J., 2015. Phylogenetic diversity and biological activity of culturable Actinobacteria isolated from freshwater fish gut microbiota. Microbiol. Res. 175, 6–15. https://doi.org/10.1016/j.micres.2015.01.009

Jin, Y., Wu, S., Zeng, Z., Fu, Z., 2017. Effects of environmental pollutants on gut microbiota.

621 Environ. Pollut. 222, 1–9. https://doi.org/10.1016/j.envpol.2016.11.045

622 Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T.,
623 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S.
624 streams, 1999-2000: A national reconnaissance. Environ. Sci. Technol. 36, 1202–1211.
625 https://doi.org/10.1021/es011055j

626 Lan, C., Love, D.R., 2012. Molecular Characterisation of Bacterial Community Structure along the
627 Intestinal Tract of Zebrafish (*Danio rerio*): A Pilot Study. ISRN Microbiol. 2012, 1–10.
628 https://doi.org/10.5402/2012/590385

629 Lieschke, G.J., Currie, P.D., 2007. Animal models of human disease: Zebrafish swim into view. Nat.
630 Rev. Genet. 8, 353–367. https://doi.org/10.1038/nrg2091

631 Liu, J., Wang, K., Wang, Y., Chen, W., Jin, Z., Yao, Z., Zhang, D., 2019. Strain-specific changes in the
632 gut microbiota profiles of the white shrimp *Litopenaeus vannamei* in response to cold stress.
633 Aquaculture 503, 357–366. https://doi.org/10.1016/j.aquaculture.2019.01.026

634 Llewellyn, M.S., Boutin, S., Hoseinifar, S.H., Derome, N., 2014. Teleost microbiomes: the state of
635 the art in their characterization, manipulation and importance in aquaculture and fisheries.
636 Front. Microbiol. 5, 207. https://doi.org/10.3389/fmicb.2014.00207

637 Loch, T.P., Faisal, M., 2015. Emerging flavobacterial infections in fish: A review. J. Adv. Res. 6, 283–
638 300. https://doi.org/10.1016/j.jare.2014.10.009

639 Mark Ibekwe, A., Leddy, M.B., Bold, R.M., Graves, A.K., 2012. Bacterial community composition in
640 low-flowing river water with different sources of pollutants. FEMS Microbiol. Ecol. 79, 155–
641 166. https://doi.org/10.1111/j.1574-6941.2011.01205.x

642 Mathis, D., Benoist, C., 2012. The influence of the microbiota on type-1 diabetes : on the threshold
643 of a leap forward in our understanding. Immunol. Rev. 245, 239–249.
644 https://doi.org/https://doi.org/10.1111/j.1600-065X.2011.01084.x

645 Maul, J.D., Schuler, L.J., Belden, J.B., Whiles, M.R., Lydy, M.J., 2006. Effects of the anibiotic
646 ciprofloxacin on stream microbial communities and detrivorous macroinvertebrates. Environ.
647 Toxicol. Chem. 25, 1598–1606.

648 Maynard, C.L., Elson, C.O., Hatton, R.D., Weaver, C.T., 2012. Reciprocal interactions of the
649 intestinal microbiota and immune system. Nature 489, 231–41.
650 https://doi.org/10.1038/nature11551

651 Mcilroy, J., Ianiro, G., Mukhopadhya, I., Hold, R., Hansen, G.L., 2018. Review article : the gut
652 microbiome in inflammatory bowel disease — avenues for microbial management. Aliment.
653 Pharmacol. Ther. 26–42. https://doi.org/10.1111/apt.14384

654 Miller, E.T., Svanbäck, R., Bohannan, B.J.M., 2018. Microbiomes as Metacommunities:
655 Understanding Host-Associated Microbes through Metacommunity Ecology. Trends Ecol.
656 Evol. 33, 926–935. https://doi.org/10.1016/j.tree.2018.09.002

657 Monteiro, S.H., Francisco, J.G., Andrade, G.C.R.M., Botelho, R.G., Figueiredo, L.A., Tornisielo, V.L.,
658 2016. Study of spatial and temporal distribution of antimicrobial in water and sediments
659 from caging fish farms by on-line SPE-LC-MS/MS. J. Environ. Sci. Heal. - Part B Pestic. Food
660 Contam. Agric. Wastes 51, 634–643. https://doi.org/10.1080/03601234.2016.1181917

661 Moriarty, D.J.W., 1997. The role of microorganisms in aquaculture ponds. Aquaculture 151, 333–
662 349. https://doi.org/10.1016/S0044-8486(96)01487-1

663 Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P. R.,
664 O'Hara, R. B., Simpson, G. L., Solymos, P., Stevens, M. H. H., Szoecs, E.&, Wagner, H., 2016.
665 Vegan: community ecology package. [WWW Document]. URL https://cran.r-
666 project.org/web/packages/vegan/index.html (accessed 4.10.19).

667 Olsen, I., 2015. Biofilm-specific antibiotic tolerance and resistance. Eur. J. Clin. Microbiol. Infect.
668 Dis. 34, 877–886. https://doi.org/10.1007/s10096-015-2323-z

669 Parks, D.H., Chuvochina, M., Waite, D.W., Rinke, C., Skarshewski, A., Chaumeil, P.-A., Hugenholtz,
670 P., 2018. A standardized bacterial taxonomy based on genome phylogeny substantially
671 revises the tree of life. Nat. Biotechnol. 36, 996–1004. https://doi.org/10.1038/nbt.4229

672 Parlapani, F.F., Michailidou, S., Pasentsis, K., Argiriou, A., Krey, G., Boziaris, I.S., 2018. A meta-
673 barcoding approach to assess and compare the storage temperature-dependent bacterial
674 diversity of gilt-head sea bream (*Sparus aurata*) originating from fish farms from two
675 geographically distinct areas of Greece. Int. J. Food Microbiol. 278, 36–43.
676 https://doi.org/10.1016/j.ijfoodmicro.2018.04.027

677 Pimentel, T., Marcelino, J., Ricardo, F., Soares, A.M.V.M., Calado, R., 2017. Bacterial communities
678 16S rDNA fingerprinting as a potential tracing tool for cultured seabass *Dicentrarchus labrax*.
679 Sci. Rep. 7, 1–10. https://doi.org/10.1038/s41598-017-11552-y

680 Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2012.
681 The SILVA ribosomal RNA gene database project: improved data processing and web-based
682 tools. Nucleic Acids Res. 41, D590–D596. https://doi.org/10.1093/nar/gks1219

683 R Core Team, 2016. R: A language and environment for statistical computing [WWW Document]. R
684 Found. Stat. Comput. Vienna, Austria. URL http://www.r-project.org/

685 Rawls, J.F., Samuel, B.S., Gordon, J.I., 2004. Gnotobiotic zebrafish reveal evolutionarily conserved
686 responses to the gut microbiota. Proc. Natl. Acad. Sci. U. S. A. 101, 4596–4601.
687 https://doi.org/10.1073/pnas.0400706101

688 Roeselers, G., Mittge, E.K., Stephens, W.Z., Parichy, D.M., Cavanaugh, C.M., Guillemin, K., Rawls,
689 J.F., 2011. Evidence for a core gut microbiota in the zebrafish. ISME J. 5, 1595–1608.
690 https://doi.org/10.1038/ismej.2011.38

691 Rognes, T., Flouri, T., Nichols, B., Quince, C., Mahé, F., 2016. VSEARCH: a versatile open source tool
692 for metagenomics. PeerJ 4, e2584. https://doi.org/10.7717/peerj.2584

693 Rogosky, A.M., Moak, P.L., Emmert, E.A.B., 2006. Differential predation by *Bdellovibrio*
694 *bacteriovorus* 109J. Curr. Microbiol. 52, 81–85. https://doi.org/10.1007/s00284-005-0038-6

695 Romero, J., Ringø, E., Merrifield, D.L., 2014. The Gut Microbiota of Fish, in: Aquaculture Nutrition.
696 John Wiley & Sons, Ltd, Chichester, UK, pp. 75–100.
697 https://doi.org/10.1002/9781118897263.ch4

698 Roose-Amsaleg, C., Laverman, A.M., 2016. Do antibiotics have environmental side-effects? Impact
699 of synthetic antibiotics on biogeochemical processes. Environ. Sci. Pollut. Res. 23, 4000–
700 4012. https://doi.org/10.1007/s11356-015-4943-3

Rosado, D., Pérez-Losada, M., Severino, R., Cable, J., Xavier, R., 2019. Characterization of the skin and gill microbiomes of the farmed seabass (*Dicentrarchus labrax*) and seabream (*Sparus aurata*). Aquaculture 500, 57–64. https://doi.org/10.1016/j.aquaculture.2018.09.063

Rud, I., Kolarevic, J., Buran, A., Berget, I., Calabrese, S., 2017. Deep-sequencing of the bacterial microbiota in commercial-scale recirculating and semi-closed aquaculture systems for Atlantic salmon post-smolt production. Aquac. Eng. 78, 50–62. https://doi.org/10.1016/j.aquaeng.2016.10.003

Sandoz, K.M., Rockey, D.D., 2010. Antibiotic resistance in Chlamydiae. Future Microbiol. 5, 1427–1442. https://doi.org/10.2217/fmb.10.96

Santos, L., Ramos, F., 2018. Antimicrobial resistance in aquaculture: Current knowledge and alternatives to tackle the problem. Int. J. Antimicrob. Agents 52, 135–143. https://doi.org/10.1016/j.ijantimicag.2018.03.010

Schmieder, R., Edwards, R., 2011. Quality control and preprocessing of metagenomic datasets. Bioinformatics 27, 863–864. https://doi.org/10.1093/bioinformatics/btr026

Schubert, M., Lindgreen, S., Orlando, L., 2016. AdapterRemoval v2: Rapid adapter trimming, identification, and read merging. BMC Res. Notes 9, 1–7. https://doi.org/10.1186/s13104-016-1900-2

Semova, I., Carten, J.D., Stombaugh, J., Mackey, L.C., Knight, R., Farber, S. a, Rawls, J.F., 2012. Microbiota regulate intestinal absorption and metabolism of fatty acids in the zebrafish. Cell Host Microbe 12, 277–288. https://doi.org/10.1016/j.chom.2012.08.003

Shahi, N., Sharma, P., Pandey, J., Bisht, I., Mallik, S.K., 2018. Characterization and pathogenicity study of Chryseobacterium scophthalmum recovered from gill lesions of diseased golden mahseer, Tor putitora (Hamilton, 1822) in India. Aquaculture 485, 81–92. https://doi.org/10.1016/j.aquaculture.2017.11.018

Shannon, C.E., Weaver, W., 1964. The mathematical theory of communication., MD computing: computers in medical practice. https://doi.org/10.2307/3611062

Sonnenschein, E.C., Nielsen, K.F., D'Alvise, P., Porsby, C.H., Melchiorsen, J., Heilmann, J., Kalatzis, P.G., López-Pérez, M., Bunk, B., Spröer, C., Middelboe, M., Gram, L., 2017. Global occurrence and heterogeneity of the Roseobacter-clade species *Ruegeria mobilis*. ISME J. 11, 569–583. https://doi.org/10.1038/ismej.2016.111

Sowani, H., Kulkarni, M., Zinjarde, S., Javdekar, V., 2017. Gordonia and Related Genera as Opportunistic Human Pathogens Causing Infections of Skin , Soft Tissues , and Bones, 1st ed, The Microbiology of Skin, Soft Tissue, Bone and Joint Infections. Elsevier Inc. https://doi.org/10.1016/B978-0-12-811079-9.00007-0

Stephens, Z.W., Burns, A.R., Stagaman, K., Wong, S., Rawls, J.F., Guillemin, K., Bohannan, B.J.M., 2015. The composition of the zebrafish intestinal microbial community varies across development. ISME J. 1–11. https://doi.org/10.1038/ismej.2015.140

Stride, M.C., Polkinghorne, A., Nowak, B.F., 2014. Chlamydial infections of fish : Diverse pathogens and emerging causes of disease in aquaculture species. Vet. Microbiol. 170, 19–27. https://doi.org/10.1016/j.vetmic.2014.01.022

741 Tilg, H., Kaser, A., 2011. Gut microbiome, obesity, and metabolic dysfunction. J. Clin. Invest. 121,
742 2126–2132. https://doi.org/10.1172/JCI58109

743 Wallace, K.N., Akhter, S., Smith, E.M., Lorent, K., Pack, M., 2005. Intestinal growth and
744 differentiation in zebrafish. Mech. Dev. 122, 157–173.
745 https://doi.org/10.1016/j.mod.2004.10.009

746 Widenfalk, A., Bertilsson, S., Sundh, I., Goedkoop, W., 2008. Effects of pesticides on community
747 composition and activity of sediment microbes - responses at various levels of microbial
748 community organization. Environ. Pollut. 152, 576–584.
749 https://doi.org/10.1016/j.envpol.2007.07.003

750 Xia, J.H., Lin, G., Fu, G.H., Wan, Z.Y., Lee, M., Wang, L., Liu, X.J., Yue, G.H., 2014. The intestinal
751 microbiome of fish under starvation. BMC Genomics 15, 266. https://doi.org/10.1186/1471-
752 2164-15-266

753 Xue, S., Xu, W., Wei, J., Sun, J., 2017. Impact of environmental bacterial communities on fish health
754 in marine recirculating aquaculture systems. Vet. Microbiol. 203, 34–39.
755 https://doi.org/10.1016/j.vetmic.2017.01.034

756 Zhao, X., Wang, J., Zhu, L., Wang, J., 2019. Field-based evidence for enrichment of antibiotic
757 resistance genes and mobile genetic elements in manure-amended vegetable soils. Sci. Total
758 Environ. https://doi.org/10.1016/j.scitotenv.2018.10.446

759 Zhou, L., Limbu, S.M., Shen, M., Zhai, W., Qiao, F., He, A., Du, Z.Y., Zhang, M., 2018. Environmental
760 concentrations of antibiotics impair zebrafish gut health. Environ. Pollut. 235, 245–254.
761 https://doi.org/10.1016/j.envpol.2017.12.073

762 Zou, S., Xu, W., Zhang, R., Tang, J., Chen, Y., Zhang, G., 2011. Occurrence and distribution of
763 antibiotics in coastal water of the Bohai Bay, China: Impacts of river discharge and
764 aquaculture activities. Environ. Pollut. 159, 2913–2920.
765 https://doi.org/10.1016/j.envpol.2011.04.037

766

Supplementary data

The impact of antibiotic exposure in water and zebrafish gut microbiomes: a 16S rRNA gene-based metagenomic analysis

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Table S1: PCR products, concentration used. Total volume of 25 µL

PCR products	Conc.
Forward primer* Bakt_341F 5'- CCTACGGGNGGCWGCAG -3'	0.3 µM
Reverse primer** Bakt_805R 5'- GACTACHVGGGTATCTAATCC -3'	0.3 µM
DNA template	12.5 ng

* Herlemann *et al*., (2011); ** Klindworth *et al.*, (2013)

Table S2: PCR program used to amplify gut and water samples

	Temperature	Time
1st step	95 ºC	3 min
	98 ºC	20 s
2nd step*	55 ºC	30 s
	72 ºC	30 s
3rd step	72 ºC	5 min

*The second step corresponds to 25 cycles

Table S3: Number of reads and OTUs per sample after singletons removal

		Reads		OTUs	
	Conc. (µg/L)	Unassigned	Bacteria + Archaea	Unassigned	Bacteria + Archaea
GUT	0	2514	46090	681	232
	0	2097	103472	503	343
	0	2599	120028	589	316
	10	15548	41590	1745	273
	10	12763	65378	1442	310
	10	11479	71265	1563	341
	10000	12478	70889	1571	247
	10000	19623	69387	1976	303
	10000	11490	53224	1353	239
WATER	0	361	53243	50	269
	0	111	46973	31	279
	0	359	60295	47	308
	10	162	92494	32	437
	10	149	91266	30	369
	10	111	84899	13	309
	10000	1274	72732	50	351
	10000	119	70703	18	382
	10000	2478	79669	70	336

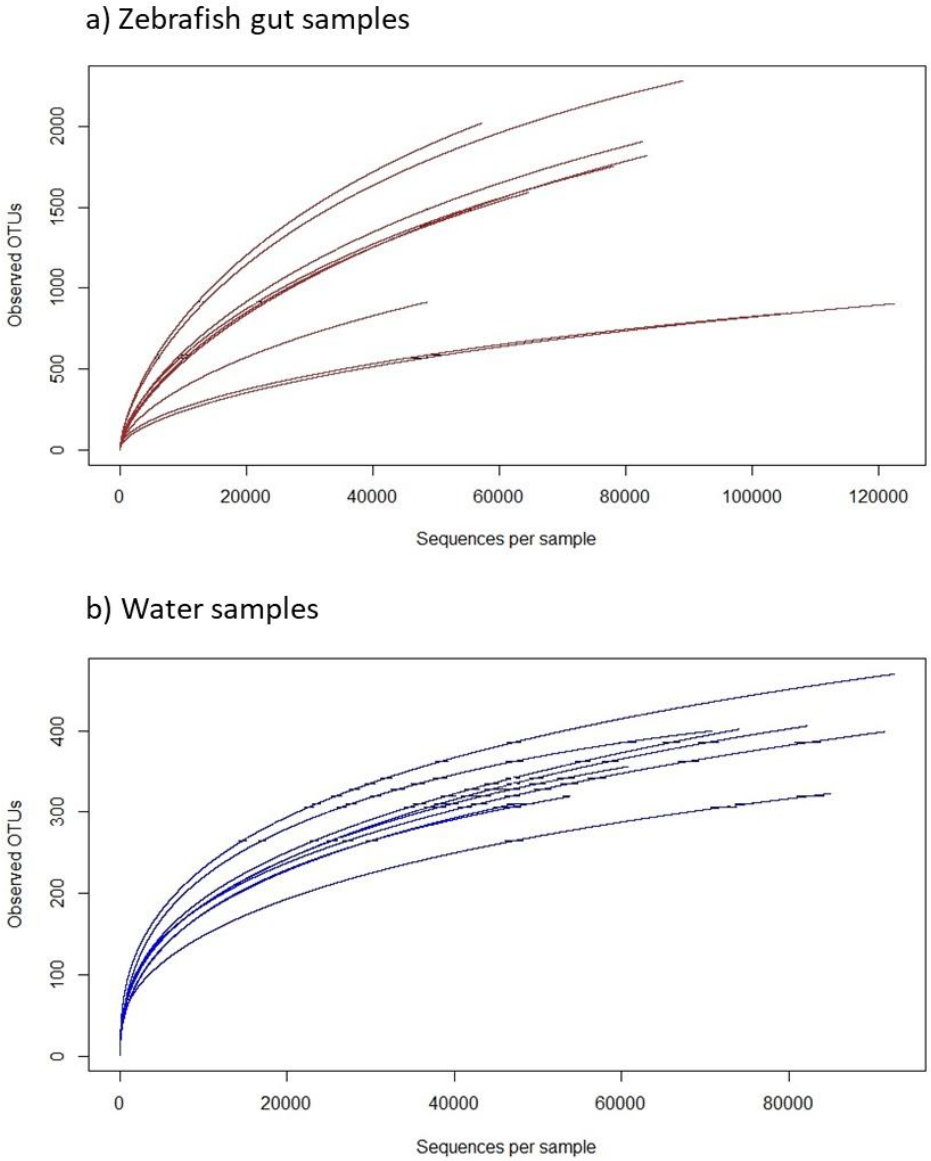
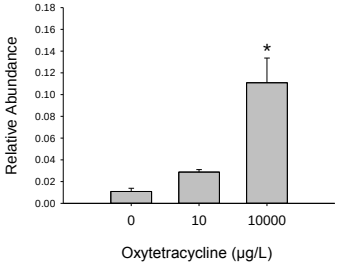
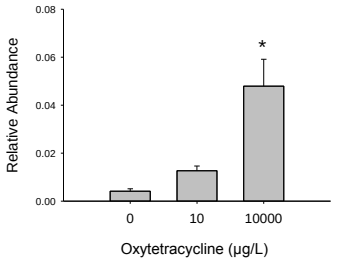
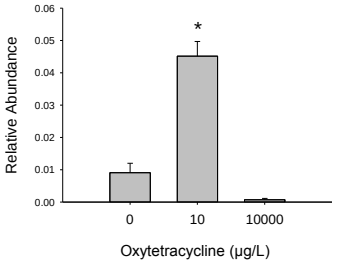
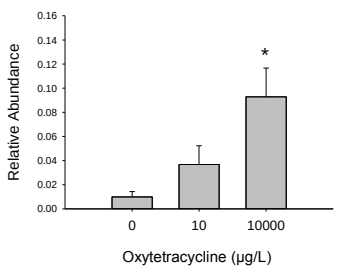
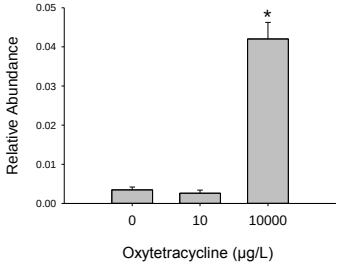
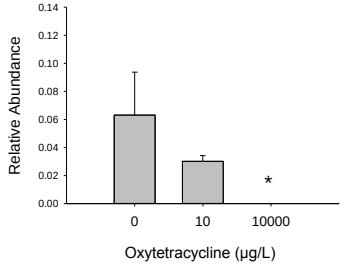
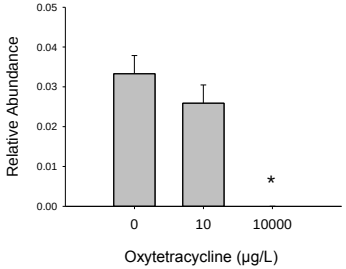
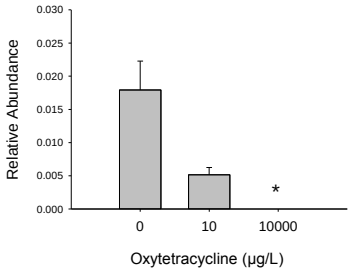
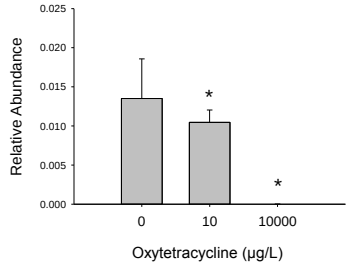
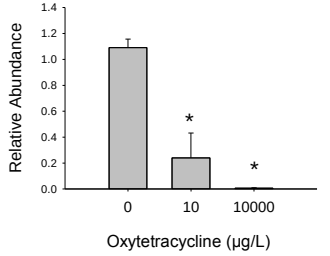
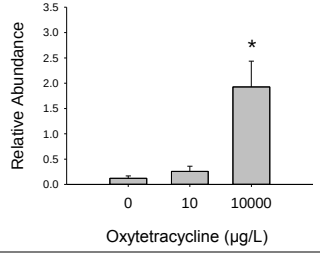


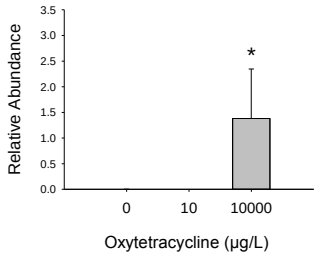
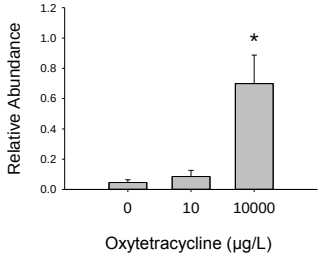
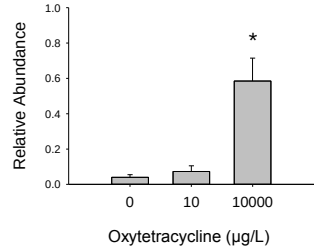
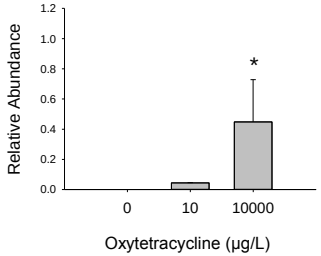
Fig. S1: Rarefaction curves (without singletons) for: a) zebrafish gut samples and b) water samples

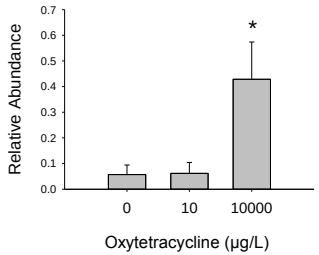
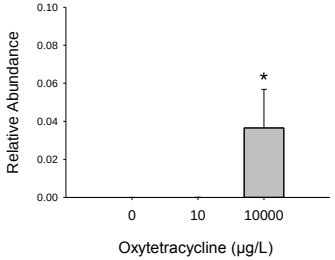
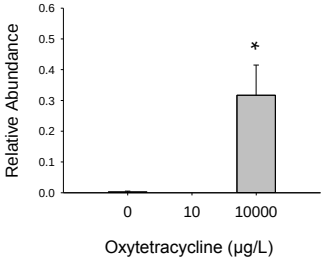
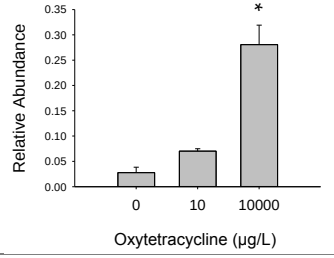
Table S4: OTUs significantly affected by OTC exposure of zebrafish gut. Asterisks (*) indicate differences in the relative abundance towards the respective control (↓ decrease; ↑ increase) ($p \leq 0.05$; Dunnett's test).

OUT id	F	p	Abundance (↑/ ↓)	Taxonomic affiliation	Graphical representation
OTU_25054	16.003	0.004	↑	Proteobacteria Alphaproteobacteria Rhodobacterales Rhodobacteraceae uncultured	
OTU_24710	12.352	0.007	↑	Proteobacteria Alphaproteobacteria Rhodobacterales Rhodobacteraceae ----	
OTU_35219	57.071	< 0.001	↑	Proteobacteria Alphaproteobacteria Rhodobacterales Rhodobacteraceae *Defluviimonas*	

OTU_7849	6.490	0.032	↑	Actinobacteria Actinobacteria Corynebacteriales Nocardiaceae *Gordonia*	
OTU_32606	79.856	< 0.001	↑	Proteobacteria Alphaproteobacteria Rhizobiales Beijerinckiaceae *Bosea*	
OTU_7123	H= 5.600	0.050	↓	Planctomycetes Planctomycetia Pirellulales Pirellulaceae *Pirellula*	
OTU_7574	21.909	0.002	↓	Proteobacteria Gammaproteobacteria Betaproteobacteriales Chromobacteriaceae *Crenobacter*	

OTU_7855	7.448	0.004	↓	Proteobacteria Gammaproteobacteria Alteromonadales Shewanellaceae *Shewanella*	
OTU_9167	5.337	0.047	↓	Proteobacteria Alphaproteobacteria Reyranellales Reyranellaceae *Reyranella*	
OTU_7240	23.669	0.001	↓	Planctomycetes Planctomycetia Pirellulales Pirellulaceae *Pirellula*	
OTU_41647	11.175	0.009	↑	Actinobacteria Actinobacteria Corynebacteriales Nocardiaceae *Gordonia*	

OTU_31612	H= 6.764	0.011	↑	Proteobacteria Alphaproteobacteria Rhizobiales Rhizobiaceae -----	
OTU_26247	10.814	0.010	↑	Actinobacteria Actinobacteria Corynebacteriales Nocardiaceae *Gordonia*	
OTU_26531	15.454	0.004	↑	Actinobacteria Actinobacteria Corynebacteriales Nocardiaceae *Gordonia*	
OTU_25507	H= 5.793	0.050	↑	Proteobacteria Alphaproteobacteria Rhodobacterales Rhodobacteraceae *Rhodobacter*	

OTU_33018	5.632	0.042	↑	Proteobacteria Alphaproteobacteria Rhizobiales Rhizobiaceae *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*	
OTU_33056	H= 6.720	0.050	↑	Proteobacteria Alphaproteobacteria Rhizobiales Xanthobacteraceae uncultured	
OTU_24656	H= 6.720	0.050	↑	Proteobacteria Alphaproteobacteria Rhizobiales Rhizobiaceae *Aminobacter*	
OTU_32536	34.187	<0.001	↑	Actinobacteria Acidimicrobiia Microtrichales uncultured uncultured bacterium	

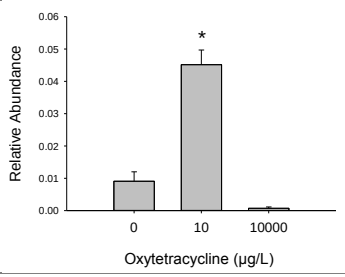
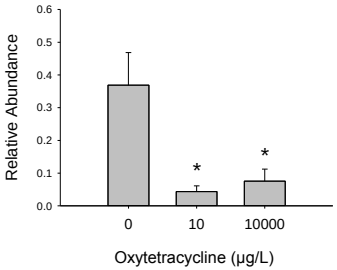
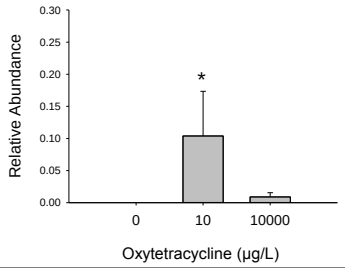
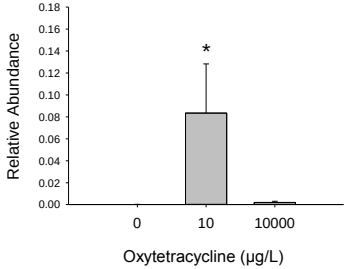
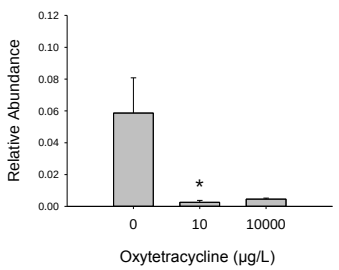
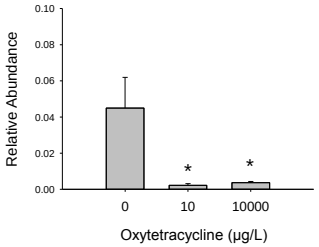
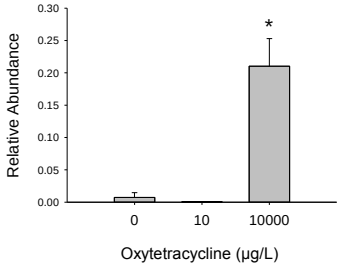
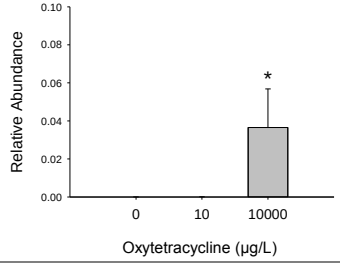
OTU_34457	40.201	<0.001	↑	Proteobacteria Alphaproteobacteria Rhodobacterales Rhodobacteraceae *Defluviimonas*	

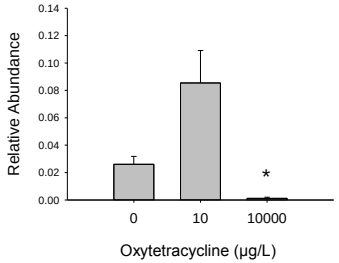
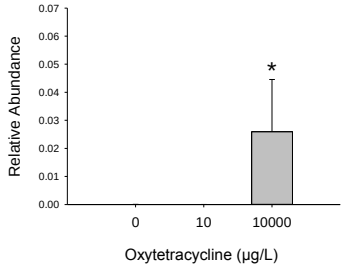
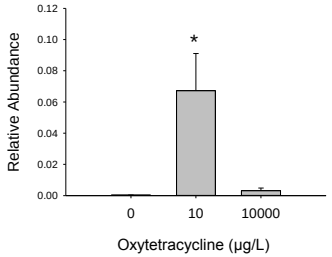
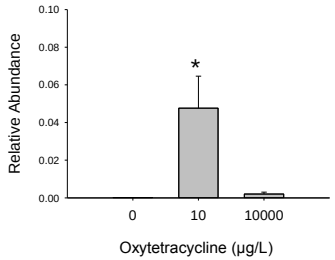
Table S5: Predicted functional genes/ proteins (KEGG ID) significantly affected by OTC in zebrafish gut and water microbiomes. Differences in relative abundance towards the control (↓ decrease; ↑ increase) are indicated ($p \leq 0.05$; Dunnett's test).

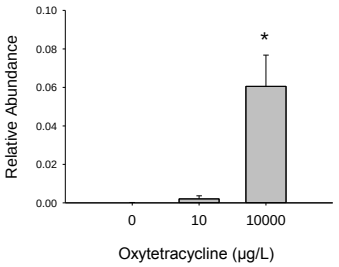
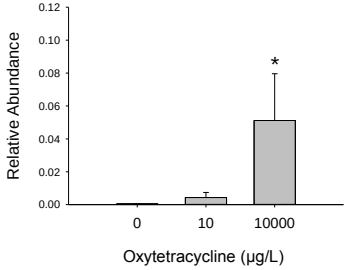
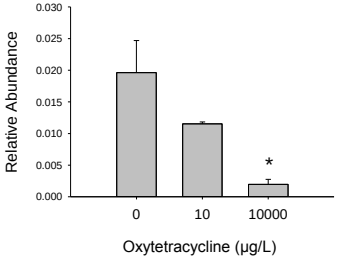
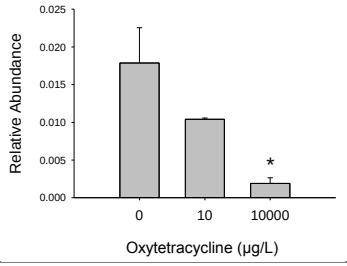
Sample type	Description	KEGG ID	Tendency	F	p
GUT	ABC.PE.P; peptide/nickel transport system permease protein	K02033	↑	28.853	<0.001
	ABC.PE.S; peptide/nickel transport system substrate-binding protein	K02035	↑	30.386	<0.001
	tRNA-Leu; tRNA Leu	K14228	↓	12.092	0.008
	tRNA-Met; tRNA Met	K14230	↓	9.367	0.014
	tRNA-Arg; tRNA Arg	K14219	↓	11.724	0.008
	tRNA-Ser; tRNA Ser	K14233	↓	11.884	0.008
	tRNA-Gly; tRNA Gly	K14225	↓	10.020	0.012
	tRNA-Ala; tRNA Ala	K14218	↓	6.899	0.028
	tRNA-Val; tRNA Val	K14237	↓	10.403	0.011
	5SrRNA, rrf; 5S ribosomal RNA	K01985	↓	7.510	0.023
	23SrRNA, rrl; 23S ribosomal RNA	K01980	↓	8.002	0.020
	16SrRNA, rrs; 16S ribosomal RNA	K01977	↓	7.750	0.022
	GST, gst; glutathione S-transferase [EC:2.5.1.18]	K00799	↑	13.991	0.006
	tetB; MFS transporter, DHA2 family, metal-tetracycline-proton antiporter	K08168	↓	H = 7.20	0.004
	tet35; tetracycline resistance efflux pump	K18218	↓	9.436	0.014
	tetV; MFS transporter, DHA3 family, tetracycline resistance protein	K18215	↓	6.088	0.036
	tetM, *tetO;* ribosomal protection tetracycline resistance protein	K18220	↓	H = 5.956	0.025
WATER	chrA; chromate transporter	K07240	↓	7.495	0.023
	TC.FEV.OM; iron complex outermembrane recepter protein	K02014	↑	H=6.489	0.011
	frc; formyl-CoA transferase [EC:2.8.3.16]	K07749	↓	6.164	0.035
	kpsS, lipB; capsular polysaccharide export protein	K07265	↑	H=6.489	0.011

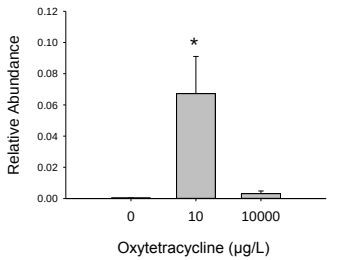
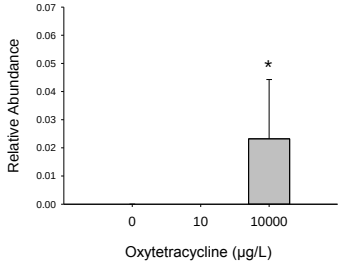
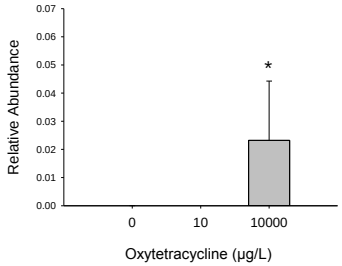
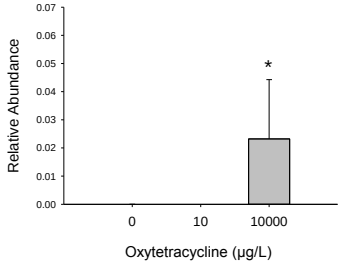
Table S6: OTUs significantly affected by OTC in exposure water. Asterisks (*) indicate differences in the relative abundance towards the respective control (↓ decrease; ↑ increase) ($p \leq 0.05$; Dunnett's test).

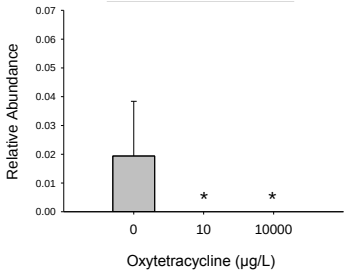
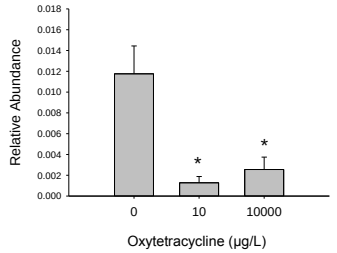
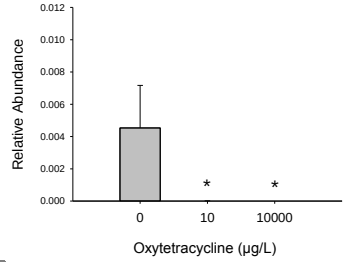
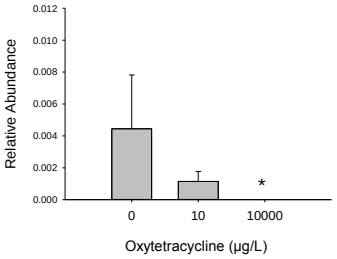
OUT id	F	p	Abundance (↑/ ↓)	Taxonomy affiliation	Graphical representation
OTU_8190	H = 5.600	0.050	↓	Proteobacteria Gammaproteobacteria Betaproteobacteriales Burkholderiaceae *Polynucleobacter*	
OTU_7687	H = 6.489	0.011	↑	Proteobacteria Gammaproteobacteria Betaproteobacteriales Burkholderiaceae *uncultured*	
OTU_6796	H = 7.200	0.004	↑	Proteobacteria Gammaproteobacteria Betaproteobacteriales Burkholderiaceae *Acidovorax*	

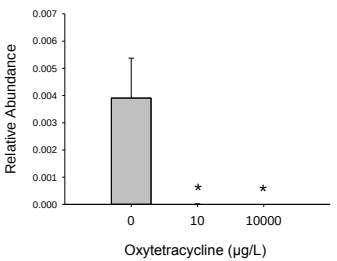
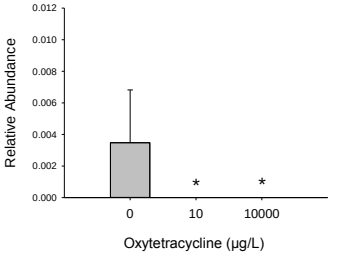
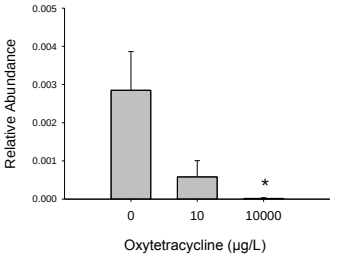
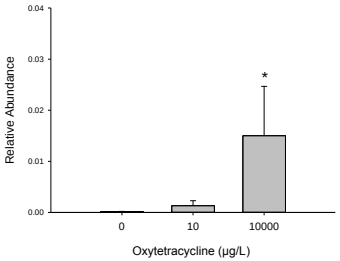
OTU_7923	H = 6.489	0.011	↓	Proteobacteria Gammaproteobacteria Betaproteobacteriales Burkholderiaceae *Limnobacter*	
OTU_6849	H= 5.956	0.025	↑	Proteobacteria Gammaproteobacteria Betaproteobacteriales Burkholderiaceae *Limnobacter*	
OTU_24583	22.672	0.002	↑	Proteobacteria Alphaproteobacteria Rhizobiales Rhizobiales Incertae Sedis uncultured	
OTU_19538	H= 6.543	0.011	↑	Proteobacteria Alphaproteobacteria Rhizobiales Hyphomicrobiaceae *Hyphomicrobium*	

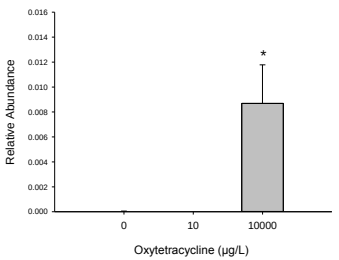
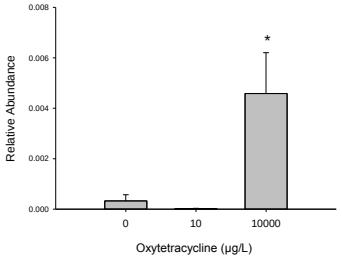
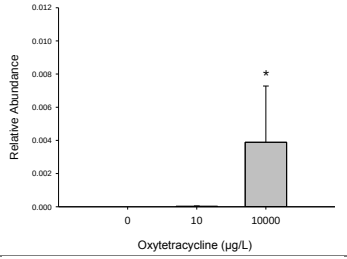
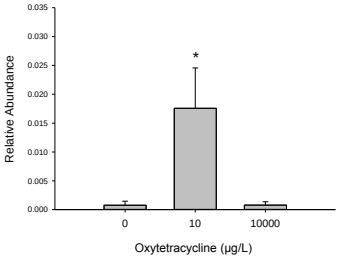
OTU_8339	9.496	0.014	↓	Bacteroidetes Bacteroidia Flavobacteriales Weeksellaceae *Chryseobacterium*	
OTU_7691	H= 6.161	0.025	↑	Bacteroidetes Bacteroidia Flavobacteriales Crocinitomicaceae *Fluviicola*	
OTU_6634	H= 6.489	0.011	↑	Proteobacteria Deltaproteobacteria Myxococcales Phaselicystidaceae *Phaselicystis*	
OTU_4880	H=7.200	0.004	↑	Proteobacteria Deltaproteobacteria Myxococcales Phaselicystidaceae *Phaselicystis*	

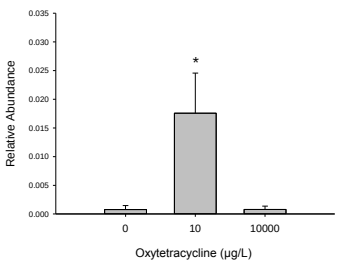
OTU_7515	H= 5.600	0.050	↑	Chlamydiae Chlamydiae Chlamydiales Parachlamydiaceae *Candidatus Protochlamydia*	
OTU_7969	H= 5.956	0.025	↑	Bacteroidetes Bacteroidia Cytophagales Spirosomaceae *Runella*	
OTU_8489	8.800	0.016	↓	Chloroflexi Anaerolineae Caldilineales Caldilineaceae uncultured	
OTU_24365	H= 5.600	0.050	↓	Chloroflexi Anaerolineae Caldilineales Caldilineaceae uncultured	

OTU_7538	H= 7.200	0.004	↑	Proteobacteria Deltaproteobacteria Myxococcales Phaselicystidaceae *Phaselicystis*	
OTU_7846	H= 6.720	0.050	↑	Planctomycetes Planctomycetacia Pirellulales Pirellulaceae *Pirellula*	
OTU_8242	H= 5.793	0.050	↑	Bacteroidetes Bacteroidia Chitinophagales Chitinophagaceae uncultured	
OTU_8449	H= 6.006	0.025	↑	Bacteroidetes Bacteroidia Chitinophagales Chitinophagaceae uncultured	

OTU_8650	H= 5.793	0.050	↓	Bacteroidetes Bacteroidia Flavobacteriales Flavobacteriaceae *Flavobacterium*	
OTU_6535	10.968	0.010	↓	Proteobacteria Gammaproteobacteria Betaproteobacteriales Burkholderiaceae *Polynucleobacter*	
OTU_33311	H= 6.764	0.011	↓	Proteobacteria Alphaproteobacteria Rhizobiales Pleomorphomonadaceae *Prosthecomicrobium*	
OTU_20626	H= 5.647	0.050	↓	Bacteroidetes Bacteroidia Chitinophagales uncultured uncultured bacterium	

OTU_8611	H= 6.720	0.050	↓	Proteobacteria Gammaproteobacteria Xanthomonadales Xanthomonadaceae *Lysobacter*	
OTU_20619	H= 6.720	0.050	↓	Bacteroidetes Bacteroidia Flavobacteriales Crocinitomicaceae *Fluviicola*	
OTU_7240	5.595	0.043	↓	Planctomycetes Planctomycetacia Pirellulales Pirellulaceae *Pirellula*	
OTU_9362	H= 5.600	0.050	↑	Bacteroidetes Bacteroidia Cytophagales Spirosomaceae *Runella*	

OTU_38765	H= 6.720	0.050	↑	Proteobacteria Alphaproteobacteria Rhizobiales Rhizobiales Incertae Sedis uncultured	
OTU_25509	7.280	0.025	↑	Proteobacteria Alphaproteobacteria Azospirillales Azospirillales Incertae Sedis *Stella*	
OTU_24165	H= 6.764	0.011	↑	Bacteroidetes Bacteroidia Cytophagales Spirosomaceae *Flectobacillus*	
OTU_8407	5.674	0.041	↑	Bacteroidetes Bacteroidia Chitinophagales Chitinophagaceae *Sediminibacterium*	

OTU_7428	H= 5.600	0.050	↑	Bacteroidetes Bacteroidia Chitinophagales Chitinophagaceae *Sediminibacterium*	

References

Herlemann DPR, Labrenz M, Jürgens K, et al (2011) Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. ISME J 5:1571–1579. doi: 10.1038/ismej.2011.41

Klindworth A, Pruesse E, Schweer T, et al (2013) Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res 41:1–11. doi: 10.1093/nar/gks808