Universidade de Aveiro
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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia e Ecologia das Alterações Globais, realizada sob a orientação científica da Doutora Isabel da Silva Henriques, Professora auxiliar do Departamento das Ciências da Vida da Universidade de Coimbra e da Doutora Paula Inês Borralho Domingues, Investigadora auxiliar do Departamento de Biologia da Universidade de Aveiro

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palavras-chave

Tetraciclinas, *Danio rerio*, comportamento, actividade enzimática, comunidades bacterianas, resistência a antibióticos, recuperação

resumo

Para dar resposta à crescente necessidade de produção de alimentos, algumas indústrias têm assistido a um grande desenvolvimento, como é o caso das aquaculturas. De modo a atingir as metas pretendidas, os organismos são comummente mantidos em condições sub-ótimas (ex. altas densidades) favorecendo o aparecimento e disseminação de doenças. Para combater este problema, são utilizados antibióticos de largo-espectro como é o caso da oxitetraciclina (OTC). Uma vez que grande parte do composto consumido não é metabolizado pelo organismo, o antibiótico é excretado na sua forma ativa e libertado para o meio ambiente. Uma vez no meio ambiente, os antibióticos podem ter efeitos nos organismos não alvo como por exemplo nos peixes, no seu microbioma e nas comunidades bacterianas da água. Assim, este trabalho teve como objetivo estudar o impacto de antibióticos, nomeadamente da OTC, no peixe-zebra, numa análise integrada e dirigida a vários níveis de organização biológica. Para isso, numa primeira experiência, expuseram-se peixes-zebra adultos a OTC por dois meses. Foram analisados os efeitos a nível sub-individual (ex. atividade enzimática), do indivíduo (ex. comportamento) e do microbioma tanto dos organismos como da água de exposição (ex. estrutura, função e seleção de bactérias e genes de resistência à OTC). Em seguida, foi realizada uma experiência para verificar a reversibilidade dos efeitos observado. Neste caso os organismos foram expostos por dois meses nas mesmas condições e, seguidamente, foram transferidos para água limpa e assim mantidos durante um mês. Nesta experiência, foram analisados efeitos a nível sub-individual (ex. atividade enzimática), e no microbioma dos organismos e da água de exposição. Os efeitos foram avaliados tanto durante o período de exposição (cinco dias e dois meses de exposição) como no período de pós-exposição (cinco dias e um mês pós-exposição).

Os resultados permitiram verificar que a OTC tem um impacto nos vários níveis de organização. A nível sub-individual, foi observada uma inibição dos mecanismos de destoxificação e a redução da energia celular disponível. A nível do indivíduo, a OTC provocou uma alteração do comportamento dos peixes, verificando-se hiperatividade como resposta ao stress químico. A nível das comunidades bacterianas, verificou-se uma alteração tanto a nível da estrutura como das funções das comunidades. Por exemplo, na água, os resultados sugeriam a seleção de bactérias que estão relacionadas com o ciclo do azoto. No período de pós-exposição foi possível verificar que tanto a nível das reservas energéticas como a nível do microbioma dos peixes e da água de exposição, as diferenças entre grupos expostos e grupo controlo diminuíram, sugerindo uma possível recuperação. A OTC demonstrou ter também um papel na seleção de bactérias resistentes a antibióticos e genes de resistência. Nomeadamente foi observada a seleção de géneros bacterianos indicados como potenciais patogénicos de peixes e humanos. O potencial patogénico de alguns isolados foi confirmado em ensaios *in vivo*.

No geral este estudo demonstrou que a presença de OTC na água tem um efeito no peixe-zebra com consequências nos vários parâmetros analisados. O uso de parâmetros sensíveis, que podem funcionar como sinais de alerta precoce, permitiu observar efeitos mesmo após exposição a baixas concentrações do composto. Os efeitos observados ao nível dos organismos (ex. alteração do comportamento, reservas de energia) podem comprometer a capacidade dos peixes de capturar presas e evitar predadores, levando a consequências posteriores ao nível da população. Também, a seleção de potenciais bactérias patogénicas e/ou filotipos bacterianos relacionados com o ciclo do azoto pode vir a ter um impacto ao nível do ecossistema. Assim, o nosso trabalho demonstra a importância de mais estudos interdisciplinares para uma compreensão mais profunda do real impacto dos antibióticos no meio ambiente.

keywords

Tetracyclines, *Danio rerio*, fish behavior, enzymatic activity, antibiotic resistance, recovery

abstract

Due to high food demand, aquaculture is one of the fastest growing industries. Hence, to supply all the needs, organisms are commonly grown under stress conditions (e.g. overcrowded) which favor the emergence and spread of infectious diseases. To combat this problem, broad-spectrum antibiotics such as oxytetracycline (OTC) are used. Since organisms are not able to absorb all the antibiotic consumed, it is known that a large part of these compounds is excreted in its active form and released to the environment. Once in the environment, antibiotics can have effects on non-target organisms such as fish, their microbiome, and the bacterial communities in the water. Thus, this work aimed to study, the impact of antibiotics, namely OTC, on zebrafish in an integrated analysis at various levels of biological organization. For this purpose, in a first experience, adult zebrafish were exposed to OTC for two months via water. The analysis of the effects was performed at a sub-individual level (e.g. enzyme activity), individual (e.g. behavior) and microbiome (e.g. structure, function, and selection of bacteria resistant to OTC and resistance genes). Then, to verify the reversibility of effects in a non-continuous exposure scenario, after a long-term exposure period, organisms were transferred and kept in clean water for one month. In this experiment, the analysis of the effects was performed at a sub-individual level (e.g. enzyme activity), and in the microbiome (e.g. structure, function, and selection of bacteria resistant to OTC and resistance genes). Analyses during the exposure (five days and two months of exposure) and in the post-exposure period (five days and one month of post-exposure) were also performed.

During the exposure period, our results revealed that OTC had an impact in organisms at several biological organization levels. At a sub-individual level, a decrease in the detoxification mechanisms and cellular energy available was observed. At individual level, an increase of stress response was observed through the increase of hyperactivity of fish. At bacterial communities' level, OTC exposure induced changes at structure and functional level in both fish gut and water. For instance, in water samples ou results suggested the selection of some bacterial phylotypes that play a role in the nitrogen cycle. Regarding the post-exposure period, in both parameters analyzed (energetic reserves and bacterial communities' structure and function) differences between the exposed and control group were minimized indicating a recovery in the fish gut and water. The role of OTC in the selection of antibiotic-resistant bacteria and antibiotic resistance genes was also verified. Namely, the selection of resistant bacteria indicated as potential pathogens to humans and fish was observed. Moreover, the pathogenic potential of some isolates was tested and confirmed *in vivo*.

Overall, our work demonstrated that the water contamination with the antibiotic has an impact in zebrafish with consequences at several levels analyzed. The use of sensitive endpoints, that may act like early warning signals, allowed to assess effects at low concentrations. The effects observed at organisms' level (e.g. behavior change, energy reserves) can compromise fish ability of prey capture and predator avoidance, leading to further consequences at population level. Moreover, the selection of potential pathogens and/or bacterial phylotypes related with nitrogen cycle may have an impact at ecosystem level. Hence our work reinforces the importance of interdisciplinary studies for a deeper understanding of the real impact of antibiotics in the environment.

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List of acronyms

1mPE	1 Month Post-Exposure
2mE	2 Months of Exposure
5dE	5 Days of Exposure
5dPE	5 Days Post-Exposure
AchE	Acetylcholinesterase
ANOVA	Analysis of Variance
ARB	Antibiotic Resistant Bacteria
ARGs	Antibiotic Resistance Genes
Cat	Catalase
CEA	Cellular Energy Allocation
CFUs	Colony Forming Units
DGGE	Denaturing Gradient Gel Electrophoresis
EA	Energy Available
EC	Energy Consumption
ETS	Electron Transport System
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GSP	*Pseudomonas Aeromonas* Selective Agar
GST	Glutathione S-transferase
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC	Lethal Concentration
LDH	Lactate Dehydrogenase
MFc	Membrane Fecal Coliform agar
OECD	Organization for Economic Co-operation and Development
OTC	Oxytetracycline
OTU	Operational Taxonomy Unit
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
PERMANOVA	Permutational Multivariate Analysis of Variance
QS	Quorum Sensing
TD	Total distance
TET	Tetracycline
Tf	Total food ingestion
TG	Total Glutathione
Ti	First Feeding
WWTP	Wastewater treatment plants

Chapter 1

Introduction

1. Introduction

Nowadays, due to our lifestyle and improvement of life quality, the use of pharmaceuticals is widely disseminated not only in human but also in veterinary medicine. Antibiotics are used to treat health problems and can be defined as compounds used to kill or inhibit the growth or metabolic activity of microorganisms (Brandt et al. 2015). These compounds can be natural, synthetic, or semisynthetic. Currently, several antibiotic classes exist, divided by their target, mode of action and/or chemical structure. Table 1 presents some examples.

Table 1: Antibiotic classification and mode of action (main examples) (adapted from Grenni et al. 2018).

Mode of action		Class		Antibiotics (examples)	Uses
Inhibition of cell wall synthesis		β-Lactam	Penicillins	Ampicillin; Amoxicillin; Ticarcillin	Human/ Veterinary
			Cephalosporins	Cefepime; Ceftazidime;	Human/ Veterinary
			Carbapenems	Imipenem; Meropenem	Human
			Monobactams	Aztreonam	
		Glycopeptides		Vancomycin; Bleomycin	Human / Veterinary
Inhibition of Protein synthesis	Targeting the 30S ribosomal sub-unit	Aminoglycosides		Gentamycin; Amikacin	Human / Veterinary
		Tetracyclines		Tetracycline; Oxytetracycline; Tigecycline	Human / Veterinary
	Targeting the 50S ribosomal sub-unit	Chloramphenicol		Chloramphenicol	Veterinary
		Macrolides		Erythromycin; Azithromycin	Human / Veterinary
		Lincosamides		Lincomycin; Clindamycin	Human / Veterinary
Inhibition of DNA synthesis		Quinolones and Fluoroquinolones		Ciprofloxacin; Nalidixic acid	Human / Veterinary
		Sulfonamides		Sulfamethoxazole; Sulfapyridine	Human / Veterinary

Inhibition of RNA synthesis	Rifamycines	Rifampicin	Human

The consumption of antibiotics is widely distributed for human medicine, veterinary use, agriculture and livestock production (Grenni et al. 2018; Kovalakova et al. 2020). In 2014 it was estimated that in the 28 EU/European Economic Area Member States (EEA MSs) 3821 tons of antibiotics (active substance) were sold for human use (ECDC et al. 2017). According to World Health Organization (WHO), in 2015 the human median antibiotic consumption varied between 17.9 and 38.2 Defined Daily Doses (DDD; average maintenance dose per day of an antimicrobial substance) per 1000 inhabitants per day in the European Region (WHO World Health Organization 2018). Yet, most of antibiotic sales regarded animal use (Fig. 1 and Fig. 2) (ECDC, 2017; Szymańska et al., 2019). Also in USA, 70 % of antibiotics sold are consumed by animals and only 30% by humans (O'Neill 2015).

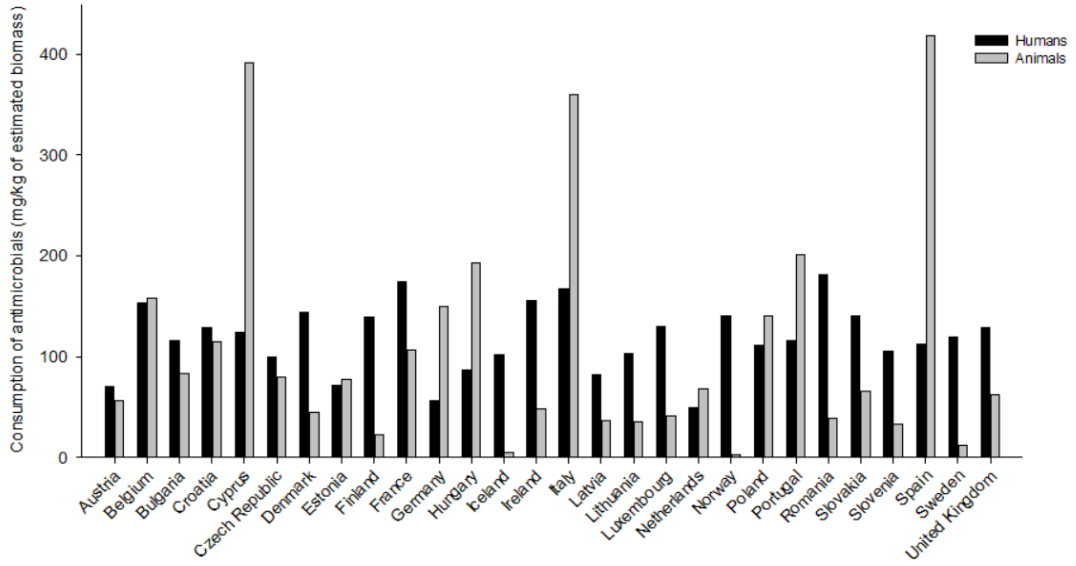

Fig. 1: Overall consumption of antimicrobial agents (mg/kg of estimated biomass) for humans and food-production animals by country in 2014 in 28 EU/EEA MSs (ECDC, 2017).

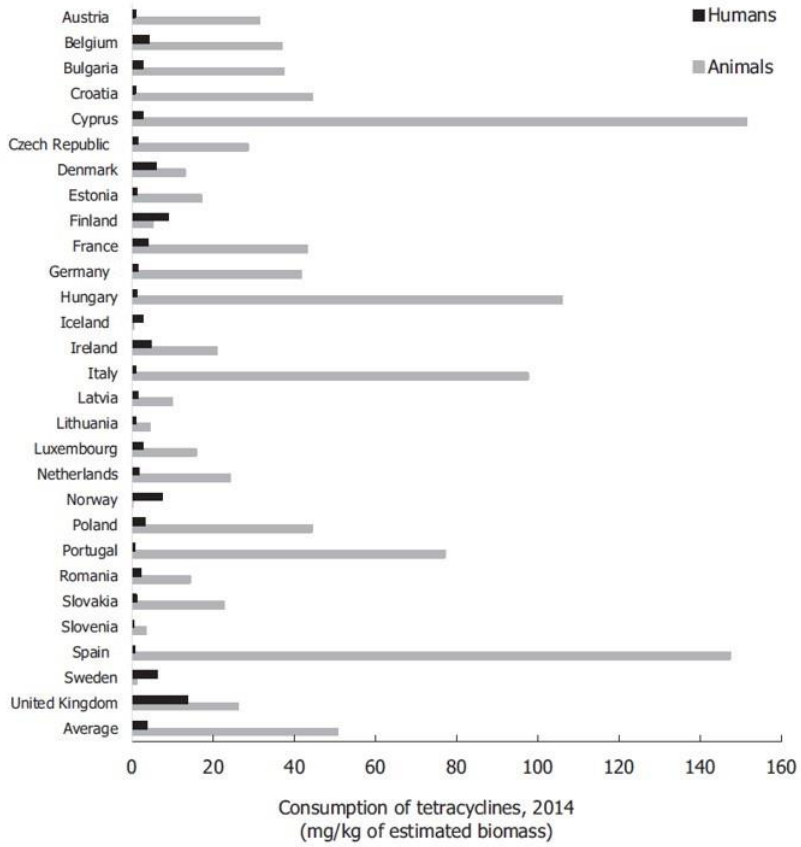

Fig. 2: Tetracyclines consumption (mg/kg of estimated biomass) for humans and food-production animals by country in 2014 in 28 EU/EEA MSs (ECDC, 2017).

Tetracyclines were among the most used antibiotics for livestock in 2017 in Europe (30.4%), followed by Penicillins (26.9%) and Sulfonamides (9.2%) (European Medicines Agency 2019). In USA, Tetracyclines class represented 66% of sales of antimicrobials for food-production animals in 2018, followed by Penicillins (12%) and macrolides (8%) (FDA 2018).

The problematic of extensive use of antibiotics is not only related with the consequences to organisms itself but also related with environmental contamination. Antibiotics are bioactive substances that are not completely metabolized by organisms. Hence, around 30 – 90% of these compounds may be excreted on its unaltered form and/or active metabolites (Massé et al. 2014; Carvalho and Santos 2016). Consequently, antibiotics may reach the environment through several routes like wastewater treatment

plants (WWTP) effluents, landfill, runoff and leaching from farmland fertilized with manure or livestock waste (Fig. 3) (Li 2014; Carvalho and Santos 2016). Therefore, worldwide antibiotics have been detected at concentrations ranging from ng/L to µg/L in the aquatic environments like groundwater (Cabeza et al. 2012), rivers (Matongo et al. 2015), lakes (Liu et al. 2018b) and marine environments (Arpin-Pont et al. 2016).

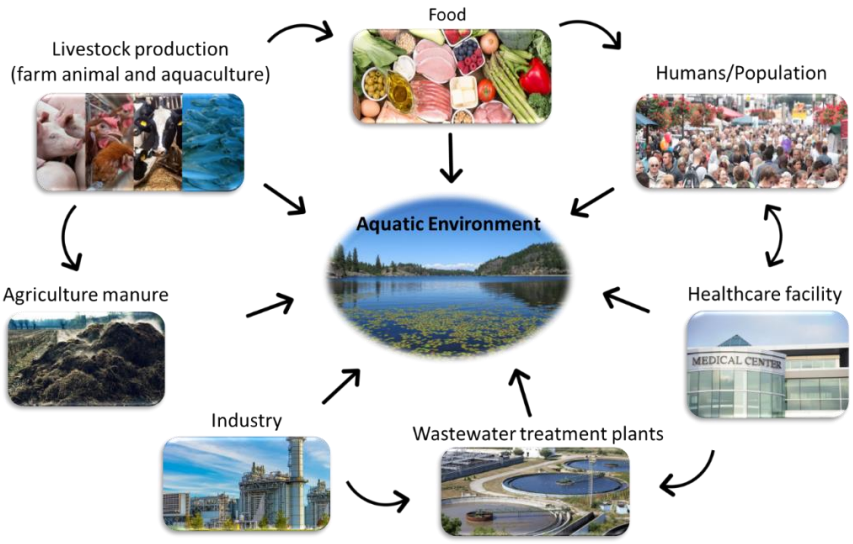

Fig. 3: Routes of antibiotic contamination in the environments.

Since these compounds are continuously released and may persist in the environment, antibiotics are considered micropollutants of concern (Bilal et al. 2020). Aquatic organisms are in special risk as they may be exposed to antibiotics during long periods or even their entire life. Therefore, several works were devoted to study the impact of antibiotics in aquatic organisms (Carlsson et al. 2013; Desbiolles et al. 2018; Liu et al. 2018a; Yang et al. 2020). Nevertheless, several questions remain to delve. For instance, most of the ecotoxicology works aimed to study the impact in the organism itself and few is known about the impact on organism's microbiome. Moreover, the role of antibiotics in the selection of resistant bacteria within organism's microbiome, with potential pathogenic effect remain to be explored.

In the present work, we will focus on the Tetracyclines class, which includes some of the most used antibiotics in food-production and therefore the most detected in the environment. This chapter aims to overview/summarize topics related with environmental

concentrations and ecotoxicological effects on non-target organisms of Tetracyclines exposure.

2. Tetracyclines

2.1 Mode of action and uses

Tetracyclines were discovered in the 1940s and firstly used as therapeutic in 1950s (Roberts 2003). Since then, Tetracyclines class has been widely used in both human and livestock. Among the most used antibiotics of this class are the chlortetracycline ($C_{22}H_{23}ClN_2O_8$; CTC), oxytetracycline ($C_{22}H_{24}N_2O_9$; OTC) and tetracycline ($C_{22}H_{24}N_2O_8$; TET) (Agwuh and MacGowan 2006; Pulicharla et al. 2017). These antibiotics have a broad-spectrum efficacy and can be used not only against Gram-positive and Gram-negative bacteria but also against chlamydia, mycoplasma and protozoan parasites (Chopra and Roberts 2001). Its mode of action consists in the inhibition of bacterial protein synthesis. These molecules bind to the 30S ribosomal subunit and therefore prevent the association of aminoacyl-tRNA with the bacterial ribosome (Fig. 4) (Chopra and Roberts 2001).

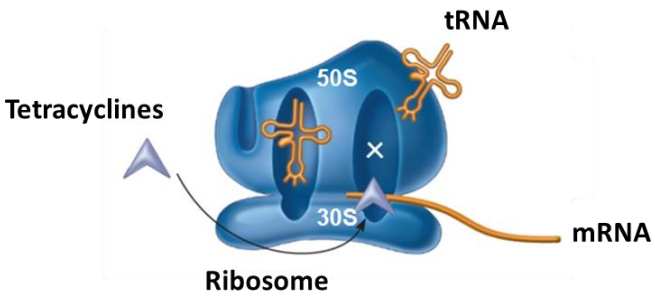

Fig. 4: Tetracyclines mode of action (adapted from https://moleculeoftheday.tumblr.com/post/154296297857/molecule-of-the-day-tetracycline).

Tetracyclines are considered one of the antibiotic classes that includes the cheapest compounds and then one of the most used in food-production animals (Roberts 2003; Daghrir and Drogui 2013). Worldwide is estimated that thousands of tons of tetracyclines are produced and sold for food-producing animals outweighed the amount used for humans

(ECDC et al. 2017; FDA 2018). Tetracyclines are frequently used to treat gastrointestinal, respiratory, and skin infections in both livestock and domestic animals (e.g. pigs, sheep, goats, dogs) (Sarmah et al. 2006; Hopman et al. 2019; Oh et al. 2019). In the aquaculture industry these compounds are also extensively used to treat infections in several species with economic interest like salmonids (e.g. *Salmo* spp., *Oncorhynchus* spp., *Salvelinus alpinus*), Nile tilapia (e.g. *Oreochromis niloticus*), sea bream (e.g. *Sparus aurata*) and catfish (e.g. *Ictalurus punctatus*) (Tuševljak et al. 2013; Leal et al. 2018). In fact, fish infections represent around 15-20 % of production loss in aquaculture in China, one of the major aquaculture producers (Mo et al. 2017). Therefore, Tetracyclines are broadly used in livestock in both health and sick organisms, in a therapeutic and prophylactic way to prevent economic loss (Fig. 5). Although the use of antibiotics as growth promotors was banned from Europe since 2006 (O'Neill 2015), some countries (e.g. Canada, USA and Australia) still use it for this purpose (Laxminarayan et al. 2015; O'Neill 2015). In addition, new EU regulations, which will come into force in 2022, have banned the prophylactic use of antibiotics in animals (Regulation (EU) 2019/6).

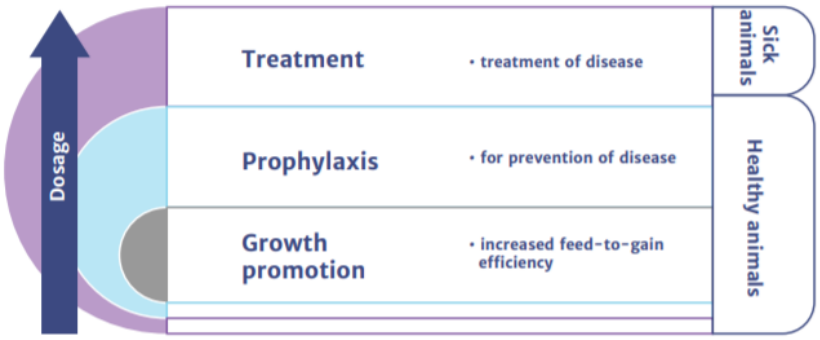

Fig. 5: Uses of antibiotics in livestock production (retrieved from O'Neill 2015).

2.2 Environmental fate and concentrations

Antibiotics may be administrated to organisms through feed, water, water baths or injection (Armstrong et al. 2005; FDA 2018). For instance, OTC is usually administrated by food pellets at a range of 50 to 100 mg of OTC per kg of body weight per day during 3 to 21 days (Lundén and Bylund 2000; Oliveira et al. 2013). Part of the antibiotic ingested is absorbed by the organisms, however, a big amount is excreted. For instance, OTC is referred as poorly metabolized (Jaime Romero et al. 2012; Leal et al. 2018). Therefore, more than 50 % of this compound is excreted in feces and urine (Cravedi et al. 1987). Moreover, the dispersion and deposition of food excess also contribute to the environmental contamination. Hence, it is estimated that 80% of antibiotics consumed in aquaculture may enter the aquatic environment (Cabello et al. 2013). Therefore, Tetracyclines have been detected around the world in several aquatic reservoirs (Table 2).

Table 2: Occurrence of Tetracyclines in aquatic environments. TET: tetracycline; OTC: oxytetracycline; CTC: chlortetracycline; WWTP: wastewater treatment plant; ND: not detected or below detection limit.

Country	Type of sample/ place	Compound	Concentration range ng/L (min. – max.)	Reference
Spain	surface water (Llobregat river)	TET	5.92 – 17.10	Osorio et al. (2015)
	surface water (Ebro river)	TET	5.92 – 27.40	Osorio et al. (2015)
	surface water (Júcar river)	TET	ND – 5.92	Osorio et al. (2015)
	surface water (Guadalquivir river)	TET	ND – 5.92	Osorio et al. (2015)
Poland	surface water (rivers and lakes)	TET	20 – 50	Gbylik-Sikorska et al. (2014)
	surface water (Drwęca River)	OTC	45 – 110	Harnisz et al. (2015b)
		TET	16 – 34	Harnisz et al. (2015b)
Luxembourg	surface water (Alzette river)	OTC	ND – 2	Pailler et al. (2009)
		TET	ND – 8	Pailler et al. (2009)
	surface water (Mess river)	OTC	ND – 7	Pailler et al. (2009)
		TET	ND – 7	Pailler et al. (2009)
	WWTP- effluent	OTC	ND – 5	Pailler et al. (2009)
		TET	ND – 24	Pailler et al. (2009)
Portugal	Surface water (Tagus river)	TET	ND – 23	Valcárcel et al. (2011)
	WWTP- raw	TET/OTC/CTC	ND- 37400	Novo et al. (2013)
	WWTP- treated	TET/OTC/CTC	ND - 7000	Novo et al. (2013)
Italy	surface water (Po river)	OTC	ND – 19.20	Calamari et al. (2003)
China	surface water (Baiyangdian lake)	OTC	4.64 – 90.30	Cheng et al. (2014)

		TET	8.07 – 85.19	Cheng et al. (2014)
	surface water (Bosteng lake)	OTC	ND – 6.60	Liu et al. (2018b)
		TET	ND – 2.84	Liu et al. (2018b)
	surface water (Taihu lake)	OTC	ND – 72.8	Xu et al. (2014)
		TET	ND – 87.90	Xu et al. (2014)
	surface water (Bohai Bay)	OTC	4.10 – 270	Zou et al. (2011)
		TET	3.60 – 30	Zou et al. (2011)
	surface water (Yangtze River delta)	OTC	ND – 241	Cui et al. (2018)
		TET	ND – 95	Cui et al. (2018)
	surface water	OTC	ND – 51.50	Cheng et al. (2019)
		TET	ND – 31.40	Cheng et al. (2019)
	surface water (Hanjiang river)	OTC	ND – 9.8	Hu et al. (2018)
		TET	ND - 12	Hu et al., (2018)
Hong Kong	tap water	OTC	ND – 600	Li et al. (2017)
USA	surface water (Choptank river)	OTC	ND – 47	Arikan et al. (2008)
		TET	ND – 5	Arikan et al. (2008)
	surface water (Cache la Poudre river)	OTC	ND – 1210	Kim and Carlson, (2007)
	surface water	OTC	ND – 340	Kolpin et al. (2002)
		TET	ND – 110	Kolpin et al. (2002)
Canada	WWTP effluent	TET	157 – 977	Miao et al. (2004)

Some studies have quantified Tetracyclines in aquaculture environments: OTC concentration in water varied between 14 to 7993 ng/L in Brazil (Monteiro et al. 2016), 108 to 120 ng/L in Poland (Harnisz et al. 2015b) and 17.8 to 15163 ng/L in China (Chen et al. 2015); while TET was detected at concentrations of 2305 ng/L in an aquaculture in China (Chen et al. 2015). In Portugal, OTC was detected in an aquaculture near Caima river (Pereira et al. 2015), at a mean concentration of 3 ng/L in fish tank; 11.9 ng/L in the exit of the aquaculture, and 9 ng/L in the downstream river (250 m from the aquaculture) (Pereira et al. 2015).

Also, due to feed excess and fish excretion (Shi et al. 2012), tetracyclines deposit in sediments near aquaculture systems. For instance, OTC was detected at concentrations between 0.75 to 4.36 ng/g in China (Yuan et al. 2019) and 17 to 5477 ng/g in Brazil (Monteiro et al. 2016). TET concentration varied between 0.84 to 5.61 ng/g in China (Yuan et al. 2019) and 10 to 72 ng/g in Brazil (Monteiro et al. 2016).

The detection of Tetracyclines residues in fish tissues was also observed in some works. The maximum residue limit (MRL) for Tetracyclines was set by EU as 100 μg/kg for fish muscle in food-producing animals (EC 2010). After a post-dosing period (after the end of Tetracyclines treatment), values of 32 ng/g (OTC; 19 days post-dosing) in coho salmon (Meinertz et al. 2001), 32 ng/g (OTC; 21 days post-dosing) and 27 ng/g (TET; 21 days post-dosing) in crucian carp (Kowalski 2008) and 540 ng/g (TET; 4 days post-dosing) in crucian carp (Deng et al. 2012) were observed.

The persistence of these compounds in the environment may vary according to environments' physical and chemical properties (e.g. pH, salinity, and temperature) or abiotic degradation (e.g. photolysis, hydrolysis, and biodegradation). It was reported that OTC may persist in water and sediments for days (Daghrir and Drogui 2013; Leal et al. 2018). Yet, in a study conducted by Liu et al. (2019b), in a simulated stream, the persistence of OTC showed to be lower in water (0.13 days of half time) than in sediment (disappeared in 20 days). Tetracyclines by-products may also constitute an environmental problem. Depending on the degradation processes and the compound formed, toxicity for exposed organisms can be considerable (Leal et al. 2018). Nevertheless, most of studies only consider the mother compound.

3. Impacts in the aquatic organisms and microorganisms

3.1 Ecotoxicological studies

Due to their persistence in the environment, Tetracyclines may affect non-target organisms. Indeed, aquatic organisms may be of concern since they are unavoidably exposed. Exposure for short or long-term periods may occur threatening organism's fitness and survival and ultimately affecting populations and ecosystems.

Several studies have been devoted to understanding the impact of Tetracyclines, namely OTC exposure in organisms like duckweed, algae, crustacean and fish. For instance, growth inhibition was reported for the green algae *Chlorella vulgaris* (72h-EC_{50} = 7.05 mg/L) (Eguchi et al. 2004), *Pseudokirchneriella subcapitata* (96h-EC_{50} = 3.1 mg/L) (Zounková et al. 2011) and *Selenastrum capricornutum* (72h-EC_{50} = 0.342 mg/L) (Eguchi et al. 2004). In crustacean, immobilization was reported in *Moina macrocopa* (48h-EC_{50} = 126.7 mg/L) (Park and Choi 2008) and *Ceriodaphnia dubia* (48h-EC_{50} = 18.65 mg/L) (Isidori et al. 2005); reproduction inhibition in *Daphnia magna* (48h-EC_{50} = 86 mg/L) (Zounková et al. 2011) and mortality in *Artemia parthenogenetic* (48h-LC_{50} = 805.99 mg/L) (Ferreira et al. 2007) and *Portunus trituberculatus* (larvae) (48h-LC_{50} > 100 mg/L) (Ren et al. 2017).

Even though, usually pointed as not so sensitive to antibiotic exposure as other aquatic organisms, fish have been calling special attention (Kovalakova et al. 2020). Indeed, several reports have demonstrated that Tetracyclines may compromise fish health (Table 3). Analysis at several biological organization level has been performed after chronic or acute exposure. In general, Tetracyclines exposure impairs physiological functions, enzymatic activity, antioxidant defense and immunological response (Table 3). Worth to note that even environmental concentrations may induce changes in fish.

Table 3: Effects of Tetracyclines (oxytetracycline: OTC and tetracycline: TET) in fish after an acute or chronic exposure. LC: lethal concentration; EC: effects concentration.

Compound	Organism	Type of exposure	Concentration (µg/L)	Parameter/endpoint	Reference
OTC	*Danio rerio* (embryos)	Acute	150000	Hatching delay; Glutathione S-transferase activity decrease; Lactate dehydrogenase activity increase.	Oliveira et al. (2013)
OTC	*Danio rerio* (embryos)	Acute	5000	Oxidative stress induction; Antioxidant capacity reduction.	Yu et al. (2019)
OTC	*Danio rerio* (embryos)	Chronic	5	Body length, weight and body mass index decrease; Heart rate increase; Disruption of thyroid system.	Yu et al. (2020)
OTC	*Danio rerio*	Chronic	0.42	Non-specific immune reactions change; Increase of oxygen consumption rate.	Zhou et al. (2018)
OTC	*Danio rerio*	Chronic	100	Thyroid hormone and serotonin homeostasis change.	Li et al. (2020)
OTC	*Oncorhynchus mykiss*	Acute	50000	Gills and liver histological changes; Antioxidant activity change; DNA damage.	Rodrigues et al. (2017a, b)
		Chronic	5	Gills and liver histological changes; Antioxidant activity change; DNA damage.	Rodrigues et al. (2017a, b)
OTC	*Sparus aurata*	Acute	400	Liver histological change;	Rodrigues et al.

				EROD activity decrease; DNA damage.	(2018, 2019)
		Chronic	4	Gills histological change; Lipid peroxidation (TBARS levels) increase; DNA damage.	Rodrigues et al. (2018, 2019)
OTC	*Oreochromis niloticus*	Acute	8	DNA damage.	Botelho et al. (2015)
OTC	*Labeo rohita*	Chronic	80000	Lactate dehydrogenase activity increase; Hematological change	Ambili et al. (2013)
OTC	*Oreochromis niloticus*	Chronic	0.42	Effects on growth performance; Alteration nutrient digestibility; Innate immunity suppression.	Limbu et al. (2018)
OTC	*Oryzias latipes*	Acute	110100	96h-LC_{50}	Park and Choi (2008)
TET	*Gambusia holbrooki*	Acute	0.50	Gills histological change; Antioxidant activity change.	Nunes et al. (2015)
TET	*Danio rerio* (embryos)	Acute	20	Delayed yolk sac absorption; Swim bladder deficiency.	Zhang et al. (2015)
TET	*Brachydanio rerio*	Acute	260500	24h-LC_{50}	Wang et al. (2020)
TET	*Danio rerio*	Chronic	100	Dysregulations in hepatic metabolic pathways	Keerthisinghe et al. (2020)

Table 3 presents studies regarding exposure via water, however, other studies report OTC effects after exposure via food. As an example, the induction of oxidative stress response and suppression of the specific and nonspecific immune system was reported in rainbow trout *Oncorhynchus mykiss* (Yonar et al. 2011; Yonar 2012) and in the silver catfish *Rhamdia quelen* (Pês et al. 2018).

Yet, although ecotoxicological effects of antibiotic exposure are documented in literature, several questions remain to answer. Namely, the effects of antibiotics at other sensitive endpoints like fish energetic metabolism and fish locomotor behavior are missing. Moreover, additional studies considering other environmental scenarios like an intermittent exposure or a post-exposure period are still needed to understand the real impact of antibiotic exposure. Besides, few studies devoted to understanding the impact of Tetracyclines exposure in organism's microbiome and studies considering effects of antibiotic exposure in both organism and microbiome in a holistic perspective are absent.

3.2 Effects in the organism's microbiome

Organisms microbiome may be defined as the collective genomes of all microorganisms (bacteria, archaea and fungi), symbiotic and pathogenic, living in and on a specific niche (e.g. organisms' gut) (Berg et al. 2020).

Microbiome plays an important role in host health and survival. It was reported that organism's microbiome is involved in host nutrient processing, organ development, immune function, metabolic processes and behavior in fish (Banerjee and Ray 2017; Abreu et al. 2019). Each organism has its own "core microbiome" that remains relatively constant and is associated with a specific host genotype (Berg et al. 2020). Yet transient microbiome may vary according organisms age (Kanther and Rawls 2010) and feed regime/ nutritional status (Ni et al. 2014). Therefore, host-microbiome relation is a dynamic balance (De Schryver and Vadstein 2014). Indeed, in last years a holistic approach has been raising regarding host-microbiome interaction (Simon et al. 2019). The holobiont concept considers that host and microbiome co-evolve as a single unit. Hence, the disease state results from a dysbiosis and low microbiome diversity while the healthy state is linked to eubiosis and high diversity (Berg et al. 2020).

It is known that a chemical exposure may affect the organism's microbiome balance with severe consequences to the host (Jin et al. 2017). For instance, it was reported that pesticides, metals, nanoparticles and antibiotics exposure may affect community structure of host-associated microbiome (Catron et al. 2019; Bertotto et al. 2020). In literature the effects of Tetracyclines exposure, namely in fish microbiome, were reported. Zhou et al. (2018) exposed zebrafish to 0.420 μg/L of OTC for 6 weeks. These authors observed a lower bacterial richness and a change in the structure of gut bacterial communities of exposed organisms. Li et al. (2020), which exposed juvenile zebrafish until adulthood to 1 and 100 μg/L of OTC, noticed changes in bacterial communities structure, namely in the balance between Proteobacteria and Firmicutes. Limbu et al. (2018) revealed that Nile tilapia exposed via water (0.420 μg/L) or orally (80 mg/kg/day) to OTC presented microbiota dysbiosis. Kim et al. (2019a) observed a reduction on microbial diversity of olive flounder fish exposed to OTC by oral feed (80 mg/kg/day). The same result, reduction of diversity, was observed by Navarrete et al. (2008) in the Atlantic salmon feed with OTC (75 mg/kg/day).

Microbiome diversity is important to maintain host' biological process and welfare (De Schryver and Vadstein 2014). Healthy organisms usually present higher microbial diversity than diseased ones. Although antibiotics are used to treat diseases it may also have an impact in the organism's welfare. Overall, studies demonstrated that Tetracyclines exposure may decrease microbiome diversity and change bacterial communities' structure. Nevertheless, effects at bacterial communities' functional level are usually not considered. Therefore, more studies are needed considering a more holistic approach, including organism-microbiome-environment.

3.3 Effects in the water microbiome

Water bacterial communities are involved in several environmental processes. Namely, the decomposition of organic matter, nutrient and carbon cycle. Changes in these bacterial communities may compromise water quality altering parameters like pH, ammonia and oxygen content (Moriarty 1997). On the other hand, variation in temperature, pH or river flow may also impact bacterial communities (Mark Ibekwe et al. 2012).

Antibiotics may play a role as a selective factor in natural bacterial communities. The inhibition of growth or the disappearance of some bacterial groups may affect the ecosystem. Several studies have demonstrated that antibiotic exposure, for instance to sulfonamides and fluoroquinolones, may decrease microbial respiration/catabolic activity, and nitrification and denitrification processes in water (Grenni et al. 2018). Regarding Tetracyclines class, changes in bacterial communities structure such as the shift in the dominance of specific bacterial families was observed due to TET exposure (Zhang et al. 2013; Huang et al. 2015; Eckert et al. 2019). Moreover, Klaver and Matthews (1994) showed that high OTC concentration inhibits the nitrification process; Islam and Gilbride (2019) observed an increase in the ammonia and nitrogen rate removal due to TET presence. Katipoglu-Yazan et al. (2013) showed an inhibition of organic carbon removal and nitrification process due to TET exposure.

Changes in the nitrogen cycle may be of concern for aquatic animals. The decrease of nitrification, ammonia oxidation (anammox) or denitrification processes may lead to accumulation of toxic compounds like nitrate, nitric oxide and nitrous oxide. Since these compounds may be toxic to aquatic organisms like fish it is important to understand the role of antibiotics in the selection of bacterial communities that play a role in the nitrogen cycle (Camargo et al. 2005; Kim et al. 2019b).

4. The problem of Antibiotic Resistance

One of the most reported impacts of antibiotic exposure in natural bacterial communities is the direct or indirect selection of antibiotic resistance. When exposed to antibiotics, bacteria can resist due to intrinsic (e.g. membrane permeability) or acquired mechanisms (e.g. through mutation or horizontal gene transfer). The share of genetic material may occur between phylogenetically related bacteria and/or between phylogenetically distant bacteria (Grenni et al. 2018). Therefore, antibiotic resistance genes (ARGs) are considered emerging pollutants and antibiotic resistance is one of the most important global public health concerns (Grenni et al. 2018; Kovalakova et al. 2020). In fact, the persistence of these compounds in the environment at concentrations below the

minimal inhibitory concentrations may aggravate this phenomenon (Grenni et al. 2018). It is estimated that every year, thousands of people (around 700000) die in Europe and USA due to antibiotic-resistant infections (O'Neill 2016; ECDC 2019). Furthermore, predictions point to an increase to 10 million deaths per year by 2050 worldwide (O'Neill 2014, 2016).

The "One Health" initiative has been highlighting the need for more caution in antibiotic use. The integration of multi-disciplinary perspectives like medicine/public health and veterinary medicine/agriculture may allow us to understand the global perspective of the real impact that antibiotics may have (Fig. 6). Hence, the connection between animal–human–ecosystems interfaces may be one important approach to be achieved. Also, the implementation of better politics like surveillance, appropriate sanitary measures, disease monitoring and vaccination may allow us to reach the best practices and quality parameters.

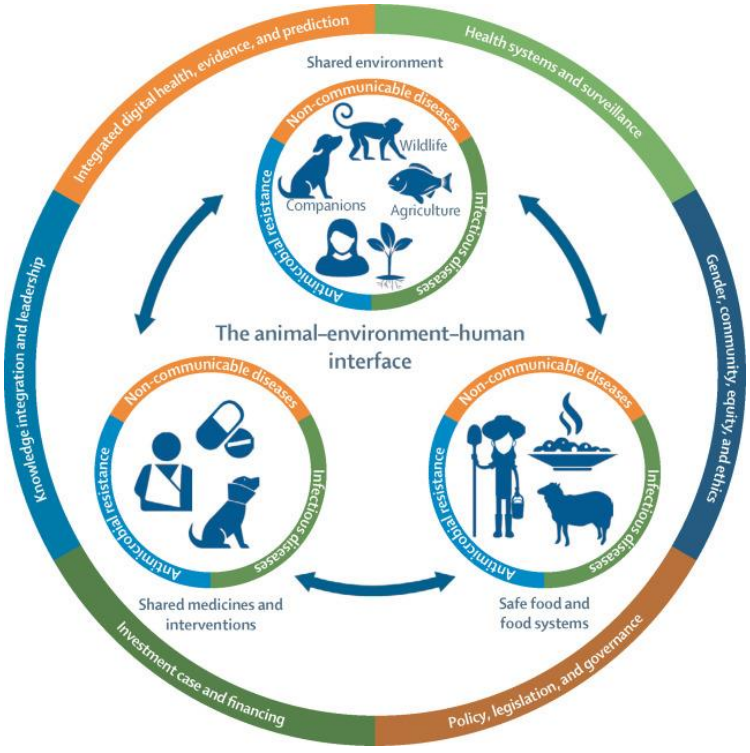

Fig. 6: The One Health concept (retrivied from Gables et al. 2020).

Tetracycline resistance genes may be classified in three main mechanisms: efflux pumps (e.g. *tet*A, *tet*B and *tet*C), ribosomal protection (*tet*M, *tet*S and *tet*O) and enzymatic

inactivation (e.g. tetX) (Roberts 2005; Wang et al. 2019b). *tet* resistance genes (e.g. *tet*A, *tet*C, *tet*G and *tet*M) are generally associated with transferable elements like plasmids and transposons, which facilitate its spread (Roberts 2005; Henriques et al. 2008; Wang et al. 2019b). Indeed, tetracycline (*tet*) and sulfonamide (*sul*) resistance genes are the most frequently detected ARGs in aquatic environments (Tuševljak et al. 2013; Alves et al. 2020). The presence of *tet* genes was reported in several environments like aquaculture residues (Huang et al. 2017; Suzuki et al. 2019), WWTP effluents (Harnisz et al. 2020; Osińska et al. 2020), rivers (Harnisz et al. 2015a, 2020) and lakes (Liang et al. 2020).

As discussed in the previous section, Tetracyclines exposure may change microbial communities' structure, which may allow the growth of Tetracyclines resistant bacteria (TET-ARB). Usually, the increase of bacteria like *Nitrospira*, *Dechloromonas* and *Rhodobacter*, which play a role in the nitrogen cycle, has been reported (Liu et al. 2019a; Zhang et al. 2019). Interestingly, these genera were found to be positively correlated with several *tet* genes (Liu et al. 2019a). Moreover, some authors established a relationship between the use of Tetracyclines and the selection of ARB and possible ARB-pathogens to fish and humans (e.g. *Aeromonas*, *Enterobacter* and *Pseudomonas*) (Santos and Ramos 2018; Wang et al. 2019b; Jia et al. 2020). Indeed, food-animals are pointed has a reservoir of resistance genes and resistant pathogens to humans (Serrano 2005). In a study conducted by Furushita et al. (2003), it was demonstrated that TET-ARB collected from three different fish farms in Japan carried *tet* resistance genes with high sequence similarities with those genes present in human clinical isolates. Moreover, Rhodes et al. (2000) showed that Tetracyclines resistance encoding plasmids may disseminate between different *Aeromonas* (fish isolates) and *E. coli* (human isolates) species isolated from human and aquaculture environments. Also, studies conducted by Henriques et al. (2008) and Azevedo et al. (2013) in Ria de Aveiro, an estuarine environment harboring several aquaculture farms, detected several *tet* genes in *Aeromonas, Pseudomonas* and *E. coli* isolates.

The transmission of ARB and ARGs from environment to humans and vice-versa may facilitate the spread of zoonotic antibiotic-resistant pathogens that threatens both animals and human health. Nevertheless, few studies were conducted in a controlled environment, as a microcosm approach. This methodology permits to reduce other environmental variabilities and allow a better correlation between the role of antibiotic use and the selection of ARB and ARGS. Moreover, most studies were restricted to the analyses of

water bacterial communities and therefore, the selection of ARB and ARGs within organism's microbiome is poorly understood.

5. Zebrafish: the model organism

5.1 Origin and morphology

Zebrafish (*Danio rerio*) is a small freshwater fish belonging to the *Cyprinidae* family. This fish is native from Southern Asia (e.g. India, Bangladesh and Nepal), being typically found in shallow ponds, canals, and edges of streams (e.g. rice fields) (Spence et al. 2008). Its omnivorous diet is based on zooplankton, phytoplankton, insects, arachnids and organic matter (e.g. plants material, spores, etc.) (Spence et al. 2008).

Zebrafish is characterized by its fusiform shape with dark blue longitudinal stripes. It has sexual dimorphism: males tend to have larger anal fins while the females have rounded body shape (Fig. 7). Zebrafish fertilization occurs externally with each female laying hundreds of eggs per spawn (Lawrence 2007; Spence et al. 2008). Eggs are demersal and optically transparent, having synchronous and fast development. After two to three days post-fertilization embryos hatch and become a free-swimming larva. This fish reaches the mature age (adult stage) at 3 to 4 months old (Fig. 7) (Spence et al. 2008).

In the laboratory, zebrafish may be maintained in small aquaria (3.5L) in a recirculatory system. Due to its small size, not exceeding 4 cm, it is possible to have numerous individuals in a small space, being usually kept in a range of 7 to 10 fish per liter. Its maintenance conditions may vary between facilities since it supports a wide range of temperature (24 to 30 ºC), salinity (0.25 – 0.75 ppt), and pH (7- 8) (Lawrence 2007).

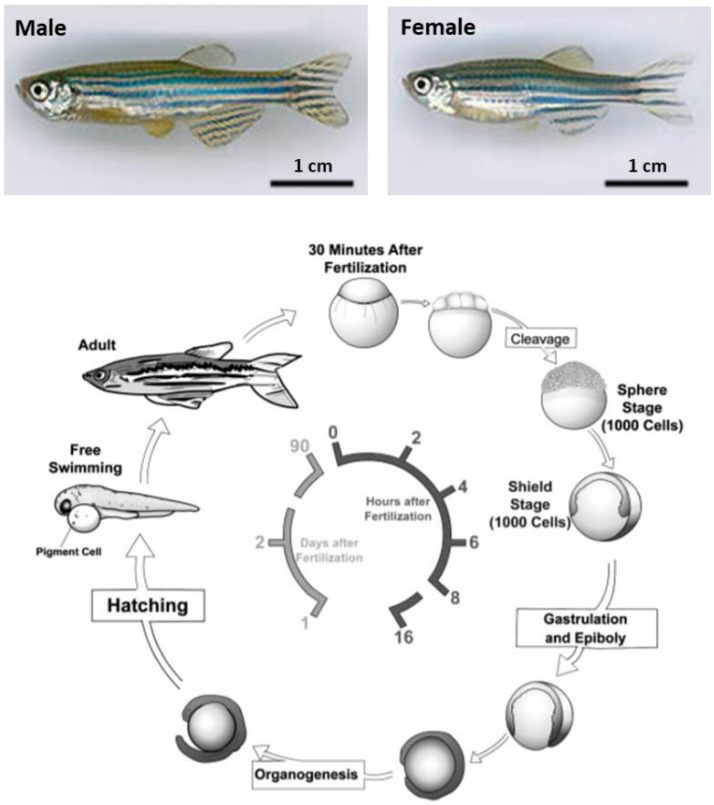

Fig. 7: Zebrafish morphology and life cycles (retrivied from Costa and Shepherd 2009).

5.2 Zebrafish as a model organism

Zebrafish is a model organism extensively used worldwide. Since the first publication in 1947, this organism has gained attention and nowadays, more than 3000 papers are published per year (Scopus: www.scopus.com). Areas like neurosciences (Abreu et al. 2020); behavior (Correia et al. 2019); ecotoxicology (Almeida et al. 2019); immunology (Lv et al. 2020) genetic (Seritrakul and Gross 2019) reproduction (Chen et al. 2021) and microbiology (Torraca and Mostowy 2018) have been referring zebrafish has a suitable model organism. In fact, zebrafish represents numerous advantages in relation to other models like rodents. Namely, its small size and low maintenance cost allows to have numerous organisms in a small space. It has a high fecundity rate, which permits the testing of a wide range of compounds at the same time. Moreover, its rapid development allows to monitor all the embryonic development (e.g. organ development) and to reduce the experiment consuming time (Garcia et al. 2016). Also, 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). In fact, zebrafish shares around 70% of orthologue genes with humans and has several homologous organs (Howe et al. 2013; Garcia et al. 2016). Also, zebrafish core microbiome was already identified which allows to delve the relation host–microbiome (Roeselers et al. 2011; Tarnecki et al. 2017).

Therefore, due to its several advantages, numerous tools and methodologies have been developed and optimized for zebrafish, transforming this small organism in a powerful tool in research. Hence, in this work we used the zebrafish model to understand the impact of tetracycline in organism itself as also in organism's microbiome.

Chapter 2

Hypotheses, Goals and Thesis structure

Oxytetracycline is one of the most used antibiotics in animal food-production like aquaculture. It is known that organisms are not able to absorb all the antibiotic consumed (Cravedi et al. 1987) and thus, through excretion and food excess these compounds may reach the aquatic environment. Since antibiotics may be still bioactive (Carvalho and Santos 2016), effects in non-target organisms are expected. Although some studies have already shown the possible effects of long-term exposure to oxytetracycline on fish, there are few information regarding sensitive endpoints like behavior and energetic metabolism. Moreover, in their natural environment organisms may not be always continuously exposed to antibiotics and recovery periods may occur. Nonetheless, few is known about organism's capacity to recover in a post-exposure scenario.

Organism's microbiome plays an important role in host health and welfare (Nayak 2010; Miller et al. 2018). However, organism-microbiome relationship may be affected by a chemical exposure (Jin et al. 2017). Nevertheless, the impact that oxytetracycline exposure may have in organism's microbiome and the consequences to the host are still unknown. Besides, the role of antibiotic exposure in the selection of antibiotic resistance genes and resistant bacteria with potential pathogenicity behavior is still poorly understood.

Therefore, based on the statements above, this study was developed to fill these knowledge gaps. To do that the following hypothesis and goals were stablished:

1. Hypotheses

I. Oxytetracycline may induce changes in zebrafish behavior and energetic metabolism.
II. Organisms microbiome and water bacterial communities are affected by oxytetracycline exposure.
III. A post-exposure period may allow the organisms and its microbiome to recover.
IV. Long-term exposure to oxytetracycline may select antibiotic-resistant bacteria and/or antibiotic resistance genes.

2. Goals

The main goal of this study was to evaluate the effects of long-term exposure to oxytetracycline in zebrafish at several biological organization levels, during exposure and in a post-exposure period. To test the proposed hypothesis and to achieve the main goal, the following specific goals were stablished:

I. Evaluate the effect of oxytetracycline long-term exposure in zebrafish behavior and biochemical markers.
II. Evaluate the impact of oxytetracycline long-term exposure in zebrafish microbiome and water bacterial communities.
III. Determine if organisms can recover in a post-exposure scenario at energetic reserves and microbiome level.
IV. Isolate and characterize antibiotic-resistant bacteria, after long-term exposure to oxytetracycline, selected from zebrafish and water samples.
V. Evaluate the pathogenicity of selected antibiotic-resistant bacteria and the selection of antibiotic resistance genes.
VI. Evaluate the role of OTC exposure in the prevalence of tetracycline resistance genes (e.g. *tet*A) in water and fish gut microbiome, through quantitative PCR (qPCR).

3. Thesis structure

This thesis is composed by seven chapters. The Chapters 3 to 6 are presented as scientific papers. Namely, Chapters 3 and 4 were published in the international journals Chemosphere and Ecotoxicology and Environmental Safety, respectively. Chapter 5 and 6 were submitted to the international journals Environmental Pollution and Chemosphere, respectively. The description of each chapter is summarized below:

Chapter 1: Introduction: The impact of tetracycline in the aquatic environment

In this chapter an overview and contextualization about the tetracycline use and its impact in the aquatic organisms and environment is given. Topics like tetracycline environmental concentration, its impacts on non-target organisms (e.g. fish and water microbiome) and its role in the selection of antibiotic-resistant bacteria are addressed.

Chapter 2: Hypothesis, Goals and Thesis Structure

The hypothesis to test and goals to achieve in this thesis are pointed in this chapter. It also contains a picture of thesis structure and chapter description.

Chapter 3: Long-term effects of oxytetracycline exposure in zebrafish: A multilevel perspective

In this study, the long-term impact of oxytetracycline in zebrafish was accessed. After two months of exposure, via water, analysis at several biological organizations like fish behavior, biochemical markers and microbiome was performed. Results revealed that even low concentrations of oxytetracycline may impair fish fitness. Effects at fish behavior (e.g. hyperactivity), enzymatic activity (e.g. decrease of oxidative stress enzymes) and fish gut and water bacterial communities' structure (e.g. DGGE analysis) were observed.

Chapter 4: The impact of antibiotic exposure in water and zebrafish gut microbiomes: A 16S rRNA gene-based metagenomic analysis

Based on our previous work (Chapter 3) this chapter intended to achieve a deeper analysis of fish gut and water bacterial communities at composition and functional level. To do that we used a 16S rRNA gene metagenomics approach through illumina technology. Our results showed that oxytetracycline impacts both fish gut and water at alpha and beta diversity level. Namely, it was observed the selection of bacteria that plays a role in the nitrogen cycle as also

the selection of bacteria intrinsically resistant to antibiotics belonging to phylogenetic groups pointed as fish pathogens.

Chapter 5: Zebrafish and water microbiome recovery after oxytetracycline exposure

In this chapter, the recovery capacity of organisms exposed to oxytetracycline was evaluated. To do that, organisms were exposed for two months of exposure, via water, to oxytetracycline. After this period, fish were transferred to clean water and kept for reversibility of effects. Analysis was performed at fish energetic reserves level and fish gut and water microbiome at four different sampling points: during exposure (5 days and 2 months of exposure) and in the post-exposure period (5 days and 1 month of post-exposure). Generally, our results indicate that after exposure ceased, a re-adjust of fish fitness seems to occur. At both energetic reserves and microbiome level, effects were attenuated, and control and exposed organisms became more similar suggesting a recovery.

Chapter 6: Tetracycline resistance in water and zebrafish bacteria following antibiotic exposure

In this study the role of tetracycline in the selection of antibiotic resistance genes (ARGs) and antibiotic-resistant bacteria (ARB) was assessed through a microcosm assay. Samples from fish (skin and gut) and exposure water were collected at three different sampling points: during exposure (5 days and 2 months of exposure) and in the post-exposure period (5 days of post-exposure). Culture methods (e.g. isolation and characterization of antibiotic resistance genotypes and phenotypes; zebrafish pathogenicity test) and independent-culture methods (e.g. quantitative PCR targeting tetracycline resistance genes- qPCR) were used for an integrated analysis to understand oxytetracycline impact. Overall, our results revealed the selection of ARB and ARGs due to oxytetracycline exposure. Moreover, the selection of multidrug resistant (MDR) bacteria with potential pathogenic effect to zebrafish embryos was also observed.

Chapter 7: Final remarks

In this chapter a general discussion of the work was addressed as also a discussion of the general limitations of the work. Moreover, since along the present work some additional questions were raised, future work was also indicated.

Chapter 3

Long-term effects of oxytetracycline exposure in zebrafish: a multilevel perspective

Long-term effects of oxytetracycline exposure in zebrafish: A multilevel perspective

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Abstract

Oxytetracycline (OTC) is a broad-spectrum antibiotic widely used in livestock production. Like many other pharmaceuticals, OTC is not completely metabolized by the organism and thus, increasing amounts of the compound are being detected in the aquatic environment. The assessment of the environmental risk of pharmaceuticals is hindered by their very low concentrations and specific modes of action and thus relevant exposure scenarios and sensitive endpoints are needed. Thus, this work aimed to study the long-term effect of OTC exposure in zebrafish (at behavior and biochemical levels) and associated bacterial communities (fish gut and water bacterial communities). Results revealed that at behavioral level, boldness increase (manifested by increased exploratory behavior of a new environment) was observed in fish exposed to low OTC concentrations. Moreover, changes in fish swimming pattern were observed in light periods (increased stress response: hyperactivity and freezing) probably due to photo-sensibility conferred by OTC exposure. Effects at biochemical level suggest that long-term exposure to OTC interfere with cellular energy allocation mainly by reducing lipids levels and increasing energy consumption. Moreover, evidences of oxidative damage were also observed (reduced levels of TG, GST and CAT). The analysis of water and gut microbiome revealed changes in the structure and diversity of bacterial communities potentially leading to changes in communities' biological function. Some of the effects were observed at the lowest concentration tested, 0.1 μg/L which is a concentration already detected in the environment and thus clearly demonstrating the need of a serious ecotoxicological assessment of OTC effects on non-target organisms.

Key words: antibiotic**;** microbiome; behavior assay; enzymatic activity

1. Introduction

Worldwide, antibiotics are commonly used to prevent or treat diseases in livestock production and aquaculture. These compounds, are usually administered through food, to both sick and healthy animals (Sapkota et al. 2008; Rodgers and Furones 2009). The excess of food and the animals' feces and urine are the main entry routes into the environment (Shi et al. 2012). Indeed, it is estimated that 80% of the ingested antibiotics reach the environment in their original form (not metabolized) (Armstrong et al. 2005; Ben et al. 2008). Antibiotics were already detected in both terrestrial and aquatic systems (waste water effluents and natural waters) at concentrations ranging from ng/L to µg/L (Fent et al. 2006; Chen et al. 2011a). In addition, due to their continuous release, antibiotics may be considered persistent contaminants with unclear ecological effects in the environment (Zhang et al. 2015).

Tetracyclines are among the most frequently used antibiotics, representing nearly 32% of antibiotic consumption in livestock in the European Union in 2015 (European Medicines Agency 2017). Tetracyclines family includes large spectrum antibiotics, being effective both against gram-positive and gram-negative bacteria. They act by inhibiting the protein synthesis by avoiding the combination of aminoacyl-tRNA with the bacterial 30S ribosomal subunit (Brodersen et al. 2000; Pioletti 2001). Although antibiotics are used to kill or inhibit bacteria growth, they can also affect non-target organisms directly or indirectly. In fact, some ecotoxicological studies have reported toxic effects of tetracyclines, namely oxytetracycline (OTC), to aquatic organisms, in particular fish. For instance, OTC has been shown to delay hatching in zebrafish embryos (Oliveira et al. 2013); to induce tissue damage in gill and liver of rainbow trout (Rodrigues et al. 2017a); and to increase oxidative stress in muscle of silver catfish (Pês et al. 2018). Nonetheless, these studies mostly reported effects of acute exposures to relatively high concentrations of OTC and information about the long-term effects of sub-lethal concentrations (e.g.: ng/L to µg/L) is missing. This information is essential to accurately predict ecological risk. OTC was already detected at concentrations of 340 ng/L in surface

water in USA (Kolpin et al. 2002), 7 ng/L in Mess river water in Luxembourg (Pailler et al. 2009) and 19.2 ng/L in Po river water in Italy (Calamari et al. 2003). Moreover, concentrations ranging from 14 to 7993 ng/L were also detected in aquaculture systems in Brazil (Monteiro et al. 2016).

Thus, this work aims at studying the effects of a long-term exposure (2 months) to OTC using adult zebrafish. Considering that environmental concentrations are usually very low, a battery of highly sensitive endpoints was selected so that effects could be properly assessed. This battery includes behavioral parameters based on fish locomotion, biochemical markers, and analysis of the microbiome associated to the fish gut and to the water. This multi-level approach allows the assessment of OTC effects at different levels of biological organization including at individual and sub-individual levels contributing to a more comprehensive characterization of risk.

Organisms' microbiome plays an important role in the host health and survival (Shi and Walker 2004; Nayak 2010). For example, intestinal microbiome can protect the host against pathogens, help in the immune system and organ development (Rawls et al. 2004). Yet, this is not a static association and may change due to several internal (e.g. age, nutritional and health status) or external factors (e.g. environmental conditions and chemical stress). Changes in fish microbiome due to antibiotic exposure have been described in literature, but the vast majority of the studies concern exposure via food (Navarrete et al. 2008; He et al. 2012; Liu et al. 2012). For instance, changes in gut bacterial communities composition were showed in hybrid tilapia (He et al. 2011), Atlantic salmon (Navarrete et al. 2008) and zebrafish (Brugman et al. 2009) exposed to antibiotics by feed intake. In opposition, in this work we will assess changes in the microbiome of fish due to exposure through the water which better represents the scenario of environmental exposure. Changes in gut and water bacterial communities will be assessed through analysis of structure and diversity.

The interest in zebrafish behavior assays has been raising due to their high sensitivity, and usefulness as a tool in ecological risk assessment (Sison et al. 2006; Egan et al. 2009; Andrade et al. 2016). Behavior tests in fish have been derived from rodent methodologies and are based on the fish swimming behavior (Champagne et al. 2010). When fish are faced to a mild stress such as a novel environment or a sudden change in light conditions, different swimming

patterns translating anxiety-like behavior (e.g hyperactivity, erratic swimming and freezing) can be observed. In the literature, some authors devoted to study the impact of some drugs like antibiotics in these stereotyped behaviors as a marker of neuronal disruption. Hence, in this work, the novel tank test and reaction of fish to sudden changes in light conditions were used to assess behavioral disruption in zebrafish adults exposed to OTC. In addition, to determine the physiological status of the organism, a battery of biochemical markers was chosen. It is known that antibiotics, namely OTC may affect fish metabolism and oxidative stress (Rodrigues et al. 2017a; Nakano et al. 2018). Thus, analysis of energetic metabolism parameters (cellular energy allocation: CEA; total energy available: Ea; energy consumption: Ec), as also analysis of enzymatic activity related with antioxidant defense (total glutathione: TG; glutathione S-transferase: GST; catalase: CAT) neurotransmission (acetylcholinesterase: AchE) and anaerobic metabolism (lactate dehydrogenase: LDH) were used to access the impact of long-term exposure to OTC in zebrafish.

2. Materials and Methods

2.1 Zebrafish culture

Zebrafish (*Danio rerio*) were obtained from the zebrafish culture established at the Biology Department of University of Aveiro (Aveiro, Portugal). The fish were kept under controlled conditions in a recirculation system as described by Domingues et al. (2016). Water parameters were the following: temperature of 27 ºC; pH 7.5 ± 0.5; conductivity 800 ± 50 and dissolved oxygen ≥ 95%. Adult fish were feed once a day with artificial diet Gemma Micro 500 (Skretting®, Spain). The photoperiod cycle was 14:10 h (light:dark). Zebrafish adults, four months old, were selected for the experimental assays as recommended by OECD guideline 230 (OECD 2009).

2.2 Zebrafish exposure

Oxytetracycline hydrochloride (CAS number: 2058-46-0), was purchased from Sigma Aldrich. Stock solutions were prepared by dissolving oxytetracycline in zebrafish recirculation system water and used to prepare the test solutions. A test was performed to assess degradation of OTC along the time in our exposure conditions (Section 2.6). During four days, test solutions were collected daily and OTC concentration was analyzed to achieve the degradation pace.

Zebrafish adults were exposed to four concentrations (0, 0.1, 10, 10000 µg/L) of oxytetracycline hydrochloride during two months under a semi-static condition. The concentrations 0.1 and 10 µg/L were chosen based on previous works reporting environmental concentrations (Osorio et al. 2015; Monteiro et al. 2016; Carvalho and Santos 2016). In addition, the higher OTC concentration (10000 µg/L) was used in order to elucidate the mode of action of OTC in our exposure scenario. The experiment was performed in 1 L aquaria containing 6 fish per replicate (6 replicates) in a total of 36 fish per concentration. Temperature, photoperiod and water physicochemical parameters were kept similar to the culture conditions. During the experiment, medium was renewed every three days to maintain constant exposure concentrations and water quality parameters (Table S1).

Fish were weighed immediately before (0 h) and after the exposure period (2 months) and the pseudo-specific growth rate (R) calculated using Eq. 1 (OECD 2000). At the end of the test, fish were used for behavior analysis (section 2.3) before being euthanized by anesthetic overdose (tricaine methane sulfonate, Metacain, MS-222; CAS number: 886-86-2) followed by spinal cord severing. For biochemical markers (section 2.4), fish trunk and heads from 8 fish per treatment were dissected, snap frozen separately and stored at -80 ºC until analyses. For microbiome analysis (section 2.5), fish guts (25 guts per treatment) were sampled individually in aseptic conditions, placed in sterile microtubes, snap frozen and stored at -80ºC until DNA extraction.

Eq. 1 $$R = \frac{log_e W_2 - \overline{log_e W_1}}{t_2 - t_1} \times 100$$

R: "pseudo" specific growth rate

W_1: weight of a particular fish in the beginning of the test

W_2: weight of a particular fish in the end of the test

t_1: time at start of the test

t_2: time at end of the test

$log_e W_1$: logarithm of an individual fish weight in the beginning of the test

$log_e W_2$: logarithm of an individual fish weight in the end of the test

$\overline{log_e W_1}$: average of the logarithms of the values W_1 for the fish in the tank in the beginning of the test.

2.3 Behavior assay

2.3.1 Feeding test

Six fish per treatment were placed individually in an aquarium with 7 cm of water height. After 3 min of acclimation, 2 % fish body weight of food was provided to each fish (Gemma Micro 500). Time until first feeding action (Ti) and time to total food ingestion (Tf) were recorded until a maximum of 20 min (Chollett et al. 2014; Domingues et al. 2016).

2.3.2 Novel tank/ Exploratory swimming test

Nine fish per treatment were placed individually in a new aquarium, representing a new environment, and their movement recorded during 5 min with a digital camera (Samsung ES70) placed 18.5 cm distant from the aquarium. Movement of fish (swimming time) was analyzed discriminately in three different zones (layers): bottom of the aquarium (0-3 cm height); middle (3-6 cm height) and top (6-9 cm height) (Domingues et al. 2016). The schematic diagram is represented in Fig. S1. Additionally, the time until first exploring movement and the number of transitions between each aquarium zone was also calculated (Egan et al. 2009).

2.3.3 Light/dark Swimming test

Nine fish per treatment were transferred individually to a small aquarium with 8 x 13 cm (L x W). The fish were then placed into the ZebraBox tracking system (Viewpoint Life Sciences, Lyon, France) left for 5 min in the light for acclimation, and then their movement was recorded for 12 min. During this period, light/dark cycles of 2 min were performed for stimulation/inhibition of fish movement. Total distance moved by fish (TD) in each period of time was calculated as described by Andrade et al. (2016). A transparent background and a threshold of 88 were set. Three types of movements were considered: low velocity (hypoactivity and inactivity) for movements below 8mm/s; medium velocity for movements between 8 and 40 mm/s (normal activity) and high velocity (hyperactivity) for movements above 40 mm/s. Fish path angle was also recorded and 8 classes of angles were defined as described by Zhang et al. (2017) and represented in Fig. S2. Angles were calculated through the vector of fish swimming direction and the turn path performed by the animal. Angles of low amplitude (classes 4 and 5) indicate straightforward movements while high amplitude angles (1, 2, 7 and 8) indicate movements with significant changes of direction and suggest erratic swimming behaviour, a measure of stress. Angles of medium amplitude (class 3 and 6) indicate average turns.

2.4 Biochemical analysis

On the day of analysis, samples obtained as described in section 2.3 were defrosted in ice, 1 mL of ultra-pure water was added for each 100 mg of fish tissue and homogenized with a sonicator (KIKA Labortechnik U2005 Control). Samples were then divided in aliquots (300 µL) to determine available energy reserves (Ea) and energy consumption (Ec). The remaining volume was doubled with 0.2 M K-phosphate buffer, pH 7.4, centrifuged (4◦C, 10000 *g*, 20 min) to isolate the post mitochondrial supernatant (PMS) and used to determine enzymatic activities (TG; GST; CAT; AchE and LDH). The enzymatic activity was analyzed in 96 wells microplates, in quadruplicate using spectrophotometric methods (Thermo Scientific Multiskan Spectrum, USA).

2.4.1 Cellular energy allocation (CEA)

The CEA was calculated as the relation between Ea and Ec following the Verslyske et al. (2003) formula and expressed in mJ/mg wet weight. The Ea consists in the sum of estimated proteins, carbohydrates and lipids content by converting them into energetic equivalents through the energy combustion (proteins: 24 kJ/g; carbohydrates: 17.5 kJ/g and lipids: 39.5 kJ/g) as described by De Coen and Janssen (1997). Total protein, carbohydrates and lipids content were determined as described by Rodrigues et al. (2015). Briefly, protein content was measured at 520 nm using the Bradford (1976) method and bovine serum albumin to determine the standard curve; carbohydrates were measured at 492 nm using glucose as standard curve and lipids were measured at 375 nm using tripalmitin to determine the standard curve. The Ec was calculated based on electron transport energy (ETS) following De Coen and Janssen (1997) method. The Ea and Ec activities were expressed in mJ/mg wet weight and mJ/mg wet weight/hour respectively.

2.4.2 Enzymatic activities

The enzymatic activity of TG, GST and CAT, were determined as described by Rodrigues et al. (2015) and ChE and LDH activity were determined as described in Domingues et al. (2010). Briefly, TG activity was measured at 412 nm according to Tietze (1969) and Baker et al. (1990); GST activity was determined at 340 nm according to Habig and Jakoby (1981); CAT activity was measured at 240 nm according to Clairborne (1985); ChE activity was determined at 414 nm according to Ellman et al. (1961) and LDH activity was measured at 340 nm, according to Vassault (1983). Protein quantification was determined at 595 nm using γ-globulin as standard, according to Bradford (1976) method.

2.5 Molecular analysis of microbiome

2.5.1 DNA extraction

Total fish gut DNA was extracted individually (1 gut = 1 extraction) from samples collected as described in section 2.2 using the commercial kit PowerSoil® DNA isolation kit (MOBIO laboratories, CA, USA), according to manufactures instructions.

To water bacterial communities' analysis, in the end of experience, 100 mL of each water condition (in triplicate) were filtered using 0.22 µm hydrophilic PVDF durapore membrane filter (Merck Millipore; Massachusetts, EUA). Total DNA extraction was performed as described by Henriques et al. (2004) using the commercial kit Genomic DNA Purification kit (Thermo Fisher Scientific; Massachusetts, EUA).

2.5.2 Polymerase chain reaction (PCR)

The 16S rDNA V3 region of the fish gut and water bacterial communities was amplified through a nested polymerase chain reaction (nested PCR) and single PCR respectively. The PCR mixture (25µL) contained: nuclease-free water (16.25 µL), NZYTaq 2x Green Master Mix (6.25 µL; 2.5 mM MgCl2; 200 µM dNTPs; 0.2 U/µL DNA polymerase) (NZYTech, Portugal), the primers 338F (5′-GACTCCTACGGGAGGCAGCAG-3′) (0.75 µL of a 10 µM solution) and 518R (5′-ATTACCGCGGCTGCTGG-3′) (0.75 µL of a 10 µM solution) with a GC clamp attached to the forward primer (Muyzer et al. 1993). The temperature profile started with the template denaturation (94 ºC for 5min) followed by 35 cycles denaturation (92 ºC for 30 s), annealing (55 ºC for 30 s) and extension (72 ºC for 30 s), finishing with a final extension step (72 ºC for 30 min). The nested-PCR was conducted with the same PCR mixture and temperature profile as the first PCR. The reactions were conducted in Bio-Rad Thermal Cycler (Bio-Rad Laboratories, CA, USA) and included a positive and negative control.

PCR products were verified by electrophoresis (1.5% agarose gel) and stained with ethidium bromide (15 min). Then pools of 5 PCR products per replicate (5 replicates per treatment) were prepared and used for DGGE analysis.

2.5.3 Denaturing gradient gel electrophoresis (DGGE)

A polyacrylamide gel (8%) with a linear denaturing gradient ranging from 35% to 60% (100% representing 7 M Urea and 40% formamide) was used to observe PCR amplicons profile. Also, a DGGE marker composed by 8 bands was applied to gel extremities as a reference of the analysis quality (Henriques et al. 2004). Electrophoresis was conducted in DCode™ System (Bio-Rad, Hercules, CA, USA) following Henriques et al. (2006) method. Gel images were captured by the Molecular Imager® Gel Doc™ XR + System with Image Lab™ Software (Bio-Rad, Hercules, CA, USA). DGGE band patterns were analyzed through Bionumerics Software (Applied Maths, Belgium) and cluster analysis obtained by UPGMA method (group average method) applying DICE correlation.

2.6 Chemical analysis

Methanol and acetonitrile (LiChrosolv® Hypergrade) were purchased from Merck (Darmstadt, Germany). Formic acid (LC/MS grade) to acidify the mobile phases was purchased from Labicom (Olomouc, Czech Republic). Ultrapure water was produced using an Aqua-MAX-Ultra System (Younglin, Kyounggi-do, Korea). The analytical standards were of high purity (mostly 98%). The internal standard tramadol (OCD3) was acquired from Lipomed AG. A stock solution of each compound was prepared in methanol at a concentration of 1 mg/mL. A spiking mixture of each was prepared by diluting the stock solution with methanol to a final concentration of 1 μg/mL. All stock and spiking solutions were stored at -20 °C.

2.6.1 Sample preparation

The thawed water samples were filtered through a syringe filter (0.45 μm, regenerated cellulose, Labicom, Olomouc, Czech Republic). All water samples were spiked with the internal standard to achieve a concentration of 250 ng/L.

All sets of samples were analyzed by automated online solid phase extraction liquid chromatography with a heated electrospray ionization (H-ESI) high resolution (HRMS) and tandem mass spectrometry detection (on-line-SPE-LC/HRMS).

2.6.2. Analytical procedure

An Accela 1250 LC pump (Thermo Fisher Scientific, San Jose, CA, USA) coupled with an Accela 600 LC pump (Thermo Fisher Scientific) and a HTS XT-CTC autosampler (CTC Analytics AG, Zwingen, Switzerland) was used. A hybrid quadrupole/orbital trap Q-Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) coupled with Accela 1250 LC and Accela 600LC pumps (Thermo Fisher Scientific, San Jose, CA, USA) and HTSXT-CTC autosamplers (CTC Analytics AG, Zwingen, Switzerland), were used to separate and detect the target analyte. Hypersil GOLD aQ column (50 mm length, 2.1 mm i.d., 5- µm particles particle size (Thermo Fisher Scientific, San Jose, CA, USA), preceded by a guard column (10 mm × 2.1 mm i.d, 3 mm particles) of the same packing material and from the same manufacturer, was used for chromatographic separation of target compounds.

A heated electrospray ionization (H-ESI) was used to ionize the target compounds. The spray voltage was set to static: positive ion (3500 V), negative ion (2500 V). Nitrogen (purity >99.999%) was used as a sheath gas (40 arbitrary units), auxiliary gas (10 arbitrary units) and sweep gas. The vaporizer was heated to 270 °C and the capillary to 250 °C. A detailed description of pharmaceutical standards and LC gradients has been provided by Fedorova et al. (2014). Data were evaluated by the Trace Finder (Thermo Fisher Scientific, San Jose, CA, USA).

2.7 Statistical analysis

A linear mixed effects analysis using maximum likelihood estimation was performed to investigate the effect of OTC, light/dark stimulus and time (cycle) on the behavioral endpoints (swimming activity parameters). In the mixed model, concentration of the toxic, stimulus and time were considered fixed effects, while fish ID was considered a random effect, to take into consideration repeated measures. Interactions between fixed effect factors were also included in the model. Further post-hoc tests were conducted using Bonferroni procedure. The analysis was computed using statistical software IBM SPSS Statistics for Windows, Version 24.0 (IBM Corp. 2016).

Sigma plot V.12.5 (SysStat software Inc., CA, USA) was used for one-way ANOVA to determine effects in biochemical activity. When normality test failed, a Kruskal-Wallis test followed the appropriate post hoc (Dunns or Holm-Sidak) was used. A significant level of 0.05 was considered to deduce statistical significance.

DGGE band matrix was analyzed in PRIMER v6 software (Primer-E Ltd., Plymouth, UK) (Clarke and Gorley 2006). Band position and intensity was used to calculate species richness, the Shannon-Wiener diversity (Shannon and Weaver 1964) and Pielou's evenness index. A one-way ANOVA followed by the Dunnett's method was used to discriminate differences in indexes. Further, two-dimensional Principal Coordinate Analysis (PCoA) was performed based on Dice index. Using DGGE band matrix, differences in bacterial communities' structures between treatments were also evaluated through PERMANOVA based on 999 permutations.

3. Results

The present study shows the effects of OTC in zebrafish at multi-level endpoints. Chemical analysis was performed to determine the OTC degradation along time. Nominal concentrations varied between 87% and 170% after 0 h of degradation (initial concentration) and between 58% and 103% after 96 h of degradation (Table S1).

During the exposure period, no mortality occurred in any treatment and no differences in the fish growth rate were observed among treatments ($F= 1.180$; $p= 0.320$, data not shown).

3.1 Behavior assay

OTC effect on fish feeding behavior is shown in Fig. 1. OTC did not have a clear effect in the time for first feeding action-Ti, probably due to high variability of data. On the other hand, in the time to total food ingestion- Tf it is possible to observe an inverted U-shape response with an increase in Tf time at the lowest concentrations (0.1 µg/L: $F= 7.677$, $p= 0.001$) with some fish exceeding the feeding time.

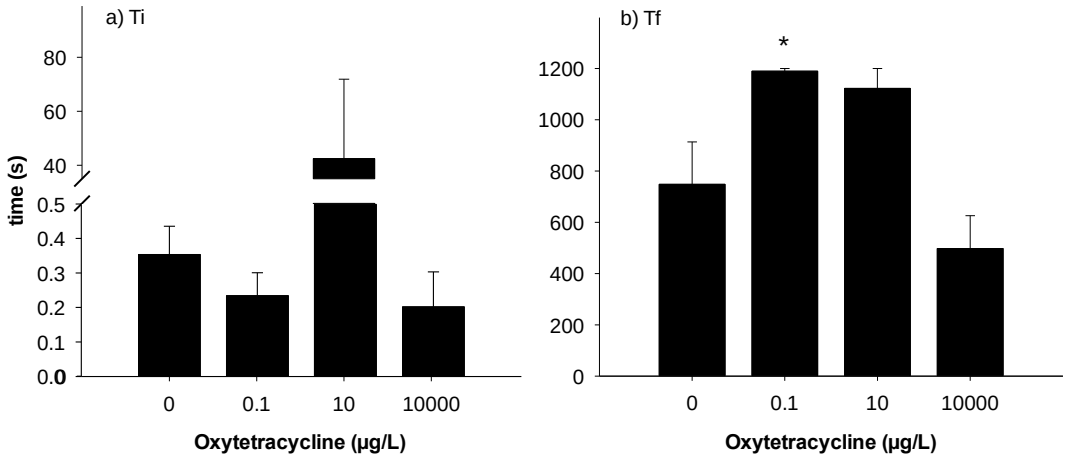

Fig. 1: Oxytetracycline exposure effect on: a) the time for the first feeding action (Ti) and b) the time for total food ingestion (Tf) (mean values ± standard error). Asterisks mean significantly different from the respective control ($p < 0.05$; Dunnett's test).

In the novel tank test, control fish seem to spend more time in the bottom of the aquarium than the exposed fish (Fig. 2 a) although this trend was not statistically significant (Table S2). The time fish took to start exploring the aquarium decreased at the lowest concentrations (Fig. 2 b; Table S2) and was similar to control at the highest. Moreover, fish exposed to OTC tend to enter the aquarium top zone more often although not statistically significative (Fig. 2 c; Table S2).

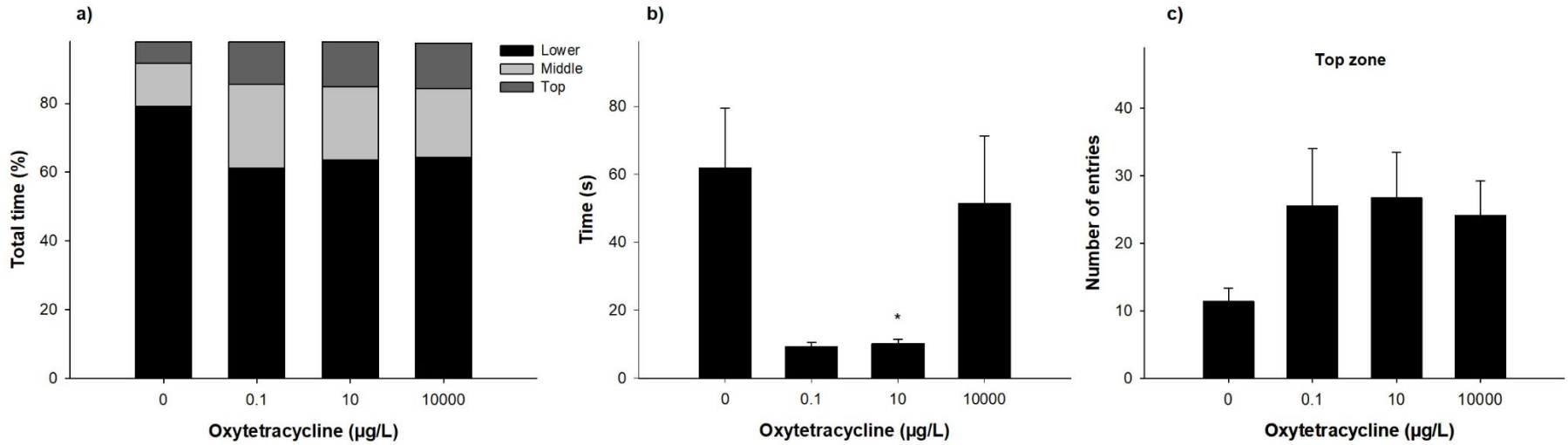

Fig. 2: Exploratory swimming activity of zebrafish exposed to oxytetracycline (mean values ± standard error): a) total time spent in each aquarium zone (lower; middle and top); b) time to fish start to explore the aquarium; c) number of entries in the top zone of the aquarium. Asterisks (*) indicate differences towards control ($p < 0.05$; Dunn's test).

In the light/dark swimming test control fish did not seem to react differently to light or dark when analyzing the distance swam by fish (Fig. S3; Table S3). However, regarding to type of movement, control fish respond to light/ dark stimulus through an increase in the % of distance swam during the dark period, at medium velocities (Fig.3; Table S3). Nevertheless, regardless of the light/dark stimulus, OTC exposure increased fish locomotion especially at concentrations of 0.1 and 10000 µg/L where distance swam by fish increased 35.1 and 30.4 % respectively (Fig. S3).

Effects of OTC on the locomotion became increasingly evident when discriminating movements in three classes (slow, medium and high velocity movements, Fig. 3). The distance swam in medium velocity movements decreased significantly with concentration ($p<0.001$) while distance swam in high velocity movements clearly increased with OTC ($p<0.001$; Table S4). This is more evident in the light periods. Worth to note that differences among light and dark were observed for the 3 types of movements (increased activity in the light for low and high velocity movements; increased activity in the dark for medium velocity movements). Furthermore, in the medium velocities movements an interaction was found between stimulus and concentration ($F= 3.321$; $p=0.021$): locomotion was strongly decreased by concentration in the light periods while in the dark no effects of the compound were perceived. In medium velocities movements we also observed an effect of cycle: the locomotor response increased along the cycle ($F= 6.645$; $p<0.001$).

The analysis of the time spent swimming in each of the 3 types of movements (Fig. S4) provided additional information on OTC effects. The time spent in slow movements (which actually also includes inactivity) increases with the concentration in light periods but not in the dark. This is however only partially supported by statistics which revealed a $p= 0.051$ for this interaction (Table S4). The time spent in medium velocity movements followed the same pattern as the distance with a dose dependent decrease in the time fish spent swimming in the light periods but not in the dark. The time fish spent swimming in high velocity movements increased with OTC concentrations ($F= 6.946$; $p<0.001$) regardless of the stimulus, however, unlike the distance moved in this type of movement, this effect did not follow a dose-dependent pattern (Fig. S4 c).

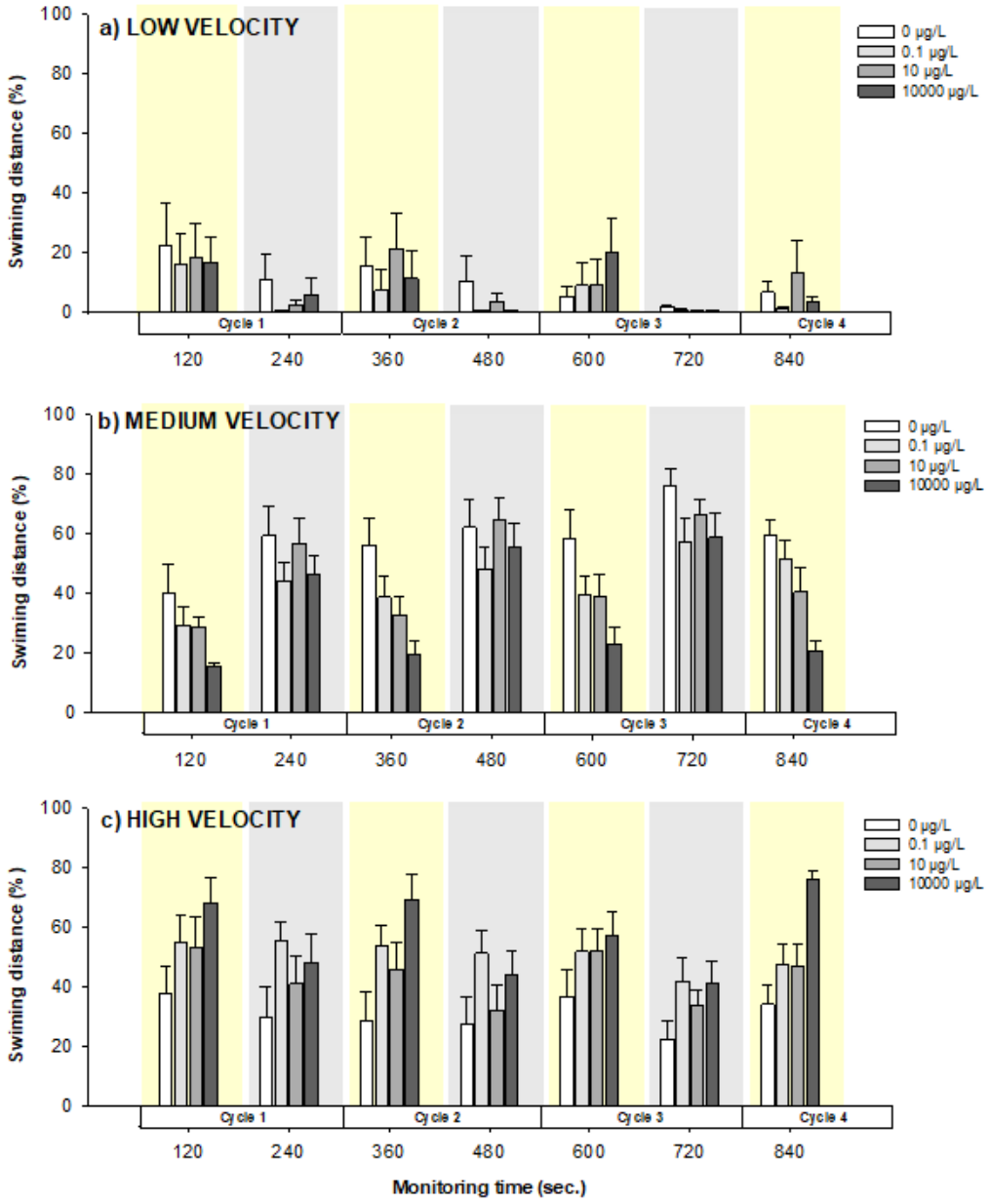

Fig. 3: Swimming distance travelled by zebrafish exposed to oxytetracycline according to the type of movement (mean values ± standard error): a) low velocity; b) medium velocity and c) high velocity. Yellow zones represent light periods and grey zone represents dark periods. Horizontal bar indicates a cycle: transition from light to dark period. Statistical analyses are presented in Table S3 and S4.

The measurement of the angles during the fish path showed that, in the control, fish swim predominantly in a trajectory with angles between -10º and 10º (classes 4 and 5; See Fig. S5). Worth to note that within these classes a predominance (40 %) of positive angles (moving towards the right side: 0 to 10º- class 5) was observed in detriment of the negative angles (25%) of the same amplitude (0 to -10º- class 4). The transition of light/dark induced changes in the proportion of angles of classes 2 and 7 where a higher proportion of angles in these classes were observed in the light periods and a lower proportion in the dark periods. On the other hand, proportion of angles of smaller amplitude (class 5) decreased in the light and increased in the dark ($p= 0.045$). Regarding the effects of OTC, the total number of angles tends to decrease with the concentration in the light but not in the dark indicating an interactive effect between the concentration and the stimulus (Table S4; Fig. S5). This decrease is apparently due to the decrease in the angles of classes 4 and 5 ($p< 0.05$). Number of angles per distance travelled (mm) was then calculated to ease comparison and analysis revealed a dose dependent decrease in angles of small amplitude (classes 4 and 5) in light periods (Fig. 4). The high amplitude angles (classes 1, 2, 7 and 8) did not increase though.

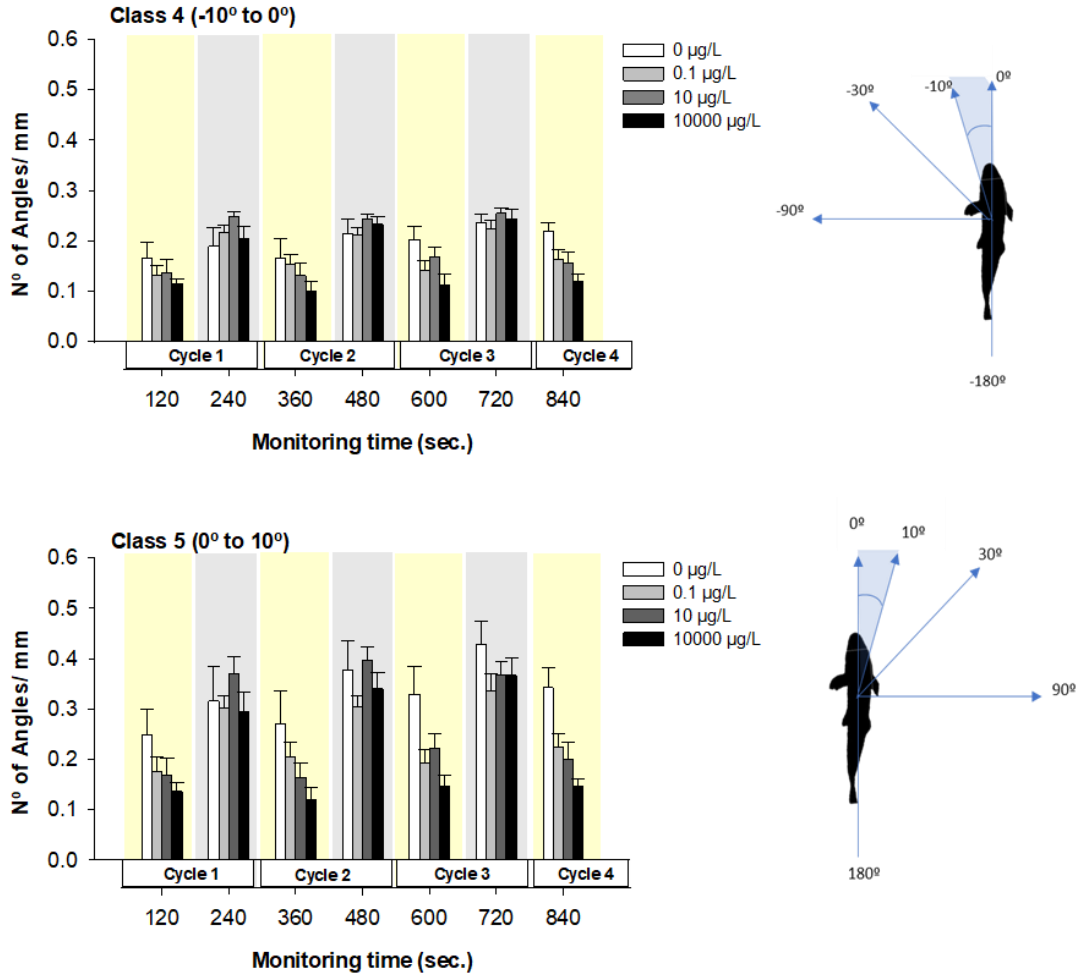

Fig. 4: Angle frequency (mean values ± standard error) and schematic representation of the respective angle. Yellow zone represents light periods and grey zone represents dark periods. Horizontal bar indicates a cycle: transition from light to dark period. The angles in figure are not at scale. Statistical analyses are presented in Table S3 and S4.

3.2 Biochemical analysis

The Ea fractions and the total Ea and Ec are represented in Table 1. Proteins and lipids are the major components of fish body followed by carbohydrates. Exposure to OTC seemed to significantly increase the contents of proteins (p= 0.013) and Ec levels (p= 0.003) and to significantly decrease the contents of lipids and Ea levels (p= 0.040; Table S5). Regarding to CEA (Fig. 5), a dose-dependent decrease was observed (F= 5.211; p=0.006). Similarly, the

exposure to OTC decreased the activities of oxidative stress enzymes especially at the highest concentrations (Table S5; Fig. 5). No effects of OTC exposure on AChE or LDH activities were found (Table S5; data not shown).

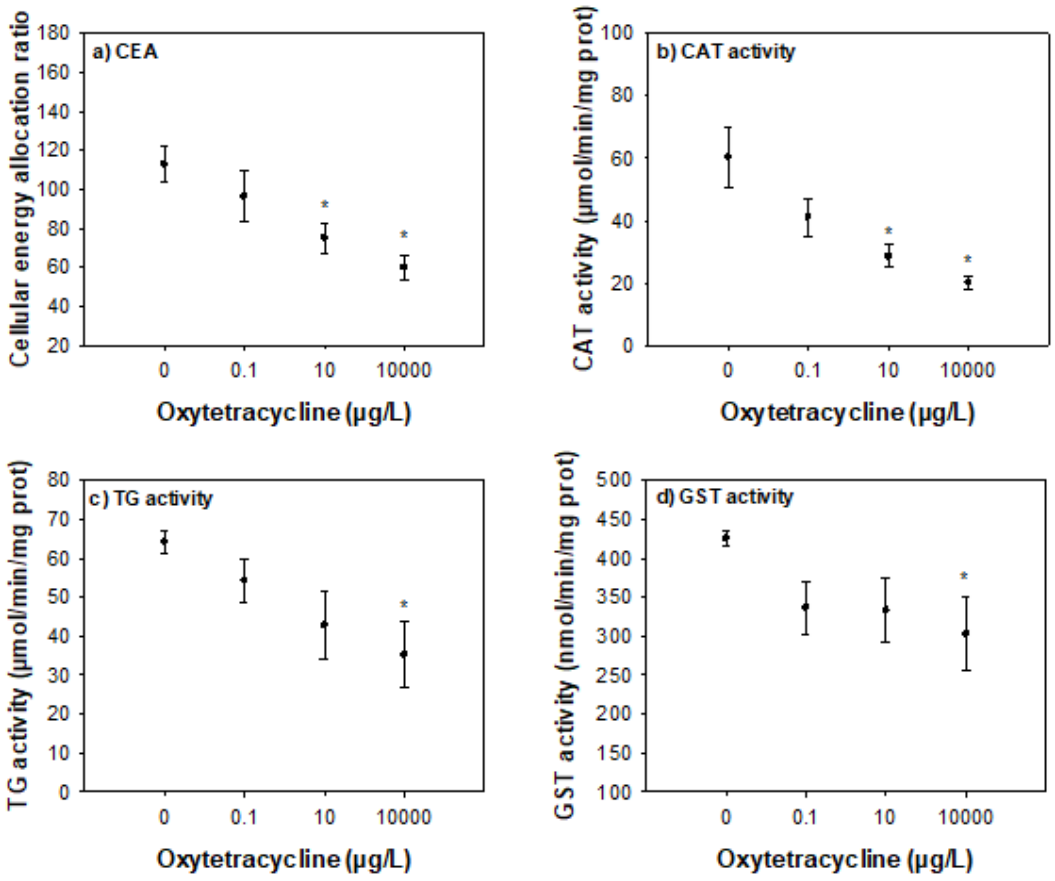

Fig. 5: Enzymatic activities (mean values ± standard error) of zebrafish exposed to oxytetracycline: a) cellular energy allocation (CEA) ratio; b) Catalase (CAT) activity; c) Total glutathione (TG) activity; d) Glutathione-S-transferase (GST) activity. Asterisks (*) indicate differences towards the respective control ($p < 0.05$; Dunnett's test).

Table 1: Energy available fractions; total energy available (Ea); sum of proteins, lipids and carbohydrates) and energy consumption (Ec). Values presented are mean ± standard deviation.

Samples (µg/L)	Ea fraction (%)			Ea (mJ/mg)	Ec (mJ/ mg/h)
	Protein	Lipids	Carbohydrates		
0	53.74 ± 8.65	44.40 ± 8.91	1.87 ± 0.41	7414.20 ± 1414.18	67.84 ± 18.16
0.1	67.89 ± 15.05*	33.04 ± 14.27*	2.05 ± 0.67	5931.93 ± 1683.62	65.37 ± 15.96
10	71.58 ± 6.15*	26.57 ± 6.44*	2.00 ± 0.70	5463.89 ± 968.35*	76.81 ± 20.04
10000	67.86 ± 6.26	29.60 ± 6.26	2.54 ± 0.36	5550.58 ± 1564.83	106.70 ± 30.56*

Asterisks (*) indicate differences towards the respective control ($p < 0.05$; Dunn's or Dunnett's test).

3.3 Molecular analysis of microbiome

The impact of OTC in both the bacterial communities of fish gut and exposure water was assessed. Cluster analysis showed that bacterial communities of both water and gut changed after OTC exposure, and that the variation was higher in the highest concentration tested (10000 µg/L). At this concentration both gut and water bacterial profiles form a distinct group, sharing around 50% and 45% of similarity, respectively, with the profiles from other concentrations (Fig. 6 a) and Fig. 7 a)). Concerning to Principal Coordinates Analysis (PCOA), although there is some variability among samples, the spatial separation of the samples corresponding to the highest concentration (10000 µg/L) from the other treatments remains evident (Fig. 6 b) and Fig. 7b)). PERMANOVA analysis of zebrafish gut bacterial communities' structure showed a significant effect ($p < 0.05$) of OTC exposure in the two highest concentrations tested (10 and 10000 µg/L). In water bacterial communities' structure, were also found statistically significant effects ($p = 0.01$) due to OTC exposure but only in the highest concentration (10000 µg/L).

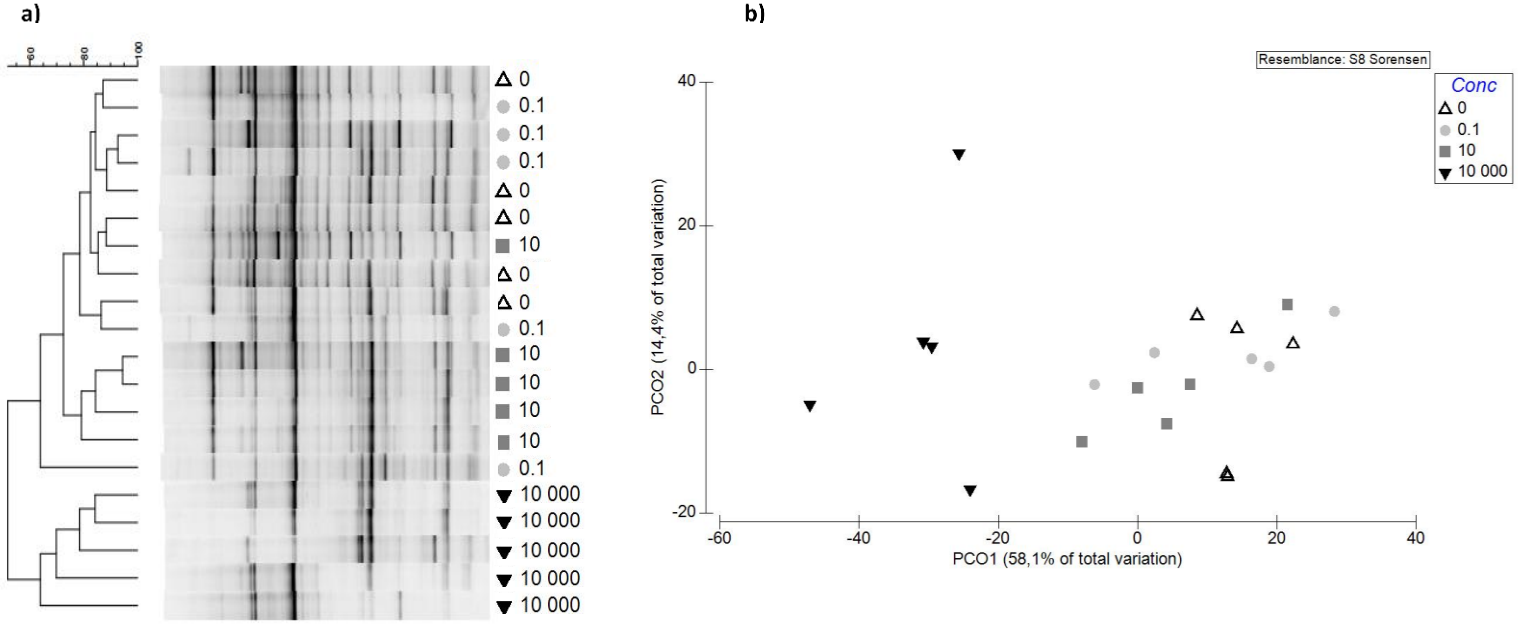

Fig. 6: PCR–DGGE analysis of the zebrafish gut exposed to oxytetracycline: a) UPGM dendrogram and b) PCoA based on Dice correlation similarity matrices. Concentrations are in µg/L.

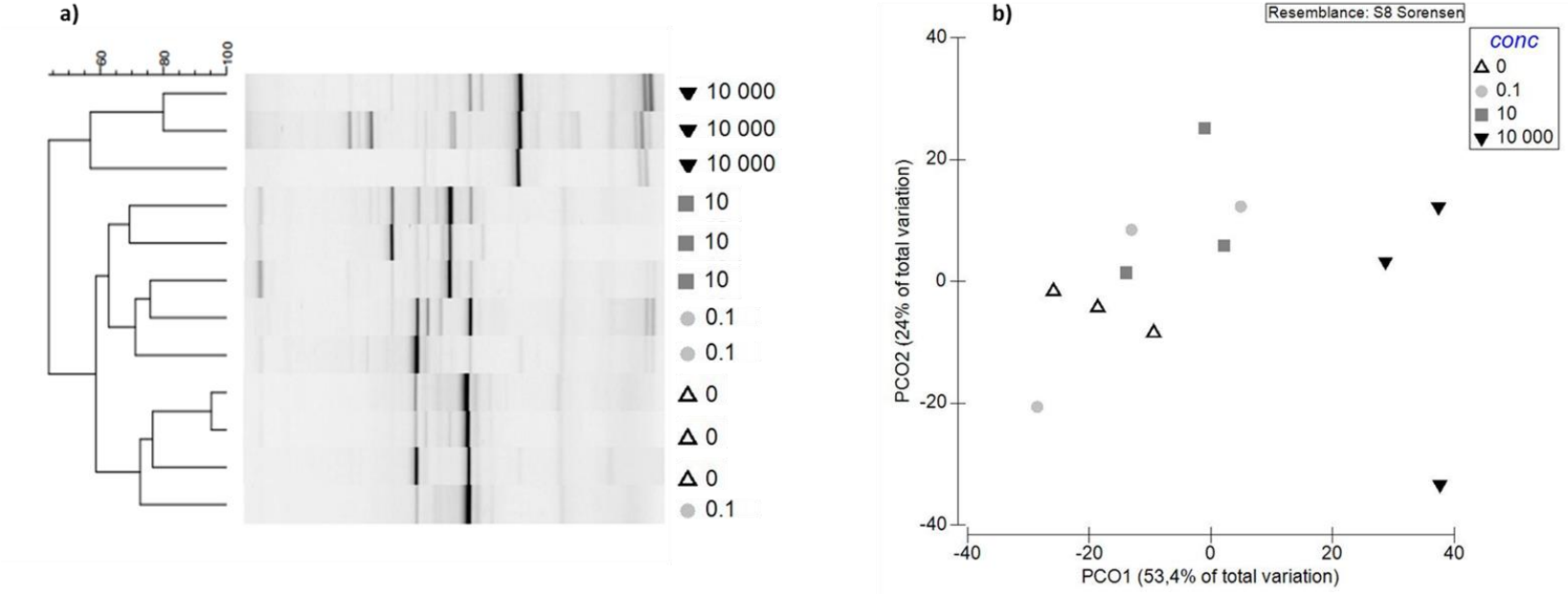

Fig. 7: PCR–DGGE analysis of the water bacterial communities exposed to oxytetracycline: a) UPGM dendrogram and b) PCoA based on Dice correlation similarity matrices. Concentrations are in µg/L.

The analysis of species richness (S) and diversity index (H) (Table 2) reveals a decrease in both parameters, regarding fish gut bacterial communities at the highest OTC concentration (10000 µg/L), with significant differences towards the control (S: $F= 25.272$, $p< 0.001$; H: $F= 18.488$, $p< 0.001$). On the other hand, no statistically significant differences were observed in species richness and diversity index in water bacterial communities. Pielou's evenness index (J) (Table 2) shows a high equitability in fish gut microbial communities, while in water bacterial communities was observed a high heterogeneity. Nevertheless, in both gut and water, J was not significantly affected by OTC exposure.

Table 2: Species richness (number of bands; 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 (DGGE based profile). Values presented per mean ± standard deviation.

Samples	Oxytetracycline (µg/L)	Species richness (S)	Shannon - Wiener diversity index (H')	Pielou's evenness index (J)
Zebrafish gut	0	21 ± 2	2.77 ± 0.14	0.91 ± 0.03
	0.1	18 ± 3	2.58 ± 0.17	0.89 ± 0.02
	10	17 ± 2	2.53 ± 0.22	0.89 ± 0.04
	10 000	10 ± 2*	1.94 ± 0.20*	0.86 ± 0.04
Water	0	11 ± 2	1.40 ± 0.18	0.58 ± 0.10
	0.1	12 ± 4	1.56 ± 0.32	0.63 ± 0.06
	10	14 ± 2	1.74 ± 0.20	0.66 ± 0.06
	10 000	19 ± 7	1.98 ± 0.62	0.68 ± 0.13

Asterisks (*) indicate differences towards the respective control ($p< 0.05$; Dunnett's test).

4. Discussion

In this work several parameters were used to determine fish physiological status (growth rate and feeding test) and anxiety/stress response (novel tank and light/dark swimming test) after a long-term exposure to OTC. Fish feeding behavior has been used to assess the fitness of the organisms, representing an important physiological process correlated to the growth, reproduction status and organism survival (Domingues et al. 2016; Sun et al. 2016). In this study, OTC did not seem to affect the fish perception of food but increased the time fish, from the lowest concentrations, required to eat all the food supplied. According to some authors (Toften and Norwegian 1997; Houlihan et al. 2002) this may be related with the fact that OTC turns food less palatable. The lack of the same response in fish exposed to the highest concentration is, however, unexpected. Nevertheless, reduction of food intake at lowest concentrations did not impact fish growth rate. Similar results were reported for zebrafish exposed to OTC for 6 weeks (Zhou et al. 2018) and for salmonids fed with OTC supplemented food for 65 days (Toften and Norwegian 1997). These last authors reported that after an initial decrease in food intake, fish seems to adapt to the compound and started to increase food ingestion.

The novel tank test and the light/dark test have been used to study anxiety-like behavior in zebrafish. The novel tank approach explores the zebrafish tendency for diving and remaining in the bottom of a new aquarium (unfamiliar space) before starting to explore the entire space (upper layers) (Champagne et al. 2010; Maximino et al. 2010). Some chemicals, namely anxiolytics, have the capacity to modify this behavior, and consequently, fish tend to explore the novel space sooner and spend less time in the bottom (Levin et al. 2007; Bencan et al. 2009). In our work, similar results were obtained, with OTC exposed fish tending to spend less time in the bottom and exploring the aquarium sooner than control fish. Nonetheless, similarly to the observed in the feeding test, this effect was not observed at the highest concentration. Although non-dose dependent patterns of response are characteristic of some anxiolytic compounds (Gerlai et al. 2000; Bencan et al. 2009; Ibrahim et al. 2014), data variability may have prevented the observation of clear trends and thus, future works should increase the number of samples (N) for data robustness.

A sudden switch from light to darkness, is frequently used in zebrafish larvae as a startle to test stress, anxiety-like behavior or neuronal disruption when embryos are exposed to chemical stress (Wang et al. 2014; Peng et al. 2016; Henriques et al. 2016). In zebrafish larvae, the startle response is characterized by a sudden increase in larvae movement (hyperactivity) (Burgess and Granato 2007). In adults, freezing, hyperactivity and erratic movements may occur as a response to stress (Speedie and Gerlai 2008) but it is not clear from literature if the sudden transition from light to dark works as a startle. Our data suggest that response in adults is not as evident as in larvae. Apparently, videos of fish movement (not shown), show that adult fish seem to increase speed, freezing and erratic swimming in the light. Although this is also observable in the graphs, data variability prevented full confirmation of these trends. Nevertheless, results suggest a slight higher stress response in the light contrary to the verified in the larvae. The dark preference by zebrafish was observed in other behavior studies where zebrafish were allowed to choose between light or dark compartments (Serra et al. 1999; Maximino et al. 2007). This behavior may not reflect what is observed in the natural environment as in the lab a higher light intensity is used when compared to nature where fish inhabiting small streams and ponds are shaded by trees and vegetation above the water and/or by aquatic plants.

OTC exposed organisms seem to increase stress behavior when compared to control in light periods (increase in distance moved particularly in high velocity movements (hyperactivity) and longer freezing moments). In addition, the increased swimming distance in high velocity movements was not accompanied by an increase of time swimming in high velocity movements which was constant. This behavior means that within high velocity movements fish swam faster as concentration increased. Angle data revealed a dose dependent decrease of low amplitude angles (class 4 and 5). This result was expected if accompanied by an increase in large amplitude angles, which was not verified (it would have shown erratic swimming). In this case we think that high speed movements performed in the light at high OTC concentrations may have prevented zebrabox to detect angles in straightforward movements.

Behavior alterations identified in our work suggest neuronal disruption by OTC as also reported in mammals (Snavely and Hodges 1984; Said et al. 1995). However, the lack of

effects in ChE activity, suggests that the cholinergic system is not involve in the toxicity mechanism. Photo-sensibilization is indicated by some authors as an effect of OTC and justifies the increased stress response to the light stimulus (Mark Stacell & David G. Huffman 1994; Roberts et al. 2017).

Biochemical markers are a sensitive tool, responsive to many types of chemicals (Smolders et al. 2003; Verslycke et al. 2003) while energy metabolism related parameters like CEA, have also been considered important to determine organism energy status. The body of adult zebrafish is mainly constituted by proteins followed by lipids and carbohydrates (Smolders et al. 2003). Lipids and carbohydrates are a fast source of energy being firstly consumed when organisms are under chemical stress. Moreover, when organisms are exposed to a moderate stress condition, it is expected an increase in protein synthesis triggered by defense processes (e.g. chemical detoxification and antioxidant defense), while the other sources of energy decrease (Smolders et al. 2003). This agrees with the obtained results (lipid content decrease and proteins content increase). Moreover, the Ec increase reflects a general response to the chemical exposure (Olsen et al. 2007; Gandar et al. 2017; Meireles et al. 2018) or increased basal metabolism due to cellular energy demand (e.g: detoxification processes) (De Coen and Janssen 1997; Gandar et al. 2017). Thus, in this work, we would expect that the observed Ec increase could be due to the triggering of the antioxidant system but, instead, decreased activities of the enzymes TG, GST and CAT were observed. According to Massarsky et al. (2017) the response of antioxidant enzymes depends on the intensity of the oxidative stress. Under extreme oxidative stress, overwhelming of the antioxidant defense system may occur, leading to proteins, lipids and DNA damage. Hence, the effects observed in our study may reflect oxidative damage due to the long-time exposure to OTC. In literature, several authors reported an increase in antioxidant defense in some fish species which were exposed to OTC for a short period of time (Elia et al. 2014; Nakano et al. 2018). On the other hand, after a long exposure period to OTC, a decrease in enzymatic activity was observed, for instance in rainbow trout (Yonar et al. 2011), silver catfish (Pês et al. 2018) and zebrafish (Zhou et al. 2018) which is in agreement with our results. Thus, our data suggest that after a long-time exposure OTC seems to induce oxidative damage in zebrafish probably due to OTC

accumulation, inducing tissue damage and consequently a decrease in the activity of this enzymes.

The study of organisms' microbiome changes has been highlighting the impact of bacterial communities in hosts health (Dimitroglou et al. 2011; Gioacchini et al. 2014; Banerjee and Ray 2017). Since they are designed to kill bacteria, antibiotics are expected to directly affect the microbiome, however long-term consequences for the host are still unclear (Croswell et al. 2009; Liu et al. 2012). In this study, a strong impact in fish gut bacterial communities structure was observed in zebrafish exposed to the highest concentration of OTC. Furthermore, a reduction on species richness and diversity was also observed. OTC is a broad-spectrum antibiotic, hence, a change in bacterial communities' diversity after antibiotic exposure was expected. It is known that under antibiotic pressure, some bacteria may present intrinsic or develop/acquire antibiotic tolerance or resistance mechanisms. These bacteria will be more adapted and resist better to antibiotic pressure. The changes in bacterial communities' structure observed in our work, may then represent an increase in tolerant or resistant bacteria and a decrease in sensitive or susceptible bacterial communities.

Similarly to fish gut, water bacterial communities' structure was also impacted by the presence of OTC. Our results indicate a clear effect in water bacterial communities at the highest concentration of OTC (10000 µg/L) although changes in richness and diversity were not observed. Changes in water bacterial communities due to antibiotic exposure were already reported (Huerta et al. 2013; Novo et al. 2013). Hence, since in our work OTC exposure was made via water, it was also expected a modulation in water bacterial communities. Moreover, due to aquatic organism's closest contact with the surrounding environment, water bacterial communities may also shape the organism's microbiome. Also, the shift of bacterial communities' may lead to a change in the biological function of the microbiome. Future works should assess the bacterial phylogenetic groups affected by OTC exposure as well as the impact of these changes to the host and environmental equilibrium.

5. Conclusion

In this work, the long-term impact of OTC in zebrafish was studied. At behavioral level, boldness increase (manifested by increased exploratory behavior of a new environment) was observed in fish exposed to low OTC concentrations. Moreover, changes in fish swimming pattern were observed in light periods (increased stress response: hyperactivity and freezing) probably due to photo-sensibility conferred by OTC exposure. In nature, effects in behavior parameters may lead to direct feeding, reproductive of predator-prey interaction impairment ultimately compromising population fitness. Effects at biochemical level suggest that long-term exposure to OTC interfere with cellular energy allocation mainly by reducing lipids levels and increasing energy consumption. Moreover, evidences of oxidative damage were also observed (reduced levels of TG, GST and CAT). The analysis of water and gut microbiome revealed changes in the structure and diversity of bacterial communities potentially leading to changes in communities' biological function, with higher ecological effects. Also, long-time exposure to low chemical concentrations may carry on antibiotic resistant bacteria selection, rising environmental and health risk concern.

Some of the effects were observed at the lowest concentration tested, 0.1 µg/L which is a concentration already detected in the environmental and thus clearly demonstrating the need of a serious ecotoxicological assessment of OTC effects on non-target aquatic organisms.

6. Acknowledgements

This work was supported by CESAM (UID/AMB/50017 - POCI-01-0145-FEDER-007638), FCT/MCTES through national funds (PIDDAC), and the co-funding by the FEDER, within the PT2020 Partnership Agreement and Compete 2020. Support was also given by FCT to Isabel Henriques (FCT Investigator Programme – IF/00492/2013), to Ana Rita Almeida (PhD grant PD/BD/127811/2016), to Inês Domingues (Post-Doc grant

SFRH/BPD/90521/2012) and to Marta Tacão (Post-Doc grant SFRH/114855/2016). Support was also given to O. Golovko and V. Zlabek by the Ministry of Education, Youth and Sports of the Czech Republic through projects “CENAKVA” (No. CZ.1.05/2.1.00/01.0024), “CENAKVA II” (No. LO1205 under the NPU I program) and by the Czech Science Foundation (No. 16-06498S). The authors wish also to thank to Catarina Amorim for all the support in DGGE data analysis.

7. Supplementary data

Table S1: % of Nominal concentration (0.1, 10, 10 000 µg/L) of OTC along time.

	% of Nominal concentration		
Time (h)	0.1	10	10 000
0	170	86.72	103.15
24	76	73.19	136.43
48	66	74.73	Nd
72	114	42.17	94.99
96	103	58.45	61.66

Nd. not determined

Table S2: Novel tank statistical analysis (one-way ANOVA) according to the time that fish spent in each area; the time that fish spent to start to explore the aquarium and number of entries in the top area.

Obs.	**Aquarium zone**	**DF**	**Test-value**	**p**
Time spent in each area	Bottom	3	F= 1.559	0.220
	Middle	3	F= 2.356	0.092
	Top	3	H= 1.718	0.633
Time to explore	-	3	H= 12.194	**0.007***
Number of entries	Top	3	H= 3.093	0.378

Obs.= Observation; Asterisks (*) indicate differences towards the respective control ($p < 0.05$).

Table S3: Results of the mixed model analysis (Stimulus*Cycle) of control fish swimming behavior: total distance; to the type of movement; time of swimming and path angles performed.

			Est.	**Cycle**
Distance	TD	DF	1	3
		F	0.052	0.372
		p	0.820	0.774
	Low velocity (distance)	DF	1	3
		F	1.009	1.618
		p	0.323	0.206
	Medium velocity (distance)	DF	1	3
		F	4.237	1.865
		p	**0.045***	0.155
	High velocity (distance)	DF	1	3
		F	1.201	0.130
		p	0.278	0.941
Time	Low velocity (time)	DF	1	3
		F	0.889	1.882
		p	0.351	0.155
	Medium velocity (time)	DF	1	3
		F	2.248	2.970
		p	0.141	**0.048***
	High velocity (time)	DF	1	3
		F	0.304	0.590
		p	0.584	0.626
Angles	Class 1	DF	1	3
		F	0.634	1.473
		p	0.434	0.248
	Class 2	DF	1	3
		F	2.306	1.536
		p	0.136	0.226

Class 3	DF	1	3
	F	5.488	4.542
	p	**0.023***	**0.010***
Class 4	DF	1	3
	F	2.242	1.717
	p	0.141	0.182
Class 5	DF	1	3
	F	4.210	1.685
	p	**0.045***	0.190
Class 6	DF	1	3
	F	5.357	5.139
	p	**0.025***	**0.006***
Class 7	DF	1	3
	F	0.005	0.850
	p	0.945	0.486
Class 8	DF	1	3
	F	0.587	1.640
	p	0.449	0.206

Conc. =concentration; Asterisks (*) indicate statistically differences ($p < 0.05$).

Table S4: Results of the mixed model analysis (Concentration*Stimulus*Cycle) of fish swimming behavior according to the type of movement; time of swimming and path angles performed.

			Conc.	**Est.**	**Cycle**	**Conc. x Est.**	**Conc. x Cycle**
Distance	TD	DF	3	1	3	3	9
		F	5.182	0.376	0.044	0.177	0.520
		p	**0.002***	0.541	0.988	0.912	0.857
	Low velocity	DF	3	1	3	3	9
		F	1.220	15.402	2.213	0.378	0.604
		p	0.304	**0.000***	0.092	0.769	0.791
	Medium velocity	DF	3	1	3	3	9
		F	14.785	66.047	6.645	3.321	0.337
		p	**0.000***	**0.000***	**0.000***	**0.021***	0.960
	High velocity	DF	3	1	3	3	9
		F	16.357	13.340	0.907	1.326	0.509
		p	**0.000***	**0.000***	0.440	0.267	0.866
Time	Low velocity	DF	3	1	3	3	9
		F	1.978	32.247	2.268	2.640	0.718
		p	0.119	**0.000***	0.086	**0.051**	0.691
	Medium velocity	DF	3	1	3	3	9
		F	6.477	64.786	8.717	4.157	0.701
		p	**0.000***	**0.000***	**0.000***	**0.007***	0.706
	High velocity	DF	3	1	3	3	9
		F	6.946	0.331	1.024	0.206	0.250
		p	**0.000***	0.566	0.385	0.892	0.986
Angles	Class 1	DF	3	1	3	3	9
		F	1.259	5.500	1.227	0.830	1.424
		p	0.291	**0.021***	0.305	0.480	0.189
	Class 2	DF	3	1	3	3	9
		F	2.125	24.466	2.009	0.425	0.635
		p	0.098	**0.000***	0.118	0.735	0.764

	DF	3	1	3	3	9
Class 3	F	9.865	46.971	7.096	0.832	1.425
	p	**0.000***	**0.000***	**0.000***	0.478	0.189
	DF	3	1	3	3	9
Class 4	F	4.328	93.502	2.886	4.510	0.402
	p	**0.005***	**0.000***	**0.039***	**0.004***	0.931
	DF	3	1	3	3	9
Class 5	F	11.172	100.735	3.236	3.121	0.437
	p	**0.000***	**0.000***	**0.025***	**0.027***	0.912
	DF	3	1	3	3	9
Class 6	F	7.535	44.467	7.283	1.204	1.616
	p	**0.000***	**0.000***	**0.000***	0.310	1.23
	DF	3	1	3	3	9
Class 7	F	1.860	5.370	0.585	0.555	0.771
	p	0.146	**0.024***	0.627	0.646	0.644
	DF	3	1	3	3	9
Class 8	F	1.691	7.410	1.591	0.966	1.191
	p	0.171	**0.007***	0.196	0.411	0.307

Conc. =concentration; Est.=stimulus; Asterisks (*) indicate differences towards the respective control ($p < 0.05$).

Table S5: Statistical analysis (one-way ANOVA) of biochemical assay: Ea; proteins; lipids; carbohydrates; Ec; CEA; TG; GST; AChE and LDH.

Biomarker	Test-value	p
Ea	F= 3.184	**0.040***
Proteins	H= 10.713	**0.013***
Lipids	H= 10.709	**0.013***
Carbohydrates	F= 1.911	0.153
Ec	F= 5.854	**0.003***
CEA	F= 5.211	**0.006***
TG	H= 8.060	**0.045***
GST	H= 8.733	**0.033***
CAT	H= 15.569	**0.001***
AChE	F= 0.639	0.597
LDH	F= 0.310	0.818

Asterisks (*) indicates differences towards the respective control ($p < 0.05$).

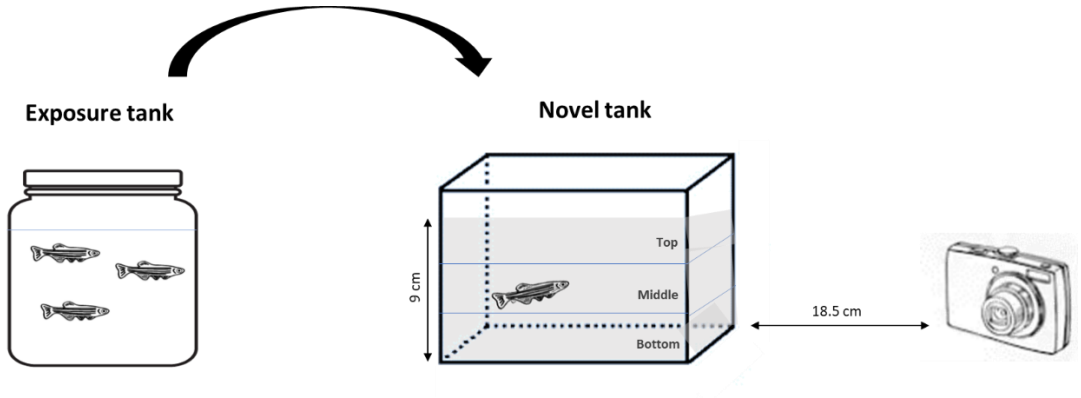

Fig. S1: Schematic representation of Novel tank /Exploratory swimming test. Image components are not at scale.

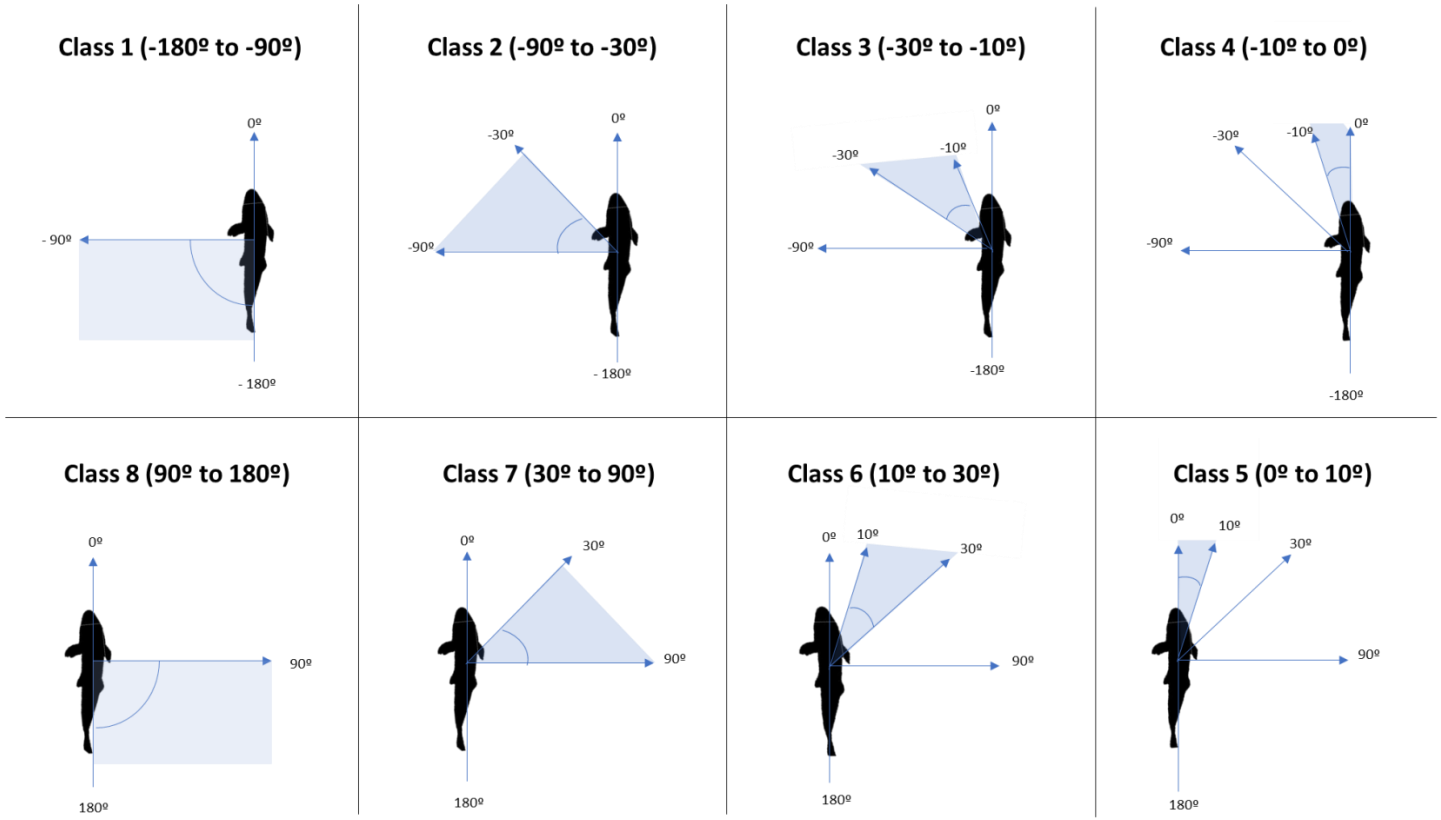

Fig. S2: Schematic representation of fish swimming direction changes by angle class. Negative angle represents left side rotation and positive angle represents right side rotation. The angles in figure are not at scale.

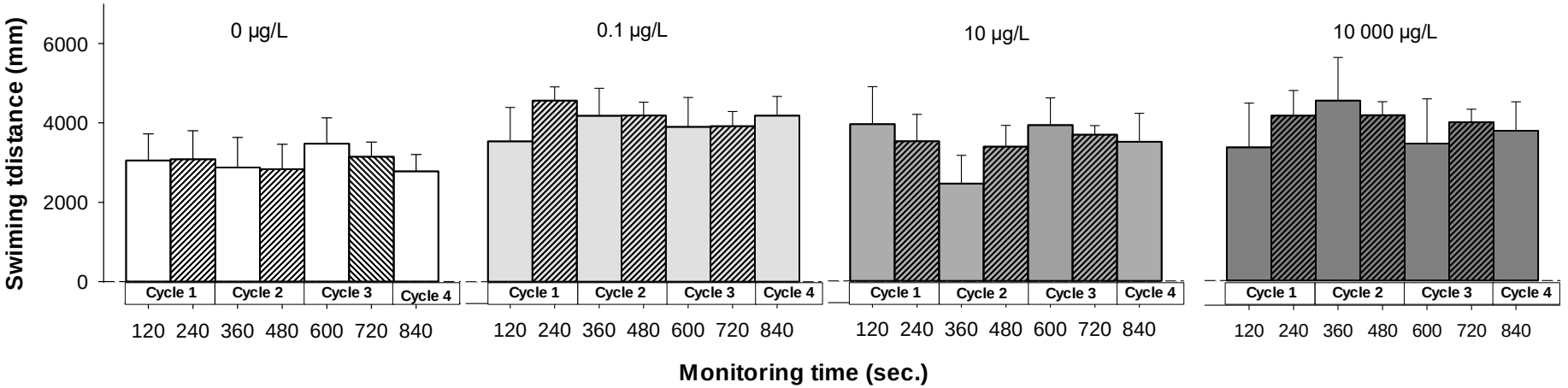

Fig. S3: Total distance traveled by zebrafish exposed to oxytetracycline (mean values ± standard error). Clean bars represent the light period and striped bars represent the dark periods. Horizontal bar indicates a cycle: transition from light to dark period. Statistical analyses are presented in Table S3 and S4.

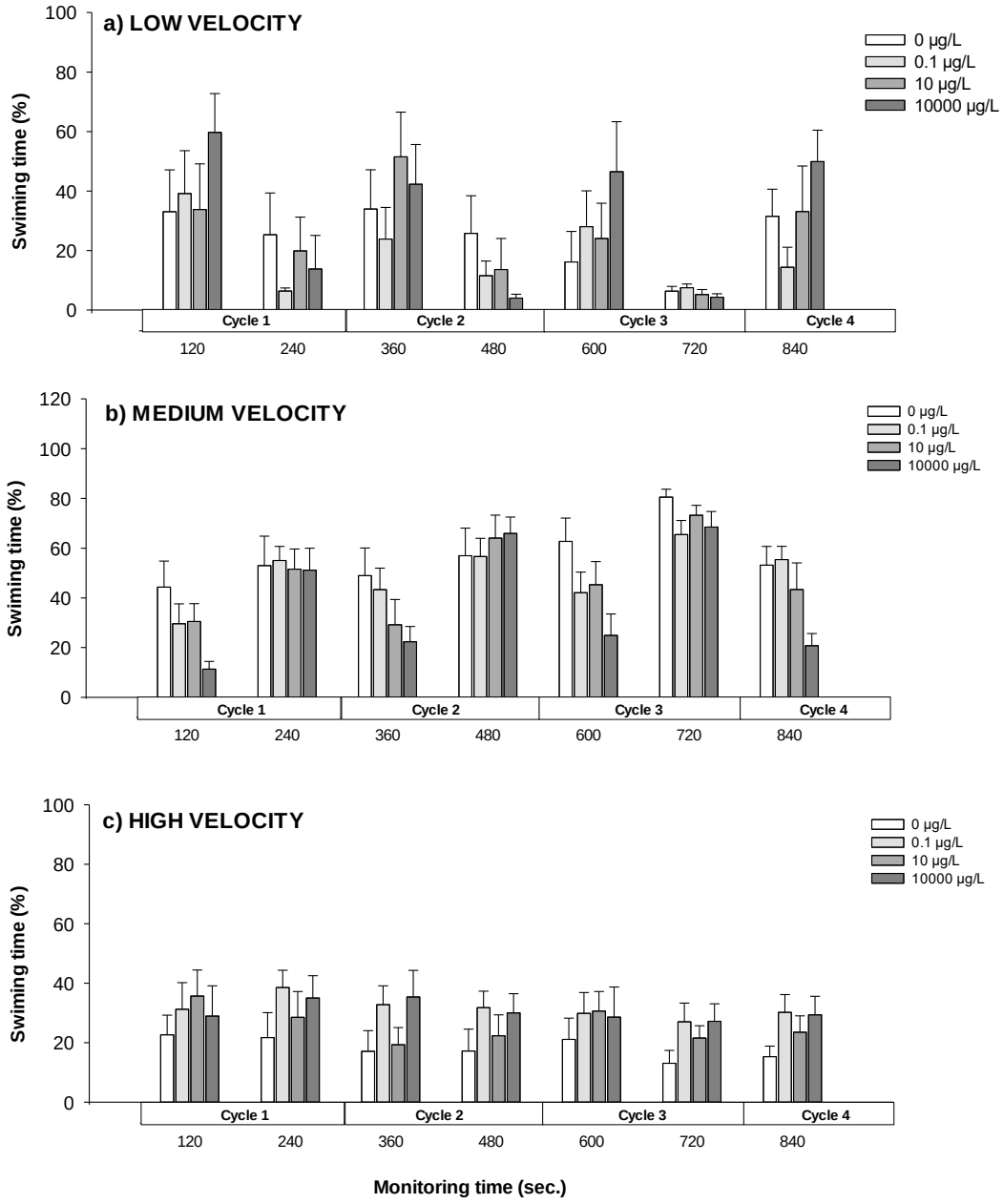

Fig. S4: Swimming time performed by zebrafish exposed to oxytetracycline (mean values ± standard error) within each velocity type: a) low velocity; b) medium velocity and c) high velocity. Yellow zones represent light periods and grey zone represents dark periods. Horizontal bar indicates a cycle: transition from light to dark period. Statistical analyses are presented in Table S3 and S4.

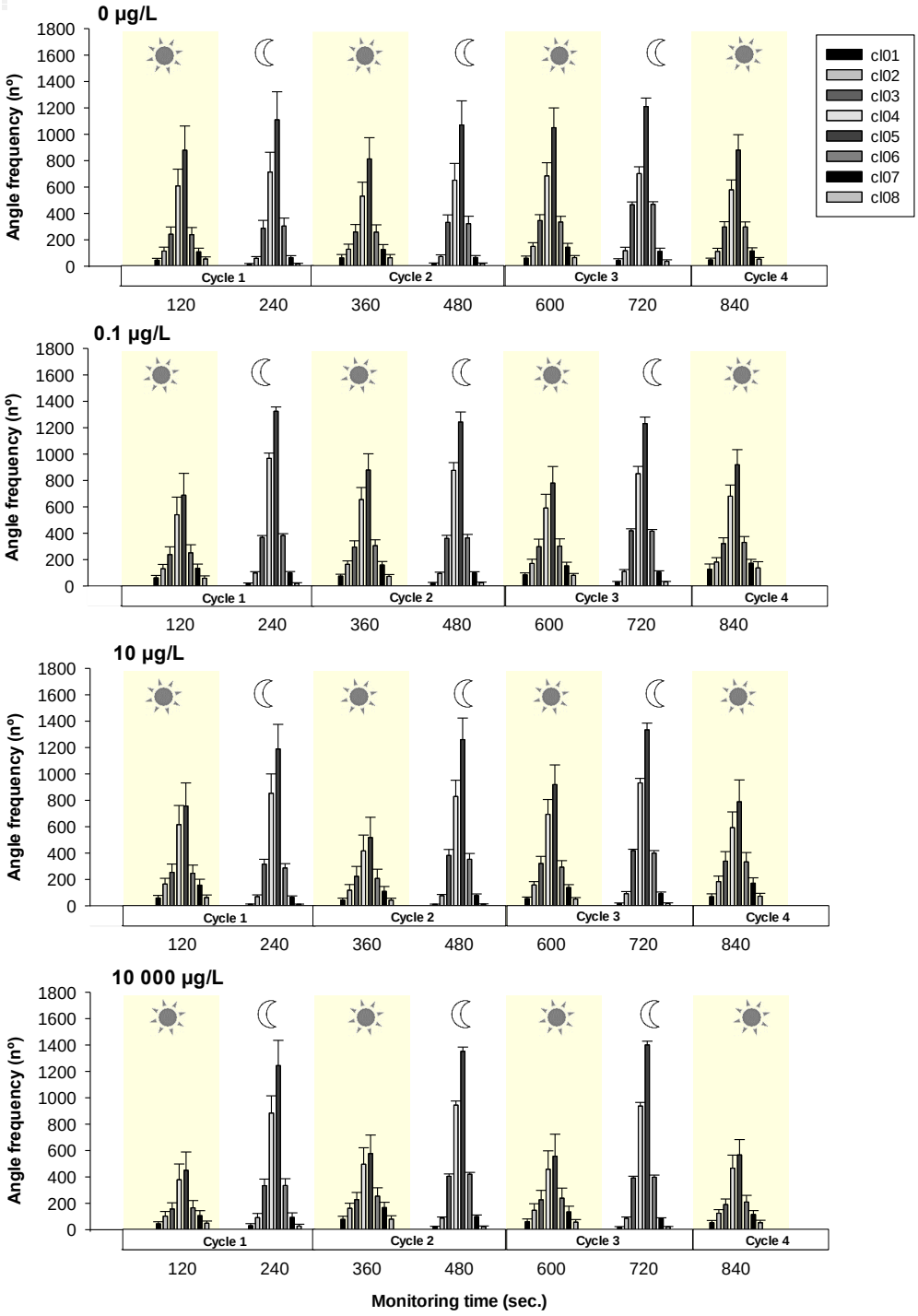

Fig. S5: Angles frequency (mean values ± standard error) performed by zebrafish exposed to oxytetracycline during the startle test. Yellow zones represent light periods and grey zone represents dark periods. Classe 1 (-180º a -90º) / Classe 2 (-90º a -30 º) / Classe 3 (-30 º a -10 º) / Classe 4 (-10 º a 0º) / Classe 5 (0 º a 10 º) / Classe 6 (10 º a 30 º) / Classe 7 (30 º a 90 º) / Classe 8 (90 º a 180 º). Statistical analyses are presented in Table S3 and S4.

Chapter 4

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

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 the rapidly 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 may reshape 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 only 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* are linked to some human infection disease. Nevertheless, in both gut and water, it was observed a decrease in Gamaproteobacteria, probably due to OTC mode of action. *In silico* functional metagenomic analysis revealed that OTC exposure selected general

detoxification mechanisms. In addition, the abundance of functional genes involved in Quorum Sensing (QS) increased under OTC exposure suggesting that QS may help bacteria to survive OTC stress. Thus, 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*

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). 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, immunological conditions, environmental stress and chemical exposure (Romero et al. 2014; Jin et al. 2017). 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, affect metabolism of fatty acid absorption, and modulate anxiety-related behavior (Rawls et al. 2004; 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) 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 (Maul et al. 2006; Mark Ibekwe et al. 2012). 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 (Chapter 3). 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). 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 (0, 10 and 10000 µg/L) for two months under a semi-static condition as described in the Chapter 3. 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 5'- CCTACGGGNGGCWGCAG -3' and Bakt_805R 5'- GACTACHVGGGTATCTAATCC -3') 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 (DNA template: 12.5 ng; Primers: 0.3 µM; total volume: 25µL) with the following conditions: denaturation step at 95 ºC for 3 min, 98 ºC for 20 s; extension step (25cycles) at 55 ºC for 30 s and 72 ºC for 30 s; final extension step at 72 ºC for 5 min) . 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) 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 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.

Sequences were processed using VSEARCH and QIIME pipeline 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). 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) 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') 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 from the R software.

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. Also, differences among bacterial communities' structure were assessed using PERMANOVA followed by the pairwise statistical possible permutation test Monte Carlo (MC) sampling with 999 unrestricted permutations.

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 S1). 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)).

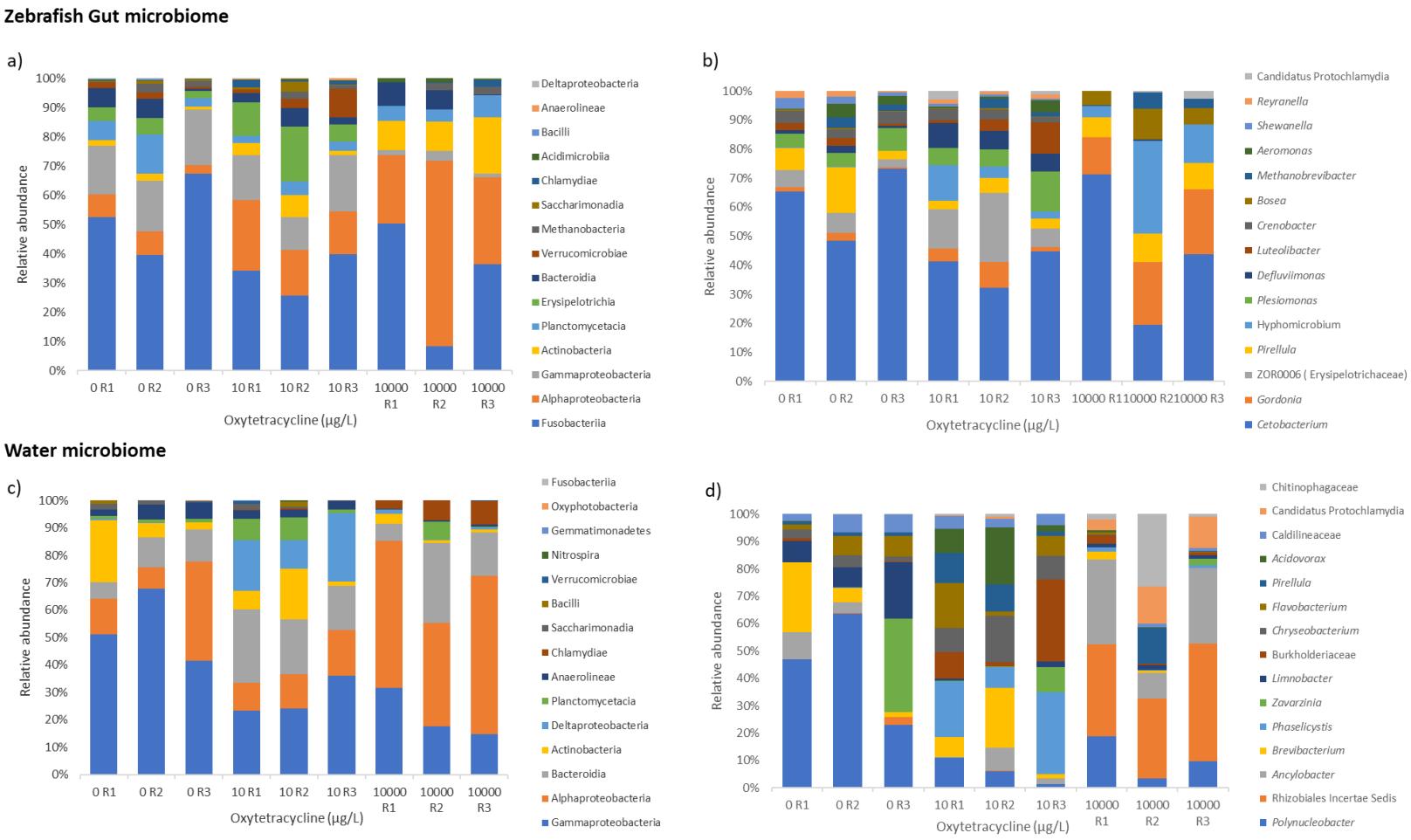

Fig. 1: Relative abundance of top 15 most abundant classes [a) and c)] and top 15 most abundant genera [b) and d)] of zebrafish gut and water microbiomes exposed to OTC. Genera classified as "uncultured" were indicated with the respective family name. Results of three replicates (n= R1, R2 and R3) are 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. Statistical analysis confirmed the significant difference between the control group and samples exposed to the highest antibiotic concentration (Table S2).

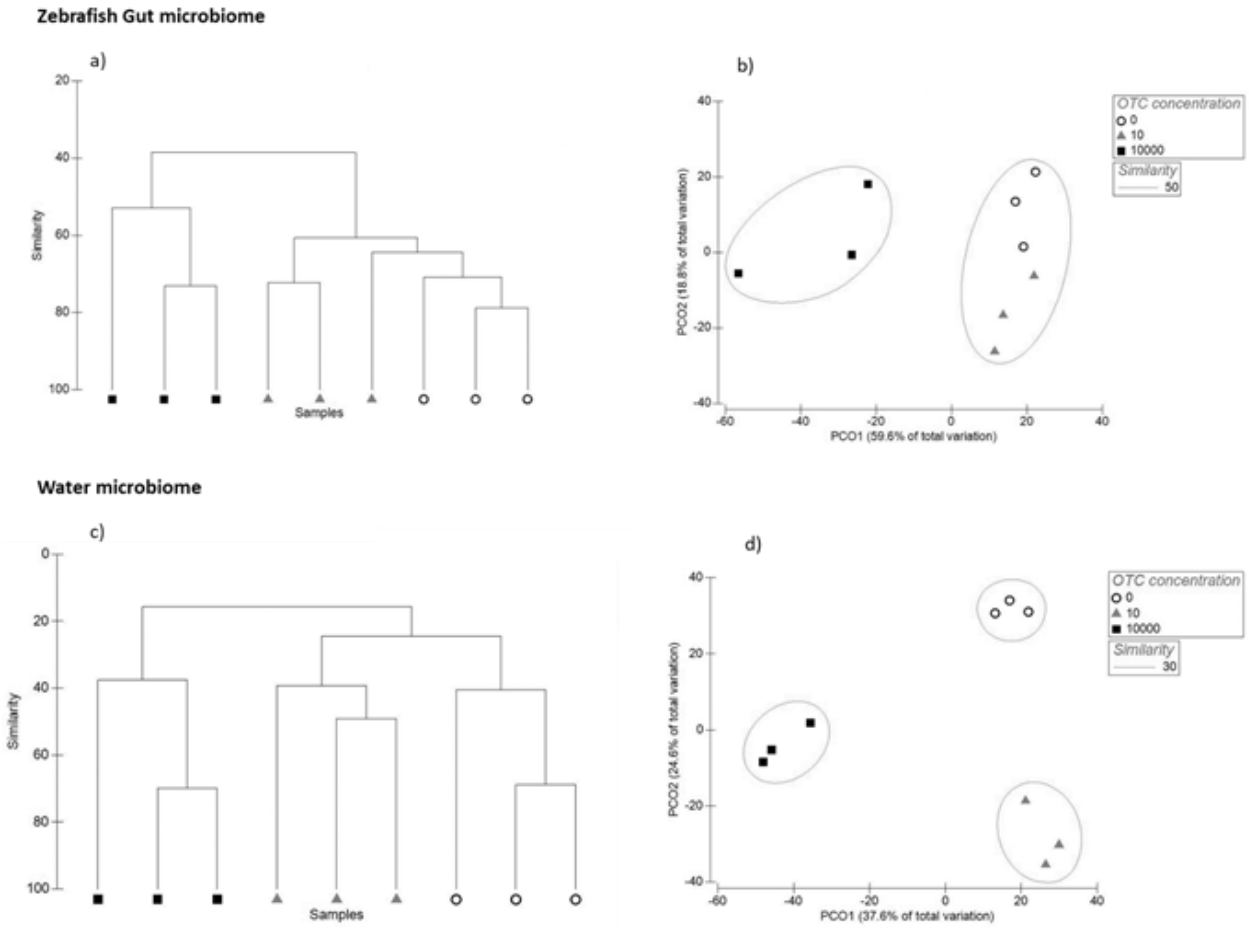

Fig. 2: Changes in zebrafish gut and water microbiomes exposed to oxytetracycline: a) and c) Dendrograms; b) and d) PCoA based on Bray-Curtis similarity matrices. Concentrations are in µg/L. Statistical analysis of differences among treatments is represented in Table S2.

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 S3). 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 S3). 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 S3).

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 (*) and bold color 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
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*

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

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*

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

The function inference analysis revealed that overall, the bacterial communities' metabolism may be affected by OTC. A significant decrease in glycolysis/gluconeogenesis and oxidative phosphorylation was predicted. Also, the biosynthesis of amino acids seems to be affected and a decrease in sequences of tRNA was predicted (Leu; Met; Arg; Val; Ser; Gly and Ala) was predicted (Table 3 and Table S4). From our results, it was also predicted an increase in the detoxification mechanisms, due to a predicted increase in the metabolism of xenobiotics by cytochrome P450; drug metabolism- cytochrome P450 and GST. On the other hand, a decrease in tetracycline resistance mechanisms, namely in the prevalence of determinants such as *tetB*, *tet35*; *tetV*; *tetM* and *tetO*, was predicted for samples exposed to OTC. Our results suggest that Quorum Sensing (QS) cellular communication may also be promoted upon antibiotic exposure (Table 3 and Table S4).

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 (SD). 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	Treatment (µg/L)
GUT	Metabolic pathways	ko01100	↓	20.805	0.002	10000
	Biosynthesis of secondary metabolites	ko01110	↓	15.752	0.004	10000
	2-Oxocarboxylic acid metabolism	ko01210	↑	6.770	0.029	10000
	Aminoacyl-tRNA biosynthesis	ko00970	↓	6.512	0.031	10000
	Quorum sensing	ko02024	↑	20.536	0.002	10000
	Purine metabolism	ko00230	↓	11.066	0.010	10000
	Ribosome	ko03010	↓	8.251	0.019	10000
	Pyrimidine metabolism	ko00240	↓	6.010	0.037	10000
	Oxidative phosphorylation	ko00190	↓	6.947	0.027	10000
	Glycolysis / Gluconeogenesis	ko00010	↓	18.267	0.003	10000
	Glycine, serine and threonine metabolism	ko00260	↑	6.550	0.031	10000
	Alanine, aspartate and glutamate metabolism	ko00250	↓	11.255	0.009	10000
	Fatty acid biosynthesis	ko00061	↑	5.756	0.040	10000
	Metabolism of xenobiotics by cytochrome P45	ko00980	↑	11.770	0.008	10000
	Drug metabolism - cytochrome P450	ko00982	↑	10.929	0.010	10000
WATER	Two-component system	ko02020	↑	H= 5.600	0.050	10 10000
	Quorum sensing	ko02024	↑	H= 5.600	0.050	10 10000
	Glyoxylate and dicarboxylate metabolism	ko00630	↓	182.266	<0.001	10
	Sulfur metabolism	ko00920	↓	17.525	0.003	10

3.2 Water microbiome

Bacterial communities of exposure water were also affected by OTC presence at class level (Fig. 1-c)). In control samples, the classes Gammaproteobacteria (53.5 ± 13.3%), Alphaproteobacteria (18.9 ± 15.0%), Actinobacteria (10.1 ± 10.9%) and Bacteroidia (9.6 ± 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)). Statistical analysis confirmed significant differences in bacterial communities' structure between control and both concentrations tested (Table S2).

In water samples, 32 abundant OTUs were significantly affected by the presence of OTC (Table 4 and S5). 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 S5). 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 S5).

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).

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 (*) and bold color 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*
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*

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

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 (Chapter 3). 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 (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) (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 (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 (Chapter 3). 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 (Chen et al. 2011b). 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. 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, the exposure to the lowest concentration of OTC (10 µg/L) may favor the increase of some bacterial groups with potential biocontrol in fish diseases.

Nevertheless, at the highest OTC concentration, other classes were affected in water samples, namely the Alphaproteobacteria and Chlamydiae, being observed an abundance increase. Chlamydiae is a known pathogen of both mammalians and fish organisms. Members of this group are agents of epitheliocystis disease, responsible for economic loss in fish's aquacultures (Blandford et al. 2018). Also, the increase of this group may be of high concern since it was reported that Chlamydiae may acquire tetracycline resistance genes (*tet*) when exposed to selective pressure (Sandoz and Rockey 2010). Regarding Alphaproteobacteria, Rhizobiales was the most affected order by OTC exposure. This bacterial group, specially the genus *Hyphomicrobium*, is particularly abundant in biofilms (Chee and Liu 2007). The biofilm formation is usually a mechanism of stress response. For instance, in the presence of antibiotics, biofilms can provide bacterial cells a higher capacity to resist to antibiotic exposure (Olsen 2015).

Bacterial communities of fish larval stages tend to reflect more the surrounding environment, being more influenced by external factors, such as feed regime and water quality. On the opposite, adult fish tend to have a more specific and stable microbiome (Stephens et al. 2015). Yet, in our work, the long-term exposure to OTC induced a significant change in bacterial communities of zebrafish gut, namely, the increase of Actinobacteria and Alphaproteobacteria abundance. Both classes are usually found in the gut microbiome of freshwater organisms like fish (Romero et al. 2014). In particular, the Rhodobacteraceae family (Alphaproteobacteria class) was pointed as playing a role in organisms health being usually more abundant in healthy fish (Xue et al. 2017). Indeed, some tropodithietic acid-producing clades were indicated as being a potential probiotic agent due to its effects against pathogens (Sonnenschein et al. 2017). Regarding Actinobacteria, this class presents a global interest in biotechnological application due to its capacity to synthetize secondary metabolites capable of removing xenobiotics (Alvarez et al. 2017). For instance, *Gordonia* genus has been indicated has a recalcitrant pollutant degrader (Alvarez et al. 2017). Thus, these microbiome changes may have provided the 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 (Romero et al. 2014). 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 (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 (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 (Roose-Amsaleg and Laverman 2016).

The interpretation of our results must consider that laboratory organisms grown in controlled conditions were used. In natural environment genetic variation among a fish population is expected to be higher, possible affecting the microbiome response to antibiotic

exposure. Also, other stressful factors (e.g. exposure to other environmental contaminants) may interact, possible exacerbating the antibiotic effects. On the other hand, in their environment organisms may not be continuously exposed to the antibiotic and more complex exposure scenarios may occur. Nonetheless, this study represents a baseline and brings valuable insights into effects of OTC long-term exposure in water and fish microbiome. 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 only 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 resulting from OTC mode of action. 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. 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*). Thus, more studies should be made for a deeper analysis of the water and zebrafish gut resistome, since stressed bacterial communities may react differently when the pressure is removed.

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7. Supplementary data

Table S1: 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

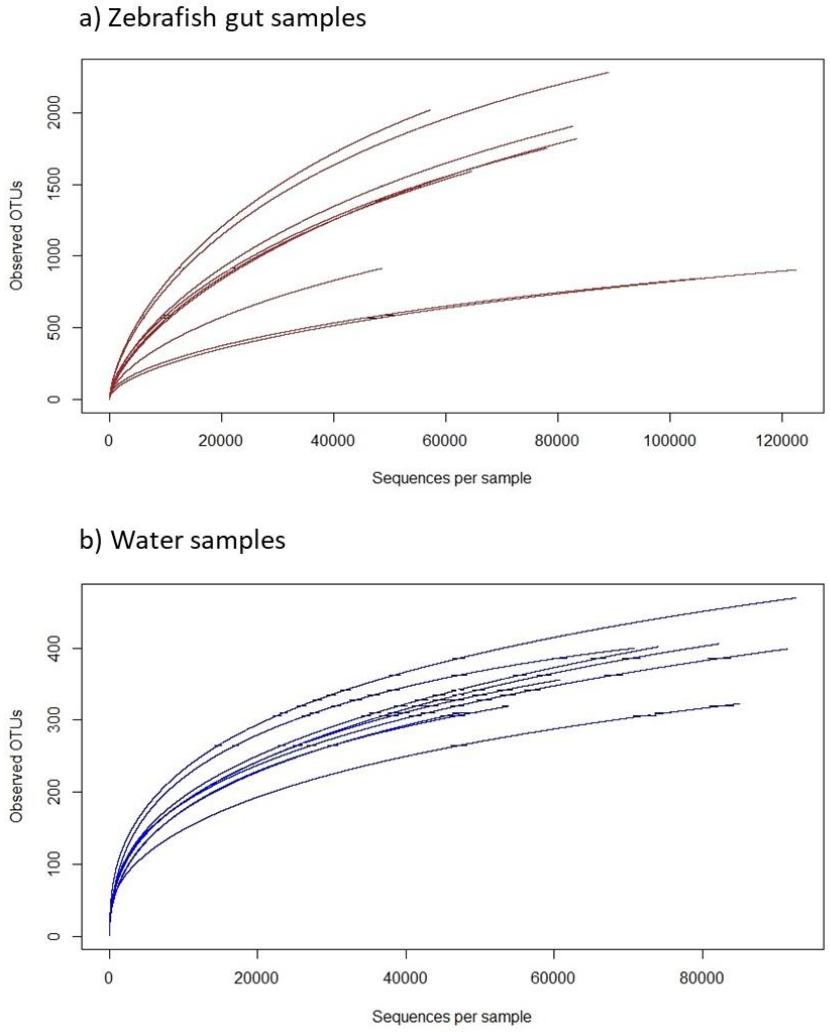

Fig. S1: Rarefaction curves (without singletons) for: a) zebrafish gut samples and b) water samples.

Table S2: Monte Carlo permutation test representing the significant differences among bacterial communities' structure of the different treatment.

	Conc. (µg/L)	**t**	**P**
GUT	0 x 10	1.7442	0.076
	0 x 10000	2.7685	0.015
	10 x 10000	2.5247	0.023
WATER	0 x 10	1.9214	0.034
	0 x 10000	2.3119	0.021
	10 x 10000	2.2668	0.017

Table S3: 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
OTU_25054	16.003	0.004	↑	Rhodobacteraceae
OTU_24710	12.352	0.007	↑	Rhodobacteraceae
OTU_35219	57.071	< 0.001	↑	*Defluviimonas*
OTU_7849	6.490	0.032	↑	*Gordonia*
OTU_32606	79.856	< 0.001	↑	*Bosea*
OTU_7123	H= 5.600	0.050	↓	*Pirellula*
OTU_7574	21.909	0.002	↓	*Crenobacter*
OTU_7855	7.448	0.004	↓	*Shewanella*
OTU_9167	5.337	0.047	↓	*Reyranella*
OTU_7240	23.669	0.001	↓	*Pirellula*
OTU_41647	11.175	0.009	↑	*Gordonia*
OTU_31612	H= 6.764	0.011	↑	Rhizobiaceae
OTU_26247	10.814	0.010	↑	*Gordonia*
OTU_26531	15.454	0.004	↑	*Gordonia*
OTU_25507	H= 5.793	0.050	↑	*Rhodobacter*
OTU_33018	5.632	0.042	↑	*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*
OTU_33056	H= 6.720	0.050	↑	Xanthobacteraceae
OTU_24656	H= 6.720	0.050	↑	*Aminobacter*
OTU_32536	34.187	<0.001	↑	Microtrichales
OTU_34457	40.201	<0.001	↑	*Defluviimonas*

Table S4: 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	Treatment (µg/L)
GUT	ABC.PE.P; peptide/nickel transport system permease protein	K02033	↑	28.853	<0.001	10000
	ABC.PE.S; peptide/nickel transport system substrate-binding protein	K02035	↑	30.386	<0.001	10000
	tRNA-Leu; tRNA Leu	K14228	↓	12.092	0.008	10000
	tRNA-Met; tRNA Met	K14230	↓	9.367	0.014	10000
	tRNA-Arg; tRNA Arg	K14219	↓	11.724	0.008	10000
	tRNA-Ser; tRNA Ser	K14233	↓	11.884	0.008	10000
	tRNA-Gly; tRNA Gly	K14225	↓	10.020	0.012	10000
	tRNA-Ala; tRNA Ala	K14218	↓	6.899	0.028	10000
	tRNA-Val; tRNA Val	K14237	↓	10.403	0.011	10000
	5SrRNA, rrf; 5S ribosomal RNA	K01985	↓	7.510	0.023	10000
	23SrRNA, rrl; 23S ribosomal RNA	K01980	↓	8.002	0.020	10000
	16SrRNA, rrs; 16S ribosomal RNA	K01977	↓	7.750	0.022	10000
	GST, gst; glutathione S-transferase [EC:2.5.1.18]	K00799	↑	13.991	0.006	10000
	tetB; MFS transporter, DHA2 family, metal-tetracycline-proton antiporter	K08168	↓	H = 7.20	0.004	10000
	tet35; tetracycline resistance efflux pump	K18218	↓	9.436	0.014	10000
	tetV; MFS transporter, DHA3 family, tetracycline resistance protein	K18215	↓	6.088	0.036	10 10000
	tetM, *tetO;* ribosomal protection tetracycline	K18220	↓	H = 5.956	0.025	10000

	resistance protein						
WATER	chrA; chromate transporter	K07240	↓		7.495	0.023	10
	TC.FEV.OM; iron complex outermembrane recepter protein	K02014		↑	H=6.489	0.011	10
	frc; formyl-CoA transferase [EC:2.8.3.16]	K07749	↓		6.164	0.035	10
	kpsS, lipB; capsular polysaccharide export protein	K07265		↑	H=6.489	0.011	10

Table S5: 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
OTU_8190	H = 5.600	0.050	↓	*Polynucleobacter*
OTU_7687	H = 6.489	0.011	↑	Burkholderiaceae
OTU_6796	H = 7.200	0.004	↑	*Acidovorax*
OTU_7923	H = 6.489	0.011	↓	*Limnobacter*
OTU_6849	H= 5.956	0.025	↑	*Limnobacter*
OTU_24583	22.672	0.002	↑	Rhizobiales Incertae Sedis
OTU_19538	H= 6.543	0.011	↑	*Hyphomicrobium*
OTU_8339	9.496	0.014	↓	*Chryseobacterium*
OTU_7691	H= 6.161	0.025	↑	*Fluviicola*
OTU_6634	H= 6.489	0.011	↑	*Phaselicystis*
OTU_4880	H=7.200	0.004	↑	*Phaselicystis*
OTU_7515	H= 5.600	0.050	↑	*Candidatus Protochlamydia*
OTU_7969	H= 5.956	0.025	↑	*Runella*
OTU_8489	8.800	0.016	↓	Caldilineaceae
OTU_24365	H= 5.600	0.050	↓	Caldilineaceae
OTU_7538	H= 7.200	0.004	↑	*Phaselicystis*
OTU_7846	H= 6.720	0.050	↑	*Pirellula*
OTU_8242	H= 5.793	0.050	↑	Chitinophagaceae
OTU_8449	H= 6.006	0.025	↑	Chitinophagaceae
OTU_8650	H= 5.793	0.050	↓	*Flavobacterium*
OTU_6535	10.968	0.010	↓	*Polynucleobacter*
OTU_33311	H= 6.764	0.011	↓	*Prosthecomicrobium*
OTU_20626	H= 5.647	0.050	↓	Chitinophagales
OTU_8611	H= 6.720	0.050	↓	*Lysobacter*
OTU_20619	H= 6.720	0.050	↓	*Fluviicola*
OTU_7240	5.595	0.043	↓	*Pirellula*
OTU_9362	H= 5.600	0.050	↑	*Runella*
OTU_38765	H= 6.720	0.050	↑	Rhizobiales Incertae Sedis
OTU_25509	7.280	0.025	↑	*Stella*
OTU_24165	H= 6.764	0.011	↑	*Flectobacillus*
OTU_8407	5.674	0.041	↑	*Sediminibacterium*
OTU_7428	H= 5.600	0.050	↑	*Sediminibacterium*

Chapter 5

Zebrafish and water microbiome recovery after oxytetracycline exposure

Zebrafish and water microbiome recovery after oxytetracycline exposure

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Submitted to Environmental Pollution

Abstract

Oxytetracycline (OTC) is a broad-spectrum antibiotic widely used in animal production, including aquaculture, which results in significant contamination of aquatic environments. In this work we intend to assess the extent to which previously observable effects are reversible after a recovery period. To do that, zebrafish adults were exposed to 10000 µg/L of OTC via water exposure. Analysis of effects was done at 5 days (5dE) and 2 months (2mE) of exposure and recovery was assessed at 5 days (5dPE) and 1 month (1mPE) after exposure ceased. Overall, our results reported impacts at energetic reserves and bacterial communities' level and showed that these impacts were significant at 5dE. At energetic reserves level, the observed effect in cellular energy allocation (CEA) was dependent on the exposure time: initially CEA increased probably due to defense mechanisms activation, while after 2mE CEA decreased, probably due to a high energy demand imposed by the prolonged exposure stress. Regarding the post-exposure period, at energetic reserves level, organisms seem to recover and no differences between exposed and control group were observed. At microbiome level, a strong impact in bacterial communities' structure from both water and zebrafish gut was observed at 5dE. In zebrafish gut microbiome, OTC effects were attenuated after exposure ceases, indicating a recovery. On the other hand, in water bacterial communities, exposed communities remained significantly different towards the control, in terms of structure and species richness. At functional level during exposure, 12 and 13 pathways were predicted to be affected in zebrafish gut and water microbiomes respectively, while after exposure ceases few pathways remained significantly affected. Hence, our results suggest a recovery of the fish

fitness as well as of the water and intestine microbiomes after exposure ceases. Even so, some of the effects caused by OTC remain significant after this recovery period.

Keywords: antibiotics; bacterial communities; *Danio rerio*; energetic reserves

1. Introduction

Tetracyclines, namely oxytetracycline (OTC), are amongst the most consumed antibiotics around the world. Due to its broad-spectrum and low-cost, OTC has been extensively used in veterinary medicine and food production, representing 30% of antibiotics consumed in Europe in 2017 (European Medicines Agency 2019). In aquaculture, OTC is usually applied through food to both sick and healthy animals. Nevertheless, around 90% of this compound is not metabolized and may be excreted still bioactive through feces and urine (Cravedi et al. 1987). Wastewater Treatment Plants (WWTP) are not specialized in the removal of pharmaceutical compounds from effluents and, in the case of OTC around 70% may reach the environment (Watkinson et al. 2009). Thus, due to its continuous release and persistence, tetracyclines have been detected in several aquatic environments around the world (Daghrir and Drogui 2013). In fact, OTC was not only detected in aquaculture systems at concentrations ranging from 315 to 15163 ng/L in China (Zou et al. 2011; Harnisz et al. 2015b; Monteiro et al. 2016), 14 to 7993 ng/L in Brazil and 110 ng/L in Poland , but it has also been detected in surface waters at concentrations of 340 ng/L in USA (Kolpin et al. 2002), 287 ng/L in Dou river in China (Zou et al. 2011), and 7 ng/L in Mess river in Luxembourg (Pailler et al. 2009). Effects of such concentrations on non-target aquatic organisms and ecosystems remains largely unknown.

In our previous works, the toxic effect of long-term exposure to OTC in zebrafish adults was evaluated at several biological organization levels. After a long-term exposure (2 months), it was observed that OTC induced changes in fish behavior (e.g. fish boldness increase), energetic reserves (e.g. decrease in cellular energy allocation) and detoxification mechanisms (e.g. decrease of glutathione S-transferase (GST) and catalase (CAT) activities) (Chapter 3).

Moreover, effects in alpha and beta-diversity were also observed in the fish gut and water bacterial communities (Chapter 4). Namely, it was observed a slight increase of diversity in water bacterial community and the selection of intrinsically antibiotic-resistant bacteria belonging to phylogenetic groups pointed as fish pathogens. In literature, other studies also revealed that chronic exposure to OTC led to increased oxygen consumption rate and decreased non-specific immune reactions in zebrafish exposed to 0.42 ug/L (Zhou et al. 2018); induced significant histological damages, namely in gill in Sparus aurata at 4 μg/L (Rodrigues et al. 2019) and caused genotoxic damage (e.g. genetic damage and abnormalities in the nucleus of erythrocytes) in Sparus aurata at 0.4 and 4 μg/L (Rodrigues et al. 2018). Moreover, changes were observed in thyroid hormone and serotonin homeostasis in zebrafish exposed to 100 μg/L of OTC (Li et al. 2020); a negative feedback control of the hypothalamic–pituitary–thyroid axis in zebrafish at 5 μg/L of OTC (Yu et al. 2020) and a dysregulations in hepatic metabolic pathway in zebrafish exposed to 100 μg/L of tetracycline (TET) (Keerthisinghe et al. 2020).

Nonetheless, in their natural environment, organisms are not continuously exposed to contaminants being subjected to pulsed exposures with potential recovery time between exposures. However, most of the studies focus on the effects during or at the end of exposure, not examining other environmental scenarios. In what concerns antibiotics/ antimicrobials, few studies analyzed post-exposure effects in fish: Yan et al. (2018) revealed that after a 7 days of recovery, the effects of the antibiotic sulfamethazine (SMZ) in the antioxidant system (superoxide dismutase: SOD and malondialdehyde: MDA) of zebrafish were reversed; Narrowe et al. (2015) demonstrated that fathead minnows' gut bacterial communities returned to basal levels, regarding alpha and beta diversity, after two weeks of recovery after exposure to the antimicrobial triclosan. On the other hand, Carlson et al. (2017) showed that bacterial communities of Gambusia affinis exposed to rifampicin antibiotic were not able to recover in terms of diversity or composition after 1-week of recovery.

To our knowledge the effects of OTC were never analyzed in a post-exposure scenario. Hence, there is a lack of information about possible reversibility of these effects in the organism itself, in its microbiome and surrounding water bacterial communities. For this reason, our study was conducted to fill this knowledge gap. After OTC long-term exposure (2

months), zebrafish adults were transferred to clean water and kept for assessment of reversibility of effects after 5 days and 1 month of post-exposure. Moreover, since in our previous work OTC effects were only assessed after 2 months of exposure, analysis of possible early effects was also conducted after 5 days of exposure. Sensitive endpoints were analyzed, like organisms' energetic reserves and microbiome changes.

2. Materials and Methods

2.1 Zebrafish culture

Zebrafish (*Danio rerio*) adults were obtained from the zebrafish culture established at the Biology Department of University of Aveiro (Aveiro, Portugal). The fish were kept under controlled conditions (temperature 27 ºC; pH 7.5 ± 0.5; conductivity 800 ± 50 µS and dissolved oxygen ≥ 95%) in a recirculation system as described by Domingues et al. (2016). The photoperiod cycle was 14:10 h (light:dark). Zebrafish adults, were selected for the experimental assays as recommended by OECD guideline 230 (OECD 2009).

2.2 Zebrafish exposure and sample collection

A total of 72 adults were used in the experiment; 36 used as control organisms and 36 exposed to oxytetracycline (10000 µg/L) for two months under a semi-static condition as described in Chapter 3. In each treatment the fish were randomly divided in 12 aquaria (1L) with 3 fish each in a controlled temperature room. Fish were feed daily with artificial diet Gemma Micro 500 (Skretting®, Spain; amount corresponding to 2% of fish body weight). Water renewal was performed every three days to maintain both water quality and OTC concentration constant (Chapter 3). Concentration was chosen based on our previous works that reported significant effects of OTC exposure at energic and microbiome level of both zebrafish and water (Chapter 3 and 4). Although it is not an environmental concentration, a high OTC concentration was used to help understanding the OTC effects in a worst-case

scenario. Moreover, since the aim of our study was to verify the reversibility of the observed effects in a post-exposure scenario, based on our previous works, a high OTC concentration was used. Control animals were kept in identical conditions without antibiotic. After exposure, organisms were transferred to new aquaria with clean water (culture water) to ensure no OTC residues and kept for reversibility of effects during a month. Samples were collected throughout the experiment at four sampling moments: 5 days and 2 months of exposure (5dE and 2mE), and 5 days and 1 month of post-exposure (5dPE and 1mPE). All sampling points were chosen based on literature. Namely, exposure period was chosen based on our previous works that reported effects at enzymatic activity and microbiome level after 2mE (Chapter 3 and 4); The post-exposure period was chosen based on reports showing recovery after 1 week after the end of exposure at biochemical level (Yan et al. 2018) while at microbiome level organisms seemed to need more time (≥2 weeks) (Narrowe et al. 2015; Carlson et al. 2017). In each sampling period, 3 replicates (N= 9 fish) per concentration were euthanized with tricaine overdose (tricaine methane sulfonate, Metacain, MS-222; CAS number: 886-86-2) followed by spinal cord severing. Then, fish trunks were used for energetic reserves analysis (section 2.3) and zebrafish guts were removed aseptically for microbiome analysis (section 2.4). In addition, water samples were also collected at the same time points for microbiome analysis (section 2.4). Exposure water (100 mL in triplicate) was filtered using 0.22 µm hydrophilic PVDF durapore membrane filter (Merck Millipore; Massachusetts, EUA). Samples were then stored at -80 ºC until analysis.

2.3 Energetic reserves analysis

Samples mentioned in the section 2.2 were processed as described in Chapter 3. In brief, after sample defrosting, 100 mg of fish tissue was added to 1mL of ultra-pure water and homogenized with a sonicator (KIKA Labortechnik U2005 Control). Then, samples were divided in aliquots according to Chapter 3 to determine available energy reserves (Ea) and energy consumption (Ec). Analyses were performed in 96-well microplates, in quadruplicate using spectrophotometric methods (Thermo Scientific Multiskan Spectrum, USA).

The relation between Ea and Ec was used to calculate cellular energy allocation (CEA) ratio, according to the Verslycke et al. (2003) formula. The Ea was calculated through the sum

of estimated proteins, carbohydrates and lipid after converting them into energetic equivalents (24 kJ/g proteins; 17.5 kJ/g carbohydrates and 39.5 kJ/g lipids) according to De Coen and Janssen (1997). Total lipid, carbohydrates and protein content were determined as described by Abe et al. (2018). The Ec was calculated following De Coen and Janssen (1997) method, considering the electron transport energy (ETS). The Ea activity was expressed in mJ/mg wet weight and the Ec activity was in mJ/mg wet weight/hour.

2.4 Molecular analysis of microbiome

2.4.1 DNA extraction

Pools of 3 guts (3 guts = one extraction) were made for fish gut DNA extraction. Briefly, the commercial kit PowerSoil® DNA isolation kit (MOBIO laboratories, CA, USA), was used following manufactures instructions. For water samples, total DNA was extracted using the commercial kit Genomic DNA Purification kit (Thermo Fisher Scientific; Massachusetts, EUA) as described by Henriques et al. (2004).

2.4.2 Illumina high-throughput sequencing

Microbiome profiling using the V3-V4 region of the 16S rDNA was achieved through Illumina MiSeq sequencing performed by Eurofins Genomics (Ebersberg, Germany) with the following primers: 357F-forward TACGGGAGGCAGCAG and 800R-reverse CCAGGGTATCTAATCC primers. Sequence processing analysis was also performed by Eurofins Genomics (Ebersberg, Germany) according to their standard protocols as follows. Reads were quality-filtered to remove reads with less than 285 bp, ambiguous bases ("N") and sequences with an average quality lower than Q30. Sequences were then processed using the UCHIME script from VSEARCH package to identify and discard chimeric reads (Rognes et al. 2016). Briefly, to identify Operational Taxonomic Units (OTUs), high-quality reads were processed using Minimum Entropy Decomposition (MED) partitioning the marker gene datasets into OTUs (Eren et al. 2015). Taxonomy assignment was achieved by DC-MEGABLAST alignments of representative OTU sequences to the NCBI database (NCBI_nt; release: 2019-08-02). To consider a reference sequence it was applied a minimal of 70%

identity across at least 80% of the representative sequence (Altschul et al. 1990). Bacterial taxonomy unit abundances were normalized using lineage-specific 16S gene copy number to improve estimation (Angly et al. 2014).

2.4.3 *In silico* metagenome analysis

Functional profile inference was calculated based on the OTU sequences and OTU abundance table using the Piphillin software (Iwai et al. 2016). OTUs were matched against the Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/) version 88.1 (KEEG Oct2018). A list of KEGG orthologs (KO) and their abundance for each sample was obtained using an identity cut-off of 97%.

2.5 Statistical analysis

Statistical significance among differences at energetic reserves (Ea, Ec and CEA) and microbiome level (indexes, genus and OTU level and KO abundance) were assessed using a t-test with the Sigma plot V.12.5 (SysStat software Inc., CA, USA) software. Data normality was tested through Shapiro-Wilk test. When normality fails, the non-parametric Mann-Whitney test was used. A significance level of 0.05 was considered.

Bacterial communities' structure was analyzed through a cluster and principal coordinate analysis (PCoA) using a Bray-Curtis distance matrix based on a transformed Log(x +1) OTU abundance table, obtained through PRIMER v6 software (Primer-E Ltd., Plymouth, UK). The OTU abundance table was also used to calculate diversity indexes: species richness (S; number of OTU), diversity (H′; Shannon-Wiener index) and evenness (J; Pielou's evenness index). R software was used to obtain the rarefaction curve through the vegan package (rarecurve function) (Oksanen et al. 2016). Differences among bacterial communities' structure at each sampling time, were assessed using PERMANOVA followed by the pairwise Monte Carlo (MC) test (999 unrestricted permutations).

3. Results

The present study shows the effects of OTC during and after exposure at fish energetic reserves and microbiome level (zebrafish gut and water). No mortality was observed during the experiment.

3.1 Energetic reserves

In the Fig. 1, the energy available (Ea), energy consumption (Ec) and cellular energy allocation (CEA) are represented. After 5dE, OTC significantly increased the Ea level and CEA while a significant decrease in Ec was observed (Table S1). On the other hand, after 2mE, a significant increase of Ec and a decrease in CEA were observed (Fig. 1). After exposure ceased (5dPE and 1mPE), no statistically significant differences were observed between exposed and control zebrafish.

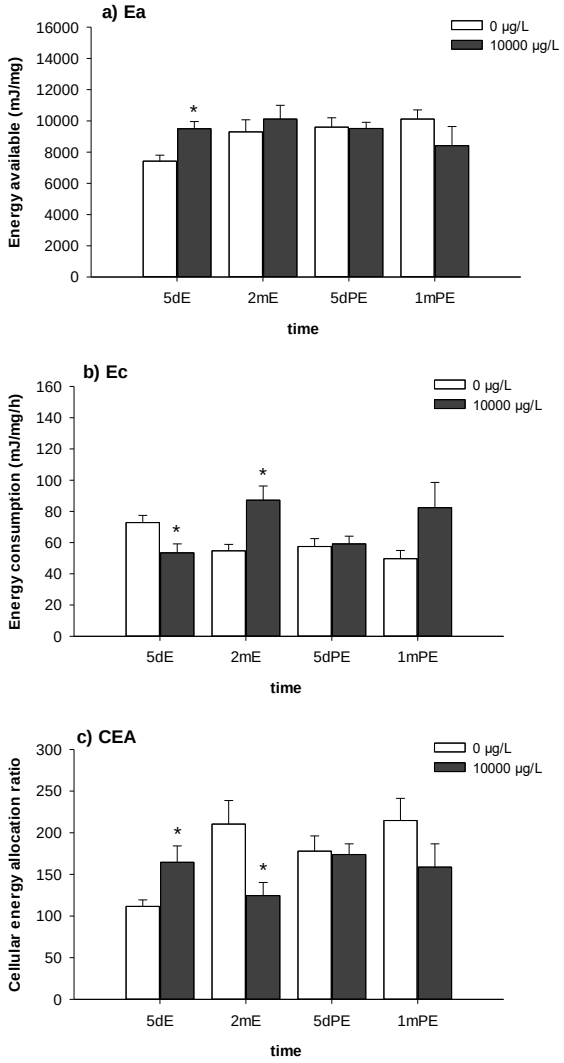


Fig. 1: a) Total energy available (Ea); b) energy consumption (Ec) and c) cellular energy allocation (CEA) of zebrafish exposed to oxytetracycline at each sampling time (5 days of exposure: 5dE; 2 months of exposure: 2mE; 5 days of post-exposure: 5dPE and 1 month of post-exposure: 1mPE). Values presented are mean ± standard deviation. Asterisks (*) indicate significant differences towards the respective control ($p \leq 0.05$; t-test) in each sampling time. Statistical analysis of differences among treatments is represented in Table S1. Data is normal except for CEA: 5dE and 1mPE

.

3.2 Microbiome analysis

At microbiome level, a total number of 518017 and 866195 sequence reads were obtained from both zebrafish gut and water bacterial communities, respectively (Table S2). The rarefaction curves indicate a good estimation of the community richness since each sample's curve tends to saturation (Fig. S1).

3.2.1 Zebrafish gut microbiome

The overall impact of OTC in both exposure and post-exposure period, at OTU structure level, is presented in the cluster and PCoA analysis (Fig. 2). After 5dE, OTC significantly affected the community structure (P= 0.014; Table S3) with samples of exposed organisms sharing low similarity with the control group (36% of similarity; Fig. 2 – a)). Although there is some variability among samples, the same tendency was observed after 2mE (64% of similarity) although differences between control and exposed samples were not significative (Table S3). Regarding the post-exposure period (5dPE and 1mPE), exposed and control communities shared similarity levels of 68% at 5dPE and 58% at 1mPE. This can be observed in both cluster and PCoA analysis (Fig. 2- a) and b)). Diversity indexes (Table S4) revealed no significant impact of OTC in species richness, evenness or diversity in both exposure and post-exposure periods in gut samples.

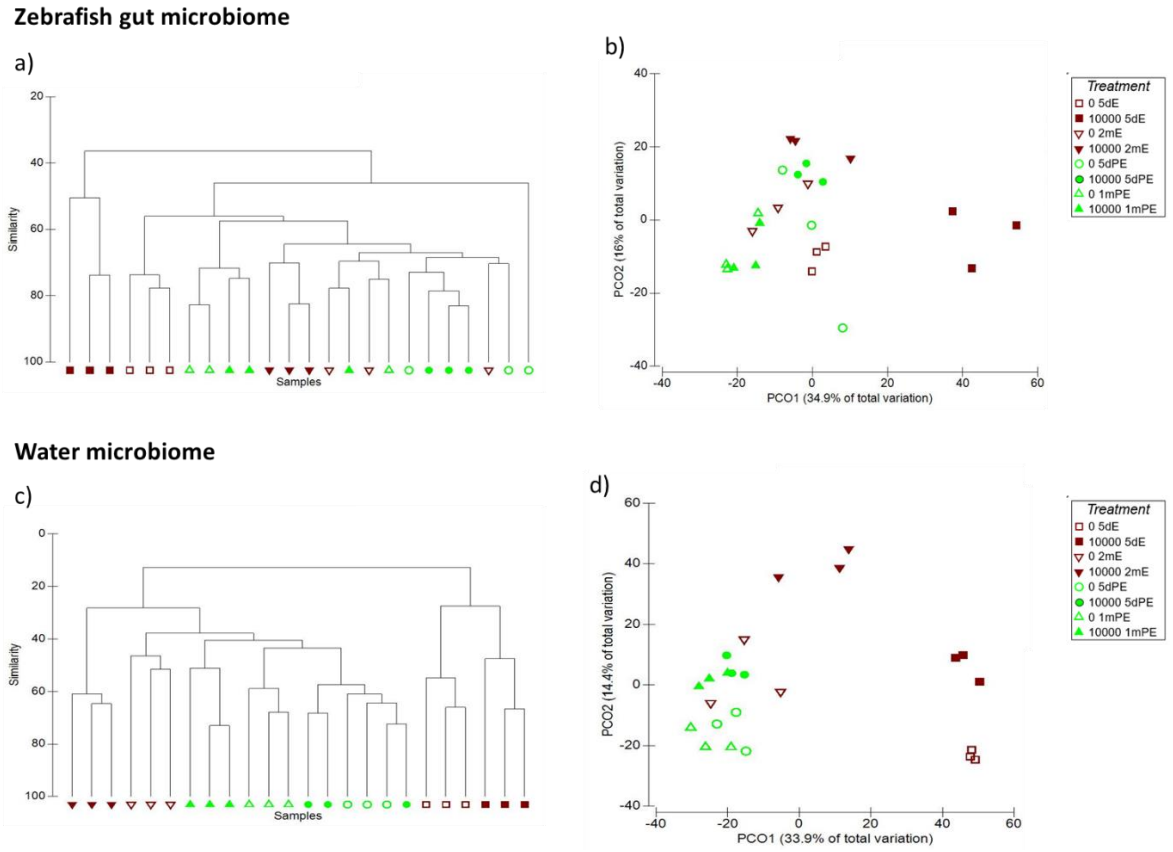


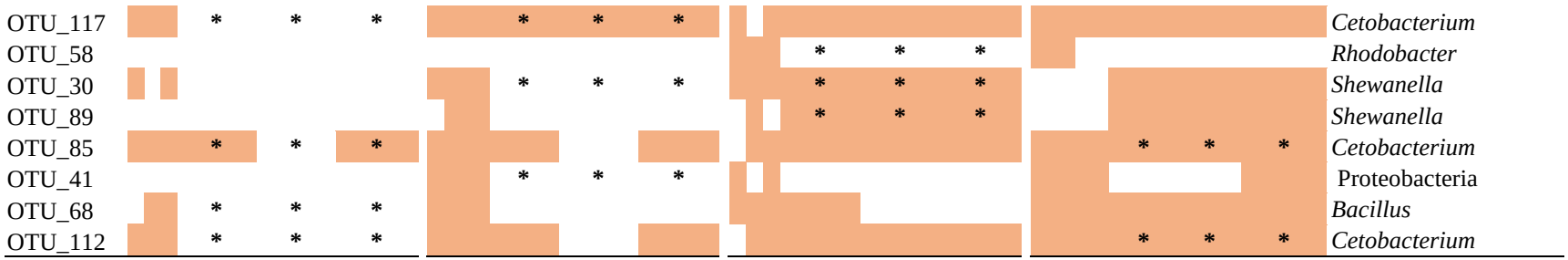
Fig. 2: Dendogram [a) and c)] and PCoA based on Bray-Curtis similarity matrices [b) and d)] representing changes in zebrafish gut and water microbiomes exposed to oxytetracycline at each sampling time (5 days of exposure: 5dE; 2 months of exposure: 2mE; 5 days of post-exposure: 5dPE and 1 month of post-exposure: 1mPE). Statistical analysis of differences among treatments is represented in Table 1. Concentrations are in µg/L.

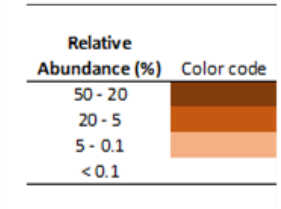
The most abundant OTUs that were significantly affected by OTC are represented in the Table 1 and Table S5. During the exposure period, OTC significantly affected the abundance of 25 OTUs (5dE: 5 OTUs increased and 16 OTUs decreased abundance; 2mE: 1 OTUs increased and 6 OTUs decreased abundance). Namely, the relative abundance of the OTUs affiliated with the genus *Cetobacterium* (e.g. OTU_1, OTU_60, OTU_71, OTU_79, OTU_81, OTU_88, OTU_101, OTU_71, OTU_85, OTU_112) significantly decreased at 5dE with only one OTU affiliated with this genus (OTU_117) significantly increasing at 2mE (Table S5). Moreover, the same effect occurred in OTUs affiliated to the class Gammaproteobacteria

(*Aeromonas*: OTU_4; *Shewanella*: OTU_45, OTU_30; *Plesiomonas*: OTU_72 and Enterobacterales: OTU_22). On the other hand, an abundance increase was observed for OTUs affiliated with the Alphaproteobacteria class (Rhodobacteraceae: OTU_31; *Mesorhizobium*: OTU_28; Rhizobiaceae: OTU_123), Gammaproteobacteria (*Pseudomonas*: OTU_18) and Betaproteobacteria (*Variovorax*: OTU_42) (Table S5). Regarding the post-exposure period (5dPE and 1mPE), a significant effect was observed for only 16 OTUs (5dPE: 5 OTUs increased and 1 OTU decreased in abundance; 1mPE: 8 OTUs decreased in abundance). Similar to the exposure period, the OTUs affiliated with the genus *Cetobacterium* showed significantly abundance decreases in the exposed communities (OTU_11; OTU_71; OTU_81; OTU_63; OTU_84; OTU_86; OTU_85; OTU_112) (Table S5). The relative abundance increase of OTUs affiliated with *Shewanella* (OTU_30 and OTU_89) and *Bacteroides* (OTU_19; OTU_130 and OTU_149) was observed only in 5dPE (Table S5).

Table 1: The top 30 most abundant OTUs affected significantly by OTC exposure in zebrafish gut (t-test). Asterisks indicates significant differences towards the respective control ($p \leq 0.05$) at each sampling time (5 days of exposure: 5dE; 2 months of exposure: 2mE; 5 days of post-exposure: 5dPE and 1 month of post-exposure: 1mPE); color code represents the relative OTU abundance. OTC concentrations are in µg/L. When affiliation to genera was not possible, affiliation to a higher taxonomic level is presented. Statistical analysis is represented in Table S5.

	5 days of exposure (5dE)			**2 months of exposure (2mE)**			**5 days of post-exposure (5dPE)**			**1 month of post-exposure (1mPE)**			**Taxonomy afiliation**
#OTU ID	0	10000	10000	0	10000	10000	0	10000	10000	0	10000	10000	
OTU_1		*	*										*Cetobacterium*
OTU_16		*	*								*	*	*Crenobacter*
OTU_4		*	*		*	*							*Aeromonas*
OTU_9											*	*	*Gemmobacter*
OTU_22					*	*							Enterobacterales
OTU_11											*	*	*Cetobacterium*
OTU_15		*	*										*Bacillus*
OTU_60		*	*										*Cetobacterium*
OTU_31		*	*										*Rhodobacteraceae*
OTU_45		*	*		*	*							*Shewanella*
OTU_72					*	*							*Plesiomonas*
OTU_120		*	*										*Cetobacterium*
OTU_71		*	*								*	*	*Cetobacterium*
OTU_79		*	*										*Cetobacterium*
OTU_81		*	*								*	*	*Cetobacterium*
OTU_88		*	*										*Cetobacterium*
OTU_18		*	*										*Pseudomonas*
OTU_28		*	*										*Mesorhizobium*
OTU_19								*	*				*Bacteroides*
OTU_63											*	*	*Cetobacterium*
OTU_123		*	*										*Rhizobiaceae*
OTU_42		*	*										*Variovorax*
OTU_84											*	*	*Cetobacterium*
OTU_130								*	*				*Bacteroides*
OTU_86											*	*	*Cetobacterium*
OTU_149								*	*				*Bacteroides*
OTU_101		*	*										*Cetobacterium*


OTU_117
OTU_58
OTU_30
OTU_89
OTU_85
OTU_41
OTU_68
OTU_112
Cetobacterium
Rhodobacter
Shewanella
Shewanella
Cetobacterium
Proteobacteria
Bacillus
Cetobacterium


Relative Abundance (%)
Color code
50 - 20
20 - 5
5 - 0.1
< 0.1

The structure of zebrafish gut microbiome at genera level during and after exposure ceased is represented in the Fig. 3. During the exposure, OTC significantly affected the relative abundance of 11 genera (Table 2). Namely, 8 genera were affected at 5dE: 3 genera increased in abundance (*Gemmobacter*, *Pseudomonas* and *Mesorhizobium*) and 5 genera decreased (*Cetobacterium*, *Crenobacter*, *Aeromonas*, *Bacillus* and *Shewanella*). At 2mE, 5 genera were significantly affected: *Aeromonas*, *Plesiomonas*, *Shewanella* and two unidentified genera affiliated with *Enterobacteriales* and Proteobacteria decreased in abundance (Fig. 3 and Table 2 and S6). In the post-exposure period (5dPE and 1mPE), OTC significantly affected the relative abundance of 4 genera, namely 1 genus at 5dPE that increased in abundance (*Shewanella*) and 3 genera at 1mPE: 1 genus increased (*Crenobacter*) and 2 genera decreased (*Cetobacterium* and *Gemmobacter*).

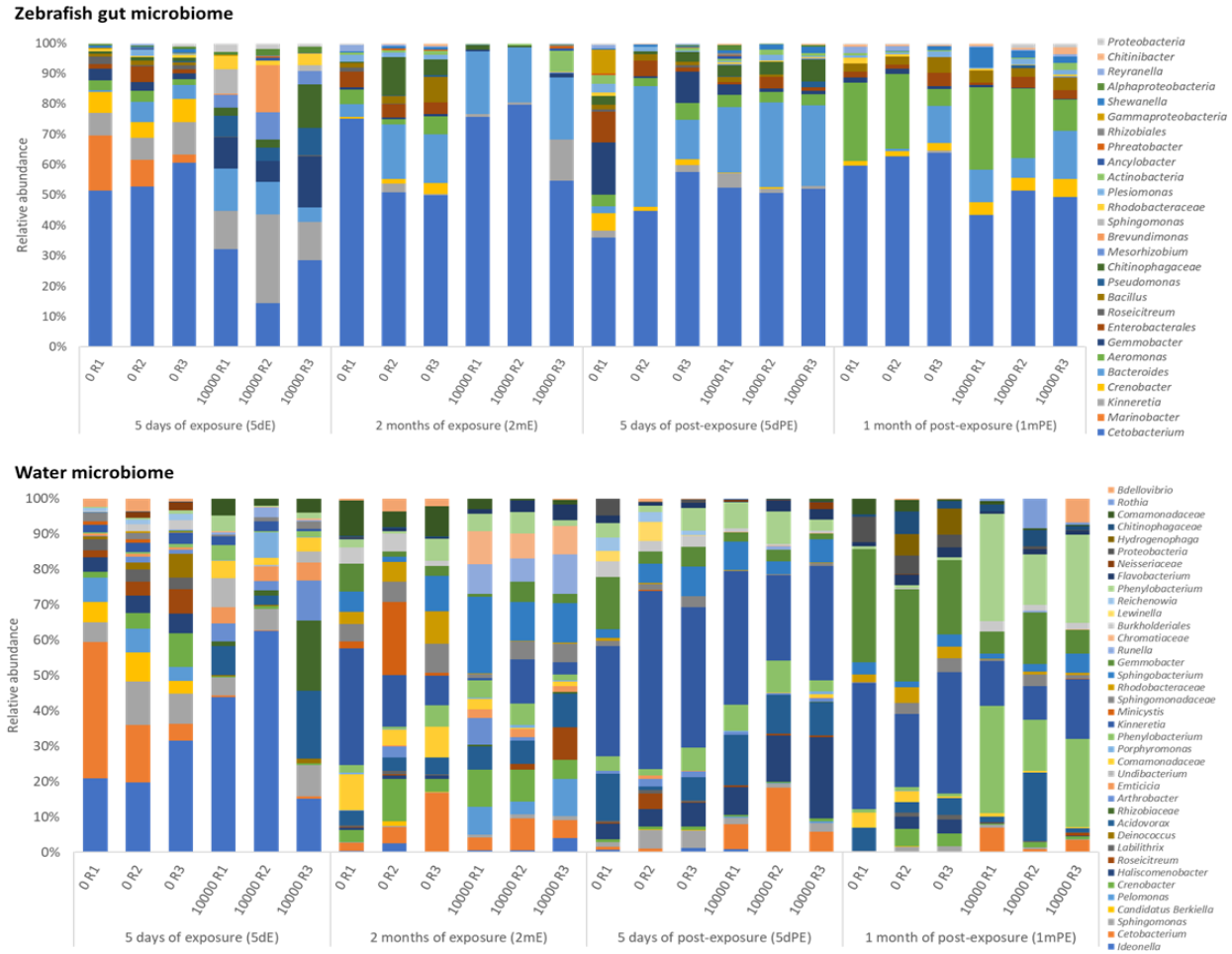


Fig 3: Relative abundance of top 10 most abundant genera in each sampling time (5 days of exposure: 5dE; 2 months of exposure: 2mE; 5 days of post-exposure: 5dPE and 1 month of post-exposure: 1mPE) of zebrafish gut and water microbiomes. When affiliation to a genus was not possible, affiliation to a higher taxonomic level is presented. Results of three replicates (n= R1, R2 and R3) are presented for each concentration (0 and 10000 µg/L) at each sampling time. Statistical analysis of differences among treatments is represented in the Table S5.

Table 2: Statistical significantly affected genera whose abundance was significantly affected in at least one of the sampling times (5 days of exposure: 5dE; 2 months of exposure: 2mE; 5 days of post-exposure: 5dPE and 1 month of post-exposure: 1mPE) of zebrafish gut and water microbiomes. Values represent the variation of relative abundance (%) towards the respective control (decrease: red color; increase: green color) in each sampling time ($p \leq 0.05$; t-test). White color represents no statistically significant effects. Statistical analysis of differences among treatments is represented in the Table S3. When affiliation to a genus was not possible, affiliation to a higher taxonomic level is presented.

Sample type	Taxonomic Affiliation	5dE	2mE	5dPE	1mPE
GUT	*Cetobacterium*	-28.8			-13.8
	Crenobacter	-6.3			2.7
	Aeromonas	-2.8	-4.2		
	Gemmobacter	8.3			-1
	Enterobacterales		-4.5		
	Bacillus	-1.1			
	Pseudomonas	6.4			
	Mesorhizobium	5.7			
	Plesiomonas		-1.8		
	Shewanella	-0.7	-0.7	1.0	
	Proteobacteria		-0.4		
WATER	*Sphingomonas*		0.8		
	Candidatus Berkiella	-4.5			
	Pelomonas	-4.4	6.6	0.3	
	Haliscomenobacter	-3.7	-0.9		
	Roseicitreum	-3.3			
	Labilithrix	-2.5	-0.5	-0.6	
	Acidovorax	10.1	2.6		
	Emticicia	4.2	1.8		
	Comamonadaceae	3.6	-6.6		
	Rhodobacteraceae		-6.0		-2.4
	Runella		7.9		
	Gemmobacter				-13.0
	Chromatiaceae	0.4	7.6	0.3	
	Burkholderiales		-3.8	-2.6	2.7
	Reichenowia	-1.1		-2.5	
	Proteobacteria				-4.6
	Chitinophagaceae	-0.1	-0.3		
	Rhizobiaceae	7.5			

In relation to the function inference analysis (Table 3 and S7), our results revealed that during exposure, 12 functional pathways were predicted to be significantly affected by OTC. Namely, 1 pathway after 5dE (abundance decrease) and 11 pathways after 2mE (2 pathways increased and 9 pathways decreased) (Table 3). A significant decrease of pathways related with global and overview maps (e.g. biosynthesis of amino acids), nucleotide metabolism (e.g.

pyrimidine metabolism), translation (e.g. aminoacyl-tRNA biosynthesis: tRNA-Leucine, Methionine, Valine and Serine) and energy metabolism (e.g. oxidative phosphorylation) was predicted (Table 3 and S7). Also, the same tendency in bacterial secretion system, two-component system and flagellar assembly was predicted (Table 3). After exposure ceased, 3 functional pathways were predicted to be significantly affected. Namely, 1 pathway after 5dPE (decreased of abundance) and 2 pathways after 1mPE (decreased of abundance). For instance, the decreasing tendency in some functions like carbon and pyruvate metabolism was predicted (Table 3).

Table 3: Predicted pathway changes of zebrafish gut and water microbiomes exposed to OTC at each sampling time (5 days of exposure: 5dE; 2 months of exposure: 2mE; 5 days of post-exposure: 5dPE and 1 month of post-exposure: 1mPE). Values represent the variation of relative abundance (%) towards the respective control (decrease: red color; increase: green color) in each sampling time ($p \leq 0.05$; t-test). White color represents no statistically significant effects.

Sample type	KEGG pathway		KEGG ID	5dE	2mE	5dPE	1mPE
GUT	Global and overview maps	Metabolic pathways	ko01100		-0.36	-0.26	
		Biosynthesis of amino acids	ko01230		-0.14		
		Carbon metabolism	ko01200				-0.05
	Nucleotide metabolism	Purine metabolism	ko00230	-0.07			
		Pyrimidine metabolism	ko00240		-0.32		
	Carbohydrate metabolism	Glyoxylate and dicarboxylate metabolism	ko00630		0.24		
		Pyruvate metabolism	ko00620				-0.02
	Translation	Aminoacyl-tRNA biosynthesis	ko00970		-0.87		
		Ribosome	ko03010		-0.49		
	Energy metabolism	Oxidative phosphorylation	ko00190		-0.21		

	Amino acid metabolism	Valine, leucine and isoleucine degradation	ko00280		0.42			
	Membrane transport	Bacterial secretion system	ko03070		-0.55			
	Signal transduction	Two-component system	ko02020		-0.80			
	Cell motility	Flagellar assembly	ko02040[a]		-0.81			
Water	Global and overview maps	Biosynthesis of secondary metabolites	ko01110	-0.48				
		Microbial metabolism in diverse environments	ko01120	0.47				
		Biosynthesis of amino acids	ko01230					-0.07
		Carbon metabolism	ko01200[a]					0.10
	Membrane transport	ABC transporters	ko02010	1.39				
	Nucleotide metabolism	Purine metabolism	ko00230		-0.06	-0.04	-0.05	
		Pyrimidine metabolism	ko00240	-0.07	-0.07	-0.03		
	Carbohydrate metabolism	Pyruvate metabolism	ko00620	-0.24				
		Glyoxylate and dicarboxylate metabolism	ko00630	0.10	0.10			
		Propanoate metabolism	ko00640	-0.1				
		Butanoate metabolism	ko00650	-0.05				
		Glycolysis / Gluconeogenesis	ko00010			-0.03	-0.04	
		Pentose phosphate pathway	ko00030			-0.08	-0.08	
	Amino acid metabolism	Glycine, Serine and Threonine metabolism	ko00260		-0.07			
		Valine, Leucine and Isoleucine degradation	ko00280		0.13			

Translation	Aminoacyl-tRNA biosynthesis	ko00970		-0.05	-0.07	
Energy metabolism	Nitrogen metabolism	ko00910	0.09	0.06		

a = Mann-Whitney U Statistic

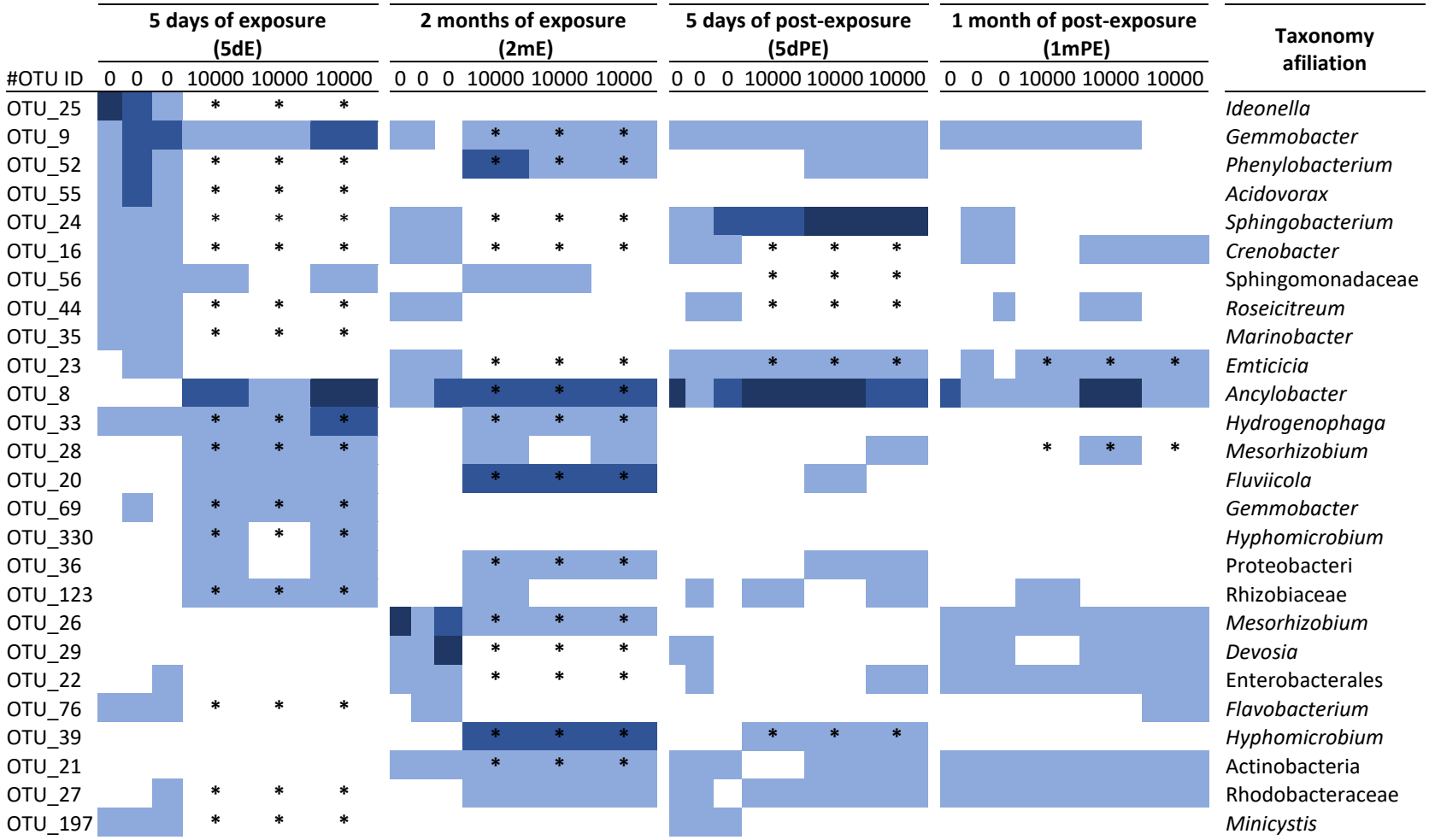
3.2.3 Water microbiome

Regarding water microbiome structure, the impact of OTC exposure is presented in Fig. 2. Similar to the fish gut, OTC effect may be observed from the 5dE with exposed samples sharing low similarity with the control samples (28% of similarity; Fig. 2 – c)). In both exposure periods (5dE and 2mE) bacterial communities were statistically different from the control (Table S3). In post-exposure period (5dPE and 1mPE) in both cluster and spatial distribution (Fig. 2- c) and d)) it was possible to observe that bacterial communities' structures became more similar with the control (58% and 41% of similarity). Nevertheless, statistical differences between exposed and control group were observed after 1mPE (Table S3). Analysis of diversity indexes revealed a significant effect of OTC in species richness after 2mE (decrease) and 1mPE (increase) (Table S4). A significant decrease in Pielou's evenness index was only observed after 5dE (Table S4).

During exposure, OTC affected 26 OTUs from the most abundant in water (Table 4 and Table S8). Namely, 16 OTUs after 5dE (5 OTUS increased and 11 OTUs decreased in abundance) and 15 OTUs after 2mE (8 OTUS increased and 7 OTUs decreased abundance) were significantly affected. The abundance decrease was observed in the OTUs affiliated with the class Alphaproteobacteria (*Roseicitreum*: OTU_44 and Rhodobacteraceae: OTU_27), Betaproteobacteria (*Acidovorax*: OTU_ 55 and *Ideonella*: OTU_25) Gammaproteobacteria (*Marinobacter*: OTU_35), Deltaproteobacteria (*Minicystis*: OTU_197), and Flavobacteria (*Flavobacterium*: OTU_76) after 5dE. Also, the same tendency was observed after 2mE in the OTUs affiliated with Sphingobacteria (*Sphingobacterium*: OTU_24), Betaproteobacteria (*Crenobacter*: OTU_16) and Gammaproteobacteria (*Aeromonas*: OTU_4). OTUs affiliated with Alphaproteobacteria (*Mesorhizobium*: OTU_26; *Devosia*: OTU_29),

Gammaproteobacteria (Enterobacterales: OTU_22) and Cytophagia (*Emticicia*: OTU_23) were only affected (abundance decrease) after 2mE. The abundance increase of OTUs affiliated with Alphaproteobacteria (*Gemmobacter*: OTU_9 and OTU_69; *Mesorhizobium*: OTU_28; *Hyphomicrobium*: OTU_330 and OTU_39; Rhizobiaceae: OTU_123; *Ancylobacter*: OTU_8), Flavobacteria (*Fluviicola*: OTU_20), Betaproteobacteria (*Hydrogenophaga*: OTU_33) and Actinobacteria (OTU_21) were observed after the exposure period (5dE and 2mE). During the post-exposure period, only 5 OTUs were affected significantly (5dPE: 2 OTUs increased and 3 OTUs decreased abundance; 1mPE: 2 OTUs increased abundance). The OTUs affiliated with the genus *Crenobacter* (OTU_16), *Roseicitreum* (OTU_44) and *Emticicia* (OTU_ 23) had an abundance decrease after 5dPE. On the other hand, the abundance increase of *Hyphomicrobium* (OTU_39) and Sphingomonadaceae (OTU_56) was observed after 5d, while the genera *Mesorhizobium* (OTU_ 28) and *Emticicia* (OTU_23) only increased in abundance after 1mPE (Table 4 and Table S8).

Table 4: The top 30 most abundant OTUs affected significantly by OTC exposure in water (t-test). Asterisks indicates significant differences towards the respective control ($p \leq 0.05$) at each sampling time (5 days of exposure: 5dE; 2 months of exposure: 2mE; 5 days of post-exposure: 5dPE and 1 month of post-exposure: 1mPE); color code represents the relative OTU abundance. OTC concentrations are in µg/L. When affiliation to genera was not possible, affiliation to a higher taxonomic level is presented. Statistical analysis is represented in Table S6.



Relative Abundance (%)	Color code
50 - 10	
10 - 5	
5 - 0.2	
<0.2	

At genus level, the most abundant genera of water at each sampling time are represented in Fig. 3. Water microbiome seemed to be more affected by OTC than zebrafish gut (Fig. 3 and Table 2 and S6). During the exposure period, OTC significantly affected the relative abundance of 16 genera. Namely, at 5dE, 12 genera were significantly affected: 5 genera increased (*Acidovorax* and *Emticicia* and unidentified genera affiliated with Comamonadaceae, Chromatiaceae and Rhizobiaceae) and 7 genera decreased (*Candidatus Berkiella*, *Pelomonas*, *Haliscomenobacter*, *Roseicitreum*, *Labilithrix* and *Reichenowia* and an unidentified Chitinophagaceae genus). At 2mE 12 genera were significantly affected: 6 genera increased (*Shingomonas*, *Pelomonas*, *Acidovorax*, *Emticicia* and *Runella* and an unidentified Chromatiaceae genus) and 6 genera decreased (*Haliscomenobacter* and *Labilithrix*; and four genera affiliated with Comamonadaceae, Rhodobacteraceae and Chitinophagaceae and Burkholderiales) in the water microbiome. In the post-exposure period, OTC significantly affected the relative abundance of 8 genera. At 5dPE, 5 genera were significantly affected: 2 genera increased in abundance (*Pelomonas* genus and a Chromatiaceae genus) and 3 genera decreased in abundance (*Labilithrix* and *Reichenowia* and one genus affiliated with Burkholderiales). After 1mPE 4 genera were affected: one genus affiliated with Burkholderiales increased in abundance while in *Gemmobacter* genus, and two genera affiliated with Rhodobacteraceae and Proteobacteria was observed a decrease in abundance (Fig. 3 and Table 2 and S6).

Regarding the function inference, during exposure, 13 functional pathways were predicted to be significantly affected by OTC in water. Namely, 8 pathways after 5dE (3 pathways increased and 5 pathways decreased abundance) and 6 pathways after 2mE (2 pathways increased and 4 pathways decreased abundance) (Table 3). For instance, the increase of microbial metabolism in diverse environments and ABC transporters pathways was only predicted in the beginning of exposure (Table 3 and S7). In the post-exposure period 7 functional pathways were predicted to be significantly affected. Specifically, 5 pathways after 5dPE (decrease in abundance) and 5 pathways after 1mPE (1 pathway increased and 4 pathways decreased in abundance) (Table 3). A significant decrease in pathways related with nucleotide metabolism and translation was predicted to occur in both exposure and post-exposure period.

4. Discussion

The evaluation of the capacity of the organisms to recover at physiological and microbiome level after chemical exposure is important to assess populations' capacity of recovery in a pulse or intermittent contamination scenario. For this reason, in our work, the effect of long-term exposure to OTC was evaluated during and after exposure ceased. Higher similarity between exposed organisms and its microbiome and the control group after a post-exposure period was interpreted as a recovery.

Energetic reserves balance represents a good estimation of organism' health status and is a useful biomarker of stress (Sokolova 2013). In our work, the energy available (Ea) increase in the beginning of exposure (5dE) may be related with the increase in protein content (Table S9). Proteins are the main source of energy in adult zebrafish and when organisms are confronted with a moderate stress, protein synthesis increase is expected due to the intensification of defense and detoxification processes. In literature, Smolders et al. (2003) showed that exposure to low stress may trigger detoxification process with no changes in overall energy budget. Nevertheless, our results indicate that at an initial exposure time to OTC, organisms may initiate compensatory mechanisms to deal with the stress resulting in cellular energy allocation (CEA) increase. Moreover, due to OTC mode of action (e.g. inhibition of ribosomes), this compound may also lead to a reduction of energy production and thus CEA increase (Freitas et al. 2018). In addition, although the same amount of food was added to each aquarium, we cannot exclude differences on food consumption among fish. On the other hand, a long-term exposure may result in a stress increase with possible metabolic processes changes. Usually, the increase of energy consumption (Ec) is associated with a general chemical response related with detoxification and reparation processes demand (Gandar et al. 2017). Thus, the increase in Ec lead to an increase in cellular energy demand and consequently a decrease in CEA. Therefore, the decrease in CEA after 2mE, also observed in our previous work, would be expected (Chapter 3). Hence, the observed effect in energetic reserves of zebrafish due to OTC exposure is related not only with the concentration used but also with the prolonged exposure time. Regarding the post-exposure period, no statistically significant differences were observed at energetic reserves levels. Both EA, Ec and CEA seem

to return to basal levels. This result suggest that organisms were able to recover at energetic reserves level after exposure ceases. Although few studies evaluated the post-exposure impact of antibiotics, our results are in agreement with a previous work conducted by Yan et al. (2018) that also reported a recovery effect at enzymatic level of zebrafish after 7 days of recover from the antibiotic sulfamethazine (SMZ) exposure.

The organisms' microbiome plays an important role in several physiological processes like immunity (Nayak 2010), metabolic function (Banerjee and Ray 2017) and behavior (Bertotto et al. 2020). Since the relation host-microbiome is dynamic, several factors can modulate microbial communities' structure. Moreover, as fish are in intimal contact with its surrounding environment, a two-way crossed impact in microbial communities may occur. In fact, in our previous work, changes due to OTC exposure in both water and zebrafish gut bacterial communities' structure were observed after 2mE (Chapter 4). In the present work we intended to elucidate if the changes observed were already noticeable after a shorter exposure period. Interestingly, changes in bacterial communities' structure due to OTC exposure were even more pronounced after five days of exposure (5dE) than after 2 months (2mE), indicating that bacterial communities may adapt to the exposure. Yet, in zebrafish gut, the relative abundance of several genera was affected during exposure, namely the *Cetobacterium* and *Bacillus*. *Cetobacterium* is one of the most abundant genera in freshwater fish gut, namely in zebrafish gut (Zheng et al. 2019; Li et al. 2020). In literature an increase of *Cetobacterium* abundance is usually reported after exposure to low antibiotic concentrations in fish (Carlson et al. 2017; Zhou et al. 2018; Li et al. 2020). However, in our work an opposite effect (abundance decrease) was observed during OTC exposure. In fact, the high OTC concentration used in our experiment (10000 μg/L) may be the reason for this contrary effect. *Cetobacterium* is identified as a vitamin B-12 producer (Tsuchiya et al. 2008) and is pointed as having the capacity of fermentation of peptides and carbohydrates (Finegold et al. 2003) and of inhibition of the growth of other bacterial species (Sugita et al. 1996). Therefore, changes of relative abundance of this genus may lead to a metabolic disorder. The *Bacillus* genus includes the most used probiotic strains in aquaculture, which were reported to influence host immunity against pathogens and to promote growth of fish (Lin et al. 2019). Thus, although OTC is usually used to prevent fish diseases, it may also lead to a decrease of potential beneficial

genera which may unbalance fish health. Indeed, fish gut microbiome may include opportunistic pathogens that induce disease when organisms face a stress condition. Possibly due to OTC susceptibility, the decrease in relative abundance of possible pathogens like *Aeromonas*, and *Plesiomonas* was observed (these genera have minimum inhibitory concentration of OTC 4 µg/mL; CLSI 2017). Moreover, decrease of relative abundance was also observed for some predicted virulence-related genes (Table S7). On the other hand, other genera intrinsically resistant to OTC, like *Pseudomonas*, increased in abundance. Nevertheless, since our study was solely based on culture-independent analyses it is not possible to determine if those phylotypes are indeed pathogens and susceptible to OTC. After exposure ceases, differences between control and exposed communities became more attenuated at structure level in zebrafish gut indicating that bacterial communities seemed to recover. Nevertheless, especially regarding water bacterial communities, after 1mPE significant differences between control and exposed communities were still observed. Indeed, bacterial communities may react differently depending on their capacity to deal with the selective pressure (e.g. susceptibility, resistance or resilience). In this work, the rise of some bacterial phylotypes in the post-exposure period was observed while in other groups, the OTC effect seems to remain (e.g. *Cetobacterium*). This response may be related with the concentration used in our test and the long-term OTC exposure. Hence, OTC may affect sensitive bacteria though not eliminating them totally and, when the selective pressure ends, some have the capacity to recover. One of the rising groups in zebrafish gut after exposure ceased was the *Bacteroides* genus. This is an important member of gut microbiome since some taxa are able to modulate host gene expression related with nutrient absorption, mucosal barrier and xenobiotic metabolism (Hooper et al. 2001). Even though its resistance to several antibiotic classes, including tetracyclines, was already reported, the increase of *Bacteroides* was only observed after exposure ceased. This result may indicate that members of this genus may survive under OTC exposure and the decrease of other taxa might have allowed the observed increase in its relative abundance. Another genus that had increased abundance after exposure ceased (5dPE) was *Shewanella.* Members of this genus are common in fish intestine, and due to its capacity to induce fish immunologic response, some *Shewanella* species have been pointed as having probiotic properties for several fish species (Wang et al. 2019a).

Nevertheless, some members of this genus may act as opportunistic pathogens of freshwater fish (Hau and Gralnick 2007).

OTC also played a role in the selection of some phylotypes from the Rhizobiales order, especially in water. This effect was not only observed during exposure, which is in agreement with our previous work (Chapter 4), but was also observed after exposure ceased. Some members of this group are involved in nitrogen cycling. For instance, *Mesorhizobium* are nitrogen-fixing organisms that hold the ability to provide nitrogen to their host, which can be incorporated into amino acids and other essential organic compounds (Carareto Alves et al. 2014). Moreover, the increase of *Hyphomicrobium* genus under OTC exposure was also observed, which may be related with its capacity to form biofilms (Chee and Liu 2007). The biofilm formation is a strategy that allows bacteria to survive to a higher antibiotic concentration than in the free-living form (Olsen 2015). Thus, probably due to this strategy, the increased relative abundance of some members of this group (e.g *Mesorhizobium* and *Hyphomicrobium*) was also observed even when the exposure ceased. Although the increase of bacteria related with nitrogen cycle was observed, the ammonia released by fish might also have had an influence in this bacteria selection. As stated in our previous work, this result may be related with our experimental design which was performed in a semi-static system (Chapter 4). Yet, nitrogen related bacterial phylotypes may present OTC resistance, which may also explain our results (Feng et al. 2020). In addition, in the present study, an increase in nitrogen metabolism was predicted during OTC exposure (Table 3). However, in literature, other studies showed that OTC exposure may inhibit nitrification and ammonia oxidation process, leading to the accumulation of potential toxic compounds to aquatic organisms (Roose-Amsaleg and Laverman 2016). Nevertheless, in our study, no differences towards the control were observed in nitrite and nitrate concentration. Therefore, a deeper study of the changes in this cycle, for instance through quantitative PCR technologies, targeting for example ammonia oxidizing and denitrification related genes (e.g *amoA*, *nirS*, *nirK*, *nosZ*, *nifH* and *narG*), should be taken in consideration in the future.

Changes in diversity indexes may be an indicator of organism health status, since healthy fish usually presents more diverse bacterial communities (de Bruijn et al. 2018). Similar to our previous work (Chapter 4), no statistically significant changes in these indexes were observed

in zebrafish gut due to OTC exposure. This is likely related with the fact that fish gut microbiomes are more stable in adults (Stephens et al. 2015). On the other hand, since exposure was performed via water, water bacterial communities were expected to be more affected than gut communities, and a decrease in species richness was in fact observed after 2mE for this community. However, since the gut microbiome is exposed to OTC through the ingested water, the exposure dose may be lower than in water. Nonetheless, in our work, OTC concentration in fish gut was not measured. The decrease in species richness was also observed in other studies, even though in fish gut, and seems to be characteristic of antibiotic exposure (Carlson et al. 2017; Zhou et al. 2018). Nevertheless, after 1mPE an opposite effect was observed and an increase in species richness occurred, comparing the exposed with the control group, at this sampling time (1mPE). This result indicates that water bacterial communities might be resilient and after a long post-exposure period appear to be capable of recovering. Nevertheless, changes in bacterial communities' diversity may also have an impact in functional pathways. As previously described, OTC exposure was performed via water. Then water bacterial communities may have been subjected to a higher selective pressure and, consequently, larger changes in the predicted pathways were observed earlier in water community than in fish gut microbiome. This indicates that changes in gut bacterial communities' function takes more time to occur, possible, due to bacterial communities' stability and host internal factors. In addition, as stated before, these results might be also related with a lower OTC exposure concentration in the fish gut. Yet, most of the predicted effects occurred during exposure, and were essentially related with a decrease in genes related with pathways like nucleotide metabolism and translation. Some of the predicted function changes, like the increase of the relative abundance of amino acid transporters, during exposure, might be related with nonspecific antibiotic resistance mechanisms. For instance, some bacteria have efflux pumps that allow the cells to reduce the intracellular antibiotic concentration. In fact, this non-specific resistance mechanism is usually more advantageous to the cells since less energy is required to resist to the antibiotic. Moreover, this strategy might also explain the decrease of relative abundance of *tet* resistance genes observed in our work, since keeping specific resistance genetic determinants may imply a higher energy cost. Nevertheless, after exposure ceased, few pathways remain affected. This may indicate that

OTC sensitive species may be replaced by other bacteria with higher resistance, with similar functions. On the other hand, this result also suggests that after a post-exposure period, bacterial communities recover their function. In fact, overall, the decrease in the number of affected OTUs and genera after a post-exposure period as also the decrease in the number of pathways that continue effected, suggest that bacterial communities recover from the exposure. Nevertheless, it is not possible to indicate the driven force of the recovery. Both parameters are related and the recovery at structure (control and exposed groups become more similar) may led to a recover in the predicted function as vice versa. Hence, in future works, the use of other methodologies like microbiome manipulation would be helpful to reach a deeper answer.

5. Conclusion

In our work, the effects of long-term exposure to OTC in zebrafish, during exposure and after a recovery period, were studied. Overall, our results indicate that exposure to OTC impacts organisms' fitness (e.g. energetic metabolism) and microbiome since the first week of exposure. Indeed, at microbiome structural level, more pronounced effects were observed after 5dE. Nevertheless, after 2mE, zebrafish gut bacterial communities seemed to adapt, and changes observed between exposed and control communities appear somewhat attenuated.

After exposure ceased, organisms seem to recover at energetic reserves level and no differences between exposed and control group were observed. Also, in fish gut and water microbiome, differences were attenuated at structural level. At predicted functional level, few pathways seemed to remain affected by OTC after exposure ceases indicating a recovery of bacterial communities. Then, all-around, our results indicate that after exposure ceased, a re-adjust of fish fitness at energetic reserves and microbiome level seems to occur. Nevertheless, in future works, the analysis of more realistic scenarios like long-term exposure as also a post-exposure period is needed for a better understanding of the real impact of antibiotics exposure.

6. Acknowledgements

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7. Supplementary data

Table S1: Statistical analysis of biochemical assay: EA; Ec and CEA at each sampling time (5dE; 2mE; 5dPE and 1mPE). Asterisks (*) indicates differences towards the respective control ($p < 0.05$; t-test). N= 9 fish per concentration.

Biomarker	Time	Test-value	p
Ea	5dE	-3.528	**0.003***
	2mE	-0.704	0.492
	5dPE	0.121	0.905
	1mPE	1.253	0.231
Ec	5dE	2.640	**0.019***
	2mE	-3.230	**0.004***
	5dPE	-0.235	0.817
	1mPE	-2.127	0.052
CEA	5dE	6.000[a]	**0.017***
	2mE	2.538	**0.025***
	5dPE	0.180	0.860
	1mPE	11.000[a]	0.142

a = Mann-Whitney U Statistic

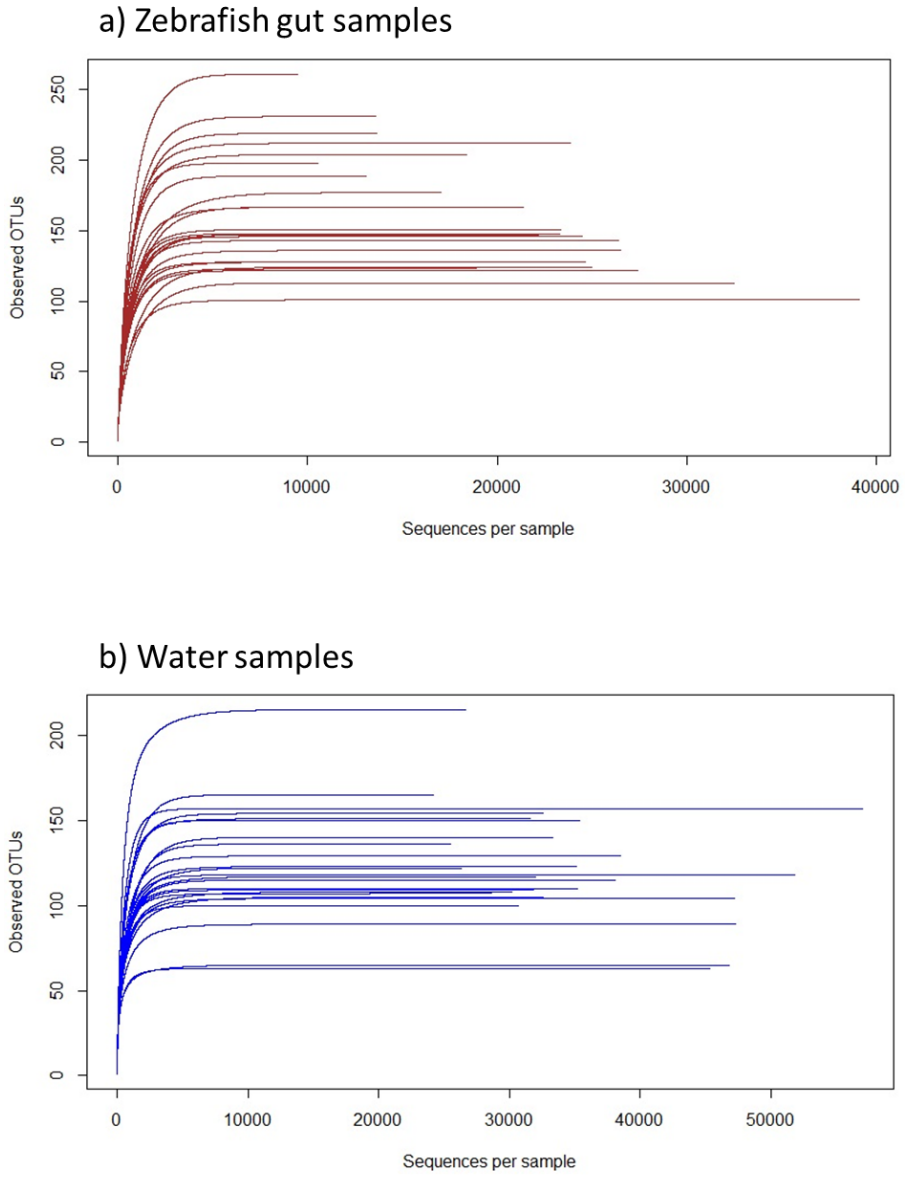

Fig. S1: Rarefaction curves (without singletons) for: a) zebrafish gut samples and b) water sample.

Table S2: Summary of sequencing analysis: Input sequences; Sequences after processing and chimera removal; Sequences assigned to OTUs; Sequences assigned to taxa; Number of reads after lineage-specific copy-number correction and OTUs at each sampling time (5 days of exposure: 5dE; 2 months of exposure: 2mE; 5 days of post-exposure: 5dPE and 1 month of post-exposure: 1mPE).

Sample	Time	Conc. (µg/L)	Input sequences	Sequences after processing and chimera removal	Sequences assigned to OTUs	Sequences assigned to taxa	Counts after lineage-specific copy-number correction	Number of OTUs
GUT	5dE	0	127477	126788	96506	96506	24552	128
		0	137320	136671	110024	110024	27457	122
		0	125605	125052	102061	102061	24722	128
		10000	131767	131460	108485	108485	32832	113
		10000	123663	123178	98708	98708	39292	101
		10000	107811	107335	82526	82526	25207	124
	2mE	0	66584	66557	49357	49357	10613	198
		0	96778	96317	76885	76885	18981	123
		0	100335	100159	73390	73390	15759	148
		10000	102070	102000	79810	79810	18543	204
		10000	133404	133329	105523	105523	24071	2012
		10000	122849	122557	99367	99367	26663	136
	5dPE	0	99642	99516	77468	77468	21843	167
		0	122028	121603	100128	100128	24670	146
		0	127598	127390	101987	101987	23448	148
		10000	126429	126316	99409	99409	23505	151
		10000	117889	117832	92979	92979	22530	147
		10000	145421	145232	112950	112950	26649	143
	1mPE	0	92910	92762	76816	76816	13152	189
		0	70152	70133	55059	55059	9518	261
		0	119691	119535	91538	91538	19176	167
		10000	109229	109064	84712	84712	13912	219
		10000	97849	97678	77820	77820	13794	231
		10000	102788	102400	82198	82198	17128	177
WATER	5dE	0	118042	118014	83112	83112	32636	154

		0	132659	132608	95784	95784	38184	115
		0	100766	100669	77111	77111	31685	151
		10000	148912	148875	117265	117265	57143	157
		10000	84764	84756	63222	63222	26762	215
		10000	88616	88565	57654	57654	24279	165
	2mE	0	118419	118405	92076	92076	35491	150
		0	97159	97155	71853	71853	26378	122
		0	108938	108926	86918	86918	33373	140
		10000	143237	141835	112302	112302	47372	89
		10000	152495	151155	121644	121644	46926	65
		10000	133474	132708	109003	109003	45450	63
	5dPE	0	124520	123133	96424	96424	38599	129
		0	89168	87633	72849	72849	28681	107
		0	105649	104155	85022	85022	35334	110
		10000	96703	95141	76430	76430	31906	109
		10000	79858	79235	61651	61651	25625	136
		10000	139720	138549	114575	114575	51952	118
	1mPE	0	158024	155343	125561	125561	47287	104
		0	129632	127301	99696	99696	32689	105
		0	110610	109356	84382	84382	30792	100
		10000	114222	111914	87387	87387	32100	117
		10000	118390	115765	88799	88799	30300	108
		10000	125489	121580	95354	95354	35251	123

Table S3: Monte Carlo (MC) permutation test representing the significant differences among control and the exposed bacterial communities' structure at each sampling time (5 days of exposure: 5dE; 2 months of exposure: 2mE; 5 days of post-exposure: 5dPE and 1 month of post-exposure: 1mPE). Asterisks (*) indicate significant differences ($p \leq 0.05$).

	Sampling time	t	P(MC)
GUT	5dE	2.679	**0.014***
	2mE	1.608	0.087
	5dPE	1.339	0.197
	1mPE	1.303	0.201
WATER	5dE	2.418	**0.014***
	2mE	2.239	**0.023***
	5dPE	1.789	0.055
	1mPE	2.229	**0.025***

Table S4: Diversity indexes - Species richness (number of OTU; S), evenness (Pielou's evenness index; J) and diversity (Shannon-Wiener index; H') - of the zebrafish gut and water bacterial communities exposed to oxytetracycline (OTU based profile) at each sampling time (5 days of exposure: 5dE; 2 months of exposure: 2mE; 5 days of post-exposure: 5dPE and 1 month of post-exposure: 1mPE). Values presented per mean (m) and standard deviation (SD); N= 3 (3fish per replicate in triplicate); Asterisks (*) indicate differences towards the respective control ($p < 0.05$; t-test). Data is normal.

	Sampling time	Conc. (µg/ L)	S		J		H'	
			m	SD	m	SD	m	SD
GUT	**5dE**	0	126	3.464	0.635	0.020	3.068	0.083
		10000	113	11.504	0.642	0.019	3.027	0.038
	2mE	0	156	38.188	0.661	0.047	3.335	0.400
		10000	184	41.761	0.611	0.012	3.173	0.179
	5dPE	0	154	11.590	0.643	0.045	3.240	0.270
		10000	147	4.000	0.651	0.008	3.246	0.057
	1mPE	0	206	49.166	0.660	0.027	3.501	0.230
		10000	209	28.355	0.662	0.012	3.531	0.145
WATER	**5dE**	0	140	21.703	0.747	0.0144	3.683	0.107
		10000	179	31.432	**0.679***	0.022	3.515	0.210
	2mE	0	137	14.189	0.707	0.046	3.473	0.179
		10000	**72***	14.468	0.768	0.036	3.273	0.025
	5dPE	0	115	11.930	0.657	0.027	3.117	0.193
		10000	121	13.748	0.623	0.024	2.986	0.132
	1mPE	0	103	2.646	0.695	0.041	3.218	0.178
		10000	**116***	7.550	0.625	0.039	2.970	0.164

Table S5: OTUs of zebrafish gut, significantly affected by OTC in an exposure and post exposure period (5 days of exposure: 5dE; 2 months of exposure: 2mE; 5 days of post-exposure: 5dPE and 1 month of post-exposure: 1mPE) ($p \leq 0.05$; t-test). Abundance variation means differences of relative abundance (%) towards the respective control. When affiliation to genera was not possible affiliation to a higher taxonomic level is presented. Data is normal.

OTU id	Sampling time	t-test	P	Abundance variation (%)	Taxonomic affiliation
OTU_1	5dE	5.297	0.006	-18.185	*Cetobacterium*
OTU_16	5dE	8.275	0.001	-6.134	*Crenobacter*
	1mPE	-4.711	0.009	2.733	
OTU_4	5dE	5.188	0.007	-2.771	*Aeromonas*
	2mE	3.626	0.022	-3.749	
OTU_9	1mPE	3.570	0.023	-0.985	*Gemmobacter*
OTU_22	2mE	11.603	<0.001	-4.537	Enterobacterales
OTU_11	1mPE	2.897	0.044	-2.912	*Cetobacterium*
OTU_15	5dE	5.910	0.004	-0.990	*Bacillus*
OTU_60	5dE	11.510	<0.001	-0.705	*Cetobacterium*
OTU_31	5dE	-2.823	0.048	2.524	*Rhodobacteraceae*
OTU_45	5dE	4.036	0.016	-0.512	*Shewanella*
	2mE	8.214	0.001	-0.212	
OTU_72	2mE	3.193	0.033	-1.068	*Plesiomonas*
OTU_120	5dE	3.634	0.022	-0.327	*Cetobacterium*
OTU_71	5dE	3.693	0.021	-0.203	*Cetobacterium*
	1mPE	7.673	0.002	-0.096	
OTU_79	5dE	4.174	0.014	-0.203	*Cetobacterium*
OTU_81	5dE	3.877	0.018	-0.168	*Cetobacterium*
	1mPE	7.245	0.002	-0.115	
OTU_88	5dE	3.057	0.038	-0.164	*Cetobacterium*
OTU_18	5dE	-5.142	0.007	6.363	*Pseudomonas*
OTU_28	5dE	-3.868	0.018	5.703	*Mesorhizobium*
OTU_19	5dPE	-3.548	0.024	5.395	*Bacteroides*
OTU_63	1mPE	3.520	0.024	-0.291	*Cetobacterium*
OTU_123	5dE	-7.137	0.002	0.267	*Rhizobiaceae*
OTU_42	5dE	-2.909	0.044	0.261	*Variovorax*
OTU_84	1mPE	4.316	0.013	-0.350	*Cetobacterium*
OTU_130	5dPE	-4.087	0.015	0.715	*Bacteroides*
OTU_86	1mPE	2.895	0.044	-0.060	*Cetobacterium*
OTU_149	5dPE	-3.654	0.022	0.549	*Bacteroides*
OTU_101	5dE	4.347	0.012	-0.153	*Cetobacterium*
OTU_117	5dE	4.671	0.010	-0.143	*Cetobacterium*
	2mE	-3.482	0.025	0.101	
OTU_58	5dPE	4.260	0.013	-0.291	*Rhodobacter*
OTU_30	2mE	4.428	0.011	-0.289	*Shewanella*

	5dPE	-3.151	0.035	0.693	
OTU_89	5dPE	-3.538	0.024	0.227	*Shewanella*
OTU_85	5dE	2.994	0.040	-0.138	*Cetobacterium*
	1mPE	5.144	0.007	-0.227	
OTU_41	2mE	2.790	0.049	-0.360	Proteobacteria
OTU_68	5dE	5.522	0.005	-0.118	*Bacillus*
OTU_112	5dE	4.091	0.015	-0.115	*Cetobacterium*
	1mPE	4.610	0.010	-0.073	

Table S6: Statistically affected genera of the zebrafish gut and water bacterial communities exposed to oxytetracycline at each sampling time (5 days of exposure: 5dE; 2 months of exposure: 2mE; 5 days of post-exposure: 5dPE and 1 month of post-exposure: 1mPE). Differences in the relative abundance towards the respective control ($p \leq 0.05$; t-test). When affiliation to genera was not possible affiliation to a higher taxonomic level is presented.

	Taxonomic affiliation	**Sampling time**	**t-test**	**p**
GUT	*Cetobacterium*	5dE	4.739	0.009
		1mPE	5.211	0.006
	Crenobacter	5dE	8.630	0.001
		1mPE	-4.711	0.009
	Aeromonas	5dE	5.159	0.007
		2mE	3.315	0.030
	Gemmobacter	5dE	-2.805	0.049
		1mPE	3.570	0.023
	Enterobacterales	2mE	11.603	<0.001
	Bacillus	5dE	5.868	0.004
	Pseudomonas	5dE	-4.898	0.008
	Mesorhizobium	5dE	-3.868	0.018
	Plesiomonas	2mE	6.667	0.003
	Shewanella	5dE	3.126	0.035
		2mE	3.893	0.018
		5dPE	-2.944	0.042
	Proteobacteria	2mE	2.790	0.049
WATER	*Sphingomonas*	2mE	-7.923	0.001
	Candidatus Berkiella	5dE	4.071	0.015
	Pelomonas	5dE	5.917	0.004
		2mE	-3.971	0.017
		5dPE	-5.578	0.005
	Haliscomenobacter	5dE	8.888	0.001
		2mE	5.385	0.006
	Roseicitreum	5dE	2.853	0.046
	Labilithrix	5dE	18.536	<0.001

		2mE	2.944	0.042
		5dPE	3.169	0.034
	Acidovorax	5dE	-3.571	0.038
		2mE	-3.135	0.035
	Emticicia	5dE	-11.387	<0.001
		2mE	-4.896	0.008
	Comamonadaceae	5dE	-3.965	0.017
		2mE	2.968	0.041
	Rhodobacteraceae	2mE	3.064	0.038
		1mPE	3.674	0.021
	Runella	2mE	-8.103	0.001
	Gemmobacter	1mPE	2.993	0.040
	Chromatiaceae	5dE	-4.141	0.014
		2mE	-10.321	0.001
		5dPE	-7.410	0.002
	Burkholderiales	2mE	3.720[a]	0.021
		5dPE	4.811	0.009
		1mPE	-3.758	0.020
	Reichenowia	5dE	4.605	0.010
		5dPE	3.357	0.028
	Proteobacteria	1mPE	4.145	0.014
	Chitinophagaceae	5dE	8.295	0.001
		2mE	6.133	0.004
	Rhizobiaceae	5dE	-22.263	<0.001

a = Mann-Whitney U Statistic

Table S7: Predicted genes/ proteins (KEGG ID) for which abundance was significantly affected by OTC in zebrafish gut and water microbiomes at each sampling time (5 days of exposure: 5dE; 2 months of exposure: 2mE; 5 days of post-exposure: 5dPE and 1 month of post-exposure: 1mPE). Values represents the variation of relative abundance (%) towards the control (decrease: red color; increase: green color) ($p \leq 0.05$; t-test). White color represents no statistically significant differences. Data is normal.

Sample type	Description	KEGG ID	5dE	2mE	5dPE	1mPE
GUT	*tetA*; MFS transporter, DHA1 family, tetracycline resistance protein	K08151	0.020			
	tet35; tetracycline resistance efflux pump	K18218		-0.013		
	atoB; acetyl-CoA C-acetyltransferase [EC:2.3.1.9]	K00626	0.068	0.244		
	fabG, OAR1; 3-oxoacyl-[acyl-carrier protein] reductase [EC:1.1.1.100]	K00059		0.156		
	tRNA-Leu; tRNA Leu	K14228		-0.070		
	tRNA-Met; tRNA Met	K14230		-0.113		
	tRNA-Val; tRNA Val	K14237		-0.157		
	tRNA-Ser; tRNA Ser	K14233		-0.054		
	23SrRNA, rrl; 23S ribosomal RNA	K01980		-0.199		
	5SrRNA, rrf; 5S ribosomal RNA	K01985		-0.198		
	16SrRNA, rrs; 16S ribosomal RNA	K01977		-0.198		
	putative transposase	K07497		-0.263		
	transposase	K07483		-0.211		
	insB; insertion element IS1 protein InsB	K07480		-0.539		
	mviM; virulence factor	K03810	-0.004	-0.010		
	hlyU; ArsR family transcriptional regulator, virulence genes transcriptional regulator	K22042	-0.007	-0.025		
WATER	*tetA*; MFS transporter, DHA1 family, tetracycline resistance protein	K08151	-0.029	-0.026		
	tetV; MFS transporter, DHA3 family, tetracycline resistance protein	K18215		0.001		
	tet35; tetracycline resistance efflux pump	K18218		-0.0001		
	tetM, tetO; ribosomal protection tetracycline resistance protein	K18220	-0.001		-0.0002	
	tetR; TetR/AcrR family transcriptional regulator, tetracycline repressor protein	K18476	-0.006			
	GST, gst; glutathione S-transferase [EC:2.5.1.18]	K00799	0.109			
	E2.3.1.9, atoB; acetyl-CoA C-acetyltransferase [EC:2.3.1.9]	K00626	-0.040	0.089		

	livK; branched-chain amino acid transport system substrate-binding protein	K01999	0.259			
	livH; branched-chain amino acid transport system permease protein	K01997	0.201			
	livG; branched-chain amino acid transport system ATP-binding protein	K01995	0.219			
	livF; branched-chain amino acid transport system ATP-binding protein	K01996	0.210			
	livM; branched-chain amino acid transport system permease protein	K01998	0.183			
	ABC.PE.P; peptide/nickel transport system permease protein	K02033	0.027	-0.143		
	ABC.SN.S; NitT/TauT family transport system substrate-binding protein	K02051	0.218			
	ABC.SN.P; NitT/TauT family transport system permease protein	K02050	0.195			
	ABC.SN.A; NitT/TauT family transport system ATP-binding protein	K02049	0.198			
	ABC.PE.S; peptide/nickel transport system substrate-binding protein	K02035		-0.137		
	ABC.PE.P1; peptide/nickel transport system permease protein	K02034		-0.148		
	ABC.PE.A; peptide/nickel transport system ATP-binding protein	K02031		-0.140		-0.162
	ABCB-BAC; ATP-binding cassette, subfamily B, bacterial	K06147		-0.051	-0.033	-0.054
	fabG, OAR1; 3-oxoacyl-[acyl-carrier protein] reductase [EC:1.1.1.100]	K00059		0.038	0.020	0.014
	ABC-2.A; ABC-2 type transport system ATP-binding protein	K01990			-0.069	-0.121
	ABC-2.P; ABC-2 type transport system permease protein	K01992				-0.083
	tRNA-Arg; tRNA Arg	K14219			-0.011	
	hlyU; ArsR family transcriptional regulator, virulence genes transcriptional regulator	K22042	0.0004	-0.0002		

Table S8: OTUs of water, significantly affected by OTC in an exposure and post exposure period (5 days of exposure: 5dE; 2 months of exposure: 2mE; 5 days of post-exposure: 5dPE and 1 month of post-exposure: 1mPE). ($p \leq 0.05$; t-test). Abundance variation means differences of relative abundance (%) towards the respective control. When affiliation to genera was not possible affiliation to a higher taxonomic level is presented. Data is normal.

OTU id	Sampling time	t-test	p	Abundance variation (%)	Taxonomic affiliation
OTU_25	5dE	2.931	0.043	-7.229	*Ideonella*
OTU_9	2mE	-7.923	0.001	0.58	*Gemmobacter*
OTU_52	5dE	5.917	0.004	-4.438	*Phenylobacterium*
	2mE	-5.458	0.005	3.994	
OTU_55	5dE	3.708	0.021	-4.348	*Acidovorax*
OTU_24	5dE	8.888	0.001	-3.727	*Sphingobacterium*
	2mE	5.385	0.006	-0.894	
OTU_16	5dE	17.922	<0.001	-2.512	*Crenobacter*
	2mE	2.944	0.042	-0.536	
	5dPE	3.169	0.034	-0.554	
OTU_56	5dPE	-13.414	<0.001	0.156	Sphingomonadaceae
OTU_44	5dE	11.181	<0.001	-1.438	*Roseicitreum*
	5dPE	3.526	0.024	-0.307	
OTU_35	5dE	3.028	0.039	-1.370	*Marinobacter*
OTU_23	2mE	3.731	0.020	-3.544	*Emticicia*
	5dPE	4.958	0.008	-2.375	
	1mPE	-3.573	0.023	2.377	
OTU_8	2mE	-3.182	0.034	2.546	*Ancylobacter*
OTU_33	5dE	-11.387	<0.001	4.157	*Hydrogenophaga*
	2mE	-5.581	0.005	1.963	
OTU_28	5dE	-3.965	0.017	3.603	*Mesorhizobium*
	1mPE	-9.606	0.001	0.176	
OTU_20	2mE	-9.869	0.001	7.192	*Fluviicola*
OTU_69	5dE	-3.849	0.018	0.536	*Gemmobacter*
OTU_330	5dE	-4.141	0.014	0.352	*Hyphomicrobium*
OTU_36	2mE	-2.978	0.041	3.243	Proteobacteria
OTU_123	5dE	-6.956	0.002	0.285	Rhizobiaceae
OTU_26	2mE	3.461	0.026	-7.148	*Mesorhizobium*
OTU_29	2mE	3.181	0.034	-6.220	*Devosia*
OTU_22	2mE	3.182	0.034	-0.802	Enterobacterales
OTU_76	5dE	3.130	0.035	-0.494	*Flavobacterium*
OTU_39	2mE	-10.830	<0.001	7.537	*Hyphomicrobium*
	5dPE	-7.410	0.002	0.261	
OTU_21	2mE	-2.892	0.045	2.156	Actinobacteria
OTU_27	5dE	2.861	0.046	-0.253	Rhodobacteraceae
OTU_197	5dE	4.803	0.009	-0.333	*Minicystis*
OTU_4	5dE	8.295	0.001	-0.053	*Aeromonas*
	2mE	6.133	0.004	-0.253	

Table S9: Fractions of energy available (Ea): Protein, Lipids and Carbohydrates (mJ/mg wet weight) at each sampling time (5dE; 2mE; 5dPE and 1mPE). Values are mean ± standard deviation. Asterisks (*) indicate differences towards the respective control ($p < 0.05$; t-test); a = Mann-Whitney U Statistic.

Sampling time	OTC (µg/L)	Protein	Lipids	Carbohydrate
5dE	0	1256.3 ± 337.8	6118.6 ± 725.7	244.1 ± 19.9
	10000	**1719.0 ± 329.8*[a]**	7183.3 ± 1224.6	**320.2 ± 118.1*[a]**
2mE	0	1518.9 ± 285.6	6705.8 ± 1887.1	311.4 ± 63.3
	10000	1421.7 ± 505.5	7515.4 ± 1544.9	**177.4 ± 16.7*[a]**
5dPE	0	1508.8 ± 396.6	7411.8 ± 1427.3	214.2 ± 46.2
	10000	1363.8 ± 451.5	7931.6 ± 1134.3	214.4 ± 44.2
1mPE	0	1015.7 ± 165.5	8035.8 ± 1325.8	272.5 ± 71.9
	10000	**1327.1 ± 219.9***	7887.5 ± 2109.4	186.0 ± 91.2

Chapter 6

Tetracycline resistance in water and zebrafish bacteria following antibiotic exposure

Tetracycline resistance in water and zebrafish bacteria following antibiotic exposure

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Submitted to Chemosphere

Abstract

Tetracycline is one of the most used antibiotics in food-production and thus, one of the most often detected in aquatic systems around the world. Since there are evidences that the use of antibiotics in food-production (e.g. aquaculture) may promote the emergence of potential antibiotic-resistant pathogens, this topic raises concern. Therefore, in this work, the selection of tetracycline-resistant bacteria and tetracycline resistance genes was evaluated following an exposure to oxytetracycline (OTC). Zebrafish adults were exposed to OTC (0.01 and 10 µg/mL) for two months via water. Then, organisms were transferred to clean water and kept for 5 days. Samples from fish (skin and gut) and water were collected during exposure (5 days and 2 months of exposure: 5dE and 2mE) and at the end of the test (5 days of post-exposure: 5dPE). The selection of antibiotic-resistant bacteria (ARB) were determined using selective media. Then ARB were isolated, identified and characterized. Moreover, the abundance of *tetA* resistance gene was estimated through qPCR in all sampling periods.

Overall, our results revealed an increase in the abundance of ARB, although only statistically significant at 5dPE in water samples. ARB affiliated to *Pseudomonas* and *Stenotrophomonas* genera, which include strains indicated as fish and humans' pathogens. The analysis of susceptibility profile revealed that *Stenotrophomonas* isolates were resistant to other antibiotics, such as trimethoprim/sulfamethoxazole (95 % resistant isolates) and chloramphenicol (100 % resistant isolates) while *Pseudomonas* isolates were resistant to ticarcillin (95 % resistant isolates) and ticarcillin/clavulanic acid (100 % resistant isolates). Also, the resistance profiles of our isolates differed according to the isolates sampling origin (water or fish skin). A multidrug resistance (MDR) phenotype was observed for 37 % of the

isolates. The inspected *tet* genes (i.e. *tetA, tetB, tetC, tetD, tetE, tetG*, *tetM* and *tetO*) were not detected in our isolates, suggesting the presence of other resistance mechanisms. However, the selection of the *tetA* gene was confirmed by qPCR in zebrafish gut samples at 5dE, at the highest OTC concentration tested. The virulence of 7 selected MDR isolates was tested using zebrafish embryos. A significantly higher embryo mortality was observed for two *Stenotrophomonas* strains, revealing a potential impact in fish health and survival. Overall, our work highlights the potential impact of antibiotic contamination in aquatic environments and its role in the selection of potential pathogenic antibiotic-resistant bacteria and antibiotic resistance genes.

Keywords: *Danio rerio,* pathogenicity test, multidrug resistance; qPCR, microcosm

1. Introduction

It is estimated that in the next decades, due to human population growth, the pressure on land and resources will increase (Cazalis et al. 2018). Thus, to supply food demand, intensive and semi-intensive systems for livestock and agriculture production are used. For instance, in aquaculture, one of the fast-growing industries in the last decades (Føre et al. 2018), fish are usually cultivated overcrowded, representing a highly stressful environment which facilitates pathogens spread. Due to its low-cost production and broad-spectrum efficacy, oxytetracycline (OTC) is one of the most used antibiotics in food production industry in Europe (European Medicines Agency, 2019). Since organisms are not able to metabolize all the ingested antibiotic, the compound is easily excreted and dispersed into the aquatic environment (Cravedi et al. 1987). Consequently, OTC has been detected not only in aquaculture system residues (Chen et al. 2015; Monteiro et al. 2016) but also in rivers and surface waters around the world (Zou et al. 2011; Harnisz et al. 2015b).

The selective pressure that antibiotics can cause in environmental bacteria, even at low concentrations, may lead to the selection of antibiotic-resistant bacteria (ARB) and to the increase of antibiotic resistance genes (ARGs) prevalence. Since ARGs can be transmitted to other bacteria through a variety of gene transfer systems (e.g. horizontal transfer via conjugation), they can be easily disseminated, especially in water systems (Tacão et al. 2012; Marti et al. 2014). Therefore, ARGs have been pointed as environmental pollutants and aquatic environments are being considered large reservoirs of ARGs (Zhu et al. 2017). In literature several works have reported the selection of ARB and ARGs by antibiotic exposure in aquatic environments. For instance, Seyfried et al. (2010) showed that the water from fish farms with recent OTC use had higher prevalence of tetracycline resistance genes (*tet*) than in water from farms not using OTC; Harnisz et al. (2015b) showed that fish farms using OTC impacted Drweca river water by increasing the diversity of *tet* resistance genes; Huang et al. (2017) revealed that the abundance of ARGs in fish culture ponds was higher than in control ponds. Yet, to our knowledge, few works devoted to studying the effect of OTC exposure in the selection of antibiotic ARB and ARGs in a controlled environment like a microcosms approach (Maruzani et al. 2018, 2020). In fact, this methodology has been pointed has an advantageous tool since this controlled environment allows to reduce confounding variables (Maruzani et al. 2018; Silva et al. 2021).

The extensive use and misuse of antibiotics may unbalance organisms' defenses, promoting the emergence of pathogens. Indeed, there are evidences that the use of antibiotics in husbandry and aquaculture systems may promote the emergence of antibiotic-resistant zoonotic pathogens that may be transferred from animal to humans (Santos and Ramos 2018). Therefore, the possible selection of antibiotic-resistant pathogens that threatens both animals and human health has been raising concern.

In our previous works, we have demonstrated that long-term exposure to OTC may not only affect the organisms itself (e.g. decrease of energetic reserves) but also induce changes in fish and water bacterial communities (Chapter 3 and 4). Moreover, although organism's microbiome and water bacterial communities seem to recover after a post-exposure period, some of the effects promoted by OTC may be still observed (Chapter 5). However, the role/influence of OTC exposure in the selection of potential pathogens harboring ARGs was

not evaluated. Consequently, in this work, we intend to study the effect of a long-term exposure to OTC in the selection of ARB and ARGs in zebrafish and exposure water using a microcosm approach. Zebrafish adults were exposed via water, for two months to two concentrations of OTC (0.01 and 10 µg/mL). Then, organisms were transferred to clean water and kept for 5 days. The selection of ARB and ARGs was evaluated through culture-dependent (e.g. isolation of OTC-resistant bacterial strains, characterization of their antibiotic resistance genotypes and phenotypes and zebrafish pathogenicity tests) and culture-independent methods (e.g. purification of environmental DNA followed by quantitative PCR (qPCR) targeting tetracycline resistance genes) for a comprehensive and integrated analysis.

2. Materials and Methods

2.1 Zebrafish culture and exposure

Zebrafish (*Danio rerio*) adults were obtained from the zebrafish culture established at the Biology Department of University of Aveiro (Aveiro, Portugal). The fish were kept under controlled conditions (temperature 27 ºC; pH 7.5 ± 0.5; conductivity 800 ± 50 and dissolved oxygen ≥ 95%) in a recirculation system as described by Domingues et al. (2016). Zebrafish adults, were selected for the experimental assays as recommended by OECD guideline 230 (OECD 2009). A total of 81 adults were exposed to two concentrations of oxytetracycline hydrochloride (0.01 and 10 µg/mL) plus a control, for two months, via water, under a semi-static condition as described in Chapter 3. After exposure, organisms were kept for five days in clean water (culture water) for recovery. During the experiment, fish were fed daily with the commercial pellet Gemma Micro 500 food (Skretting®, Spain) and water renewed every three days to ensure water quality and OTC concentrations (Chapter 3). Samples were collected during the experiment at 3 different sampling moments: 5 days and 2 months of exposure (5dE and 2mE, respectively) and 5 days of post-exposure (5dPE). At each sampling point, samples from both exposure water and fish were collected. To sample fish gut and skin bacteria, 9 fish per concentration were euthanized with tricaine overdose (tricaine methane sulfonate,

Metacain, MS-222; CAS number: 886-86-2) followed by spinal cord severing. Zebrafish fins were removed and placed in 3 mL of Lysogeny broth (LB) medium and incubated at room temperature with smooth agitation until processing (section 2.2.1); fish guts were aseptically removed and stored at -80 ºC until analysis through culture-independent methods (section 2.3.1). To select water bacteria through culture-dependent methods, water samples were immediately processed (section 2.2.1). Also, for culture-independent analysis 100 mL of exposure water were collected in triplicate and filtered using 0.22 mm hydrophilic PVDF durapore membrane filter (Merck Millipore; Massachusetts, EUA) for DNA purification (section 2.3.1). Samples were then stored at -80ºC until further analysis.

2.2 Culture-dependent analyses

2.2.1 Bacterial strains isolation

Tetracycline (TET)-resistant bacteria were selected from samples (fish skin and water) exposed to the highest concentration of OTC (10 µg/mL). Zebrafish skin bacteria, pre-incubated as described in the section 2.1 were collected by filtrating 100 µL of LB medium, through 0.45 µm pore membranes (Pall Corporation; Michigan, USA). To collect bacteria from exposure water, 100 µL of water was filtrated through 0.45 µm pore membranes (Pall Corporation; Michigan, USA). Bacteria retained in the membranes, from both fish skin and water, were then incubated for 24h at 30 ºC in the following media: Membrane Fecal Coliform agar (mFC), *Pseudomonas Aeromonas* Selective agar (GSP) and Plate Count Agar (PCA), all supplemented with 16 µg/mL of TET. After this period, colony forming units (CFUs) were quantified. Sixty antibiotic-resistant isolates from each culture media (30 isolates from fish skin and 30 isolates from water from GSP and mFC) were randomly selected, purified and stored for further analysis.

2.2.2 Isolates identification- 16S rRNA gene sequencing

To identify the bacterial isolates, the 16S rRNA gene was amplified through polymerase chain reaction (PCR) using as DNA template a whole-cell suspensions prepared in 20 µL of sterile distillated water (Araújo et al. 2017). PCR mixture (25 µL) consisted of 16.25 µL of nuclease-free water, 6.25 µL of NZYTaq 2x Green Master Mix (2.5 mMMgCl2; 200

mMdNTPs; 0.2 U/µL DNA polymerase) (NZYTech, Portugal), 0.75 µL of each primer (10mM solution) and 1 µL DNA suspension. The PCR programs and primers are described in Table S1. All reactions were conducted in the Bio-Rad Thermal Cycler (Bio-Rad Laboratories, CA, USA). A positive and a negative control were included. PCR products were separated by electrophoresis (1.5% agarose gel) and stained with ethidium bromide. Then, PCR products were purified using the NZYGelpure kit (NZYTech, Portugal), following the manufacturer's instructions and sent to Eurofins Genomics (Eurofins Genomics, Ebersberg, Germany) for Sanger sequencing. The nucleotide sequences were used for comparison with the GenBank database using the nucleotide Basic Local Alignment Search Tool (nBLAST) and aligned with their closest relatives (Altschul et al. 1990).

2.2.3 Detection of tetracycline resistance and integrase encoding genes

The occurrence of tetracycline resistance genes was inspected by PCR in the selected isolates. Different target genes were selected, namely genes encoding efflux pumps (*tet*A, *tet*B, *tet*C, *tet*D, *tet*E and *tet*G) and ribosomal protection proteins (*tet*M and *tet*O). Also, the presence of integrons, namely the integrase encoding genes *intI1* and *intI2*, was analyzed. PCR mixtures (25 µL) were obtained by adding the DNA (1µL of cell suspension as described above) to nuclease-free water (16.25 µL), NZYTaq 2x Green Master Mix (6.25 µL; 2.5 mMMgCl2; 200 mMdNTPs; 0.2 U/µL DNA polymerase) (NZYTech, Portugal) and the respective primers (0.75 µL of each in a 10mM solution) (Table S1). The PCR primers and programs are described in Table S1. PCR products were verified as described in the section 2.2.2.

2.2.4 Antibiotic susceptibility testing

The isolates antibiotic susceptibility profile was determined using the disk diffusion method on Mueller-Hinton Agar (MH) according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST, www.eucast.org) guidelines (Matuschek et al. 2014). The selection of antibiotic and disk concentrations was based on the EUCAST recommendations for the genera of the isolates. The antibiotics tested were the following: aztreonam (ATM; 30 mg), cefepime (FEP; 30 mg), ceftazidime (CAZ; 10 mg), chloramphenicol (C; 30 mg),

ciprofloxacin (CIP; 5 mg), gentamicin (CN; 10 mg), imipenem (IMI; 10 mg), ticarcillin (TIC; 75 mg), ticarcillin/clavulanic acid (TIM; 75 mg TIC + 10 mg clavulanic acid), tigecycline (TGC; 15 mg), trimethoprim/sulfamethoxazole (STX; 25 mg) (Oxoid, Basingstoke, UK). Cells of each isolate were suspended in sterile saline solution (0.9% NaCl) with an adjusted turbidity of 0.5 McFarland. The reference strain used for quality control was the *Escherichia coli* ATCC 25922. Plates were incubated at 30ºC for 18 h and the inhibition zone diameters were measured. Isolates were then classified as susceptible, intermedium or resistant according to EUCAST expert rules and CLSI breakpoints tables for the gentamicin (European Committee on Antimicrobial Susceptibility Testing 2020).

2.2.5 Zebrafish pathogenicity test

To assess strains pathogenicity, we selected potential fish pathogenic bacteria, based on the phylogenetic affiliation (i.e. *Stenotrophomonas maltophilia*) and also included isolates resistant to 4 classes of antibiotic (isolates M-A6, M-A12, M-A16, M-A18, M-P9, M-P13 and M-P14; Table S3). Selected strains were grown in LB overnight at 30ºC with agitation. The optic density (OD) of each strain was measured at 600 nm in a spectrophotometer UV mini-1240 (UV-VIS Spectrometer, Shimadzu). Bacterial concentrations were adjusted to 10^8 CFU/mL in sterile fish system water (Milligan-Myhre et al. 2011).

On the day before mating, zebrafish adults were placed in rearing aquariums and left for matting. Zebrafish eggs were collected within 30 min after natural mating and rinsed in fish system water. Then, embryos were screened using a stereomicroscope (Stereoscopic Zoom Microscope-SMZ 1500, Nikon Corpotation) to exclude unfertilized or injured embryos.

Embryos with 24 hours-post-fertilization (hpf), were manually dechorionized for exposure and pools of 20 organisms in triplicate were used for exposure to each test strain. Embryos were then exposed by static immersion for 5 hours to each strain (10^8 CFU/mL) in petri dish as described by Milligan-Myhre et al. (2011). After this period, organisms were removed and washed twice in sterile fish system water. Then, embryos were transferred to new petri dishes and kept in fish system water in a controlled temperature room. Organisms were observed daily under a stereomicroscope, during a period of four days. Mortality was recorded and dead organisms were removed daily.

2.3 Culture-independent analyses

2.3.1 Environmental DNA extraction

Total DNA extraction of zebrafish gut and water was performed as described in Chapter 5. Briefly, zebrafish gut DNA was extracted using the PowerSoil® DNA isolation kit (MOBIO laboratories, CA, USA) following the manufactures instruction. Water DNA extraction was performed using the commercial kit Genomic DNA Purification kit (Thermo Fisher Scientific; Massachusetts, EUA) as described by Henriques et al. (Henriques et al. 2004).

2.3.2 Quantitative Polymerase Chain Reaction (qPCR)

The abundance of total bacteria (using the 16S rRNA gene as proxy) and the abundance of the *tet*A resistance gene were determined by qPCR using the primer sets 338F/518R and tetAF/tetAR, respectively. PCR mixture (20 µL) consisted in 7.2 µL of ultrapure water, 10 µL NZYSpeedy qPCR Green Master Mix (NzyTech, Portugal), 0.4 µL of each primer (10mM solution) and 2 µL of DNA (50 to 100 ng). The temperature profile for the 16S rRNA gene was the following: 95 ºC for 3 min, followed by 30 cycles at 95 ºC for 15 s and 65 ºC for 30 s. The temperature profile for *tet*A was 95 ºC for 10 min, followed by 35 cycles at 95 ºC for 15 s and 60 ºC for 30 s. The melting curve for both genes was obtained from 55 to 95 ºC, with steady 0.1ºC increments at each 5 seconds. Standard curves were obtained by 10-fold dilutions in ultrapure water of plasmid DNA holding inserts of the target genes as described by Tavares et al. (2020). Concentration of the standard plasmids was determined using NanoDrop spectrophotometer (NanoDrop Technologies, USA) and converted to DNA copies (Kim et al. 2013). The amplification efficiency was calculated, and the copy numbers of each target were determined by interpolation in the corresponding curve. The copy numbers of *tet*A resistance gene was normalized by dividing it by the 16 S rRNA copy number at each sampling time.

2.4 Statistical analysis

Sigma plot V.12.5 (SysStat software Inc., CA, USA) was used to calculate statistical differences due to OTC exposure. A t-test was used to calculate OTC effects in the selection

of TET-resistant bacteria at each sampling time. The one-way ANOVA test was used to calculate differences in *tet*A gene abundance (qPCR) and pathogenic effect of our isolates. The Shapiro-Wilk test was used to test normality. A Kruskal-Wallis test followed by the appropriate post hoc (Dunn or Dunnett's test) was used when normality test failed. A significant level of 0.05 was considered.

3. Results and discussion

The present study shows the effects of long-term exposure to oxytetracycline (OTC) in the selection of tetracycline-resistant bacteria and resistance genes in both fish (skin and gut) and water.

3.1 Effects of OTC in the abundance of tetracycline-resistant bacteria

The variation of total counts of tetracycline (TET)-resistant bacteria, expressed in Log (CFU/mL), along exposure time is represented in the Fig. 1. Our results reveal the selection of tetracycline-resistant bacteria in both water and fish skin, regardless of the sampling time and culture medium. However, possibly due to the variability observed between replicates, this selection was only statistically significant for water samples at 5dPE in GSP medium ($p= 0.05$). In fact, our experimental design, namely the use of individual aquariums (each aquarium represents a replicate), in a semi-static condition may explain this result. Since the relation organism-environment is dynamic, individual characteristics of fish may regulate water bacteria. On its side, water bacterial communities may also influence organisms microbiome (Stephens et al. 2015). After 2mE, possibly due to the antibiotic effect, an overall reduction in total colony counts was observed, particularly in fish skin. On the other hand, after exposure ceased, the total counts of resistant bacteria increased. It is known that OTC may have a bacteriostatic effect not eliminating all the bacteria (Chopra and Roberts 2001). Therefore, after the exposure ceased the increase of antibiotic-resistant bacteria (ARB) would be expected. In fact, evidences of the selection of ARB due to antibiotic use were observed in aquaculture environments (Gao et al. 2012; Harnisz et al. 2015b). Nevertheless, *in situ* studies

imply that several factors may influence these results which may represent a limitation. Therefore, due to the methodology used, in our study it was possible to reduce other environmental variabilities and the evidences of OTC impact was clearer. Hence, our work comes to highlight the importance of applying a microcosm approach, to surpass these limitations and better understand the impact of antibiotic exposure.

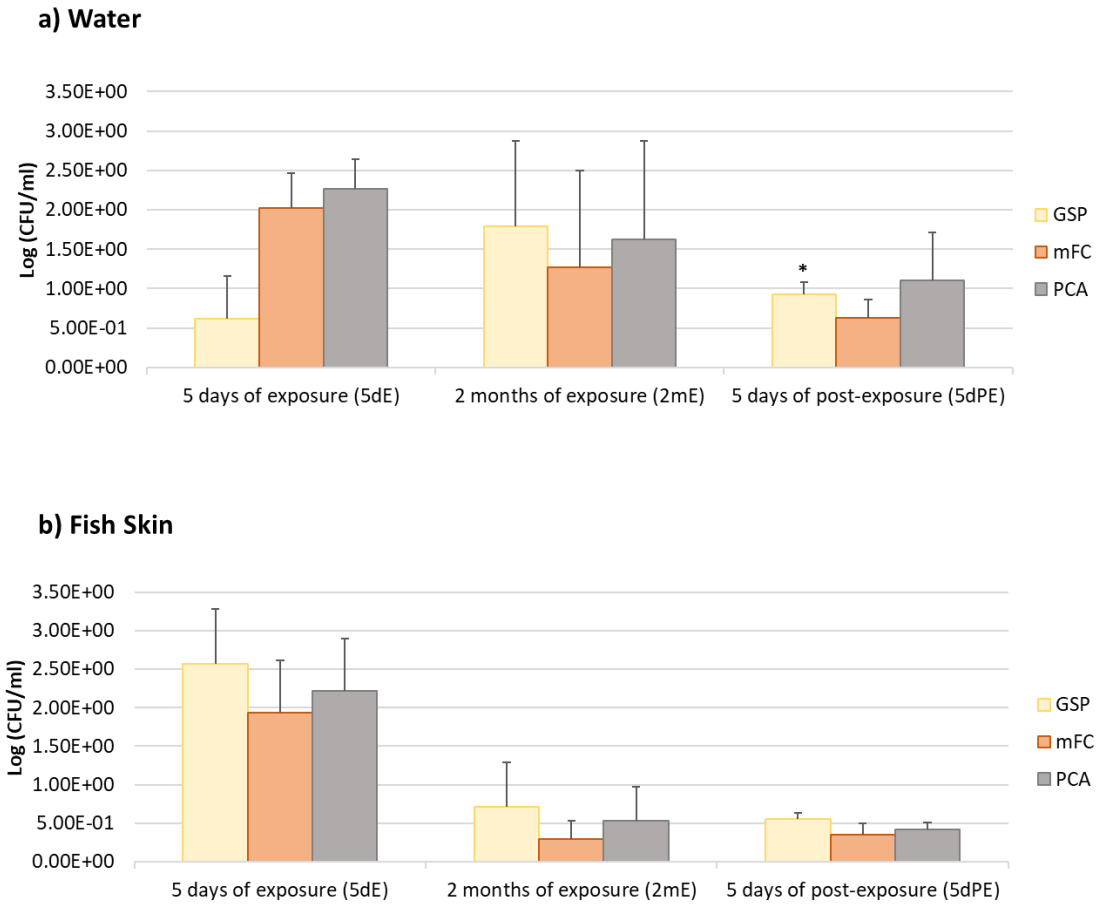

Fig. 1: Variation (difference between exposed and control) of total counts Log (CFU/mL) of antibiotic resistant bacteria at each sampling time (5 days of exposure: 5dE; 2 months of exposure: 2mE and 5 days of post-exposure: 5dPE), according to its respective selective medium (GSP, mFC and PCA) supplemented with tetracycline (TET) at 16 µg/mL. Asterisks (*) represent statistically significant differences ($p \leq 0.05$) towards the respective control.

3.2 Taxonomic affiliation of isolates

Regarding the taxonomic affiliation, in our experiment two genera were identified: *Stenotrophomonas* and *Pseudomonas*. Namely, 37 % of the isolates selected from water samples were identified as *Stenotrophomonas* (16 isolates in GSP medium and 6 isolates in mFC medium) and 63 % as *Pseudomonas* (14 isolates in GSP medium and 24 isolates in mFC medium). Isolates selected from the fish skin affiliated with *Stenotrophomonas* (35%; 17 isolates in GSP medium and 4 isolates in mFC medium) and *Pseudomonas* (65%; 13 isolates in GSP medium and 26 isolates in mFC medium). These genera present intrinsic characteristics that provide resistance against tetracyclines. For instance, it is known that *Pseudomonas* have low membrane permeability, express efflux pumps that may expel toxic compounds and are also able to produce antibiotic-inactivating enzymes (Pang et al. 2019). *Stenotrophomonas*, on the other hand, express multi-drug efflux pumps and have the capacity to form biofilms (Brooke 2012), which are known to provide additional resistance to antibiotics (Olsen 2015). In addition, it has also been reported that both *Stenotrophomonas* and *Pseudomonas* have the capacity to biodegrade tetracyclines, allowing these genera to use this antibiotic as a carbon source and to survive in the presence of this antibiotic (Wang et al. 2019b; Reis et al. 2020).

Stenotrophomonas and *Pseudomonas* are considered pathogenic to fish and humans. Although these genera are naturally present in healthy fish, the abundance of both *Stenotrophomonas* and *Pseudomonas* can significantly increase in diseased fish skin (Rosado et al. 2019). For instance, some *Stenotrophomonas* species are known to cause enteritis and cutaneous hemorrhage in fish (Geng et al. 2010). Moreover, *Stenotrophomonas* spp. may also cause septicemia and pneumonia in humans (Adegoke et al. 2017). Consequently, the selection of these genera by OTC may raise health concern.

3.3 Antibiotic resistance profile and resistance genes

Antibiotic resistance profiles of the selected isolates are represented in the Fig. 2. *Stenotrophomonas* isolates were resistant to chloramphenicol (100% in water and fish skin)

and trimethoprim/sulfamethoxazole (water: 95% and fish skin: 100%) followed by cefepime (water: 82% and fish skin: 71%). All *Stenotrophomonas* isolates revealed to be sensitive to tigecycline (100% in water and fish skin). Regarding *Pseudomonas* genus, isolates were resistant to ticarcillin/clavulanic acid (water: 100% and fish skin: 97%) and ticarcillin (97% in water and fish skin). All *Pseudomonas* isolates showed to be sensitive to gentamicin and displayed a reduced susceptibility to ciprofloxacin. These results suggest that the resistance profiles differ according to the isolates sampling origin (water or fish skin). In fact, bacterial communities of both matrices are different and therefore, influenced by distinct factors. For instance, fish mucus is a complex fluid containing several proteases, lectins and anti-microbial peptides, that plays a barrier against pathogens and harbor a highly specific bacterial community (Boutin et al. 2013; Benhamed et al. 2014; Pimentel et al. 2017). Indeed, a previous work reported that bacteria from freshwater fish mucus may present multiple antibiotic resistance phenotype (Ozaktas et al. 2012).

The isolation of strains resistant to three or more classes of antibiotics (multidrug resistant) was also verified (Table S3). Namely, *Stenotrophomonas* included a higher number of multidrug resistant isolates (water: 82% and fish skin: 76%) than what was observed for *Pseudomonas* (water: 21 % and fish skin: 5 %). Worth to note that the resistance profile of our isolates included resistance phenotypes even to those antibiotics used in clinical settings to treat human infections. For instance, trimethoprim/sulfamethoxazole is usually the primary choice to treat *Stenotrophomonas* infections in humans (Chang et al. 2015; Rizek et al. 2018). Nevertheless, in the last years the prevalence of *Stenotrophomonas* resistant to this antibiotic has been increasing (Rizek et al. 2018). In our work 97 to 100% of our isolates revealed to be resistant to trimethoprim/sulfamethoxazole. Hence, in literature, other alternatives have been studied and antibiotics like tigecycline and moxifloxacin were indicated as having a high efficacy against *Stenotrophomonas* in *in vitro* tests (Nicodemo and Paez 2007; Looney et al. 2009). Regarding *Pseudomonas*, antibiotics like colistin, aminoglycosides and ceftolozane/tazobactam are usually prescribed for clinical treatment (Bassetti et al. 2019; Horcajada et al. 2019). From the antibiotics tested in this work, few showed effective activity in our isolates. For instance, only tigecycline and gentamicin induced 100% of susceptible

profile in *Stenotrophomonas* and *Pseudomonas* isolates, respectively. Hence, this result indicates that OTC exposure may co-select for bacteria resistant to other antibiotics.

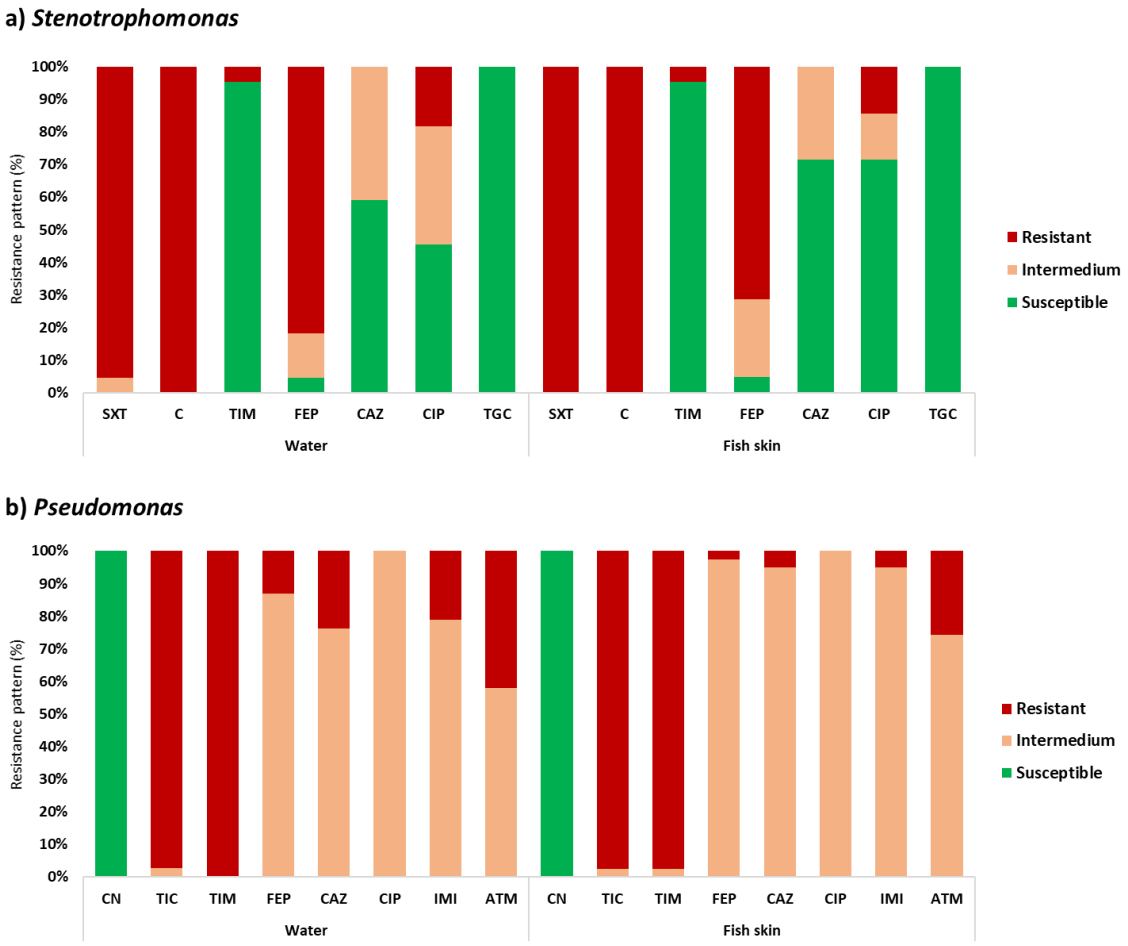

Fig. 2: Resistance pattern (susceptible, intermedium, resistant) of isolated bacteria according to the genera (*Stenotrophomonas* and *Pseudomonas*) and type of sample (water or fish skin). Aztreonam: ATM; cefepime: FEP; ceftazidime: CAZ; chloramphenicol: C; ciprofloxacin: CIP; gentamicin: CN; imipenem: IMI; ticarcillin: TIC; ticarcillin/clavulanic acid: TIM; tigecycline: TGC; trimethoprim/sulfamethoxazole: STX.

It is known that bacteria may acquire antibiotic resistance mechanisms through vertical or horizontal gene transfer. In fact, in literature, it was reported that some tetracycline resistance genes like *tet*B, *tet*C, *tet*E, *tet*S, *tet*O and *tet*M are usually associated to transferable elements (Roberts 2005; Agersø et al. 2007; Henriques et al. 2008). In our work, the presence of *tet*

resistance genes was inspected by PCR, however, no *tet* genes were detected in any of the isolates. Moreover, the search for integrases was also carried out. These elements are usually associated with mobile antibiotic resistance genes, involved in intraspecific and interspecific dissemination of resistance (Cambray, et al. 2010). Nevertheless, in our work no integrase genes (*intI*1 and *intI*2) were found. Indeed, the methodology used may favor this result. The use of selective medium supplemented with 16 µg/mL (minimal inhibitory concentration: MIC for *Enterobacteriaceae*) of TET might be high enough to not allow bacteria harboring *tet* resistance genes to grow, promoting the growth of intrinsically resistant bacteria (Lundström et al. 2016). Also, another hypothesis may be related with the fact that the proportion of ARB harboring *tet* genes may be so small that the increase of other intrinsically resistant bacteria may mask them (Lundström et al. 2016). Therefore, the absence of acquired *tet* and *intI* genes suggests a predominance of intrinsic resistance characteristics among our isolates.

3.4 Pathogenicity test

Zebrafish embryos were used to evaluate the pathogenicity of 7 multidrug *Stenotrophomonas* resistant strains. In fact, zebrafish embryos present several advantages like optical transparency and translational potential to humans, being indicated has a powerful tool to study host-pathogen interactions (Torraca and Mostowy 2018; Gomes and Mostowy 2020). Overall, all the strains tested induced mortality in zebrafish embryos (Fig. 3). Nevertheless, only two isolates revealed significant results, namely one isolate from exposure water (M-A16; p= 0.004) and one isolate from fish skin (M-P14; p= 0.018). The infection via immersion test the capacity of a pathogen to cross the fish natural barrier (e.g. fish skin), simulating the natural exposure pathway (Rowe et al. 2014). Nonetheless, it is not possible to determine the exact number of bacteria that can invade the organism (Rowe et al. 2014). Yet, the results are an indicator that our isolates have a pathogenic effect and can cause mortality in fish. Hence, future studies applying other endpoints like fish immunological response or strains' virulence factors screening may allow us to obtain a deeper understanding of the pathogenic potential of these isolates.

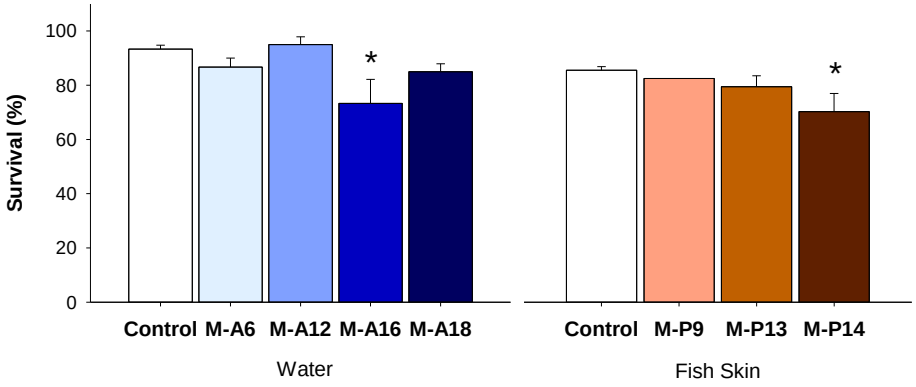

Fig. 3: Survival percentage (%) of exposed zebrafish embryos exposed to multidrug resistant strain isolated from water and zebrafish skin. Strains were obtained after 5 days post-exposure (5dPE) period, that had been previously exposed to 10 µg/mL of OTC. Asterisks (*) represent statistically significant differences ($p \leq 0.05$) towards the control.

3.5 Abundance of *tet*A gene in water and fish gut

The abundance of the tetracycline resistance gene *tet*A was assessed in zebrafish gut and water in three sampling times, namely during exposure at 5dE and 2mE and after exposure ceases at 5dPE (Fig 4). The 16S rRNA gene was quantified to estimate the absolute abundance of bacteria and to estimate the relative abundance of *tet*A gene (*tet*A/16 S rRNA gene copy number). The qPCR reaction efficiency and correlation coefficients (R^2) for both genes, 16S rRNA gene and *tet*A, are presented in Table S2. This is a very sensitive tool, used to analyze/quantify ARGs in the bacterial communities of environmental samples (Kim et al. 2013; Suzuki et al. 2019). Hence, it might be a useful complement to the culture-dependent methods (Harnisz et al. 2015a). One of the most frequently detected tetracyclines resistance gene is *tet*A. Actually, this gene was already pointed has a potential indicator of tetracyclines resistance genes abundance (Huang et al. 2017). In our work, despite the low abundance, it was possible to detect *tet*A gene in both zebrafish gut and water samples. Regarding zebrafish gut, it was possible to observe that only in samples exposed to 10 µg/mL of OTC occurred an increase in the prevalence of the *tet*A gene comparatively to the control. Indeed, this tendency can be observed in all sampling points, however, only in the first exposure period (5dE) was

observed a statistically significant result (p= 0.05). On the other hand, no statistically significant differences were observed in bacteria abundance (16S rRNA gene absolute abundance) due to OTC exposure. Concerning exposure water, at 10 µg/mL of OTC, the same tendency observed in fish gut (increase in the prevalence of the *tet*A gene) was also observed after 5dE and 5dPE, although not statistically significant regarding the respective control. Nevertheless, an opposite effect occurred after 2mE and was observed a decrease in the relative abundance of *tet*A gene towards the control, although not statistically significant. Moreover, at the same exposure time (2mE) was observed an increase of bacteria abundance (16 S rRNA gene absolute abundance increase; $p \leq 0.001$). This result may indicate that, probably occurred the selection of bacteria expressing other resistance mechanisms like intrinsic mechanisms.

Given this, our results indicate that OTC may select ARB and promote the increase of ARGs abundance, namely the *tet*A, although this increase is exposure time-dependent. In fact, previous works reported that low concentrations as 1 to 15 µg/L of TET may increase the abundance of resistance genes (Gullberg et al. 2011; Lundström et al. 2016). Moreover, it was also reported that the selection of *tet* resistance genes, also observed in this work, may also allow the co-selection of ARGs to other antibiotics (Lundström et al. 2016; Zhang et al. 2019). For instance, it was pointed the co-selection of *tet*A with *sul2* and bla_{TEM-1} possible due to the location of these genes in the same mobile genetic elements (e.g. plasmids) (Zhang et al. 2019).

Hence, the use of culture-independent methods, might be an useful methodology to complement the culture-dependent methods. This integrated approach may allow a better understanding of the antibiotic role in the selection of ARB and ARGs.

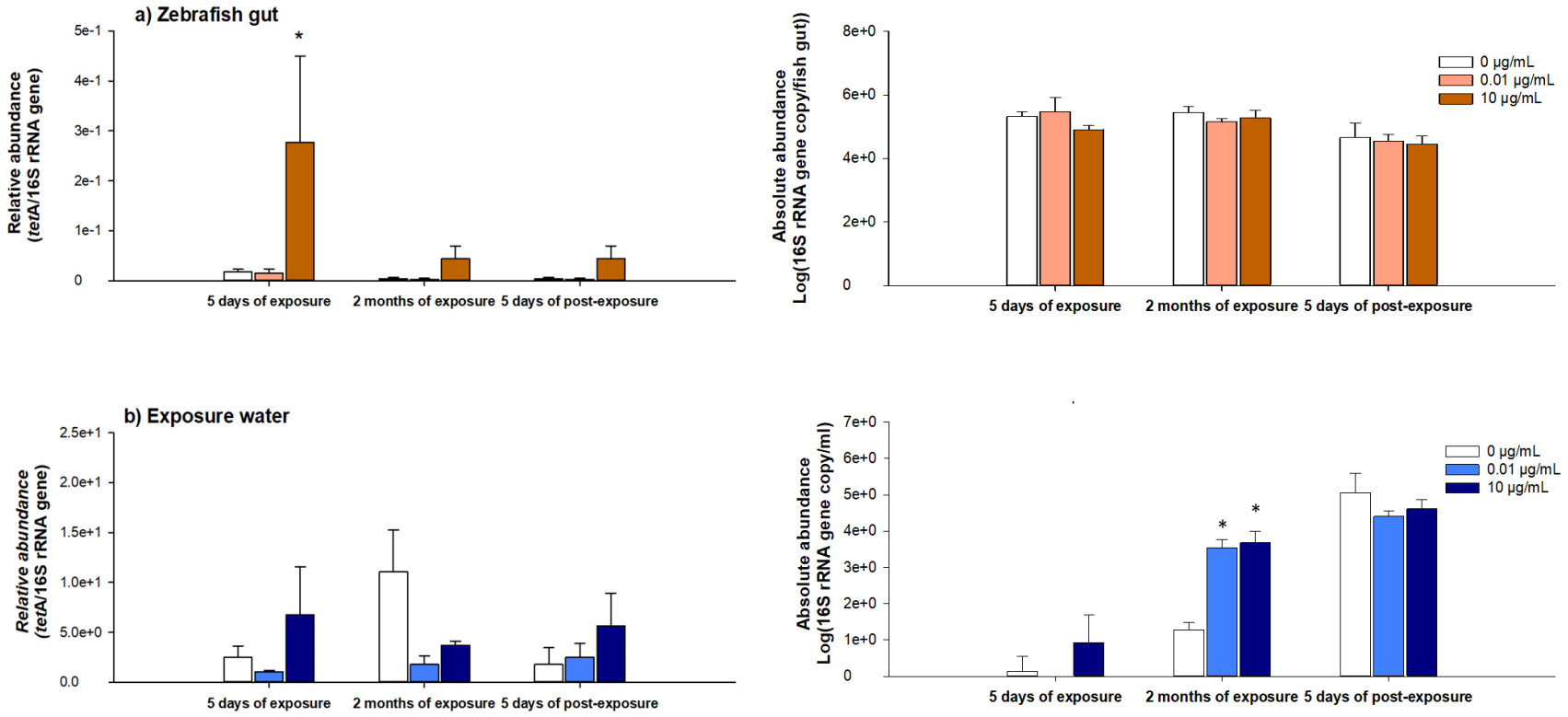

Fig 4: Relative abundance of *tet*(A) and absolute abundance of 16S rRNA gene (Log gene copy number/mL or fish gut), determined by qPCR in zebrafish gut and water exposed to OTC (0, 0.01 and 10 µg/mL) at three sampling time (5days of exposure: 5dE; 2 months of exposure: 2mE and 5 days post-exposure: 5dPE). Asterisks (*) represent statistically significant differences ($p \leq 0.05$) towards the respective control (0 µg/mL).

4. Conclusion

Our results indicate that the selection of TET-resistant bacteria from fish skin and water occurred. Moreover, the selection of *Pseudomonas* and *Stenotrophomonas* may be related with intrinsically characteristics of these genera. Regarding ARGs, no *tet* or integrase encoding genes were detected in our isolates. Although ARB selected in this work were possibly intrinsic resistant to TET, analysis of susceptibility to other antibiotics revealed the multidrug resistance (MDR) profile of some isolates. In addition, the pathogenicity test revealed that these MDR bacteria may induce mortality in zebrafish embryos. Hence, the selection of intrinsically resistant bacteria may raise concern since the resistance to other antibiotics may also occur. The analysis of *tetA* presence in bacterial community of fish gut and water, through qPCR, revealed that this gene was selected in the samples exposed to the highest concentration tested (10 µg/mL). Thus, culture-independent methods complemented results obtained using the culture-based approach and may allow a better understanding of the real impact of TET in ARB and ARGs selection.

Therefore, this study underlines the impact of an antibiotic exposure in the aquatic environment as also the role of an antibiotic exposure in the selection of a potential antibiotic-resistant pathogens.

5. Acknowledgements

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6. Supplementary data

Table S1: PCR primers and conditions of 16S, tetracycline resistance genes (*tet*) and integrases (*int*).

Target	Primer sequence	Amplicon Size	Annealing temperature (ºC)	Program	Reference
16S rDNA	27_F: AGAGTTTGATCCTGGCTCAG 1492_R: GGCTACCTTGTTACGACTT	1467	52	94 ºC - 3 min (1x) 94 ºC - 1 min 52 ºC - 1 min 72 ºC - 2 min (30x) 72 ºC–10 min (1x)	Lane (1991)
***tet*(A)**	tetA_F: GCTACATCCTGCTTGCCTTC tetA_R: GCATAGATCGCCGTGAAGAG	211	53	94 º C – 5 min (1x) 94 ºC – 30 s, 53 ºC – 30 s, 72 ºC– 30 s, (35x) 72 ºC–7 min (1x)	Nawaz et al. (2006)
***tet*(B)**	tetB_F: TCATTGCCGATACCACCTCAG tetB_R: CCAACCATCATGCTATTCCATCC	391			
***tet*(C)**	tetC_F: CTGCTCGCTTCGCTACTTG tetC_R: GCCTACAATCCATGCCAACC	897			
***tet*(D)**	tetD_F: TGTGCTGTGGATGTTGTATCTC tetD_R: CAGTGCCGTGCCAATCAG	844			
***tet*(E)**	tetE_F: ATGAACCGCACTGTGATGATG tetE_R: ACCGACCATTACGCCATCC	744			
***tet*(G)**	tetG_F: GCGCTNTATGCGTTGATGCA tetG_R: ATGCCAACACCCCCGGCG	803	55	94 ºC – 5 min (1x) 94 ºC – 30 s, 55 ºC – 30 s, 72 ºC– 30 s, (35x) 72 ºC–7 min (1x)	Ng et al. (2001)
***tet*(M)**	tetM_F: GTGGACAAAGGTACAACGAG tetM_R: CGGTAAAGTTCGTCACACAC	406			
***tet*(O)**	tetO_F: AACTTAGGCATTCTGGCTCAC tetO_R: TCCCACTGTTCCATATCGTCA	515			

***tet*(S)**	tetS_F: CATAGACAAGCCGTTGACC tetS_R: ATGTTTTTGGAACGCCAGAG	667			
intI1	intI1_F: CCTCCCGCACGATGATC intI1_R: TCCACGCATCGTCAGGC	280	55	94 ºC – 5 min (1x) 94 ºC – 30 s, 55 ºC – 30 s, 72 ºC– 30 s, (30x) 72 ºC–7 min (1x)	Kraft et al. (1986)
intI2	intI2_F: CCTCCCGCACGATGATC intI2_R: TCCACGCATCGTCAGGC	233	50	94 ºC – 5 min (1x) 94 ºC – 30 s, 50 ºC – 30 s, 72 ºC– 30 s, (30x) 72 ºC–7 min (1x)	Goldstein et al. (2001)

Table S2: The qPCR reaction efficiency (E) and correlation coefficients (R^2) for 16S and *tet*A at each sampling time (5 days of exposure: 5dE; 2 months of exposure: 2mE and 5 days of post-exposure: 5dPE).

	16S		*tet*A	
	R^2	E (%)	R^2	E (%)
5 days of exposure (5dE)	0.948	185.1	0.980	130.4
2 months of exposure (2mE)	0.979	146.4	0.993	103.8
5 days of post-exposure (5dPE)	0.975	171.0	0.974	88.8

Table S3: Resistance profile of multidrug resistant bacteria according its medium selection (GSP: G or mFC: M medium) and type of sample (water: A or fish skin: P). Aztreonam: ATM; cefepime: FEP; ceftazidime: CAZ; chloramphenicol: C; ciprofloxacin: CIP; imipenem: IMI; ticarcillin: TIC; ticarcillin/clavulanic acid: TIM; trimethoprim/sulfamethoxazole: STX.

Strain	Closest relative strain	Accession number	Antibiotic resistance phenotype
G-A1	*Stenotrophomonas maltophilia* strain APP36	MT533812.1	FEP; C; SXT
G-A5	*Stenotrophomonas maltophilia* strain APP36	MT533812.1	FEP; C; SXT
G-A6	*Stenotrophomonas maltophilia* strain APP36	MT533812.1	FEP; C; SXT
G-A8	*Stenotrophomonas maltophilia* strain CGKV/J16a-2013	MK078536.1	FEP; C; SXT
G-A9	*Stenotrophomonas sp.* ROi7	EF219038.1	FEP; C; SXT
G-A10	*Stenotrophomonas maltophilia* strain APP36	MT533812.1	FEP; C; SXT
G-A13	*Stenotrophomonas maltophilia* strain B.xNS12	MT199173.1	FEP; C; SXT
G-A15	*Stenotrophomonas maltophilia* strain APP36	MT533812.1	FEP; C; SXT
G-A16	*Stenotrophomonas maltophilia* strain APP36	MT533812.1	FEP; C; SXT
G-A17	*Stenotrophomonas maltophilia* strain APP36	MT533812.1	FEP; C; SXT
G-A18	*Stenotrophomonas maltophilia* strain APP36	MT533812.1	FEP; C; SXT
G-A19	*Stenotrophomonas maltophilia* strain APP36	MT533812.1	FEP; C; SXT
G-A25	*Stenotrophomonas pavanii* strain S1-5	MT645772.1	FEP; C; SXT
G-P4	*Stenotrophomonas maltophilia* strain APP36	MT533812.1	FEP; C; SXT
G-P5	*Stenotrophomonas maltophilia* strain APP36	MT533812.1	FEP; C; SXT
G-P8	*Stenotrophomonas maltophilia* strain APP36	MT533812.1	FEP; C; SXT
G-P21	*Stenotrophomonas maltophilia* strain APP36	MT533812.1	FEP; C; SXT
G-P22	*Stenotrophomonas maltophilia* strain Ai 4	MN880434.1	FEP; C; SXT
G-P23	*Stenotrophomonas maltophilia* strain Ai 4	MN880434.1	FEP; C; SXT
G-P24	*Stenotrophomonas maltophilia* strain APP36	MT533812.1	FEP; C; SXT
G-P25	*Stenotrophomonas maltophilia* strain APP36	MT533812.1	FEP; C; SXT
G-P26	*Stenotrophomonas maltophilia* strain APP36	MT533812.1	FEP; C; SXT
G-P27	*Stenotrophomonas maltophilia* strain APP36	MT533812.1	FEP; C; SXT
G-P28	*Stenotrophomonas sp.* NA06056	CP054931.1	FEP; C; SXT

G-P30	*Stenotrophomonas maltophilia* strain APP36	MT533812.1	FEP; C; SXT
G-A14	*Pseudomonas alcaligenes* strain clon40	MN371833.1	TIC; TIM; CAZ; IMI
G-P12	*Pseudomonas sp.* strain Isyb05	KY678894.1	TIC; TIM; CAZ; IMI
G-P16	*Pseudomonas sp.* strain Atecer7E	MT386171.1	TIC; TIM; CAZ; IMI
M-A6	*Stenotrophomonas maltophilia* strain APP36	MT533812.1	TIM; C; SXT; CIP
M-A12	*Stenotrophomonas maltophilia* strain APP36	MT533812.1	FEP; C; SXT; CIP
M-A16	*Stenotrophomonas maltophilia* strain APP36	MT533812.1	FEP; C; SXT; CIP
M-A18	*Stenotrophomonas maltophilia* strain APP36	MT533812.1	FEP; C; SXT; CIP
M-P9	*Stenotrophomonas maltophilia* strain APP36	MT533812.1	FEP; C; SXT; CIP
M-P13	*Stenotrophomonas maltophilia* strain APP36	MT533812.1	FEP; C; SXT; CIP
M-P14	*Stenotrophomonas maltophilia* strain APP36	MT533812.1	FEP; C; SXT; CIP
M-A13	*Pseudomonas sp.* Pc102	LC420219.1	TIC; TIM; FEP; CAZ; IMI
M-A14	*Pseudomonas sp.* Pc102	LC420219.1	TIC; TIM; FEP; CAZ; IMI; ATM
M-A15	*Pseudomonas alcaligenes* strain clon40	MN371833.1	TIC; TIM; FEP; CAZ; IMI; ATM
M-A19	*Pseudomonas sp.* Pc102	LC420219.1	TIC; TIM; FEP; CAZ; IMI; ATM
M-A27	*Pseudomonas sp.* Pc102	LC420219.1	TIC; TIM; FEP; CAZ; IMI
M-A28	*Pseudomonas sp.* Pc102	LC420219.1	TIC; TIM; FEP; CAZ; IMI; ATM
M-A29	*Pseudomonas sp.* Pc102	LC420219.1	TIC; TIM; CAZ; IMI

Chapter 7

Final remarks

1. General discussion

This thesis intended to contribute to the knowledge about the impact of antibiotics at several levels of biological organizations. Due to its broad spectrum and low cost, oxytetracycline (OTC) is one of the mostly used antibiotic in aquaculture (European Medicines Agency 2019) and, therefore, widely detected in the aquatic environments (Daghrir and Drogui 2013). Therefore, OTC was used for a comprehensive analysis of antibiotics impact in non-target organisms. Analyses at the organism (e.g. behavior) and biochemical levels (enzymatic activity), of the microbiome (e.g. structure and function) and antibiotic resistance selection (e.g. antibiotic-resistant bacteria and antibiotic resistance genes) were performed. Moreover, the reversibility of effects in a non-continuous exposure scenario was also delved. To test our main goal, several hypotheses were raised and are discussed in the present section.

In this work, a wide range of OTC concentrations (0.1, 10 and 10000 µg/L) was used (**Chapter 3 and 4**). The concentrations chosen were based on literature reports for environmental concentrations in surface water (0.1 µg/L) and aquaculture residues (10 µg/L) (Kolpin et al. 2002; Monteiro et al. 2016). The use of environmental relevant scenarios with realistic concentrations allowed us to have a more comprehensive analysis of the potential impacts in the aquatic system. Yet, a higher concentration was used (10000 µg/L) to allow the study of the mode of action and the dose-response effect of OTC in fish. In addition, this approach would allow to get a better knowledge about OTC adverse outcome pathway (AOP) in fish. Therefore, the use of endpoints at several biological organization level as sub-individual (e.g. enzymatic activity) and organism level (e.g. behavior) was important to assess the overall impact of OTC in fish fitness.

Oxytetracycline (OTC) may induce changes in zebrafish behavior and energetic reserves.

The conventional ecotoxicology endpoints were applied in a first approach (**Chapter 3**). The use of fish behavioral endpoints to assess the effect of chemical exposure has been

increasing of interest in the last years, especially since new tools of automatic video recording (e.g. Zebrabox, Viewpoint) were developed allowing high-throughput and accurate analysis of fish behavior (Correia et al. 2019). Several studies have been reporting chemical effects in neurobiological processes resulting in anxiety-like behavior, hyperactivity and memory impairment (Chen et al. 2020; Wronikowska et al. 2020). Moreover, these effects are very often detected at concentrations several orders of magnitude lower than other conventional endpoints like mortality (Andrade et al. 2016). We performed several tests to understand the role of OTC in fish anxiety/stress behavior. Although neurotoxicity is not the primary mode of action of OTC in fish (Nunes et al. 2015), our results revealed an important effect of this compound at behavior level, even if the variability among individuals, prevented, in some cases, the observation of a clear tendency (e.g. feeding test). We also observed that OTC did not change the activity of acetylcholinesterase (ChE), indicating that behavior changes observed were not mediated by the disruption of the cholinergic system. We advanced the hypothesis (**Chapter 3**) that behavioral changes observed may result of photo-sensitiveness induced by OTC in zebrafish. In fact, Tetracyclines are known photosensitizers that in the presence of sunlight may cause burn, hives and edema in humans (Björn 2015), skin lesions in fish (Mark Stacell & David G. Huffman 1994) and induce phototoxicity in crustaceans (Peroti et al. 2021). During the light/dark test, organisms were exposed to high light intensity which probably induced phototoxicity and therefore the hyperactivity observed. However, further studies are needed for a better understanding of these mechanisms. Worth to note that behavior represents an ecological relevant endpoint since it allows us to extrapolate our result to the population/ community level. In the case of fish, an impairment in the locomotor behavior may compromise prey capture, predator avoidance (Correia et al. 2019), reproduction and feeding success, compromising the fitness of the species. Hence, our results indicate that although no changes at physical level (e.g. deformation or mortality) were observed, the impact of OTC may later compromise the population dynamic.

On the other hand, enzymatic activity analysis is one of the most widely used tools in ecotoxicology tests, that allows to understand the global physiological status of the organism. These parameters permit to perceive how the organism deals with a stress (e.g. energy available) and what mechanisms they may be using (e.g. detoxification). Probably due to the

long exposure time, in our work, a decrease in energetic reserves (e.g. CEA) and enzymatic activity (e.g. CAT and GST) was observed. This result may be an indicator that after a long period exposed to OTC, due to ROS production and molecular damage, organisms were not able to deal with the stress and some biological processes like detoxification and antioxidant defenses decay. Hence, this effect is of concern since antibiotics are indicated as persistent compounds in nature and therefore organisms can be exposed for long periods.

Organisms microbiome and water bacterial communities are affected by oxytetracycline exposure.

The effects of long-term exposure of OTC was assessed also at microbiome level of both fish gut and water (**Chapter 3 and 4**). As discussed along our thesis, organism's microbiome plays an important role in organisms health. Moreover, the relation between host-microorganism is not static and may change due to chemical exposure. Reports have demonstrated that changes of organism's microbiome due to chemical exposure may be also a sensitive endpoint (Evariste et al. 2019). In fact, effects at bacterial community's structure were observed at low concentrations ranging from ng/L to µg/L (Zhou et al. 2018; Evariste et al. 2019). This was also observed in our work with effects of OTC being observed at 10 µg/L (**Chapter 4**). Moreover, studies have been relating the role of organism's microbiome in several diseases and/ or regulation of biological functions. Namely, bowel disease (Glassner and Abraham 2019); social behavior (Archie and Tung 2015) and metabolic disorders (Holmes et al. 2011). The impact of OTC in this work led to dysbiosis in fish gut. It is known that some bacterial phylotypes are implied in the normal functions of the intestine and nutrient metabolism (Limbu et al. 2020). Hence, changes of fish gut bacterial communities' structure may lately cause an impact in fish metabolic responses. For instance, the decrease of CEA, also observed in **Chapter 3**, may probably be related with the dysbiosis observed.

The analysis of the microbiome, in this work, was performed through two main techniques: the denaturing gradient gel electrophoresis (DGGE) and high-throughput Illumina technology. DGGE analysis was firstly used to allow a global overview of the bacterial community structure and dynamics **(Chapter 3)**. Although in this technique only the predominant bacteria

present within the community are observed (Henriques et al. 2006), it is frequently used due to its cost and time-effectiveness to obtain a general picture of the community structure and dynamics and as a first step to choose which samples should be used for a deeper analysis. Hence, the use of DGGE in our first work **(Chapter 3)**, allowed to have a wide perspective of the impact of OTC in the bacterial communities' structure of both fish gut and water. Then, Illumina technology was used for a deeper analysis of the bacterial structure and to predict the functions affected by the chemical exposure **(Chapter 4)**. This technique has a low-cost and high efficiency which allows the analysis at high-throughput of microbial ecology with a great coverage (Caporaso et al. 2012). So, both techniques were used as a complement of each one.

Zebrafish adults were an useful model to attend our goals and hypothesis, since this organism has a well-studied microbiome. This permits a better understanding of the relation microbiome-host. In addition, adult fish have a more defined and stable microbiome than other life stages (Stephens et al. 2015), allowing the reduction of the possible influence of fish internal factors and assuring that the effects observed are mostly attributed to OTC exposure. Nevertheless, some variability among individuals was observed in our work (**Chapter 3 and 4**). This variability is possibly derived from intrinsic (e.g. genetic and immunological) (Larsen et al. 2013; Tarnecki et al. 2017) and/ or external (e.g. amount of food intake and water quality) (Xia et al. 2014; Tarnecki et al. 2017) factors. Hence, it was important the use of pools of individuals in each replicate as also the use of several replicates (each aquarium as an individual replicate) to have a higher representativeness of fish microbiome diversity.

Also, in this work, the analysis of water bacterial communities was done since organisms and environment are not disconnected. In fact, aquatic organisms are always in intimal involvement with their environment influencing each other. Probably due to its exposure to higher OTC concentrations, water bacterial communities seemed to suffer higher changes (at structural and functional level) than fish gut. Nevertheless, the impact of OTC was observed in both fish gut and water microbiomes, promoting for instance the selection of bacterial phylotypes presenting intrinsic resistance mechanisms to OTC. Moreover, although OTC is used to treat fish diseases, in our work it was observed the selection of some bacterial phylotypes that are indicated as possible human and fish pathogens (e.g. *Chlamydiae* and *Gordonia*). Since OTC is one of the most used antibiotics in aquaculture industry, the

selection of these phylotypes raises health concern. Besides, the use of prophylactic therapies, where both healthy and diseased organisms are exposed to the antibiotic may enhance the problem.

Water bacterial communities may influence several parameters of water quality (e.g. pH and dissolved oxygen). Therefore, an unbalance of those communities may have an impact at ecosystem level. In our work the selection of bacterial phylotypes that have a role in the nitrogen cycle was observed. This effect may lead to the accumulation of nitrogen products with potential toxicity effect in aquatic organisms. Thus, understanding the impact of OTC exposure in water bacterial communities may help to better understand the ecological impact, not only in fish welfare but also in other aquatic organisms (e.g. plankton) and in the ecosystem balance. Therefore, additional analysis at nitrogen cycle, using other methodologies like quantitative PCR (e.g. targeting ammonia oxidizing and denitrification related genes) would help to estimate the OTC impact in aquatic system.

Overall, the use of this non-conventional endpoint (bacterial communities' analysis) was a key point in our work for an integrative and comprehensive evaluation of OTC exposure effect. Comparing with the conventional endpoints discussed above (e.g. behavior and enzymatic activity; **Chapter 3**), this parameter showed to be also a very sensitive endpoint. In fact, effects of OTC would be observed even in the lowest concentration tested in this Chapter (10 µg/L). Therefore, we believe that the use of "microbiome" as an additional endpoint should be also taken in consideration in ecotoxicology tests. Moreover, this battery of tests (behavior, biochemical and microbiome analysis) should be used as a potential early warning signal for environmental stress.

A post-exposure period may allow the organisms and its microbiome to recover.

After the analysis of the impact of OTC in a multi-level perspective (**Chapter 3 and 4**), the possible recovery of organisms after oxytetracycline exposure ceased was investigated (**Chapter 5**). Due to the complexity of our hypothesis, we used several timepoints along the experiment: two sampling points during exposure and two sampling points during the post-exposure period. This would give us a wide perspective of the organism's recovery along time.

The OTC concentration used (10000 µg/L) was selected based on the previous works and it was observed to cause an impact in the organism itself and its microbiome (**Chapter 3 and 4**). Although higher than OTC concentrations detected in surface water (**Chapter 1**), and consequently a concentration not relevant from an ecological point of view (which may be indicated as a possible limitation of our work), this concentration was chosen to assure an effect from which a recovery could be observed and to allow the study of the adverse outcome pathway of OTC.

Oxytetracycline effects in the microbiome are for some extent reversible within bacteria at ribosome level (reversible connection of OTC to the 30S ribosomal subunit (Chopra and Roberts 2001; Roberts 2003)). Therefore, at bacterial community level, we hypothesize that by the replacement of sensitive phylotypes by other more adapted, including new phylotypes with similar functions may contribute to a functional recovery. Hence, in the present work we considered that a post-exposure period may allow the organisms and its microbiome to recover. The reversibility of chemical exposure is poorly studied and, to our knowledge this is the first report showing possible recovery of zebrafish and its microbiome after OTC exposure. The complexity of "recovery" interpretation may be contributing for this gap. In our work, recovery was defined as a higher similarity between exposed organisms and the control group at each sampling time regarding the endpoint being analyzed (**Chapter 5**). In addition, recovery can also be interpreted as an adaptation and/or compensation to the new conditions (Van Straalen 2003; Lements and Ohr 2009). Overall, in our work, a recovery occurred at the parameters analyzed (energetic reserves and microbiome). Since the recovery of some phyla relative abundance and functional pathways was observed, this result is expected to have reflections in fish health (Holobiont concept). Indeed, the observed recovery of CEA (no difference between exposed and control group) may be related with the recovery of some phylotypes that are implied in metabolic process. Hence, this result strengthens our hypothesis that the impact in fish metabolic process due to OTC exposure, may be related with the alteration on fish bacterial communities (**Chapter 3, 4 and 5**). Nevertheless, in their natural habitats, the impact of abiotic (e.g. light or temperature) or biotic factors may influence the organism capacity of recovery. Notwithstanding this study provides very important clues

about the impact of this antibiotic after exposure ceases, which is a very good baseline for other more complex experimental designs.

Long-term exposure to oxytetracycline may select antibiotic-resistant bacteria.

Culture-dependent and culture-independent analyses were used to accomplish a comprehensive analysis of OTC role in the selection of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs). To achieve this goal, a microcosm approach was used. This methodology presents some advantages such as the performance in a controlled environment allowing to reduce the impact of other environmental variables in the results. On the other hand, indirect effects may not be detected (e.g inter-species interactions) (Benton et al. 2007). Nevertheless, it is a good compromise between the complexity of ecosystem and the standard tests usually performed in laboratory.

The use of specific media, namely *Pseudomonas Aeromonas* Selective agar (GSP) and Membrane Fecal Coliform agar (mFC), intended to selected fish pathogens (*Pseudomonas* and *Aeromonas*) and humans' pathogens (*Enterobacteriaceae*). Nevertheless, the specificity of these media may not be so strict, especially when supplemented with antibiotics, and therefore, other bacterial groups also grew on these media. In fact, in our work (**Chapter 6**), it was observed the selection bacteria intrinsically resistant to OTC, namely *Pseudomonas* and *Stenotrophomonas*. These results should be analyzed in light of the applied methodology. For instance the culture-dependent techniques only recover a small fraction of bacterial communities since less than 1% of bacteria are culturable (Schleifer 2004). Also, the incubation temperature used may reduce the proliferation of other bacterial groups (e.g. *Enterobacteriaceae*). Nevertheless, selection of potential pathogenic phylotypes due to OTC exposure was suggested within zebrafish and water microbiome in our previous works (**Chapter 4**). These results strengths the potential role of OTC in the selection of antibiotic-resistant pathogens. Moreover, our results revealed that 37% of our isolates presented a multidrug resistant (MDR) pattern. As already indicated in **Chapter 1**, MDR bacteria represent a global health problem, with previsions showing that MDR bacterial infections may be one of the main human death causes in 2050 (O'Neill 2014). Hence our work reinforces the

need of more interdisciplinary studies for a deeper understanding of the real impact of antibiotics in the environment.

To test the virulence potential of the multidrug resistant (MDR) strains, zebrafish embryos were exposed to our selected strains. The use of *in vivo* model was useful to understand the potential impact in the organism's survival. Moreover, the methodology used (infection through egg immersion) allowed to perceive the capacity of our isolates to invade and cause injury/mortality. Based on our results, future studies are needed to determine the quantity of bacteria that cross fish tissues and enter the organism. Also, the activation of fish immunological response and expression of immune-related genes should be clarified.

Another main concern topic is the dissemination of antibiotic resistance genes (ARGs). The potential role of the antibiotic use in the selection of ARGs has been confirmed in literature (**Chapter 1**). However, few studies devoted to understanding the role of OTC in the ARBs and ARGs selection in a controlled condition (microcosm approach). In our work, the analysis of *tet*A gene in environmental DNA (fish gut and exposure water) was performed through the independent-culture method: qPCR. This has been a powerful tool, widely used in microbial ecology due to its high sensitivity (Kim et al. 2013). Since culture-dependent methods only recover a small fraction of bacterial communities, the qPCR technique revealed to be a good complement in our work. In fact, no *tet* resistance genes was observed in our isolates, while through qPCR analysis, the selection of *tet*A was observed. Usually, ARGs are associated with mobile elements, which facilitate their spread among bacteria. Therefore, the selection of ARGs and ARBs due to OTC exposure, observed in our work, come highlight the need of urgent practices to control the dissemination of resistance.

2. Further methodological considerations

The methodology applied in our work was essential to reach our goals. The analysis at several biological organization levels allowed to have a wide perspective of the real impact of OTC in organisms. In fact, at the light of the "One health" concept, the need of studies

including an integrated analysis of antibiotics impact is urgent. In this point, it was essential the use of a microcosm-based approach to reduce the environmental confusing variables and reach a better understanding of the impact of the antibiotic exposure. Moreover, the use of several replicates (each aquarium represented a replicate) harboring several organisms, permitted to achieve a higher representativeness of microbiome diversity. The use of zebrafish model was also a key point in our work. Several reasons have contributed for this choice. Firstly its small size permits to harbor several individuals in a small space, secondly it has several specified developed tools that permit to evaluate the impact of chemicals, finally its microbiome is well studied which allowed to relate microbiome change to OTC impact.

However, some limitations should be also pointed. For instance, OTC administration to fish was performed through water contamination. Hence, fish gut exposure was restricted to the water ingested by the fish. Therefore, the concentration that fish gut bacterial communities are exposed to, may be different from the one to which the water bacterial communities are exposed. Comparison of effects between the gut and water microbiomes would be best performed if concentrations of OTC in the gut were available, however in our work OTC concentrations were only determined in water. Yet, this was the best strategy to mimic a realistic scenario where organisms are exposed to antibiotics via water. Therefore, our results revealed that even if fish gut microbiome may be exposed to lower concentrations, bacterial communities are also impacted by OTC.

OTC concentration was determined along time in water, to determine the degradation rate in our exposure conditions. It is known that OTC degradation may produce other OTC-derivative compounds like 4-epi-oxytetracycline, α-apo-oxytetracycline and β-apo-oxytetracycline (Zhao-jun et al. 2019). However, no degradation products were determined in the chemical analysis. Yet, OTC concentration was quite stable in our experimental conditions, and no great changes was observed (nominal concentrations of 58% and 103% after 96 h of degradation).

The experimental design used in our work was a semi-static condition. Each aquarium represented a different replicate, holding distinct organisms. Since each replicate was considered a microcosm or individual system it may have promoted the variability observed among replicates which sometimes difficulted the data interpretation.

3. Future work

Overall, our goals were achieved along the work. To test our hypothesis, it was of relevance the use of very sensitive endpoints that permitted to reach the impact that environmental relevant concentrations would induce at the organism itself and its microbiome (**Chapter 3 and 4**). Also, the use of non-conventional experimental design like a post-exposure period allowed to understand if an intermittent exposure will lead to a recovery (**Chapter 5**). In addition, the analysis of potential selection of antibiotic resistant bacteria and antibiotic resistance genes through both culture-dependent and culture-independent methods revealed that OTC also affects the prevalence of ARB and ARGs (**Chapter 6**). Therefore, this interdisciplinary and integrative approach allowed to reach a deeper and higher understanding about the real impact of OTC on the environment, on the organisms and on the bacterial communities. Based on our results, new hypothesis and questions were raised:

I. Since host' microbiome plays an important role in some host biological process, it remains the question if the toxic effects observed at zebrafish, namely behavior and enzymatic activity level, were due to OTC direct effect on fish or to OTC effect at microbiome level. Here, we hypothesize that the effects observed on fish were, at least in part, due to changes at fish microbiome structure. Therefore, future work using probiotic mixture would be important to investigate if this strategy may mitigate microbiome alterations due to OTC exposure, and thus providing insights to confirm our hypothesis.

II. In this work, it was selected ARB and ARGs due to OTC exposure. Nevertheless, the selection of ARGs was limited to *tet*A. Hence, in future work, a deeper analysis of ARGs selection, by investigating other *tet* resistance genes as also genes related with mobile genetic elements through independent-culture methods, would be also important.

III. The selection of multidrug resistant bacteria with potential pathogenicity effect in fish embryos was confirmed. Hence, it would be interesting to conduct a deeper analysis of the potential pathogenicity of these strains in other stages of the fish life cycle. Moreover, the analysis of potential induction of fish immunological response as also the analysis of strains virulence-related factors would be important to better understand the risks represented by these bacteria.

IV. The use of microcosm approach allowed to understand the impact that OTC may have in the organisms and bacterial communities. Yet, in the environment other factors may also play a role in the OTC behavior. Thus, the use of a more complex experimental design like a mesocosm approach including several trophic chains would reveal how the inter-specific interaction may impact OTC bioavailability.

V. In their natural environment, organisms may be subjected to irregular exposure periods. However, few is known about how the organisms and bacterial communities would react in a re-exposure. Effects of a re-exposure using an integrated analysis, with our work as a baseline, would unveil if organisms would react differently and if effects would have higher, equal, or lower severity than the first exposure.

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