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ACCUMULATION OF PHARMACEUTICALS IN AQUACULTURE MATRICES
FROM FISH TO ALGAE

Tese no âmbito do doutoramento em Biociências, área de especialização em Ecologia, orientada pela Doutora Sara Isabel Falcão Navarro Leston Ferreira, pelo Professor Doutor Marco Filipe Loureiro Lemos e pelo Professor Doutor Fernando Jorge dos Ramos, e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

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Abbreviations

AUC – area under the curve

CCα – decision limit

CCβ – detection capability

CF – ciprofloxacin

CTRL – control

DM – dry matter

EC – European Commission

EDTA – Ethylenediaminetetraacetic acid

ENR – enrofloxacin

EU – European Union

EUMOFA – European market observatory for fisheries and aquaculture products

FAO – Food and Agriculture Organization of the United Nations

FEAP – Federation of European Aquaculture Producers

FELASA – Federation of European Laboratory Animal Science Associations

FLU – flumequine

HPLC – high performance liquid chromatography

IMTA – integrated multitrophic aquaculture

LOD – limit of detection

Log K_{ow} – Octanol-water partitioning coefficient

LOQ – limit of quantification

MRL – maximum residue limit

MRPL – minimum required performance limit

MS – mass spectrometry

OTC – oxytetracycline

OXO – oxolinic acid

PES – Provasoli Enriched Solution

PCB – Polychlorinated biphenyl

PCDD/F – Polychlorinated dibenzo-p-dioxin/debenzofuran

POM – particulate organic matter

POP – persistent organic pollutant

PVDF – Polyvinylidene Difluoride

RAS – recirculating aquaculture system

SDZ – sulfadiazine

ToF – Time of flight

TRI – trimethoprim

UN – United Nations

UHPLC-MS/MS – ultra high performance liquid chromatography tandem mass spectrometry

WW – wet weight

Abstract

It is now well recognized that, to address the steep demand for fish products, aquaculture is facing unequaled growth. Larger farms and higher densities of fish usually rely on the use of pharmaceuticals to avoid disease outbreaks with associated economic losses for the producers. It is therefore of vital importance to understand the behavior of such compounds in aquaculture matrices and if residues are present upon consumption. For this reason several works on antibiotic accumulation are described in the present work.

The first chapter provides a contemporary review on integrated multitrophic aquaculture systems (IMTAs), with focus on food safety issues that could be associated with it. The principle behind IMTAs is that several species from different trophic levels are co-cultured in proximity with each other, and the wastes from one species are food for others (extractive species). However, as filter organisms capable of filtering high amounts of nutrients from the surrounding waters, these extractive species will also accumulate chemicals that are used in these systems, mostly used to treat the main species of the system (usually finfish). An overview on the types of aquaculture and types of organisms used in IMTAs can be found in the review, as well as on pesticides, antiparasitics, antifoulings, persistent organic pollutants, metals, and antibiotics that can be present in such systems and food matrices.

Chapter II and III focused on the retention of several antibiotics by the two main species produced in aquaculture in the Mediterranean region: Gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*). Based on their use in aquaculture, five antibiotics (sulfadiazine (SDZ), trimethoprim (TRI), flumequine (FLU), oxolinic acid (OXO), and oxytetracycline (OTC)) were incorporated into manufactured feeds, and their retentions in muscle tissues were analyzed. Pharmacokinetics and drug retention can vary greatly depending on several factors such as dosage regimen or mode of administration, but also with different species. Fish were placed in tanks and were manually fed with medicated feed for 7 days. Muscle samples from the dorsal area were then taken at several sampling points and analyzed for the presence of SDZ, TRI, FLU, OXO, and OTC. Antibiotic analyses on chapter II were performed following a validated multi-class quantification method developed by our working group, and in chapter III, an extension of this validation

for European seabass was presented. Concentrations in edible tissues through time in European seabass were higher than the ones present in Gilthead seabream. Nonetheless, both studies suggest that antibiotics can be present in edible tissues longer than previously reported, with associated food safety concerns. For seabream, with the exception of OTC, antibiotic concentrations were below the maximum residue limit (MRL) established 3 days after the end of feeding period. Contrarily, withdrawal times of 0, 2, and 5 days were proposed for FLU, OXO, and SDZ administration in seabass, since concentrations found in edible tissues were above the MRL established by the European Commission. Concentrations of OTC and TRI were above the MRL until the end of the experiment, and considering the elimination rates, even the 21 days of withdrawal time set for salmonids may be insufficient.

Following a more sustainable approach to aquaculture, IMTA systems will most certainly be the future in terms of fish and seafood production. In such systems, seaweeds can be co-cultured with a double purpose: to act as a bioremediator and mitigate the nutrient loading associated with fed aquaculture, and to increase profitability of aquacultures while maintaining costs. Their capacity to take up nutrients from the water also indicates seaweeds can accumulate pharmaceuticals and thus pose a risk upon human consumption. Therefore, *Ulva*'s potential to accumulate such contaminants, as well as its effects on the macroalgae were assessed for the tetracycline oxytetracycline and the quinolone enrofloxacin (ENR) in chapters IV and V, respectively. Following the recommended dosage for OTC for aquaculture (55 mg kg^{-1}), *Ulva* organisms were exposed to two concentrations (C1 0.040 and C2 0.120 mg L^{-1}) for 96h. Macroalgae presented high accumulation rates of OTC with internal concentrations above the MRL established for fish for 24h (for the highest dosage tested). Residues of OTC were still found in *Ulva* fronds at the end of the trials, and although at low concentrations, the presence of pharmaceuticals in extractive species used in IMTA systems must be considered with new legislations in sight. The work presented in the final chapter shows that *Ulva* can efficiently remove ENR from the water, indicating that it can be used for bioremediation in IMTA systems. Further works need to be addressed in order to better understand the detoxification mechanisms involved with accumulation of

pharmaceuticals, since concentrations significantly decreased after 48 and 24 h (for C1 and C2 treatments, respectively). Nonetheless, following the recommended dosages for aquaculture, residues of ENR could be detected at the end of the trial, at levels comparable to the limits established for fish. Our results suggest that legislations on pharmacologically active substances must include these extractive species, as they are gaining relevance in global diets.

Key words: accumulation; antibiotics; aquaculture; contamination; European seabass; food safety; Gilthead seabream; IMTAs; pharmaceuticals; residues; seaweed; sustainability; *Ulva*; withdrawal times.

Resumo

Atualmente é reconhecido que, de modo a fazer face à grande procura de pescado e seus derivados, o sector da aquacultura está a sofrer um crescimento sem precedentes. Aquaculturas maiores e com densidades de peixe elevadas, geralmente recorrem ao uso de produtos farmacêuticos para evitar surtos de doença que podem implicar perdas económicas significativas para os produtores. Assim, é importante compreender o comportamento destes compostos nos produtos de aquacultura, e se ainda existem resíduos aquando do seu consumo. Neste contexto, a presente tese descreve trabalhos desenvolvidos no âmbito da acumulação de antibióticos em matrizes de aquacultura.

O primeiro capítulo fornece uma revisão atualizada sobre os sistemas de aquacultura multitrófica integrada (IMTAs), com principal foco nas questões de segurança alimentar associadas a estes sistemas. O princípio fundamental dos IMTAs é o de cultivar espécies de diferentes níveis tróficos em proximidade, funcionando os desperdícios de uma espécie como alimento para as seguintes (espécies extrativas). No entanto, como organismos filtradores capazes de filtrar elevadas quantidades de nutrientes das águas envolventes, estas espécies extrativas também podem acumular produtos químicos usados nestes sistemas. Uma visão geral dos tipos de aquacultura e tipos de organismos usados nos IMTAs pode ser encontrada nesta revisão, assim como de pesticidas, antiparasitários, anti-incrustantes, poluentes orgânicos persistentes, metais pesados, e antibióticos que podem estar presentes nestes sistemas e matrizes alimentares.

Os capítulos II e III focam-se na retenção de vários antibióticos nas duas principais espécies produzidas em aquacultura na região do Mediterrâneo: a dourada (*Sparus aurata*) e o robalo (*Dicentrarchus labrax*). Tendo por base o seu uso em aquacultura, cinco antibióticos (sulfadiazina (SDZ), trimetoprim (TRI), flumequina (FLU), ácido oxolínico (OXO) e oxitetraciclina (OTC)) foram incorporados em rações experimentais, e a retenção no músculo dos peixes foi analisada. A farmacocinética e a retenção de compostos pode variar consideravelmente dependendo de diversos factores, tais como a dosagem ou o modo de administração, mas também de espécie para espécie. Os peixes foram colocados em tanques e alimentados à mão com as rações medicadas durante 7 dias. Amostras de músculo da área dorsal dos peixes

foram posteriormente retiradas a vários tempos de amostragem e analisadas para a presença de SDZ, TRI, FLU, OXO e OTC. As análises aos antibióticos no capítulo II foram feitas com base num método de quantificação multi-classe desenvolvido pelo grupo de trabalho, e no capítulo III, uma extensão dessa validação para robalo é também apresentada. As concentrações nos músculos ao longo do tempo foram mais elevadas no robalo do que na dourada. No entanto, ambos os estudos sugerem que os antibióticos podem estar presentes em tecidos comestíveis durante mais tempo do que o que está reportado, podendo implicar um risco para a saúde humana. Para a dourada, com exceção da OTC, as concentrações de antibióticos encontravam-se abaixo do limite máximo de resíduo (MRL) 3 dias após o final do período de medicação. Pelo contrário, tempos de retirada de 0, 2 e 5 dias foram propostos para a administração de FLU, OXO, e SDZ em robalo, uma vez que as concentrações encontradas nos tecidos estavam acima do limite legal estabelecido pela Comissão Europeia. As concentrações de OTC e TRI estiveram acima do MRL até ao final da experiência, e tendo em conta as taxas de eliminação calculadas, mesmo os 21 dias de tempo de retirada estipulado para salmonídeos pode ser insuficiente.

Seguindo uma abordagem mais sustentável para a aquacultura, os IMTAs serão certamente o futuro em relação à produção de pescado e seus derivados. Nestes sistemas, as macroalgas podem ser co-cultivadas com uma dupla função: atuar como biorremediadores mitigando a elevada quantidade de nutrientes associada à aquacultura dependente de ração, e ao mesmo tempo aumentar o lucro sem que aumentem os custos de produção. A sua capacidade para captar nutrientes da água é um indicador que as macroalgas podem também acumular produtos farmacêuticos, representando assim um risco para consumo humano. Assim o potencial da macroalga *Ulva* em acumular tais contaminantes, bem como os efeitos que estes podem causar, foram avaliados tanto para a tetraciclina oxitetraciclina como para a quinolona enrofloxacina (ENR), nos capítulos IV e V, respetivamente. Com base na dosagem de OTC recomendada para aquacultura (55 mg kg^{-1}), discos de *Ulva* foram expostos a 2 concentrações (C1 0.040 e C2 0.120 mg L^{-1}) durante 96h. A macroalga apresentou taxas elevadas de acumulação de OTC, com concentrações internas acima do MRL estipulado para peixe durante 24h (para a dosagem mais alta). Resíduos de OTC foram quantificados nos tecidos de *Ulva* no final dos ensaios,

e apesar das concentrações baixas, a presença de compostos farmacêuticos em espécies extrativas usadas em IMTAs tem de ser considerada com novas legislações em vista. O trabalho apresentado no último capítulo demonstra que a *Ulva* pode remover com eficiência ENR da água, o que indica que pode ser usada para biorremediação em IMTAs. Será necessário desenvolver novos ensaios laboratoriais de modo a perceber melhor os mecanismos de detoxificação que estão envolvidos na acumulação de fármacos, uma vez que as concentrações diminuíram significativamente depois de 48 e 24 h (para os tratamentos C1 e C2, respetivamente). No entanto, seguindo as dosagens recomendadas para aquacultura, resíduos de ENR puderam ser detectados até ao final dos ensaios, a níveis comparáveis aos limites estabelecidos para peixe. Os resultados obtidos sugerem que a legislação para substâncias farmacologicamente ativas deve incluir também estas espécies extrativas, uma vez que elas estão a ganhar relevância nas dietas globais.

Palavras chave: acumulação; antibióticos; aquacultura; contaminação; dourada; fármacos; IMTAs; macroalga; resíduos; robalo; segurança alimentar; sustentabilidade; tempos de retirada; *Ulva*.

CHAPTER I – Introduction

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Aerial photo of an IMTA system located in central Portugal.

Integrated multitrophic aquaculture systems – potential risks for food safety

Abstract

Background: The demand for fish and fish products is now higher than ever, and the aquaculture sector is evolving in order to face such demand. However, several problems such as nutrient loading or excessive use of resources can be associated with the intensification of aquaculture systems. Integrated multitrophic aquaculture systems (IMTAs) refer to the co-culture of different species belonging to different trophic levels, and offer a sustainable approach to aquaculture development. In these systems, organic and inorganic extractive species will feed on other species waste or on uneaten feed nutrients, acting as bioremediators.

Scope and Approach: The extractive capacity that these organisms have to take up nutrients from the water also means they will accumulate chemicals that are often administered in intensive productions. The present review describes a vast number of substances that can be found in IMTAs, either intentionally administered or resulting from cross contamination, and subsequently accumulated in species reared afterwards in these systems. The presence of such chemicals in organisms produced in IMTAs raises several food safety and human health concerns, which need to be addressed.

Key findings and conclusions: Although IMTAs still face many challenges in terms of large scale production, legislations are not yet ready to comprise co-cultivation of multiple species in proximity. Also, maximum residue limits already existent for fish must be set for other organisms also produced in IMTAs in order to protect consumer's health. An increase in extractive species consumption (e.g. seaweeds) has been noticed during the past few years, and as IMTAs gain importance as a sustainable production method, food safety issues must be tackled.

Key words: IMTA; sustainable; aquaculture; contamination; accumulation; food safety.

General overview of aquaculture industry

World fish production has been consistently growing over the last few decades. Either driven by the steep increase in human population or the fact that capture fisheries production became relatively stable since the 1980s. Presently, aquaculture production reached a peak value of $232 Billion (FAO, 2018). With world fish consumption per capita increasing from 9.0 kg in 1961 to 20.2 kg in 2015, it is now higher than that of meat from pig, poultry, bovine and ovine combined, according to FAO's (2018) latest report on *The State of World Fisheries and Aquaculture*. In 2015, fish accounted for 20% of the total protein intake to 3.2 billion people, but this can easily reach 50% in countries like Cambodia, Bangladesh, Indonesia, and most of small island developing states (Béné et al., 2015; FAO, 2018; M. D. Smith et al., 2010). Fish often represents a cheaper alternative to other animal protein sources, but for some populations it is part of traditional cultures and is often preferred. Asia is by far the largest consumer of fish accounting for 70% of the 149 million tones consumed in 2015, while Europe and United States were responsible for 20% of fish consumption.

Fish and seafood provide not only omega-3 fatty acids, but also significant levels of micronutrients including vitamins and minerals. It represents a fundamental nutritional component of easily digestible proteins, where 150g can provide up to 60% of the daily requirement for an adult (FAO, 2018). High consumption of seafood has been widely associated with an improvement in human health, reducing the risk of several diseases such as coronary heart disease, high blood pressure, and some types of cancer (Lund, 2013). These factors contribute to the high global demand of not only fish but also other aquaculture products such as bivalves and macroalgae, a growing tendency for the upcoming years.

Traditional aquacultures, predominant during the end of the 20th century and characterized by the relatively small size of the farms and low stock densities, are no longer capable to face the demand for fish products (Edwards, 2015). In order to meet such demands, aquaculture systems faced unparalleled growth (Sapkota et al., 2008), and became a much more intensive industry, with larger farms, higher densities, and new technologies applied to production (Goldburg et al., 2001). This development allowed aquaculture to overtake the contribution of wild fish for total

human fish consumption for the first time in 2013 (FAO, 2018). According to FAO, in 2018, global aquaculture production corresponded to 80 million tonnes of fish food and 30.1 million tonnes of aquatic plants. Among fish food, 67.6% were finfish, 21.4% mollusks, and 9.8% crustaceans. China has been the major producer of farmed seafood in the last decades, followed by countries like India, Indonesia, Vietnam, Bangladesh, Egypt and Norway. Mostly due to China's contribution, Asia accounted for 89.4% of the total aquaculture production in 2016. Europe is responsible for 3.7% of the global production, corresponding to 2.9 million tonnes (FAO, 2018), most of which is attributed to salmon production in Norway - approximately 46% of total production in Europe (European Environment Agency, 2018). Nonetheless, this number is expected to increase, since aquaculture is considered a strategic sector in the EU's Blue Growth Strategy (European Environment Agency, 2018).

Types of aquaculture

Fish farming can range from extensive to intensive systems, considering its feeding regime and density, and can be either land-based or water based, depending on their location and architecture (FAO, 2014; Funge-Smith & Phillips, 2000; Ottinger et al., 2016). Aquacultures can also be divided into freshwater (rivers and lakes), brackish water (estuaries, lagoons, and fjords), or marine aquacultures (coastal areas and open sea) (Bostock et al., 2010; Ottinger et al., 2016).

The principle behind extensive aquaculture is that fish are not artificially fed (Read & Fernandes, 2003; Troell et al., 2017) and the density tends to be extremely low (under 1kg m^{-3}). This is achieved by allowing the entrance of replacement water in the system, which contains some lower trophic level organisms present in the water column, sufficient to feed the farmed species. This implies very low maintenance of the system, but this also means that productivity is low. When fish food requirements are not met in this type of system, artificial feed can be used to complement the nutritional needs of the farmed species. These later are addressed to as semi-extensive aquacultures and they present a higher productivity than extensive ones (Troell et al., 2017).

In semi-intensive systems farmed fish rely very little on natural food sources. Instead, fish is fully sustained by artificial pellets and grow in relatively medium

densities, higher than in both extensive and semi-extensive systems. Since water is not treated and the water exchange is not sufficient to clear waste products, fish densities must be maintained relatively low. On the other hand, intensive systems are capable of producing very high densities of fish, which can reach 200 kg of fish per m^3 of water. Feeding can be made twice a day or almost continuous during daylight hours, with a huge nutrient loading associated with it. With the development of more intense aquaculture systems, there are several environmental problems that need to be taken into account (Read & Fernandes, 2003). According to FAO (FAO, 2018), the farming of fish artificially fed already accounts for much higher volumes than the ones produced from unfed species, increasing the pressure on the surrounding ecosystems due to nutrient loading.

In regard to location and architecture, most traditional aquacultures are still carried in conventional ponds. These rudimentary systems are usually located in estuaries or deltas and water exchange is periodic and dependent on tides (Read & Fernandes, 2003). Abiotic factors like temperature or precipitation can heavily influence fish production in these systems and this is why fish densities are kept so low. Raceways or flow-through open systems are basically upgraded conventional ponds, where water availability is not a limiting factor since these systems consist in tanks or channels that have both an inlet and outlet of water in permanence (Ottinger et al., 2016). On a more technological end of land-based aquaculture are the Recirculating Aquaculture Systems (RAS). Fish densities here are usually high since the water quality is continuously monitored and maintained, with almost 90% of the water volume reused under controlled light and temperature (van Rijn, 2013). Such systems still involve high capital investment due to the several units comprising mechanical, biological, and chemical filtration. Besides land-based aquacultures, fish can be grown in offshore cages. These floating structures create a closed compartment for fish, and are usually located in places that offer protection against harsh conditions of the open ocean (like fjords, for example). The water inside these cages is fully equilibrated with surrounding waters, and waste products or excessive feed material are continuously dissipated directly into the water bodies.

Environmental pressures from aquaculture production

With global population reaching almost 10 billion people in 2050, according to the UN World Population Prospects, there will be an increase in global demand for fish. Capture fisheries production in 2016 presented a small decrease comparing to previous years, and the tendency is for fisheries to grow at a very small rate, or even stabilize (FAO, 2018), so aquaculture production will continue to increase. One of the major concerns associated with the intensification of aquaculture is the environmental impact of fish farms in coastal regions (Goldburg et al., 2001; Ottinger et al., 2016; Peng et al., 2013; Troell et al., 2017). Such impacts will vary greatly with the type of species cultured and the type of aquaculture activity, with more intensive practices showing higher impacts on the surroundings of the production sites. Policy makers must understand the systems utilized and the resources upon which an aquaculture depends (Deutsch et al., 2007; Read & Fernandes, 2003).

One of the major impacts of aquaculture in general is the nutrient loading associated with production of fish. It is vastly known that aquaculture production causes the release of high amounts of metabolic waste products like excreta and uneaten food and this will impact the surrounding systems (Granada et al., 2016; Grigorakis & Rigos, 2011; Islam, 2005; Ottinger et al., 2016; Read & Fernandes, 2003). Up to 95% of the nitrogen and 85% of the phosphorus introduced in aquaculture systems as feed may be lost to the environment (Zhou et al., 2006). In cage aquacultures, for example, the continuous and direct discharge of solid or particulate materials, such as feces or uneaten food, will most probably lead to sedimentation on the sea floor beneath the cages (Islam, 2005; Naylor & Burke, 2005). The magnitude of such wastes can have a vast impact, and even using high amounts of water to remove excessive nutrients might not be sufficient, potentially leading to eutrophication, which can look insignificant at a global scale, but is highly impacting locally (Burford et al., 2003; Carrasquilla-Henao, Ocampo, González, & Quiroz, 2013; Edwards, 2015; Naylor & Burke, 2005; Zhou et al., 2006).

Integrated Multitrophic Aquaculture Systems

There is no food production sector nowadays completely sustainable from an energy and biodiversity point of view. They require energy and water, but also

generate waste (Diana, 2009; Troell et al., 2017). Water pollution associated with the industrialization of aquaculture has increased and negative ecological impacts on the surrounding environments are already noticed (Ottinger et al., 2016). There are, however, farming methods more sustainable than others. For example, culturing more than one species in the same water is often better than monocultures. But polycultures, despite an increase of the profitable margins due to diversification of products and with less risks than monocultures (Chopin et al., 2012), do not mitigate some of the environmental impacts often associated with large-scale aquaculture.

One farming method that offers many advantages is the integrated multitrophic aquaculture (IMTA). Contrarily to polycultures, where several species can be cultured together but all belong to the same trophic level, IMTAs include species from different trophic levels, and can minimize environmental impacts of aquaculture while delivering economic benefits, promoting the ecological approach to aquaculture (FAO, 2014; Granada et al., 2016; Kleitou et al., 2018; Troell et al., 2003; 2009). Integrated multitrophic aquaculture can thus be defined as "*a practice in which by-products from one species are recycled to become inputs for another*" (FAO, 2014). The concept of integrated aquaculture has been used in the last decade to somehow mitigate the excessive nutrient/organic loading generated by intensive aquacultures, especially in Asia (Neori et al., 2004), but nowadays works as a response to the global demand for seafood while assisting the sustainable expansion of aquaculture in coastal and marine ecosystems (FAO, 2009a; Neori et al., 2004; Troell et al., 2009). In fact, this concept was already adopted by Ryther and colleagues (Ryther, Dunstan, Tenore, & Huguenin, 1972) back in 1972, precisely to address eutrophication issues from the nutrients coming from aquaculture systems.

In order to maximize the efficiency of an IMTA, the community assembling should include, aside from a main species such as finfish or shrimp with their own dedicated feed, both filter and deposit-feeders, i.e. secondary species capable of benefit from a larger spectra of the organic particles size, reducing environmental impacts of the organic load, and optimizing yields with the diversification of products (Figure 1) (Barrington et al., 2009; Cubillo et al., 2016; Ren et al., 2012). Organic and inorganic extractive species such as shellfish and seaweeds play a major role in IMTA, reducing the waste released by the fed aquaculture component. This

ecological function (extraction) contributes to the reduction of nutrient loading by retaining and consuming the suspended small particulate organic matter (POM) (Alexander & Hughes, 2017; Barrington et al., 2009; Chopin, 2006; Edwards, 2015; FAO, 2018; Kleitou et al., 2018; Martínez-Espiñeira et al., 2015; Troell et al., 2003; 2009; van Rijn, 2013). What is commonly addressed as waste is, in fact, raw biological material used to feed lower trophic level organisms. Furthermore, what used to be an environmental problem, now adds value for culturing biomass in proximity with each other, as they are connected by nutrient and energy transfer through water (Barrington et al., 2009; Chopin, 2006).

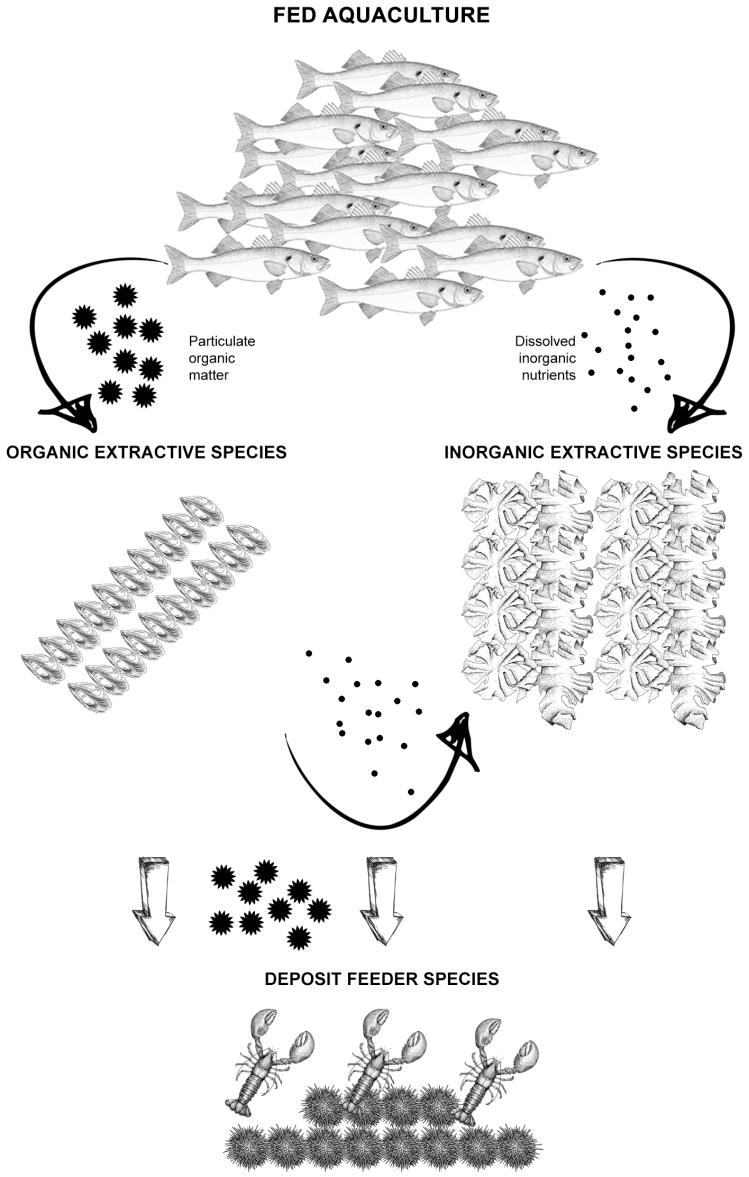

Figure I.1 – Representation of a possible IMTA system, with fed species and both organic and inorganic extractive species, as well as deposit feeder species.

Integrated multitrophic aquaculture systems usually consist in culturing fed species with extractive species, but can include aquaponics, fractionated aquacultures, integrated agriculture-aquaculture systems, integrated fisheries-aquaculture systems, algal ponds or integrated peri-urban aquaculture systems, ranging from open water to land based farms (Barrington et al., 2009; Troell et al., 2017; van Rijn, 2013). The crucial aspect is that the appropriate organisms are chosen based on their functions in the ecosystem, and the idea of IMTAs is that, besides environmental sustainability, these systems can provide economic diversification. Each species acts not only as natural biofilters, but also has their own commercial value, increasing the overall aquaculture value (Barrington et al., 2009; Chopin, 2006; Granada et al., 2016; Troell et al., 2009; 2017). In 2016, extractive species production was responsible for 49.5% of total world aquaculture production, which is an impressive number considering that they were not the main focus of the production to start with (FAO, 2018). The design of an efficient and profitable multitrophic aquaculture is an ecological engineering application of a sustained knowledge on species ecological functions and processes (Troell et al., 2009). It is important to have in mind the density, nature and seasonal cycles of all species involved, under the risk of starvation of the secondary species in the case of asynchronous cycles (Ren et al., 2012). The inclusion of local species is also highly advisable, as it decreases transport costs, as well as it reduces the risk associated with the introduction of non-indigenous species. Also, neighboring species are already adapted to the environment where aquacultures are sited, and therefore, it will imply less adjustment. Apart from these features, culture feasibility and social potential should also be regarded when choosing the secondary species to be included in the IMTA design (Ren et al., 2012). For instance, bivalves that are grown in traditional aquacultures are usually cultivated in non-fed regimes, which is often limited by the naturally available nutrients and light annual cycles (Lander et al., 2012). The growth of bivalves in a fed multitrophic context provides a continuous supply of organic matter, with an optimized secondary production through the use of wasted feed and feces from the primary target species (Lander et al., 2012). As an example, for cage-reared salmon, predictions say that 20% of the feed is wasted (and from the eaten portion, 26% is voided as feces (Lander et al., 2012)), which

could represent an important source for secondary extractive species. Taking into account that feed corresponds close to 60% of finish aquaculture operation expenses (Chopin et al., 2012), any waste reduction and recovery is highly desirable, both for environmental and economic reasons. A value of 18-26 billion €/year was estimated for mitigation services provided by the production of bivalves in aquaculture, only for the European Union (Ferreira et al., 2009).

Organisms cultivated in IMTAs

Fish production still holds the highest share in world aquaculture production, attaining 54.1 million tonnes out of the total 110.2 million tonnes registered in 2016 (FAO, 2018). However, this represents roughly half of the global aquaculture production, with aquatic plants (mainly seaweeds) representing 27% of the total production, according to the latest report on *The State of World Fisheries and Aquaculture* (FAO, 2018). Mollusks (17.1 million tonnes), crustaceans (7.9 million tonnes) and other aquatic animals (< 1 million tonnes) represent the rest of the global production. In 2016, nearly 600 species were farmed in aquaculture, either for food, pharmaceutical or ornamental use. From these, 269 are finfish. Carps are the most widely farmed finfish, with *Ctenopharyngodon idellus*, *Hypophthalmichthys molitrix*, *Cyprinus carpio*, and *Hypophthalmichthys nobilis* integrating the top 5 of the major species produced in world aquaculture, together with the Nile tilapia, *Oreochromis niloticus* (Bostock et al., 2010; FAO, 2018). All of the top major species are freshwater species, which is expected, since freshwater is the main source of farmed finfish (Naylor & Burke, 2005; Ottinger et al., 2016; Troell et al., 2014). Aside from this, some regional areas favor more specific species as is the case of the Mediterranean region for example, where two salt water species (*Sparus aurata* and *Dicentrarchus labrax*) lead the gross production (Federation of European Aquaculture Producers, 2016; Valente et al., 2011), or the case of Norway, where *Salmo salar* is by far the major species produced (FAO, 2018).

Invertebrate species with potential for IMTA production

The invertebrate species used in IMTA should exhibit simultaneously an efficient nutrient removal ability and intrinsic economic value. In traditional aquaculture, the number of invertebrate species in production is substantial: the FAO reports at least 109 mollusks, 64 crustacean and 9 species from different taxonomical groups in production during 2016 (FAO, 2018). Nevertheless, only a fraction of these has been used and tested in IMTA. Several groups of invertebrates may not be relevant for human consumption, although some of those do have economic interest. For instance, invertebrates may be incorporated in feed for primary species (Kibria & Haque, 2018), therefore performing a feedback circuit for nutrients and energy. This translates into several economic advantages such as reduction of feeding cost and reduction of the organic matter load of aquaculture ponds to neighboring areas. Other species may reach different markets, such as bait for fisheries, ornamentals or as food for aquarium species (FAO, 2018; Fernandez-Gonzalez et al., 2018).

Mollusks

As extractive organisms that are able to filter organic particles, mollusks are the most tested organisms in IMTA context (Granada et al., 2016), and they belong to a vast group of organisms capable of providing both an economic and a mitigation value. Mussels (mytilidae) such as *Mytilus edulis* or *M. trossulus* are particularly effective in coastal temperate areas and present high potential to be used as secondary species in IMTAs (Ren et al., 2012; Sarà et al., 2009). Subtidal mussel populations are often associated with continuous growth, and its aquaculture production in suspended structures is globally disseminated. Their capacity to filter not only plankton but also uneaten fish food increases the possibility of a continuous growth during all year, decreasing the grow-out period for a crop to have commercial value. Lander et al (2012) tested *M. edulis* growth at different distances from salmon aquaculture cages and suggested that growth of the mussels closest to cages optimized its growth, avoiding the effects of winter food shortage (GK Reid et al., 2010). Salmon production can be easily adapted to accommodate structures or rafts where mussels can grow attached.

Chopin and colleagues (2012) presented an IMTA concept where the mussels *M. edulis*, *M. trossulus* and *M. galloprovincialis*, the scallop *Patinopecten yessoensis* or the oyster *Crassostrea gigas* could be used as the suspension feeders' component. Other bivalve species that can contribute to the overall functioning of an IMTA is the unionidae *Diplodon chilensis*, which is able to reduce chlorophyll a, phosphate and ammonia loads in salmon ponds, while the oistreoidea *Saccostrea commercialis* seems able reduce the suspended solids load and N and P compounds in the water (Granada et al., 2016). The mytilidae *Perna canaliculus* has also been used as model species in some IMTA studies (Ren et al., 2012). Nevertheless, all bivalve species that can be cultured may present potential to be used in these multitrophic systems: this include genera such as *Haliotis*, *Pecten*, *Argopecten*, *Placopecten*, *Chloromytilus* or *Tapes* (FAO, 2009b). The inclusion of snails (gastropoda) in IMTA, despite its reduced use in human diet, can represent a feedback resource, being used as feed for primary species. For instance, Kibria and Haque (2018) used *Viviparus bengalensis* both as secondary species and feed for different species of carps and catfish, with highly positive results. Furthermore, while finfish farms can be frequently associated with the use of several chemicals, mollusk production is usually chemical free (Goldburg et al., 2001).

Crustaceans

Some groups of crustaceans are highly valued, and can be produced intensively in aquaculture. Nevertheless, their production is usually dependent on artificial feed, and therefore, in IMTA, they are usually considered as primary species (Chopin, 2015; FAO, 2009b). Similar to finfish production, some impacts have already been reported, such as habitat degradation, threatening ecosystem integrity, and directly competing for food and habitat with natural populations (Páez-Osuna, 2001). Some crustacean species successfully cultured in IMTA are shrimps, prawns, crabs and lobsters. For instance, the integrated growth of shrimp species such as *Penaeus merguiensis*, *P. indicus*, *P. monodon* or *Metapenaeus ensis* in rice fields in Vietnam proved to be an efficient combination, while increasing income for the traditional rice farmers (FAO, 2009b). Some crustacean species are already farmed in traditional polyculture ponds in Indonesia and Southeast Asia (shrimps like the

Penaeus vannamei, *P. stylirostris*, *P. monodon* or crabs such as *Scylla* sp.), but they also have a high potential for use in IMTAs, similarly to *Panulirus* sp., *Homarus americanus* and *H. gammarus* (FAO, 2009b). Simultaneously, small amphipods that naturally grow on aquaculture facilities can be harvested and used as natural food for farmed species, representing an extra source of income or at least a reduction in feeding expenses (Fernandez-Gonzalez et al., 2018; Guerra-García et al., 2016).

Sea cucumbers

Sea cucumbers (holothurians) are gaining interest both in traditional aquaculture and in IMTA systems. While disregarded in Europe and United States, the production increase of these species is being fueled by a demand in Japan and many parts of Asia (Granada et al., 2016), where some species can have a retail price up to US$200 per kg. These are detritus feeders that complement the size particle range consumed by filter feeders such as bivalves. The use of species from both groups can be combined in order to achieve higher removal efficiency. Holothurians are able to consume up to 70% of the settling organic material (Ren et al., 2012), removing substantial amounts of organic carbon and nitrogen wastes from fish production (Cubillo et al., 2016). Sea cucumbers have low mortality and high growth rates, with fast revenue potential (3 to 4 years to reach market size), and are therefore good candidates as secondary species in IMTA context.

Polychaetes

In an IMTA context, annelid polychaetes may represent an important asset. While presenting reduced interest for human nutrition, their economic potential can lean on the use for aquarium hobbyists, as ornamentals (FAO, 2009b; Granada et al., 2016). They can also represent food sources for other animals, incorporated in feeds for primary species in aquaculture, while some species are commonly used as bait in recreational fisheries (Brown et al., 2011; FAO, 2009b). Also, some species' mucus can be bioactive with potential for the biotechnological industry (FAO, 2009b; Granada et al., 2016). Nevertheless, their use in IMTAs is not fully implemented yet, and mostly restricted to research activities. These organisms can perform several ecological functions that contribute to the overall environmental equilibrium and

sustainability in aquaculture facilities, including biofiltration and sediment aeration, with intervention on nutrient biogeochemical processes and dynamics (Brown et al., 2011; Granada et al., 2016).

Other groups of invertebrates

There are also other groups of organisms that are able to contribute to an ITMA system. Sterling and colleagues (2016) tested the deposit feeder sea urchin *Strongylocentrotus droebachiensis* around cultured *Mytilus* sp. and verified that the presence of urchin was able to reduce fouling intensity while increasing growth. The anti-fouling function is an environmental friendly alternative to copper-based antifouling coatings and is allied with an increasing economic interest on the species (FAO, 2009b). Other sea urchin species that can present similar potential are *Echinometra lucunter, Loxechinus albus, Lytechinus variegatus, Paracentrotus lividus*, or *Psammechinus miliaris* (FAO, 2009b; Sterling et al., 2016).

Sponges (Demospongiae - Porifera) are able to filter large volumes of water, retaining large organic particles including pathogens, and therefore contribute to the improvement of water quality in aquaculture ponds (Granada et al., 2016). Some examples of species already tested and with proved potential for IMTA are *Dysidea avara, Chondrosia reniformis, Chondrilla nucula,* and *Spongia officinalis*. The incorporation of different groups, even with reduced economic interest, in such systems increases the number of ecological interactions and therefore these artificially engineered communities can have higher resilience to stressful events, insuring producers against total losses.

The use of invertebrates as secondary species in IMTAs is proving their potential to simultaneously reduce some of the environmental impacts of aquaculture while adding economic value to the operation (Granada et al., 2016). Invertebrates represent a fundamental link between trophic compartments that otherwise would be difficult to connect. While removing fine POM by filter feeders or larger particulates by deposit feeders, invertebrates also make dissolved nutrient fractions available to be absorbed by seaweeds (Hannah et al., 2013).

Seaweeds

The connection among different trophic levels starts at the nutrient zone where fed species are cultured, enriching water with nutrients both in the particulate and dissolved forms, which then flows to the areas where extractive species are reared (Barrington et al., 2009; Chopin, 2006). Next, nutrients in the form POM are filtered by invertebrate species and finally, dissolved inorganic nutrients (e.g. ammonium and phosphate) are removed by primary producers, mainly seaweeds (Barrington et al., 2009; Chopin, 2006).

Seaweeds (macroalgae) are primary producers that represent an ecologically important group serving many relevant functions that include an extensive contribution to the primary production of estuarine ecosystems, a role in nutrient cycling by converting inorganic forms of energy into biomass, which will then be transferred to the higher levels of the trophic web (Leston et al., 2011; Torres et al., 2008). The high surface area to volume ratio and high affinity for nutrients, especially nitrogen and phosphorus, favor a rapid nutrient uptake, translated into increased growth and production rates, leading to very large biomass buildup (Leston et al., 2011; Neori et al., 2004). Such characteristics represent a valuable resource in IMTA systems, and therefore, seaweeds are used as extractive species for dissolved inorganic nutrients, acting as bioremediators in these aquacultures (Chopin et al., 2001; Neori et al., 2004; Sanderson et al., 2008; Zhou et al., 2006). The fact that China has been using seaweed as bioremediator is one of the main reasons coastal eutrophication is not so critical, since the 10 million tonnes of seaweeds harvested annually are capable of retrieving hundreds of thousands of tonnes of phosphorous and nitrogen from the water (Edwards, 2015). Moreover, the role of such species is not limited to biofiltering, as seaweeds constitute a commodity with commercial value. The most recent FAO report states that 30.1 million tonnes of aquatic plants (representing USD 11.7 billion) were reared in 2016, and the vast majority comprised seaweeds (FAO, 2018). These impressive figures translate the rapid growth in seaweed farming, which was of 13.5 million tonnes in 1995. The commercial uses for seaweed are also responsible for this steep growth. In many countries, especially in East and Southeast Asia, algae are an important part of the human diet (with a focus on *Undaria pinnatifida*, *Porphyra* spp. and *Caulerpa* spp.),

and are quickly becoming a global trend. This is due to their nutritional value with high content of micronutrient minerals (e.g. iron, calcium, iodine, potassium, and selenium), vitamins (particularly A, C, and B-12), fibers, fatty acids (the only non-fish sources of natural omega-3) and antioxidant properties (FAO, 2018; Fleurence, 1999; Maehre et al., 2014; Paiva et al., 2014). Seaweeds are also farmed for agar, alginate and carrageenan extraction for use as thickener agents, which have many uses in the food, pharmaceutical, and cosmetic industries (Edwards, 2015; Fleurence et al., 2012). Other important uses for seaweed include animal feed, fertilizers, and even biofuel (FAO, 2018; Leston et al., 2016).

Nonetheless, there are serious concerns related to the use of extractive species reared in IMTA systems. Their appealing characteristics may represent at the same time a drawback. Due to the filtration capacity of these organisms, they may be able to also accumulate substances other than nutrients, including contaminants such as metals and pharmaceuticals. The use of chemicals in aquaculture is necessary to control disease outbreaks and ensure animal welfare and depending on the route of administration, dosages required and length of treatment, such pharmaceuticals may be present in high concentrations in aquaculture effluents (Álvarez-Muñoz et al., 2015; Kümmerer, 2009a; Leston et al., 2016). Depending on the use for these species, contaminant accumulation may pose a threat. For instances, algae are included in fish feeds as meals, or in other words, air or sun-dried algae are ground into powder and added to the other ingredients to form pellets. But seldom are they submitted to pre-treatment before incorporation, which means there is a potential risk of integrating contaminants in the feed. Moreover, direct consumption of invertebrates or algae cultivated as extractive species may pose a risk to human health, if these contaminants are present.

Chemical contaminants in aquaculture

The intensification of aquaculture is often associated with a heavy use of chemicals in this industry, since enclosed conditions and stress can predispose organisms to disease, resulting in economic losses for the producers (Armstrong et al., 2005; Burridge et al., 2010; Buschmann et al., 2009; Cabello, 2006; Cole et al.,

2009; Haya, 2001; Read & Fernandes, 2003; Romero et al., 2012; Sapkota et al., 2008; Weston, 2000). This, together with the lack of information given to farmers, may lead to an indiscriminate use of contaminants (Gräslund et al., 2003). Antimicrobials, antiparasitics, antifoulings and pesticides are often used in aquacultures to avoid deterioration of general quality, with several routes of administration such as incorporation in feeds, intravenous, bath treatments or applied to the infrastructures, either therapeutically or prophylactically (Burridge et al., 2010; Rodgers & Furones, 2009; Sapkota et al., 2008). Additionally, commercial feeds used in aquaculture can also represent a source of environmental chemical contaminants such as persistent organic pollutants (POPs) and metals. Similarly to nutrient loading, this chemical input may impact aquaculture's surrounding environments (Burridge et al., 2010; Granada et al., 2016; Ottinger et al., 2016). Aquaculture operations release, continuously or periodically, discharges that may contain such contaminants. Although dependent on the chemical's properties and the application method, up to 50% of the veterinary medicines used in aquacultures, on average, can end up in aquaculture surroundings and in the environment (Rico & Van den Brink, 2014; Sarmah et al., 2006), with potential effects not only to non-target organisms (Granada et al., 2016; Ji et al., 2012; Leston et al., 2014; Sarmah et al., 2006) but also affecting human supply (Justino et al., 2016).

Pesticides, antiparasitics and antifoulings

As new technologies and alternatives emerge, there is a tendency to decrease the administration of chemicals that have been used during the last decades. However, the rapid development of aquacultures is still a major challenge in terms of chemical use. Salmon aquaculture, for example, is often susceptible to sea lice and ectoparasites contamination (Burridge et al., 2010). Parasitic diseases may not cause death to organisms, but can lead to an increase in production costs or to a decrease in the product quality (Granada et al., 2016) and rejection by markets due to non-conformity. Thus, control of such infestation often relies on antiparasitic compounds, which despite being prescribed, usually end up released into the surrounding environment, affecting other species that are co-cultured in the same system. Some of the most common antiparasitic agents used are organophosphates,

hydrogen peroxide, avermectins, and pyrethroids (Burridge et al., 2010; Danaher et al., 2006; Haya et al., 2005; Lees et al., 2008). Such chemicals can be given in feed or via bath treatment, but runoff from adjacent agricultural sites represents an important source of not only pesticides but also fertilizers in IMTA systems (Bosma & Verdegem, 2011; Sapkota et al., 2008).

Fouling is a phenomenon visible on every surface that is submersed in seawater, and consists in colonization of the surfaces first by bacteria and unicellular organisms and then by multicellular eukaryotes. Aquaculture suffers significantly from its effects, and aside from putting in risk organisms health, equipment can fail when fouling is not controlled (Bazes et al., 2006; Sterling et al., 2016). Seaweed cultivation, for example, is highly affected since fouling organisms may reduce light availability and productivity. Continuous cleaning of the surfaces is labor intensive and costly, so preventive chemical methods such as sodium hypochlorite or copper-based coatings are used to treat seawater, cages and equipment used (Bazes et al., 2006; Braithwaite & McEvoy, 2005; Fletcher, 1995).

Integrated multitrophic systems can capitalize biofoulers, since these organisms also have an important role as bioengineers and microhabitat creators. While adding three-dimensional structures to otherwise flat surfaces (Robinson et al., 2011), these organisms will increase the potential for recapture and recycling of excessive nutrients. Some fouling invertebrates (particularly sessile bivalves) have commercial interest, since they are able to reduce the amount of pathogenic elements thus reducing the need for chemical treatments (Chopin et al., 2012). Incorporating other grazer organisms such as sea urchins in IMTAs might mitigate some of the fouling problems, but environmental friendly antifouling agents must also be considered (Braithwaite & McEvoy, 2005).

Persistent organic pollutants

Similar to wild fish, their farm-raised counterparts may be exposed to polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) and polychlorinated biphenyls (PCBs) arising from industrial and urban effluents, surface water runoff, contaminated sediments and air deposition. However, various studies revealed that PCDD/F and PCB concentrations and profiles in farmed fish tissues are correlated

with those in aquaculture feed (Berntssen et al., 2016; Perugini et al., 2013). These persistent organic pollutants (POPs) can represent a permanent threat to public health due to their broad spectrum of toxicity. These compounds have high chemical stability and tend to bioaccumulate in fatty tissues of living organisms because of their lipophilic nature, leading to biomagnification throughout the food web.

The composition and formulation of commercial feed employed in aquaculture can influence the quality of the raised fish. About 65% is composed by fish meal and oil, providing high amount of protein, essential fatty acids, vitamins and minerals to fish (Suominen et al., 2011). The inclusion of fish oil, usually obtained from small pelagic fish and trimmings from commercial fishery products for retail, unintentionally adds compounds accumulated in lipids (Russell et al., 2011). Thus, it is not surprising that feed may represent the main source of POPs for farmed fish (Maule et al., 2007; Suominen et al., 2011).

The presence of POPs in commercial feed applied in aquaculture may also have negative impacts in the ecosystem. Uneaten feed and large fecal particles may accumulate in sediments in the vicinity of aquaculture facilities, where they can be consumed by detritus-eating animals. Moreover, primary producers may take up contaminants accumulated in these organically rich sediments. Small particles of waste can remain in suspension and then be consumed by filter-feeding organisms. Therefore, PCDD/Fs and PCBs originated from feeds can be accumulated by organisms outside aquacultures and, consequently be transferred along the food chain (Cheney et al., 2019; Russell et al., 2011; Wang et al., 2011). In the IMTA context, this environmental impact caused by the presence of POPs in commercial feed can be mitigated, but the contaminants present in the uneaten feed and feces can be bioaccumulated by organic extractive and inorganic extractive species, potentially reentering the food chain supply.

In order to minimize the potential hazards that aquaculture practices can pose to public and ecosystem health, different strategies have been developed to reduce the concentration of POPs in commercial feed (Berntssen et al., 2016; Ginés et al., 2018; Kawashima et al., 2009; Nøstbakken et al., 2015; Sprague et al., 2010; Usydus et al., 2009). For example, the replacement of fish oil with vegetal oil can substantially reduce the load of POPs in commercial feed. Other approaches involve

the decontamination of fish oil and fishmeal, the improvement of pellet integrity with subsequent slow breakdown rates, and the optimization of feeding systems and protocols.

Metals

Elements may alter their chemical form, but they cannot be degraded over time, meaning that they are environmentally persistent and may bioaccumulate in biota (Vandermeersch et al., 2015). Metals and metalloids are naturally present in the aquatic environment as a result of various geochemical processes. However, anthropogenic activities can be an additional source of these elements. Aquaculture, in particular, can enrich aquatic sediments via copper-based antifoulants and commercial feed (e.g. Burridge et al., 2010; Farmaki et al., 2014; Liang et al., 2016). Copper-based antifouling paints are widely applied to aquaculture cages and nets to prevent the development of epibiota that decrease water quality, durability of the structures and reduce their flotation. Consequently, copper leaches into the water and accumulates in sediments nearby aquaculture sites (Burridge et al., 2010). Metals such as copper, zinc, iron and manganese are present in feed pellets either as constituents of the fishmeal or are added to fulfill mineral requirements (Squadrone et al., 2016). These essential trace elements have nutritional functions and contribute to maintaining a good health status in humans and animals, even though exposure above certain threshold concentrations have the potential to be toxic to biota. On the contrary, cadmium, lead, arsenic, and mercury are relevant for environmental and seafood safety since they have no biological functions and their intake can lead to adverse health effects (Squadrone et al., 2016). Whereas the use of fish oil for the manufacture of commercial feed is the main source of POPs for farmed fish (Russel et al., 2011), fishmeal can be responsible for the main contribution of these priority metal pollutants to feed (Rodríguez-Hernández et al., 2017). Additionally, the location of aquaculture facilities in areas where metal naturally occur or with high anthropogenic pressure can increase the contamination of farmed seafood.

The potential risk that metals released through aquaculture practices may pose to the aquatic environment and human health was reported in various studies

(e.g. Kalantzi et al., 2013; Liang et al., 2016; Russell et al., 2011; Squadrone et al., 2016). In contrast, to date, little information is available regarding the metal content of IMTA-produced organisms and the dynamics of trace elements between species in the system (Ratcliff et al., 2016).

Antibiotics

An antibiotic is, according to FAO's report on the *Responsible use of antibiotics in aquaculture* (FAO, 2005), a "drug of natural or synthetic origin that has the capacity to kill or to inhibit the growth of micro-organisms". Antibiotics are used as chemotherapeutic agents, but one of the problems associated with their use is that the same properties causing the desired effects on target organisms are also the same properties causing adverse effects to non-target organisms (Kümmerer, 2009a; Leston et al., 2013). The steep growth in aquaculture production led to an increase of antibiotic administration in these systems, with approximately 80% of the antibiotics used ending up in the environment (Cabello et al., 2013; Lalumera et al., 2004; Sapkota et al., 2008), since only a portion is absorbed or metabolized by the organisms. Furthermore, it is known that antibiotics have been extensively used in aquacultures for years, either in preventive or therapeutic dosages. Although there is a tendency to stop antibiotic use on a prophylaxis base with more strict regulations being applied, the majority of aquaculture production can take place in countries where such regulations are not followed, or are not so effective (Burridge et al., 2010; Cabello et al., 2013; Cole et al., 2009; Defoirdt et al., 2011). Also, effects may not occur in non-target organisms, but antibiotic resistance can be developed due to the long-term use of these pharmaceuticals, even at sub-therapeutic dosages, causing a big environmental impact on aquaculture's surroundings (Armstrong et al., 2005; Cole et al., 2009; Kummerer, 2004; Kümmerer, 2009b; Tuševljak et al., 2012). For example, in the US, most of the fish farms where antibiotics might be used have pond-like and tank structures, and after harvest they are not fully drained, leading to accumulation of high levels of drugs that will affect newly growing fish since they can be exposed to residues and resistant bacteria (Serrano, 2005). In China, negative effects to coastal waters and aquatic environments are already acknowledged (Cao et al., 2007; Peng et al., 2013).

The irresponsible use of such pharmaceuticals has been addressed by a number of international government agencies, since it can represent potential risks to public health (BIO Intelligence Service, 2013). The emergence of bacterial resistance is currently one of the main concerns in terms of human safety, since disease resistance can be transferred to humans through animal products (Defoirdt et al., 2011; Granada et al., 2016; Schnick, 2001). Antibiotic resistance is developed either through mutations in bacterial DNA or horizontal gene transfer mechanisms (Sapkota et al., 2008). Furthermore, the use of a specific antibiotic may increase not only levels of resistance to that specific molecule but to many others, by cross-resistance (Kummerer & Henninger, 2003; Kümmerer, 2009b). Sapkota and colleagues (2008) reported the presence of antibiotic-resistant bacteria in 13 out of the 15 top aquaculture producing countries, and these bacteria can be found at surrounding sites for longer periods of time after antibiotic administration (Cabello et al., 2013). Resistance genes have already been reported for *Vibrio* spp., *Photobacterium* spp., *Edwardsiella* spp., *Citrobacter* spp., and *Aeromonas* spp. (Granada et al., 2016; Naviner et al., 2011), with important consequences for human health safety. Although a well planned IMTA system will suffer less from potential pathological episodes, eventual antibiotics that are applied to one trophic level may pass to other trophic levels being co-cultured in the same system. This can lead to the presence of such contaminants in organisms that were not intended to take up antibiotics in the first place.

One of the most used antibiotics in aquacultures worldwide is oxytetracycline (Alday-Sanz et al., 2012; Rigos & Smith, 2013; Sapkota et al., 2008). Its broad spectrum activity makes it effective against different types of bacteria and some anaerobic organisms (Burridge et al., 2010; Serrano, 2005; Xuan et al., 2010). It is not only widely prescribed for use in aquaculture, but also in pigs, cattle or poultry production (De Liguoro et al., 2003; Kuhne et al., 2000; Sarmah et al., 2006; Sunderland, 2003; Thurman et al., 2002). Together with tetracyclines, quinolones are also among the most prescribed classes of antibiotics, and these act by passing the bacterial cell through passive diffusion via water-filled protein channels (Bermúdez-Almada & Espinosa-Plascencia, 2012; Samuelsen, 2006). Enrofloxacin, for example, is one of the main antibiotics administered in aquaculture systems worldwide (FAO,

2005; Liu et al., 2017; Quesada et al., 2013; Trouchon & Lefebvre, 2016). Oxolinic acid and flumequine are used to treat infections mainly from *Piscirickettsia salmonis*, *Aeromonas salmonicida*, and *Vibrio* bacteria (Burridge et al., 2010) and present very good assimilation rates when administered via medicated feed (Samuelsen, 2006; Samuelsen & Bergh, 2004). This makes them widely used in Europe, although being prohibited in the United States, Canada and Scotland. Quinolones can be used both as human or veterinary medicines, and presence of antibiotic resistance to these compounds in aquaculture products may directly affect human health (Schnick, 2001). Other antibiotic class with a big share in aquaculture is the sulfonamides, especially if used in combination (also known as potentiated sulfonamides), like Tribissen® which has in its composition 20% sulfadiazine and 80% trimethoprim. They are effective against both gram-positive and gram-negative bacteria and their cost is relatively low (Sapkota et al., 2008; Suzuki & Hoa, 2012), reasons for being highly prescribed.

The presence of antibiotics in edible tissues from fish is highly dependent on several aspects, such as dosage regimen or administration routes, as well as species and sizes, water salinity or even temperature (Hansen & Horsberg, 2000; Ishida, 1992; Rigos & Smith, 2013; Samuelsen, 2006). These factors will not only influence the presence of residues in fish but also in other extractive species co-cultured within the same IMTA system. Also, with several species being co-cultured together sharing the same system, antibiotics used in finfish will potentially be taken up by extractive species (Leston et al., 2013; Rosa et al., 2019), increasing the risk of food allergies or antibiotic resistance by ingesting these organisms.

Legislation

The guarantee of Food Safety, especially in Europe, in line with a high level of consumers protection and human health concerns, is currently one of the most important topics (European Commission, 2000) that captured worldwide attention. According to the European Commission, all consumers should be aware of all properties of their food such as how it is produced, processed, packaged, labeled, and sold. In terms of food-producing animals, it is of extremely importance the improvement of conditions in which the animals are produced and that minimum

welfare requirements are accomplished. Those important standards should be put into practice within EU member states and third countries from where EU imports. The implementation of safety policies implies several mandatory actions such as an effective control system to evaluate the EU standards of food safety and quality, animal health, welfare and nutrition as well as plant health, and this complexity may have a significant impact on innovation (Alexander et al., 2015).

Concerns related with food safety in general and in particular with aquaculture products are mainly related with biological and chemical safety (Bondad-Reantaso et al., 2008; FAO, 1999). Among biological hazards that can pose risks to public health are bacteria, virus, parasites, prions and biotoxins. The knowledge of pathogen sources and having control plans to manage biological risks through food and animal feed chain, along with an efficient hygiene policy, is considered an important tool to prevent food crises situations. Chemical contamination, such as the presence of pesticides and veterinary medicine residues, can have great impact in food production. The use of veterinary medicines is legal in food-producing animals and essential in the modern animal-food producing industry, only to prevent and cure diseases, although such substances can leave residues in food obtained from these animals. Each country has established regulations on the use of antibiotics on aquaculture, but the European Union has, nonetheless, banned some compounds from aquaculture practices, for food safety reasons (namely malachite green, nitrofurans and chloramphenicol). Also, pesticides and other contaminants can be found when animals are exposed to them, but rarely in levels that can become unsafe for consumers.

Considering veterinary drug residues, antibiotics are nowadays one of the main concerns since, when associated with an inappropriate usage, can have negative effects for consumers, one of them being the development and dissemination of antibiotic resistance through animals to humans. This problem is not only a chemical safety issue but also a great biological concern with the dissemination of resistance strains of bacteria (European Commission, 2017a; 2017b). To protect human health from undesirable residues of veterinary drugs in food from animal origin, a specific control was defined by official EU documents that have been set, and continuously improved, in the last decades. In 1996, the Directive

96/23/EC (EC Council Directive 96/23/EC) turned the control of food producing animals and their products through monitoring the presence of residues of veterinary medicines mandatory. EU member states are required to implement monitoring plans to detect the use of illegal compounds and the abuse of authorized ones. An equivalent level of food safety should be guaranteed also from non-EU countries from which the EU imports food products. The division of veterinary medicines into two groups was also defined in the same directive: prohibited substances and allowed compounds, with corresponding established maximum residue limit (MRL). These limits were defined after toxicological studies and bearing the minimum level of exposure possible for consumers in mind. For non-authorized substances and to harmonize analytical performance within member states control, a minimum required performance limit (MRPL) was set in accordance with specific guidelines from European Reference Laboratories (Community Reference Laboratories Guidance Paper, 2007) and the Commission Directive 2005/34/EC (EC Decision 2005/34/EC). In this document is settled that the MRPL concentration is the maximum to be accepted to be present in imported food, although this concentration is not related with toxicological data but only with analytical performance. In the specific case of antibiotics, most of them have MRL established, since they can be used in food-producing veterinary practices (although entirely forbidden as animal growth promoters since 2006 (EC Regulation 1831/2003)). The procedures regarding the establishment of MRLs on pharmacologically active substances and their classification are defined in the EU Council Regulation 470/2009/EC (EC Regulation 470/2009) repealing the previous Council Regulation (EEC) No 2377/90 (EC Council Directive 96/23/EC). The complete list of pharmacologically active substances and their MRLs in different food matrices of animal origin are listed in the Commission Regulation 37/2010 (EC Regulation 37/2010). However, unlike fish and other farmed species, which have specific legislation regarding the maximum levels of residues allowed in edible tissues, such regulations are inexistent for seaweeds for example. Macroalgae production has been expanding impressively, mainly in Indonesia (FAO, 2014) due to its vast areas with excellent conditions for cultivation and the knowledge for cultivation. Seaweeds are also gaining importance in human diets and, to prevent unintentional

contamination and inadvertent intake of unwanted substances, algae should be routinely analyzed before incorporation in any products intended for human and animal consumption, both direct and indirectly, and legislations updated accordingly.

In relation to other chemical hazards, in 2005, the Regulation 396/2005/EC (EC Regulation 396/200) established the maximum residue levels for pesticides in food. In 2006, the Regulation 1881/2006/EC (EC Regulation 1881/2006) defined the maximum levels of metals (lead, mercury and cadmium) as well as polyaromatic hydrocarbons in fish muscle, crustacean and mollusks, in accordance with the Council Regulation 315/93/EC previously set as the basic principles on contaminants in food (EC Regulation 315/93). Maximum levels for PCDD/Fs, dioxin-like PCBs and PCBs in fishery products are also established in the Regulation 1259/2011 (EC Regulation 1259/2011).

Knowing that fish consumption has increased, thus making aquaculture an important economic activity (FAO, 2014; 2018) with vast impact in the environment and human safety, an accurate control more directed to this economical field should be in place. Although legislation for food-producing animals are established there are still some gaps concerning other matrices such as water, algae, sediments and other species such as marine invertebrates. Besides improvements in IMTA production, the regulatory system deserves equal attention, and is often disregarded (Asche, 2017; Osmundsen et al., 2017). It seems only logical that new legislation frameworks consider IMTA systems, since there is a lack of dedicated aquaculture policy for co-cultivation of multiple species in proximity (Alexander et al., 2015; Kleitou et al., 2018).

Conclusions

There is no doubt that IMTA systems can be environmentally responsible and deliver higher profit margins. However, despite their enormous potential, IMTAs are still under development in most countries, at least at full commercial scales. While the increased complexity of these systems brings advantages and falls under the EU's Blue Growth Strategy, it also slows down its development as a producing sector. The integration of extractive species is seen as beneficial since they can assimilate and reutilize nutrients that would eventually impact surrounding systems, but

production issues such as systems maintenance or harvesting methods must be addressed in order to be cost effective. On the other hand, specific legislations for these complex systems must be created, with special focus on residues and contaminants, since these compounds will be present not only in fed species receiving treatment, but also on extractive species being co-cultured. Antibiotics for example, can be ingested in small concentrations through longer periods of time, resulting in antibiotic resistance, a major threat to human health. Thus, further work is needed to increase knowledge on the assimilative capacity of different extractive species. With global markets changing with an increase on the consumption of other organisms such as mussels or macroalgae, food safety issues may arise from their production in IMTA systems.

General aim and thesis outline

The intensification process that aquaculture has been facing during the last decade often relies on a heavy use of chemical compounds, in order to avoid disease outbreaks in fish growing at high densities. According to the Food and Agriculture Organization of the United Nations (FAO), "*Food security exists when all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food which meets their dietary needs and food preferences for an active and healthy life".* While aquaculture contributes to the sense of food security, providing nutritious food to people, there is a need for this food to be safe. In this context, the purpose of the present work was to enhance the knowledge on the accumulation of such pharmaceuticals, namely antibiotics, in several aquaculture matrices and address its potential impacts. The studies presented were based on experimental trials, simulating scenarios of aquaculture productions. Accordingly, several specific objectives were outlined in order to contribute to a better understanding of this situation:

- Evaluation of carcass retention of selected antibiotics by two main fish species reared in the Mediterranean region aquacultures (Gilthead seabream and European seabass);
- Investigation of the stability of selected compounds in seawater;
- Assessment of the impacts of selected compounds on the seaweed *Ulva* sp.;
- Increasing the information on the uptake of antibiotics by seaweed at two different concentrations;
- Address the uptake of selected antibiotics in target IMTA organisms and add information about public safety issues upon consumption.

Chapters II and III address antibiotic retention in muscle samples from Gilthead seabream and European seabass, respectively. Finally, in chapters IV and V, the accumulation of oxytetracycline and enrofloxacin on the seaweed *Ulva* sp. is studied. A general discussion and final conclusions is presented at the end, summing up the work developed within the scope of this thesis, as well as future research following this work.

CHAPTER II

2018. Rosa, J., Leston, S., Castro, M., Freitas, A., Barbosa, J., Pardal, M.A., Rema, P., Dias, J., Ramos, F. Evaluation of antimicrobials residues in farmed gilthead seabream (*Sparus aurata*) after administration through medicated feed. *Food Control*.
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Feed pellets used in the experimental trials

Evaluation of antibiotics residues in farmed Gilthead seabream (Sparus aurata) after administration through medicated feed

Abstract

The use of antibiotics in aquaculture is a well-known fact and merits the focus of the scientific community. In the present study, five drugs (oxytetracycline, sulfadiazine, trimethoprim, oxolinic acid and flumequine) were selected to assess their retention in muscle tissues from Gilthead seabream (*Sparus aurata*). Fish were placed in 150 L tanks at 18° C, and fed for 7 days with experimental diets containing two concentrations of each antibiotic (ranging from 5.51 to 131.16 mg kg^{-1}). Edible tissues were then analyzed through a validated multi-class quantification method (UHPLC-MS/MS). The results indicate that sulfadiazine concentrations were the highest immediately after the feeding period and decreased towards day 3. Flumequine was only detected on the first day with concentrations below the MRL. Both trimethoprim and oxolinic acid concentrations were below the MRLs 3 days after the feeding period was over (oxolinic acid was not detected in muscle samples at day 14 for prophylaxis and day 28 for both treatments). Oxytetracycline residues in muscle tissues were the highest through time, with concentrations above the MRL for 7 days (C_{day7} of 111.2 and 157.2 µg kg^{-1} for both dosages). Results suggest that these antibiotics can be present in Gilthead seabream muscle samples for longer periods than previously reported, when realistic conditions are tested. With the exception of oxytetracycline, concentrations were below the MRLs established 3 days after the feeding trial was over meaning that adverse effects related to human consumption are not likely. Nevertheless, allergic reactions or resistance to antibiotics can be developed if low concentrations of such compounds are ingested on a frequent basis, as is the case of the Mediterranean diet.

Key words: Gilthead seabream; antibiotics; medicated feed; retention; withdrawal times.

Introduction

Fish continues to be one of the most traded food commodities worldwide, with increasing consumption per capita from an average of 9.9 kg in the 1960s to 19.2 kg in 2012 (FAO, 2014). In order to face the demand for seafood products and also driven by the fact that wild life stocks are reaching their limits (Goldburg and Naylor, 2005), aquaculture systems have experienced unprecedented growth (Sapkota et al., 2008), with much more intensive cultivation methods (Goldburg et al., 2001). Concomitantly, infectious diseases pose a risk to production since they may cause problems to animal welfare as well as significant stock losses (Romero et al., 2012), regardless of the hygienic conditions (Rigos et al. 2010). In order to prevent and solve disease outbreaks in culture ponds, the use of antibiotics among other chemicals is characteristic in these intensified methods (Romero et al., 2012; Sapkota et al., 2008). Fish farmers tend to rely on the most cost effective method, which is oral administration through formulated feeds, with the drug either incorporated into food pellets or coated on the outside of the pellet, making this administration route the most used in aquaculture production.

Among the antibiotics used in aquacultures worldwide, oxytetracycline was the most administered drug in top producing countries, followed by oxolinic acid and chloramphenicol, present in 69 % of the countries examined by FAO (versus 92 % that use oxytetracycline) (Sapkota et al., 2008). Oxytetracycline is part of the tetracyclines family and is considered by many authors the most used antibiotic in aquaculture (e.g. Alday-Sanz et al., 2012; Rigos and Smith, 2013). It presents broad spectrum activity effective against diseases caused by Gram-positive and Gram-negative bacteria and even against some anaerobic organisms (Burridge et al., 2010; Serrano, 2005). This antibiotic is widely prescribed in the treatment of fish, poultry, pigs and cattle, and was reported in 11 out of 15 of the top aquaculture producing countries (Sapkota et al., 2008). Flumequine and oxolinic acid were two of the most frequently administered antibiotics in Norwegian aquaculture (Ellingsen et al., 2002), but their importance on human medicine has led to their prohibition in aquaculture in the United States, Scotland and Canada (Burridge et al., 2010). They present low minimum inhibitory concentrations for most fish pathogens and show good tissue penetration when administered orally in medicated feed (Martinsen and Horsberg,

1995; Samuelsen, 2006). Sulfonamides are another class of antibiotics administered in aquaculture although at a lower extension, but the combination of sulfadiazine and trimethoprim (as potentiated sulfonamides) is used in one third of the main producing countries (Sapkota et al., 2008). Both compounds are heavily used as veterinary drugs due to their low cost and wide range against Gram-positive and Gram-negative bacteria (Suzuki and Hoa, 2012).

Gilthead seabream (*Sparus aurata*) is according to the Federation of European Aquaculture Producers the most farmed species in the Mediterranean region, with an estimated production of 129,000 tons (Valente et al., 2011). Together with seabass, these species account for the gross production in Southern Europe, representing 94 % of marine fish aquaculture production, attaining a value of 300,000 tons in 2015 (FEAP, 2016). Several diseases such as pasteurellosis or the most common vibriosis are common in the production of these species and, are in most cases, treated with antibiotics. Improving production conditions in terms of hygiene and good disinfection can help prevent such problems, but antibiotics are still commonly used.

The present work aimed to increase the available information on carcass retention of Gilthead seabream in response to antibiotics supplemented in manufactured diets, using a validated multi-residue detection method for oxytetracycline (OTC), sulfadiazine (SDZ), trimethoprim (TRI), oxolinic acid (OXO), and flumequine (FLU); and to increase the knowledge on dose dependency, by using both prophylactic and therapeutic concentrations.

Materials and methods

Materials and reagents used

Oxytetracycline, sulfadiazine, trimethoprim, oxolinic acid and flumequine used in formulation of experimental diets were purchased from Sigma-Aldrich (Madrid, Spain). All reagents used in the extraction method were of analytical grade (or HPLC grade for solvents used in the mobile phase). The internal standards used were demethyltetracycline for tetracyclines, sulfameter for sulfonamides and trimethoprim, and lomefloxacin for quinolones,; also provided by Sigma-Aldrich.

An UHPLC system coupled to a triple quadrupole tandem mass spectrometer was used for chromatographic separation and MS detection for the quantification in muscle samples. The column used in the system was a reverse-phase Acquity HSS T3 2.1 × 100 mm, 1.8 µm particle size. Mobile phase consisted in ultrapure water acidified with 0.1 % formic acid (solvent A) and acetonitrile (solvent B). The gradient used flowing at a rate of 0.45 ml min^{-1} was as follows: 97 % to 40 % (A) in the initial 5 min, from 5 to 9 min the solvent A was reduced to 0 %, maintained for 1 min and then back up to 97 % (A) in 1 min; from 10 to 12 min the system maintained the original composition, with 97 % of solvent A and 3 % of solvent B. The mass spectrometer detector was a Xevo TQ MS – Acquity UPLC system (Waters, Milford, MA, USA), and data were analyzed using the Masslynx 4.1 software also by Waters.
For antibiotic quantification in feed, an Agilent 1100 Series LC system (Agilent Technologies, Palo Alto, CA, USA) coupled to a triple quadrupole tandem mass spectrometer Sciex API 2000 (Applied Biosystems, Foster City, CA, USA) was used. Data were analyzed by Sciex Analyst software. Chromatographic separation was achieved with an Agilent Zorbax XDB C18 2.1 x 100 mm, 3.5 µm particle size column with an Agilent Zorbax XDB C8 4 x 2.1 mm, 5 µm guard column. Mobile phase composition was the same, with the gradient going from 97 % (A) to 10% and then finishing again at 97 %, with a total run time of 11 min.

Experimental diets

A control diet was formulated according to the nutritional requirements of juvenile seabream. Main ingredients were ground (< 250 µm), mixed in a horizontal helix ribbon mixer (Mano, 100 L capacity, CPM, San Francisco, USA) and dry pelleted using a laboratory pellet press (CPM, C-300, San Francisco, USA) with a 2.4 mm die. Diets were stored at 5 °C until posterior utilization. Samples of each diet were taken for proximate composition. Two composite samples of each experimental diet were taken and chemical analyses performed according to the method described by AOAC (1990). Samples were then pooled and moisture content was determined (105 °C for 24 h). Samples were analyzed for crude protein (N x 6.25, Leco Nitrogen analyser, Model FP-528, Leco Corporation, St. Joseph, USA), crude lipid content by petroleum ether extraction (Soxtherm Multistat/SX PC, Gerhardt, Koenigswinter, Germany; 40-

60 °C), gross energy in an adiabatic bomb calorimeter (Werke C2000, IKA, Staufen, Germany). The basal diet was formulated to be isonitrogenous, isolipid and isoenergetic and presented 96.9 % dry matter (DM), 43.8 (% DM) crude protein, 16.9 (% DM) crude fat, and 22.4 MJ kg^{-1} DM gross energy.

This basal mixture was used as the basis to create 8 experimental diets. The same formulation was used but antibiotics (Oxytetracycline, OTC; Oxolinic Acid, OXO; Flumequine, FLU; Sulfadiazine, SDZ; Trimethoprim, TRI) were added as premixes in the form of powders during the manufacturing process. These diets differ from each other in concentration (two) and type (four) of antibiotic incorporated into each, corresponding to either prophylactic (P) or therapeutic (T) dosages (Table 1).

Experimental diets were formulated taking into account the most used antibiotics in aquaculture and dosages set accordingly, and duplicates of each one were analyzed for antibiotic exact concentration.

Experimental conditions

The experimental trial was conducted by trained scientists following category C recommendations from the Federation of European Laboratory Animal Science Associations (FELASA) and the European Directive 2010/63/EU of European Parliament and of the Council of European Union on the protection of animals used for scientific purposes.

This trial was performed at the Experimental Research Station (Vila Real, Portugal) at the University of Trás-os-Montes e Alto Douro (UTAD) facilities. Juveniles of *S. aurata* presented an average weight of 75.5±1.1 g and were randomly distributed into 9 fiberglass tanks (1 for CTRL diet + 4 for OTC, MIX, OXO, FLU prophylactic diet + 4 for OTC, MIX, OXO, FLU therapeutic diet) of 150 L water capacity each, with 26 fish in each tank. The tanks are part of a circulating saltwater system unit with partial renewal of water and temperature control. Water oxygen, temperature and quality were regularly monitored. Mean water temperature was 17.8±0.6 °C during the experimental feeding period and oxygen saturation was over 90 %. Ammonia, pH, nitrites, and nitrates were maintained within the recommended limits for the species. A 12:12 h light:dark cycle was applied during the trial. Fish

were acclimated to experimental conditions for 15 days, during which time all fish received the control-based (CTRL) diet to apparent visual satiety.

During a 7-day feeding period, fish were fed manually with experimental diets twice a day (9:00 and 17:00), and received a similar daily portion, which varied form 1.3 % to 1.5 % body weight per day. After this feeding period with medicated diets, fish were fed with CTRL diet during the rest of the experiment time. Three fish from each tank were sacrificed with a sharp blow to the head, and muscle samples (dorsal area) were collected from each dietary treatment at days 0, 3, 5, 7, 14 and 28. Samples of muscle tissue were frozen in liquid nitrogen and stored at -80 °C until further use.

Antibiotic analyses

The extraction and analyses of antibiotics in muscle tissues was conducted according to the validated multi-class quantification method developed by our group (Freitas et al., 2014). Limits of detection and quantification for each compound were determined as $X_0+K.\sigma_0$ and $X_0+10\sigma_0$, respectively (Relacre, 2000). *Sparus aurata* muscle samples from the dorsal area were weighed (2.0 ± 0.09 g) and homogenized in 15 mL Falcon tubes. A solution of internal standards with 10 µg mL^{-1} was added (20 µL), vortex mixed and allowed to stand in the dark for at least 10 min. Afterwards, a simple solvent extraction was performed by vortex mixing and shaking the sample with 5 mL of acetonitrile and 1 mL of 0.1 M EDTA using a Reax shaker for 20 min, followed by a centrifugation at 3100 g for 15 min. The supernatant was transferred to a new tube and evaporated to dryness under a gentle stream of nitrogen at 40 °C. The residue was dissolved with 400 µL of 0.1 % formic acid, filtered through a 0.45 µm PVDF Mini-uniprep™ and injected into the UHPLC-MS/MS under MRM-optimized conditions for each compound (for details on the Multiple Reaction Monitoring acquisition conditions for each antibiotic used, see Freitas et al. 2014). Quantification of antibiotics concentrations in feed were performed by weighing ~1.0 g of sample, followed by a simple extraction with 50:50 water:acetonitrile. Confirmation was made running triplicate samples in the UHPLC system described above, and the measured concentrations can be found in table 1.

Table II.1 - Formulation of diets used in the experiment. Ingredients are expressed in percentage of the total formulation and antibiotic concentration in mg kg^{-1} (mean values ± SE). The following dosages were prepared, for prophylactic and therapeutic treatments, respectively: Diet CTRL - no antibiotic; Diet OTC - 37.5 mg OTC kg^{-1} (P) and 75 mg OTC kg^{-1} (T); Diet MIX – 110 mg SDZ kg^{-1} (P) and 22 mg TRI kg^{-1} (P), 220 mg SDZ kg^{-1} (T) and 44 mg TRI kg^{-1} (T); Diet OXO – 6 mg OXO kg^{-1} (P) and 12 mg OXO kg^{-1} (T); Diet FLU - 6 mg FLU kg^{-1} (P) and 12 mg FLU kg^{-1} (T). Please refer to footnote for information on components.

	Diet								
Ingredients (%)	**CTRL**	**OTC**		**MIX**		**OXO**		**FLU**	
		P	T	P	T	P	T	P	T
Fishmeal 70 LT	10								
Fishmeal 65	20								
Corn gluten	11								
Soybean meal	16								
Rapeseed meal	7								
Sunflower meal	5								
Wheat meal	6								
Pea starch	6								
Fish oil	15								
Vit & Min Premix[1]	1								
Lutavit E50[2]	0,1								
Hilyses[3]	0,5								
Betaine	0,5								
Soy lecithin	0,5								
Binder[4]	0,5								
Antioxidant[5]	0,2								
L-Lysine	0,5								
DL-Methionine	0,2								
Antibacterials (mg kg^{-1})									
Oxytetracycline	-	5,51 (0,43)	16,49 (1,12)	-	-	-	-	-	-
Sulfadiazine	-	-	-	70,02 (2,58)	131,16 (5,52)	-	-	-	-
Trimethoprim	-	-	-	20,96 (1,05)	39,76 (1,67)	-	-	-	-
Oxolinic Acid	-	-	-	-	-	7,04 (0,03)	14,06 (1,39)	-	-
Flumequine	-	-	-	-	-	-	-	6,38 (1,07)	17,91 (0,83)

[1] Mineral and vitamins premix. Covered nutritional requirements of seabream (Supplied by SPAROS Lda. Olhão, Portugal)
[2] Vitamin E acetate; Premix, Portugal
[3] Hydrolyzed Yeast; Premix, Portugal
[4] Guar gum; Sorgal, Portugal
[5] Rosamox – rosemary extract, Kemin; Italy

Data treatment

The results of the antibiotic concentrations in Gilthead seabream for muscle tissues were reported as mean ± SE. The elimination time curve was analyzed by a

non-linear regression analysis (Microsoft® Excel Analysis Toolpak), assuming a first-order kinetics with the equation $C(t)=C_0e^{-\beta t}$. The elimination half-life ($t_{1/2}$) of antibiotics for muscle was calculated by $t_{1/2}=\ln 2/\beta$, where β is the elimination rate constant, obtained from the elimination curve equations (Baggot, 1997). Area under the curve (AUC) was determined following the trapezoidal rule, and was extrapolated to infinity (Ritschel, 1986).

Results

Mean concentrations of sulfadiazine, trimethoprim, flumequine, oxolinic acid and oxytetracycline retained in fish muscle tissues for three replicates at each sampling day and for both concentrations used are present in table 2. Antibiotic analyses were performed after experimental conditions were finished.

Except for FLU, antibiotics showed similar patterns of presence and degradation through time, with first-order elimination kinetics best describing the antibiotics tested (R^2 values for prophylactic and therapeutic dosages, respectively, are 0.6161 and 0.5531 for TRI, 0.5606 and 0.6449 for SDZ, 0.7537 and 0.5828 for OXO, 0.9163 and 0.8654 for OTC). Flumequine was only detected immediately after the feed administration period, with an average concentration of 13.6 μg kg^{-1} for prophylactic and 36.0 μg kg^{-1} for therapeutic dosages at day 0. Such concentrations are below MRL limits established for finfish (600 μg kg^{-1}), and only corresponded to 0.2 % of the initial concentration present in the administered pellets. Trimethoprim also presented very low percentages of antibiotic retention in muscle tissues, with 0.48 and 0.71 % for prophylactic and therapeutic dosages, respectively. Oxolinic acid concentrations in muscle samples at day 0 corresponded to 2.00 and 2.11 % of the initial concentrations present in feed. Sulfadiazine and oxytetracycline presented the highest percentages of carcass retention on day 0, with 5.20 and 6.57 % of the feed concentration for SDZ and 4.63 and 6.22 % for OTC, for prophylactic and therapeutic doses, respectively.

Table II.2 - Antibiotic concentrations in Gilthead seabream (*S. aurata*) muscle samples (μg kg^{-1}). Concentrations obtained following oral administration for Prophylactic and Therapeutic treatments (mean values ± SE, n = 3). Values in bold indicate concentrations above the MRL established by the Commission Regulation (EU) No 37/2010.

	SDZ		**TRI**		**FLU**	
	LOD	LOQ	LOD	LOQ	LOD	LOQ
	1.20	4.00	0.10	0.21	0.70	2.37
Sampling day	**P**	**T**	**P**	**T**	**P**	**T**
0	**3640.4 ± 810.0**	**8616.6 ± 3441.3**	**101.5 ± 1.9**	**281.6 ± 46.2**	13.6 ± 9.2	36.0 ± 10.5
3	94.2 ± 26.0	**246.0 ± 123.8**	25.2 ± 7.2	33.2 ± 11.7	<LOD	<LOD
5	15.6 ± 1.1	65.7 ± 8.5	15.7 ± 1.3	48.9 ± 16.8	<LOD	<LOD
7	19.3 ± 1.0	18.4 ± 6.9	18.3 ± 2.0	13.5 ± 2.5	<LOD	<LOD
14	9.4 ± 1.2	15.0 ± 2.3	18.2 ± 1.7	18.7 ± 4.0	<LOD	<LOD
28	4.2 ± 0.3	4.4 ± 0.5	8.1 ± 0.2	10.0 ± 2.2	<LOD	<LOD
	MRL 100		MRL 50		MRL 600	

	OXO		**OTC**	
	LOD	LOQ	LOD	LOQ
	0.74	2.45	5.00	8.00
Sampling day	**P**	**T**	**P**	**T**
0	**140.7 ± 29.8**	**296.5 ± 116.1**	**255.3 ± 36.8**	**1026.4 ± 47.5**
3	5.6 ± 1.29	11.1 ± 0.8	**216.8 ± 26.2**	**395.0 ± 64.8**
5	1.5 ± 0.1	2.5 ± 1.0	**141.7 ± 32.6**	**232.4 ± 81.0**
7	1.4 ± 0.6	0.7 ± 0.3	**111.2 ± 34.2**	**157.2 ± 27.5**
14	<LOD	0.7 ± 0.3	37.8 ± 8.0	63.8 ± 8.2
28	<LOD	<LOD	23.0 ± 1.0	34.6 ± 10.6
	MRL 100		MRL 100	

Table II.3 - Calculated pharmacokinetic parameters of orally administered antibiotics in Gilthead seabream (*S. aurata*) at 18 °C.

	SDZ		TRI		FLU		OXO		OTC	
	P	**T**	**P**	**T**	**P**	**T**	**P**	**T**	**P**	**T**
Dosage (mg kg^{-1})	110	220	22	44	6	12	6	12	37,5	75
Feed concentration (mg kg^{-1})	70,02	131,16	20,96	39,76	6,38	17,91	7,04	14,06	5,51	16,49
C day0 (µg kg^{-1})	3640,4	8616,6	101,5	281,6	13,6	36,0	140,7	296,5	255,3	1026,4
Antibiotic day0 (%)	5,20	6,57	0,48	0,71	0,21	0,20	2,00	2,11	4,63	6,22
$AUC_{0-\infty}$ (µg · h mL^{-1})	6492,9	14601,3	3450,1	3264,2	-	-	253,1	526,6	7051,2	10876,5
Clearence (mL h · g^{-1})	0,0108	0,0090	0,0061	0,0122	-	-	0,0278	0,0267	0,0008	0,0015
Elimination rate constant	0,1730	0,2060	0,0640	0,0860	-	-	0,3360	0,1860	0,0890	0,1100
$t_{1/2}$	4,01	3,36	10,83	8,06	-	-	2,06	3,73	7,79	6,30

Sulfadiazine concentration in edible tissues remained higher than the MRL (100 µg kg^{-1}) for at least 3 days after the feeding period for the therapeutic dose, and the mean concentrations were the highest detected among all antibiotics. The other compound tested – trimethoprim – only presented concentrations higher than the MRL established (50 µg kg^{-1}) on the last day of feed administration, decreasing 4 and 8 times their concentration from day 0 to 3, for prophylactic and therapeutic doses, respectively. Oxolinic acid presented the same behavior, significantly decreasing from 140.7 µg kg^{-1} (prophylactic) and 296.5 µg kg^{-1} (therapeutic) to 5.6 µg kg^{-1} and 11.1 µg kg^{-1} from day 0 to day 3. Oxytetracycline presented concentrations higher than the MRL established for at least one week after medicated feed administration, with concentrations higher than 100 µg kg^{-1} for both dosages tested. As expected, AUC from zero to infinity of SDZ was the highest of the antibiotics tested with a value of 14601.3 µg kg^{-1} d^{-1}, since the feed pellets were the most concentrated (versus

10876.5 µg kg^{-1} d^{-1} for OTC in therapeutic dose; see table 3). Considering the prophylactic treatment, OTC presented the highest values of AUC from zero to infinity, despite having lower initial concentrations than SDZ present on the medicated feed (7051.2 and 6492.9 µg kg^{-1} d^{-1}, respectively). Such values are due to OTC high retention in muscle samples through time. Despite presenting values lower than the MRL established for most of the days sampled, on the last sampling day, only FLU and OXO on the prophylactic dose were below the limit of detection. As expected, fish fed with CTRL diet did not present any antibiotic concentrations in muscle tissues in all samples analyzed.

Discussion

Although feeds were formulated to have dosages ranging from 6 to 220 mg kg^{-1}, we observed that measured concentrations of antibiotics in feed ranged from 5.51 to 131.16 mg kg^{-1}. The highest differences were observed in SDZ and OTC diets, where concentrations were 70.02 and 131.16 mg kg^{-1} for 110 and 220 mg kg^{-1} dosage, respectively, for SDZ and 5.51 and 16.49 mg kg^{-1} for 37.5 and 75 mg kg^{-1} dosage, respectively, for OTC. Diets were formulated in order to present the best homogeneity possible, therefore adding the drug in the beginning of the mixing process. Heat and humidity involved in the pelleting process could possibly degrade some compounds to a certain degree (Daniel, 2009). This process allowed nevertheless the correct mixture of the compounds, and despite concentrations being lower than expected, concentrations were similar in all replicates analyzed for confirmation.

Flumequine was only present in the initial sampling time (day 0), with very low concentrations in Gilthead seabream muscle. The concentration administered was too low to establish the pharmacokinetics of the antibiotic through time, both for prophylactic and therapeutic dosages. Plakas and co-authors (2000) detected lower residue concentrations of FLU in channel catfish muscle in comparison to liver, where concentrations were ~4x higher, which could indicate higher concentrations also in liver in the present study. Moreover, concentration peaks in all major tissues analyzed were observed 12-24 h after administration, and half-lives of 22 h were registered for oral dosing. The results obtained were in accordance with other

studies for FLU pharmacokinetics in seawater species, with the antibiotic rapidly decreasing to half of its initial concentration on Atlantic salmon muscle tissues during the first hours of administration (e.g. 17.6-21.3 h and 1.3 h following oral administration (Elema et al., 1994; Rogstad et al., 1993)), and 3.1 h following intravenous administration (Martinsen and Horsberg, 1995). Furthermore, Rogstad and colleagues (1993) stated that more than 98 % of the administered dose of FLU was distributed within a period of 10 h. Nevertheless, these authors have detected FLU up to 5 days after administration, contrarily to the results obtained in the present work, which could be explained by the fact that they used oral doses 4 times higher than in the present study. Also, Tyrpenou and colleagues (2003) obtained half-lives of 22.14 and 21.43 h at 18 and 24 °C, respectively, but the presence of FLU was detected 168 h after feed administration, which again might be related to the concentrations used (~6x higher than prophylactic dosage used). This rapid depletion in fish muscle was previously described, for Gilthead seabream (Malvisi et al., 1997), with FLU concentrations below limit of quantification 48 h after the end of medication. These results were also observed in other study on Gilthead seabream, with FLU reaching levels below the limit of detection at the 2^{nd} and 4^{th} day after administration, for 19.5 and 14 °C, respectively (Romero González et al., 2010). It has also been suggested that skin and bones can act as sink for quinolones, slowly releasing it to other tissues during longer periods of time (Malvisi et al., 1997). Our results indicate that, considering FLU, shorter sampling times should be considered in order to determine the pharmacokinetics of this chemical. Changing the sampling times might also increase the R^2 values of the first-order kinetics model since we could observe a more gradual decrease in residues concentration through time.

Regarding oxolinic acid, it was detected up to day 7 for prophylactic and day 14 for therapeutic dosages. Despite presenting initial concentrations similar to flumequine, oxolinic acid was present in fish tissues during longer periods, although in concentrations much lower than the MRL established by the Commission Regulation 37/2010. The highest values detected in fish muscle were similar to previous studies on Gilthead seabream (Rigos et al., 2003a). In a study comparing the pharmacokinetics after intravenous and oral administration, Rigos and co-authors (2002) did not detect OXO in the 128 h after oral administration. However,

concentrations were significantly different considering administration routes (AUC values of 134.99 and 26.75 μg h^{-1} mL^{-1} for intravenous and oral, respectively), with OXO being detected in muscle only when i.v. administration was followed. The decrease in OXO concentration from day 0 to day 3 followed the same accentuated decrease, even after a multiple 10 day in-feed administration, as in other work on the same species (Rigos et al., 2003a). Furthermore, the presence of OXO in muscle tissues might be conditioned by the unfavorable pH in the digestive tract of marine fish (Daniel, 2009; Rigos et al., 2002).

Levels of sulfadiazine remained above the MRL for 3 days, contrarily to a previous study following a multiple dosing for 5 days (Rigos et al., 2013). According to Rigos and co-authors (2013), sulfadiazine did not present an accumulative drug profile, decreasing its concentration even during the medication period, as reported in a similar work assessing the presence of sulfadimethoxine and ormetoprim residues in Gilthead seabream (Papapanagiotou et al., 2002). The realistic concentrations used in the present work were sufficient to maintain relatively high SDZ levels, while previous studies showed a much faster depletion of SDZ in edible tissues of Gilthead seabream (below limit of quantification after 1 (Papapanagiotou et al., 2002) and 4 days (Rigos et al., 2013) following administration). Trimethoprim is frequently used together with SDZ as potentiated sulfonamides, acting in synergy and blocking two sequential steps in the synthesis of bacterial folic acid (Romero et al., 2012). For this reason, studies on the depletion of trimethoprim *per se* in fish tissues are scarce, but resistance to this antibiotic is present in wastewaters and sewages sludge all over the world (Kümmerer, 2009). Also, TRI concentrations are usually measured in association with other sulfonamides, lacking specific information on the retention of this antibiotic in edible fish tissues.

Results obtained with trimethoprim and oxytetracycline might indicate that concentrations measured in muscle samples at the 1st day depended on the initial dosage administered (therapeutic concentrations led to ~40 % higher percentage of retention at day 0, while no differences were found in FLU and OXO treatments for example). Studies addressing only one concentration in Gilthead seabream (Malvisi et al., 1996; Rigos et al., 2003b; Rigos et al., 2011; Tyrpenou et al., 2003) are not sufficient for comparison, since our study suggests that higher dosages can lead to

higher percentages of antibiotic retained in edible tissues and should therefore increase the withdrawal times for these antibiotics. A recent review made by Rigos and Smith (2013) stated that such dose dependency, although easily found in crustaceans, is not well established for fish, taking into account existing studies. However, pharmacokinetic parameters can vary according to dose regimen, with multi or single oral dosage presenting different parameters, and even among seabream species differences can be registered (Rigos et al., 2003b; Rigos et al., 2004; Rigos and Smith, 2013). Further studies on the depletion of antibiotics in fish muscle tissues should be addressed using different dosages in order to establish the existence of such correlation.

Sparus aurata plays a major role in the economics of Mediterranean countries such as Portugal, Spain and Greece, and information on the carcass retention of antibiotics is imperative. Furthermore, it has been demonstrated that antibiotics presence in edible tissues can change if we consider different fish species, sizes, temperatures, freshwater or seawater and experimental protocols such as administration routes and dosage regimen (e.g. Hansen and Horsberg, 2000; Ishida, 1992; Rigos and Smith, 2013; Rigos et al., 2002; Rigos et al., 2003b; Rigos et al., 2004; Rigos et al., 2011; Rigos et al., 2013; Samuelsen, 2006), being of vital importance to address the parameters for such high-value species, and following the administration method most common in aquaculture. In a similar work, Romero González and co-authors (2010) addressed the depletion of antibiotics in Gilthead seabream. Overall, our results are in accordance with previous studies, but concentrations of OTC and OXO were detected in muscle tissues for longer periods. Also, the study of different concentrations used is of vital importance in order to understand alterations of the pharmacokinetic parameters.

Legislation is not always available some countries, and withdrawal times for different antibiotics are scarce. Potentiated sulfonamides are part of the approved aquaculture drugs with respective withdrawal times, where Romet-30®, a combination of sulfadimethoxine/ormetoprim is labeled with an withdrawal time of 42 days for salmonids and 3 days for catfish. Despite giving shorter withdrawal times for catfish due to skin removal, these periods are not sufficient for residues degradation. According to our results, sulfadiazine when used for therapeutic

treatment is still present in edible tissues in concentrations higher then the MRL established by the Commission Regulation (EU) No 37/2010.

Oxytetracycline, as one of the most used antibiotics worldwide for aquaculture, has some indications from the US Food and Drug Administration Agency, stating that "*Withdrawal times vary with indication as follows: for marking skeletal tissue in Pacific salmon, 7 days; for disease control in salmonids, 21 days; catfish, 21 days; lobster, 30 days*" – information on Terramycin ® 200 for Fish. Although without information for basses, our results suggest that even following the withdrawal times prescribed, oxytetracycline residues will be present up to fish sale or consumption. Moreover, the Pacific salmon indication of withdrawal time is 7 days, which according to the present results show levels of OTC above the maximum residue limit allowed by legislation.

Oxytetracycline concentrations in feed were the lowest of the prophylactic dosages and second lowest of the therapeutic dosages. Nevertheless, concentrations detected in muscle samples were above the MRL up to 7 days after the end of the feeding period, with a clearance value of 0.0008 and 0.0015 $\mu g \cdot h\ mL^{-1}$ for prophylactic and therapeutic dosages, respectively. Even when concentrations in muscle samples are below the MRLs established, drug residues can still be detected through longer periods of time.

Conclusion

The present study provides updated and reliable data on the retention of antibiotics, with conditions similar to aquaculture practices, since most studies addressing oral administration use forced feeding, which can reduce oral bioavailability and loss of appetite, as well as stress in animals. The relatively low concentrations used in the medicated feed were chosen according to the real dosages used in medicated pellets. With the exception of FLU, concentrations were detected and measured in edible tissues for longer periods than previously reported in other studies. MRLs are established to prevent consumer intake to exceed the toxicological values of chemicals used in feed and food products, but the presence of residues in foodstuff can generate problems of allergy and resistance in humans. Accordingly, withdrawal times are set so that residues are not present above the

MRLs when products are sold or consumed. In areas such as the Mediterranean, where fish consumption is a major source of animal protein, caution should be taken in order to minimize exposure to antibiotics. Understanding the pharmacokinetics of each individual species with the most used feeding method in aquaculture is vital to understand exposure pathways to these contaminants.

CHAPTER III

2018. Rosa, J., Leston, S., Freitas, A., Barbosa, J., Rema, P., Dias, J., Lemos, M.F.L., Pardal, M.A., Ramos, F. Tissue depletion of five antibiotic residues in farmed European seabass (*Dicentrarchus labrax*). *Aquaculture.*
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Juvenile of seabass (*Dicentrarchus labrax*)

Tissue depletion of five antibiotic residues in farmed European seabass (Dicentrarchus labrax)

Abstract

Concerns about antibiotic use led to stricter legislations and overall better practices in aquaculture production. However, depletion time periods in tissues vary greatly with different antibiotics, fish species or experimental setup. In the present work, five drugs (sulfadiazine (SDZ), trimethoprim (TRI), flumequine (FLU), oxolinic acid (OXO) and oxytetracycline (OTC)) were incorporated into medicated feed, and their retention in European seabass muscle tissues assessed. Juveniles were placed in 300 L tanks at 18° C and were manually fed with medicated feeds for a 7 day period (two concentrations per antibiotic, ranging from 6 to 220 mg kg^{-1}, which were based on previous studies on the occurrence of these antibiotics). Residues were analyzed through a multi-class quantification method (UHPLC-MS/MS). Data on residues concentration through time followed a one-compartment model, with TRI concentrations above the established Maximum Residue Level (MRL) throughout the experiment. Similarly, OTC concentrations at the highest dose were also above 100 µg kg^{-1} up to 14 days after the medication period. Results obtained for TRI might indicate the presence of a dose dependency for this antibiotic. FLU concentrations in muscle samples were the lowest through time (C_{day7} of 279.70 and 386.63 µg kg^{-1}, for prophylactic and therapeutic treatments, respectively). Half-life values of 14.37, 10.87, 5.36, 7.20 and 27.22 hours (prophylactic treatment), and 20.95, 8.41, 5.61, 11.22 and 17.99 hours (therapeutic treatment), were determined for SDZ, TRI, FLU, OXO and OTC, respectively.

Withdrawal times of 0, 2 and 5 days were determined for FLU, OXO and SDZ, but sampling times for OTC and TRI should be longer, since antibiotic concentrations were above the MRL 14 days after the end of the feeding period with medicated feed. Therefore, special attention should be given since they are the most used antibiotics in aquaculture and European seabass plays a major part in human nutrition in the Mediterranean region.

Key words: Seabass; Medicated feed; Withdrawal times; Retention; Residues.

Introduction

Aquaculture provides 20% of animal protein to almost 3 billion people, according to FAO's latest report on *The State of World Fisheries and Aquaculture* (FAO, 2014). Traditional aquacultures that were characterized by low stock densities and small farm sizes are no longer viable and intensive aquaculture systems have experienced unprecedented growth (Sapkota et al., 2008). The last 2 editions of The EU Fish Market place seabass as the main commercial marine fish species of 2013 and 2014, with the unit price reaching a 10-year peak of 100 million euros (EUMOFA, 2015; 2016). Together with Gilthead seabream, farmed seabass is one of the most consumed species in the Member States surrounding the Mediterranean (EUMOFA, 2016). It is one of the major species in terms of economic value for the Mediterranean region, where fish consumption per capita in 2030 is expected to be roughly the double of the EU-15 average (Failler, 2007).

With traditional aquacultures being replaced by more intensive systems, a higher number of fish is growing together in more confined spaces. This increases the risk of problems with animal welfare and can lead to stock losses, since disease outbreaks and infections become common (Rigos et al., 2010; Romero et al., 2012). The intensification of farming methods turned the use of chemicals such as pesticides, disinfectants, antifungals and other pharmaceuticals, like antibiotics, regular in these industries (Cabello, 2006; Romero et al., 2012; Sapkota et al., 2008), ultimately ending up as pollutants following several paths (Veach & Bernot, 2011). These contaminants can ultimately bioaccumulate in fish, following two routes of uptake: from water via gills and skin, or via the ingestion of medicated feed. Following uptake, xenobiotics are firstly distributed to high perfusion tissues, and then to the low perfusion tissues like muscle and skin (Streit, 1998). In aquacultures, antibiotics are often administered orally via medicated feeds, since it is the most cost effective method to treat a large number of fish (Rodgers & Furones, 2009). Drugs can be either incorporated into feed pellets through a premixture, or coated on the outer layer of the pellets (Samuelsen, 2006; Sapkota et al., 2008; Sekkin & Kum, 2011). One problem associated with in-feed administration of antibiotics is that fish have to be actually feeding in order for it to be effective, especially if we consider that ill fish have a tendency to stop eating (Daniel, 2009).

For decades, concern about antibiotic use in aquaculture was practically inexistent, lacking legislation and guidelines on their use. Nowadays, stricter legislation on food safety is turning aquaculture into a safer production method. Following Regulation (EC) No 470/2009 on the procedures for the establishment of residue limits in foodstuffs, the Commission Regulation (EC) No 37/2010 was adopted, and Maximum Residue Limits were set for pharmacologically active substances in foodstuffs of animal origin (EC Regulation 37/2010). More recently, Commission Regulation (EU) 2017/880 was created, mediating the extrapolation of MRLs from one or more species to other species where MRLs are not set (EC Regulation 2017/880). Also, when no suitable products are available for treatment of fish diseases, other products approved for use in food producing species can be prescribed, following the cascade system (Cascade, 2013). In these cases, the standard withdrawal period set in the EU for fish has been set at 500 degree-days. Antibiotic use is dependent on veterinary prescription in most countries, so one could assume that their use in aquaculture is decreasing. However, while global tendency is to stop the administration of these compounds for prophylaxis, the vast majority of global aquaculture production takes place in countries with few or no effective regulations (Defoirdt et al., 2011). Nonetheless, antibiotics are still commonly used as prophylactic treatment (Cabello et al., 2013; Mesalhy et al., 2014; Rico et al., 2012).

According to the European Regulation, there are currently 28 pharmacologically active substances with a therapeutic classification of anti-infectious agents that can be used for finfish production (EC Regulation 37/2010). There is, however, incomplete information regarding antibiotic use in aquaculture, since national regulatory agencies fail to collect this information (Cabello et al., 2013). Some authors contemplate only 7 antibiotics as approved for aquaculture use in Europe (BIO Intelligence Service, 2013; Rodgers & Furones, 2009). Considered by many the most administered antibiotic in aquaculture (e.g. Alday-Sanz et al., 2012; Reda et al., 2013; Rigos & Smith, 2013), OTC is widely used due to its broad spectrum activity, being effective against diseases caused not only by Gram-positive and Gram-negative bacteria (Xuan et al., 2010) but also by some anaerobic organisms (Burridge et al., 2010; Serrano, 2005). According to a review made by Sapkota and colleagues

(2008), OTC is used in 92% of the top 15 aquaculture producing countries, with OXO coming second together with chloramphenicol (which is now banned due to its potential risks (Wongtavatchai et al., 2004). In 2015, OTC was still addressed as the top antibiotic when it comes to the treatment of fish diseases (Done and Halden, 2015). Oxolinic acid and FLU are used to treat fish mainly against infections from *Piscirickettsia salmonis*, *Aeromonas salmonicida* and *Vibrio* bacteria (Burridge et al., 2010). These quinolones present good assimilation when administered via medicated feed (Samuelsen, 2006; Samuelsen & Bergh, 2004) and are still used in Europe even though they are prohibited in the United States, Scotland and Canada. Sulfonamides in combination, or potentiated sulfonamides, are also vastly used in aquaculture (for example Tribissen®, which is composed by 80% SDZ and 20% TRI) due to their low cost and wide range against both Gram-positive and Gram-negative bacteria (Sapkota et al., 2008; Suzuki & Hoa, 2012). As set in the Commission Regulation (EU) No 37/2010 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin, MRLs for OTC, SDZ, TRI, OXO and FLU are 100, 100, 50, 100 and 600 μg kg^{-1} respectively (EC Regulation 37/2010).

Despite the data available for antibiotic use, caution should be taken since this data can be biased towards countries where legislations are strong and tracking information is good (Sapkota et al., 2008). On the other hand, antibiotic presence in edible tissues is strongly dependent on administration routes and dosage regimen, not to mention different fish species and sizes, water temperatures and salinity (Hansen & Horsberg, 2000; Ishida, 1992; Rigos & Smith, 2013; Rigos et al., 2002a, 2002c, 2004b, 2013; Samuelsen, 2006). Within this view, the present work aims to increase information on the carcass retention of European seabass (*Dicentrarchus labrax*), in response to feeds supplemented with oxytetracycline, sulfadiazine, trimethoprim, oxolinic acid, and flumequine, under accurate conditions used in aquacultures. Also, knowledge about dosage dependence will be obtained by using both prophylactic and therapeutic concentrations.

Materials and methods

Materials and reagents used

The reagents used in the tissue extraction method of antibiotics were all of analytical grade (HPLC grade for solvents used in the mobile phases). Acetonitrile, methanol and formic acid were purchased from Merck (Darmstadt, Germany), and ethylenediamine tetra acetic acid (EDTA) from Sigma-Aldrich (Madrid, Spain). As internal standards, demethyltetracycline was used for OTC, sulfameter for SDZ and for TRI, and lomefloxacin for FLU and for OXO. All standards and internal standards used in the formulation of the feeds were purchased from Sigma-Aldrich (Madrid, Spain).

Medicated feeds

Medicated feeds were developed from an initial control diet (CTRL; table 1), similar to a commercial feed, formulated according to the nutritional requirements of juvenile seabass. Main ingredients were ground (<250 µm), mixed in a horizontal helix ribbon mixer (Mano, 100 L capacity, CPM, San Francisco, USA) and dry pelleted using a laboratory pellet press (CPM, C-300, San Francisco, USA) with a 3.2 mm die. Feeds were stored at 5 °C until posterior utilization. Samples of each feed were taken for proximate composition. Feeds were analyzed in duplicates as described by AOAC (2016), for crude lipid content by petroleum ether extraction (Soxtherm Multistat/SX PC, Gerhardt, Koenigswinter, Germany; 40-60 °C), crude protein (N x 6.25, Leco Nitrogen analyser, Model FP-528, Leco Corporation, St. Joseph, USA) and gross energy in an adiabatic bomb calorimeter (Werke C2000, IKA, Staufen, Germany).

Based on this control formulation, eight medicated feeds were created, by adding antibiotics, as premixes in the form of powders, during the manufacturing process. Feeds were formulated to be isonitrogenous (50.5±0.7% crude protein), isolipidic (15.9±0.4% crude fat) and isoenergetic (22.7±0.1 MJ kg^{-1} DM gross energy). These feeds vary from each other both in concentration and type of antibiotic incorporated into each, corresponding to an hypothetical prophylactic (P) and therapeutic (T) dosages. Medicated feeds were formulated based on the most used

antibiotics in aquaculture, with dosages set based on previous studies on such pharmaceuticals in the Mediterranean region aquacultures.

Table III.1 – Diet composition

Ingredients (%)	CTRL diet
Fishmeal Diamante LT[1]	35.0
Soy protein concentrate (Soycomil)	13.0
Wheat gluten[2]	6.0
Corn gluten[3]	10.0
Soybean meal 48[4]	7.5
Rapeseed meal	5.0
Wheat meal	5.9
Pea starch	4.0
Fish oil[5]	12.0
Vit & Min Premix PV01[6]	1.0
Binder[7]	0.3
Antioxidant powder (Paramega)	0.2
Sodium propionate	0.1

1- Peruvian fishmeal LT: 71% crude protein (CP), 11% crude fat (CF), EXALMAR, Peru.
2 -VITEN: 85.7% CP, 1.3% CF, ROQUETTE, France.
3 - Corn gluten feed: 61% CP, 6% CF, COPAM, Portugal.
4- Solvent extracted dehulled soybean meal: 47% CP, 2.6% CF, SORGAL SA, Portugal.
5- COPPENS International, The Netherlands.
6-Mineral and vitamins premix. Covered nutritional requirements of seabass (Supplied by SPAROS Lda. Olhão, Portugal).
7- Guar gum; Sorgal, Portugal.

Trial conditions

Trained scientists performed all experimental trials following category C recommendations from FELASA (Federation of European Laboratory Animal Science Associations; (Guillen, 2012), as well as the European Directive 2010/63/EU on the protection of animals used for scientific purposes.

The study was conducted at the Experimental Research Station (Vila Real, Portugal) of the University of Trás-os-Montes e Alto Douro (UTAD) facilities. Juveniles of *D. labrax* with an initial average body weight of 50.1±0.9 g were randomly distributed into 9 fiberglass tanks of 300 L water capacity each (30 fish per tank) that were part of a circulating saltwater system unit with partial renewal of water. Mean water temperature was 17.9±0.5 °C and oxygen saturation was over

90% throughout the experimental feeding period. Ammonia, pH, nitrites and nitrates were maintained within the recommended limits for this species. Fish were acclimated to experimental conditions for 15 days with a 12:12 h light:dark cycle and during this time all fish received CTRL feed to apparent visual satiety. After this period, fish were fed manually with the medicated feeds twice a day (9:00 and 17:00) for 7 days, receiving a similar daily ration that varied from 1.3 to 1.5% body weight per day. Three fish from each tank were sacrificed with a sharp blow to the head and muscle samples from the dorsal area collected at days 0, 1, 7, 8, 9, 10, 12 and 21. Sampling at day 7 was done 12 h after the last feeding with medicated feed. Sampling times 8a and 8b corresponds to 24 and 36 h after medication period, respectively. Samples were frozen in liquid nitrogen and stored at -80 °C until analysis.

Antibiotic residues analyses

The extraction and analyses of antibiotics in muscle samples were performed after an extension of the previous method validated for *Sparus aurata* muscle tissue by our group (Freitas et al., 2014). Validation parameters for *D. labrax* can be found in table 2, with values in accordance with the limits defined by European Commission Decision nº 657/2002 (EC, 2002). Briefly, *D. labrax* muscle samples were taken from the dorsal area of the fish (2.0 ± 0.14 g) and homogenized in 15 mL Falcon tubes. Internal standards were added (20 µL from a 10 µg mL^{-1} solution) and allowed to stand in the dark for at least 15 min. A simple solvent extraction was performed by vortex mixing and shaking the sample with 5 mL of acetonitrile and 1 mL of 0.1 M EDTA using a Reax shaker for 20 min, followed by a centrifugation at 3100 *g* for 15 min. The supernatant was transferred to a new tube and evaporated to dryness under a gentle stream of nitrogen at 40 °C. The residue was redissolved with 400 µL of 0.1% formic acid, filtered through a PVDF mini-uniprep™ and injected into the UHPLC-MS/MS under Multiple Reaction Monitoring optimized conditions for each of the analyzed compound. Chromatographic separation and MS detection for quantification in fish muscle samples was achieved with an UHPLC system coupled to a Xevo TQ MS – Acquity UPLC system (Waters, Milford, MA, USA) triple quadrupole tandem mass spectrometer. This system is constituted by a vacuum degasser, an

autosampler and a binary pump equipped with an analytical reverse-phase column Acquity HSS T3 2.1 × 100 mm, 1.8 µm particle size. Mobile phase consisted in ultrapure water acidified with 0.1% formic acid (solvent A) and acetonitrile (solvent B), at a flow rate of 0.45 mL min^{-1} and the gradient program was as follows: from 97% to 40% (A) in the first 5 min, and then down to 0% in the next 4 min. Proportion was changed to the initial one in 1 min and maintained for 2 more minutes. Total run time was 12 min. Data were analyzed using Masslynx 4.1 software by Waters.

Table III.2 – Extension of the method validation

Compound	**MRL**	**CCα**	**CCβ**	**Repeatability**	**Reproducibility**	**Recovery**	**LOD**	**LOQ**
	µg kg^{-1}	µg kg^{-1}	µg kg^{-1}	%RSD	%RSD	%	µg kg^{-1}	µg kg^{-1}
Sulfadiazine	100	115.42	130.83	5.46	8.18	109.14	5.46	8.18
Trimethoprim	50	115.14	130.28	12.50	18.75	102.49	2.90	9.60
Oxytetracycline	100	117.18	134.36	5.78	8.67	96.57	3.21	10.70
Flumequine	600	628.86	657.72	14.35	21.52	82.35	0.18	0.59
Oxolinic acid	100	111.57	123.14	11.84	17.75	102.79	0.32	1.08

Data treatment

Antibiotic concentrations in European seabass muscle tissues were reported as mean ± SE, and the elimination rates were analyzed for best fit to a one or two-compartment pharmacokinetic model by a non-linear regression analyses and least square fitting (Ritschel, 1976); Graphpad Prism® 6 Software). A first-order elimination kinetics was assumed, and the exponential equation $C(t)=C_0e^{-\beta t}$ used, where β is the elimination rate constant. Elimination half-life ($t_{1/2}$) of antibiotics for muscle was calculated by $t_{1/2}=\ln 2/\beta$ (Baggot, 1977). Area under the curve (AUC) was determined following the trapezoidal rule directly on the measured concentrations (Ritschel, 1976); Graphpad Prism® 6 Software). Elimination characteristics of antibiotics were calculated by using values from day 7 onwards since it was the last day of medication.

Results

Antibiotic analyses were performed after the trial was over. Data on the residues through time could be satisfactorily fit to a one-compartment model (R^2 > 0.85), showing similar patterns of presence and degradation in muscle samples through time. The calculated pharmacokinetic parameters for all antibiotics in European seabass muscle samples are present in Table 3. As expected, SDZ presented the highest AUC values (64828 and 106591 μg·d mL^{-1} for P and T, respectively) since medicated pellets presented the highest dosage among all antibiotics. Contrarily, FLU presented the lowest AUC values (1505 and 1960 μg·d mL^{-1}), followed by OXO (with 5513 and 6955 μg·d mL^{-1}), being the ones with lowest dosages in medicated feed. AUC values in TRI and OTC treatments increased from 10999 to 68732 μg·d mL^{-1} and from 4868 to 32448 μg·d mL^{-1}, respectively, when we doubled the initial concentration in medicated feed. Flumequine treatments presented the highest elimination rates (3.1020 and 2.9630 for P and T, respectively), leading to a fast decrease of concentrations through time. Half-life values varied from 5.36 h in FLU to 27.22 h in OTC treatments.

Table III.3 – pharmacokinetic parameters

	SDZ		**TRI**		**FLU**		**OXO**		**OTC**	
	P	**T**	**P**	**T**	**P**	**T**	**P**	**T**	**P**	**T**
Dosage (mg kg^{-1})	110	220	22	44	6	12	6	12	37.5	75
C day7 (μg kg^{-1})	10375.91	15351.65	1864.43	11458.70	279.70	386.63	1449.67	1680.40	616.95	5683.17
antibiotic day7 (%)	9.43	6.98	8.47	26.04	4.66	3.22	24.16	14.00	1.65	7.58
$AUC_{0-\infty}$ (μg.d mL^{-1})	64828	106591	10999	68732	1505	1960	5513	6955	4868	32448
Clearence (mL $d.g^{-1}$)	0.0017	0.0021	0.0020	0.0006	0.0040	0.0061	0.0011	0.0017	0.0077	0.0023
Elimination rate constant (d^{-1})	1.1580	0.7942	1.5310	1.9790	3.1020	2.9630	2.3090	1.4830	0.6111	0.9249
$t_{1/2}$ (h)	14.37	20.95	10.87	8.41	5.36	5.61	7.20	11.22	27.22	17.99

Degradation pattern of SDZ, TRI, FLU, OXO and OTC in muscle samples can be found in Figure 1, for both concentrations tested, at each sampling day (mean ± SE). Both TRI (P and T dosage) and OTC (T dosage) presented values higher than the MRL established (50 and 100 μg kg^{-1}, respectively) until the end of the experimental trial (105.50 and 156.30 μg kg^{-1} for TRI and 232.35 μg kg^{-1} for OTC at day 21). Samples taken immediately after the medication period was over presented 8.47 and 26.04% of TRI initial pellet concentration, and 1.65 and 7.58% of OTC initial pellet concentration (P and T dosages, respectively). Flumequine presented the lowest values in muscle samples through time, with concentrations below the MRL established (600 μg kg^{-1}) immediately after the end of the medication period (C_{day7} of 279.70 and 386.63 μg kg^{-1}, for P and T, respectively), which corresponded to 4.66 and 3.22% of the initial feed concentration. Sulfadiazine presented high percentages in muscle samples during the medication period, only decreasing to values below the MRL (100 μg kg^{-1}) 5 days after the end of medication period (30.72 and 5.70 μg kg^{-1} for P and T, respectively). Oxolinic acid concentrations in muscle samples were below the MRL (100 μg kg^{-1}) 36 h after the end of medication period. All antibiotics tested were present in seabass muscle samples up to 14 days after the end of medicated feeding period: FLU concentrations were 2.15 and 2.75 μg kg^{-1}, OXO concentrations were 4.45 and 5.00 μg kg^{-1}, SDZ concentrations were 30.72 and 8.33 μg kg^{-1}, for P and T dosages, respectively. OTC concentration for P dosage was 76.00 μg kg^{-1}.

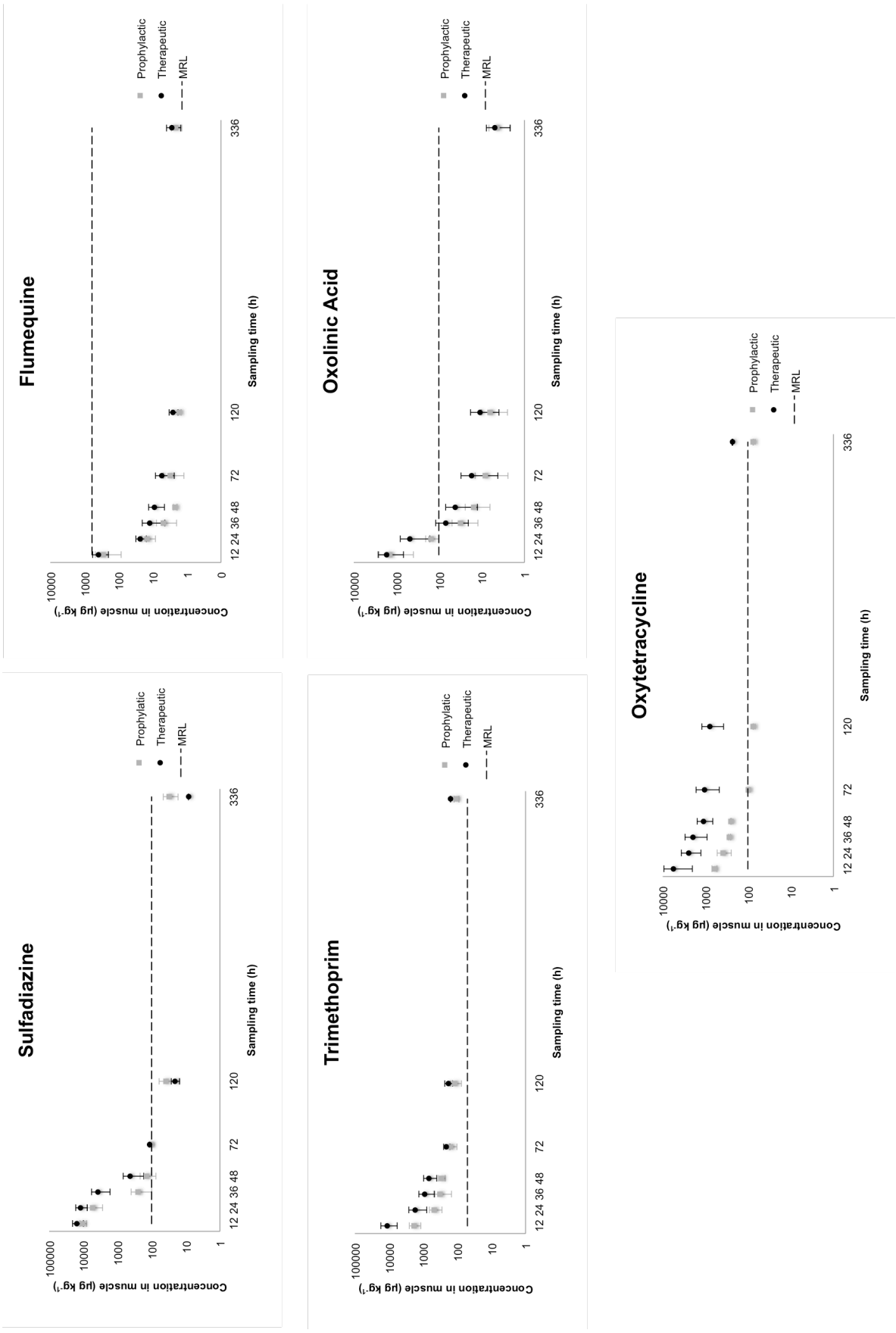


Figure III.1 – Antibiotic concentrations in European seabass muscle samples through time. Concentrations obtained following oral administration for Prophylactic and Therapeutic treatments (mean values ± SE, n=3). The MRLs established by the Commission Regulation (EU) No 37/2010 are plotted as dashed lines.

Discussion

Dosages used were selected based on several works in order for them to be realistic. Concentrations of FLU and OTC were selected based on a study on antibiotics used in aquaculture in Italy (Lalumera et al., 2004). By direct comparison with sulphamerazine, also addressed in that study, we established the therapeutic dosage for SDZ at 220 mg kg^{-1}. Since the usual proportion of TRI usage on potentiated sulfonamides is 1/5 in relation to sulfonamides, a dosage of 44 mg kg^{-1} was selected for TRI. Oxolinic acid dosage was set based on a document by the European Medicines Agency (2005) for food producing species, which recommended 12 mg kg^{-1} as the dosage for finfish. Antibiotic use as prophylactic treatment is becoming more and more outdated, although some of them are still used in this context (Cabello et al., 2013; Mesalhy et al., 2014; Rico et al., 2012), and therefore we decided to assess the retention of half the dosages recommended for each antibiotic addressed in the present study.

In general, antibiotic concentrations found in muscle samples were higher than previously reported (Table 4). In a similar work with Gilthead seabream, concentrations of the same antibiotics 14 days after the medication period was over were much lower than reported in the present study (Rosa et al., 2018). Concentrations of FLU were the lowest detected through time, with concentrations below the MRL (600 µg kg^{-1}) during all the experiment. This was expected since the dosage established for FLU was the lowest (6 and 12 mg kg^{-1}). However, following the same dosage, OXO concentrations in muscle were above the MRL established (100 µg kg^{-1}) 24 h after the end of medication period. Despite being less noticeable in muscle tissues than in liver for example, concentrations of FLU were higher than OXO in a study with Atlantic salmon (Rogstad et al., 1993). Such differences between tissues were also observed by Plakas and colleagues (2000), where concentrations ≈4x higher were detected in liver, in channel catfish samples. Similarly, a previous work made by our group for Gilthead seabream also detected higher concentrations of OXO than FLU through time (Rosa et al., 2018), but in general concentrations were lower than in the present work, rapidly decreasing below detection levels (3 days for FLU and 7 and 14 for OXO, P and T treatments, respectively). Despite the low concentrations of FLU through time, concentrations of 2.15 and 2.75 µg kg^{-1} (for P

and T, respectively) were still detected 14 days after the medication was over, unlike works for similar species such as Gilthead seabream (Malvisi et al., 1997; Rosa et al., 2018; Tyrpenou et al., 2003). Considering the dosage used for FLU and the elimination half-lives determined we could assume that if the dosages were higher we would have found higher concentrations in muscle samples through time. In a study following FLU intravascular injection, $t_{1/2}$ values tend to be higher (10.71 h versus 5.36 and 5.61 h obtained in the present work, even with similar dosages (Rigos et al., 2002d)). Nonetheless, comparable half-life values were determined in European seabass for other quinolones such as danofloxacin (Vardali et al., 2017) or enrofloxacin (Intorre et al., 2000). Considerable differences were found previously between pharmacokinetic parameters for FLU and OXO, following intravascular administration (Samuelsen, 2006), with elimination half-life varying from 10.71 h for FLU (Rigos et al., 2002d) to 55 or 315 h for OXO, depending on the temperature (Rigos et al., 2002b). For Atlantic salmon, $t_{1/2}$ values of OXO were lower than the ones determined for FLU (Rogstad et al., 1993). Such differences were not detected in the present study, but $t_{1/2}$ values for FLU did not change between dosages (5.36 and 5.61 h for P and T, respectively), while in OXO, $t_{1/2}$ values increased slightly from 7.20 to 11.22 h with the increase in dosage. Half-life values of 86.64 h (Poher et al., 1997) and 315 (13 °C) or 55 h (22 °C) (Rigo et al. , 2002b) for OXO in European seabass were previously reported, but with medication through intravascular injection. Works addressing the same compounds but in other species like Atlantic salmon (e.g. Martinsen & Horsberg, 1995) or Gilthead seabream (e.g. Romero González et al., 2010; Rosa et al., 2018), tend to present different half-life values from the ones reported in the present study, which can be attributed to the specific physiologies of each species (Streit, 1998; Rigos and Smith, 2013).

Table III.4 – Different studies on pharmacokinetic parameters of the studied antibiotics.

Antimicrobial	Species	Dosage (mg kg^{-1})	Administration	Temperature (°C)	Concentrations in muscle (µg kg^{-1})	$t_{1/2}$ (h)	Reference
Flumequine	***Dicentrarchus labrax***	**6 (P) and 12 (T)**	**In feed, oral**	**17.9**	**2.15 (P) 2.75 (T)**	**5.36 (P) 5.61 (T)**	**Present study**
Flumequine	*Sparus aurata*	6 (P) and 12 (T)	In feed, oral	17.8	13.6 (P) 36 (T)	-	Rosa et al., 2018
Flumequine	*Sparus aurata*	12	In feed, oral	25 - 28	3.9		Malvisi et al., 1997
Flumequine	*Sparus aurata*	35	In feed, oral	18 and 24	22 (18°C) 12 (24°C)		Typpeneu et al., 2003
Flumequine	*Dicentrarchus labrax*	10	intravascular injection	18	-	10.71	Rigos et al., 2002c
Danofloxacin	*Dicentrarchus labrax*	10	In feed, oral	16 and 27	22.6 (16°C) 25.3 (27°C)	16.87 (16°C) 21.13 (27°C)	Vardali et al., 2017
Enrofloxacin	*Dicentrarchus labrax*	5	Oral gavage	15	70	25	Intorre et al., 2000
Oxolinic Acid	***Dicentrarchus labrax***	**6 (P) and 12 (T)**	**In feed, oral**	**17.9**	**4.45 (P) 5.00 (T)**	**7.20 (P) 11.22 (T)**	**Present study**
Oxolinic Acid	*Sparus aurata*	6 (P) and 12 (T)	In feed, oral	17.8	1.4 (P) 0.7 (T)	2.06 (P) 3.73 (T)	Rosa et al., 2018
Oxolinic Acid	*Dicentrarchus labrax*	15	intravascular injection	13 and 22	-	315 (13°C) 55 (22°C)	Rigos et al., 2002b
Oxolinic Acid	*Dicentrarchus labrax*	10	intravascular injection	15.2	-	86.64	Poher et al., 1997
Oxolinic Acid	*Salmo salar*	25	In feed, oral	10.2	-	18.2	Martinsen and Horsberg, 1995
Oxolinic Acid	*Sparus aurata*	30	In feed, oral	14 and 19.5	23 (14°C) <LOQ (19.5°C)	146.64 (14°C) 89.76 (19.5°C)	Romero González et al., 2010
Sulfadiazine	***Dicentrarchus labrax***	**110 (P) and 220 (T)**	**In feed, oral**	**17.9**	**30.72 (P) 8.33 (T)**	**14.37 (P) 20.95 (T)**	**Present study**
Sulfadiazine	*Sparus aurata*	110 (P) and 220 (T)	In feed, oral	17.8	4.2 (P) 4.4 (T)	4.01 (P) 3.36 (T)	Rosa et al., 2018
Sulfadiazine	*Sparus aurata*	25	In feed, oral	24 - 26	18 (FishOil) 13 (PlantOil)	-	Rigos et al., 2013
Sulfadiazine	*Sparus aurata*	25	In feed, oral	24 - 26	27.7 (FishOil) 19.8 (PlantOil)	-	Zonaras et al., 2016
Sulfadiazine	*Sparus aurata*	30	In feed, oral	14 and 19.5	<LOQ (14°C) <LOQ (19.5°C)	40.08 (14°C) 30.24 (19.5°C)	Romero González et al., 2010
Trimethoprim	***Dicentrarchus labrax***	**22 (P) and 44 (T)**	**In feed, oral**	**17.9**	**105.4 (P) 156.3 (T)**	**10.87 (P) 8.41 (T)**	**Present study**
Trimethoprim	*Sparus aurata*	25	In feed, oral	24 - 26	66.5 (FishOil) 74.9 (PlantOil)	-	Zonaras et al., 2016
Trimethoprim	*Sparus aurata*	30	In feed, oral	14 and 19.5	11 (14°C) 11 (19.5°C)	306.96 (14°C) 214.56 (19.5°C)	Romero González et al., 2010
Trimethoprim	*Sparus aurata*	22 (P) and 44 (T)	In feed, oral	17.8	8.1 (P) 10.0 (T)	10.83 (P) 8.06 (T)	Rosa et al., 2018
Ormetoprim	*Sparus aurata*	50	In feed, oral	26	70	-	Papapanagiotou et al., 2002
Oxytetracycline	***Dicentrarchus labrax***	**37.5 (P) and 75 (T)**	**In feed, oral**	**17.9**	**76.0 (P) 232.35 (T)**	**27.22 (P) 17.99 (T)**	**Present study**
Oxytetracycline	*Dicentrarchus labrax*	40	intravascular injection	13.5 and 22	2600 (13.5°C) 5300 (22°C)	Last sampling point was 128 h after medication	69 (13.5C) 9.65 (22°C)	Rigos et al., 2002a
Oxytetracycline	*Dicentrarchus labrax*	75	In feed, oral	19 - 25	40	Detected 60 days after medication	1238.4	Malvisi et al., 1996

Sulfadiazine concentrations in muscle samples were above the MRL established (100 µg kg^{-1}) 3 days after the medication period was over. Withdrawal times should be longer than 3 days for this species and this compound. To the authors' knowledge, information on SDZ retention in seabass is scarce, lacking information on withdrawal times already set for this particular species. Several works already studied SDZ retention in other species such as Gilthead seabream with even slower degradation rates (calculated withdrawal times of 108 and 113 h (Rigos et al., 2013b) or 5 and 6 days (Zonaras et al., 2016). In these studies, however, dosages used were much lower than the ones used in the present study (25 mg kg^{-1}). Furthermore, an accumulative drug profile can be identified in the present work, with concentrations highly increasing during medication period, contrarily to what was described by both Rigos and colleagues (2003) or Zonaras and co-authors (2016). A previous work by our group determined similar half-life times for SDZ in Gilthead seabream (Rosa et al., 2018). Contrarily, other works calculated longer half-life times for the same species (1.67 and 1.26 days, for trials at 14 and 19.5°C (Romero González et al., 2010).

Potentiated sulfonamides are widely used in aquaculture, being the combination of SDZ and TRI the most commonly used in veterinary medicine (Rigos & Troisi, 2005), and TRI was the only antibiotic addressed in the present study with concentrations higher than the MRL established (50 µg kg^{-1}), for both concentrations, up to at least 14 days after the medication period was over (concentrations of 105.40 and 156.30 µg kg^{-1} at the end of trial, for P and T, respectively). Longer sampling periods should be made in order to assess when concentrations would be below the MRL and thus in accordance with legislation. However, withdrawal times must not be shorter than 14 days, which might not be the case for Romet® (sulfadimethoxine + ormetoprim 5:1) for example, with withdrawal times varying from 5 to 40 days, depending on the country of registration. Other works addressing TRI retention in Gilthead seabream reported different results, depending on the type of medicated feed (plant based or fish based; concentrations of TRI below the MRL 4 days after last dose (Zonaras et al., 2016), or the temperature of the trials (14.0 and 19.5 °C; concentrations below MRL 1 day after last dose (Romero González et al., 2010)). In a similar experimental setup

in seabream, concentrations of TRI were below the MRL 2 and 3 days after last feeding period, for both dosages (Papapanagiotou et al., 2002; Rosa et al., 2018).

Based on the difference between AUC values and percentages of retention in the first day from P to T treatment, TRI and OTC retention might follow a dose dependency pattern. More concentrations should be addressed in order to assess this possibility, since this dose dependency can be easily found in crustaceans, but it's not well established in fish (Rigos & Smith, 2013). Studies addressing only one concentration of each antibiotic are not sufficient for this type of comparison, but our study suggest that this dependency can occur in TRI and OTC.

Oxytetracycline is one of the most studied antibiotic since it is the most used in top producing countries (Rigos & Smith, 2013; Sapkota et al., 2008), with several studies addressing pharmacokinetic parameters for other species (e.g. (Jacobsen, 1989; Rigos et al., 2003, 2004a, 2006, 2011; Wang et al., 2004). Studies on the OTC retention in European seabass showed similar results to the ones present in this work, with determined half-times of 69 and 9.65 h at 13.5 and 22 °C (Rigos et al., 2002a). Nevertheless, concentrations found in the present study were much lower than the ones reported in one study by Rigos and colleagues (2002a), where OTC was administered via intravascular injection. On a study for this species following the same administration method and same dosage used, a similar pattern was observed in muscle samples, with high concentrations during medication period, slightly decreasing through time (Malvisi et al., 1996). Contrarily to the present work however, this study presented muscle concentrations below the MRL established (100 µg kg^{-1}) 10 days after the end of the medication period.

No withdrawal times specific for basses could be found for any of the antibiotics addressed in this experiment, and it has already been demonstrated that different species will have different retention times and pharmacokinetic parameters. Presence in edible tissues will also vary significantly if we consider different temperatures, routes of administration or dosage regimen (Hansen & Horsberg, 2000; Ishida, 1992; Rigos & Smith, 2013; Rigos et al., 2013; Samuelsen, 2006). However, based on the withdrawal times set for Terramycin®200 (OTC) for fish, for instance, the withdrawal time for Pacific Salmon is 7 days, and concentrations would still be above the MRL established in the legislation.

Considering that OTC presented the lowest elimination rate constants, even the 21 days of withdrawal time set for salmonids or catfish could be insufficient, even after extrapolation to other species, as stated in the Commission Regulation (EU) 2017/880. Following the simpler (non-parametric) approach to estimate the withdrawal times (Concordet and Toutain, 1997), SDZ and OXO withdrawal times should be set at 5 and 2 days, respectively. Since FLU concentrations were below the MRL established on the first sampling point, a withdrawal period of 0 days could be sufficient for this antibiotic. Both TRI and OTC concentrations were above the MRL until the end of the experiment, which was 14 days after medication period, and for this reason, no withdrawal times can be set. However, if half of the OTC dosage is used, a withdrawal time of 5 days could be set for OTC.

Portugal is the largest consumer of fish products in European Union, and only after Iceland and Japan globally, with a consumption of 55.6 Kg per capita per year (Monteiro, 2012), and according to FEAP latest record, seabasses are the most produced species of the Marine Mediterranean region, accounting for 49% of the whole production (Federation of European Aquaculture Producers, 2016). The results obtained need to be considered for the establishment of withdrawal times for this particular species due to its importance in regions such as the Mediterranean.

Information on the retention of antibiotics in edible tissues of this species is of vital importance, since concentrations potentially harmful for human ingestion were found in the present study. By using conditions similar to the ones used in aquacultures, we were able to get reliable and updated data on the pharmacokinetic parameters of the antibiotics chosen. Oral feeding, contrarily to parenteral administration or forced feeding, might lead to lower ingestion of medicated feed. This is however, the most realistic scenario when trying to assess the retention of antibiotics in muscle samples. Based on the results obtained, withdrawal times should be updated and set when they are not available. Even following the cascade system for off-label use, where a 500 degree-days withdrawal period should be set for fish, we observed that OTC concentrations in muscle samples would be above MRL established even after 28 days if the correct dosage is given to fish. Furthermore, all antibiotics were detected until the last sampling point and in

concentrations much higher than previously reported, which could ultimately lead to allergy or resistance problems, even though these values are below the MRL.

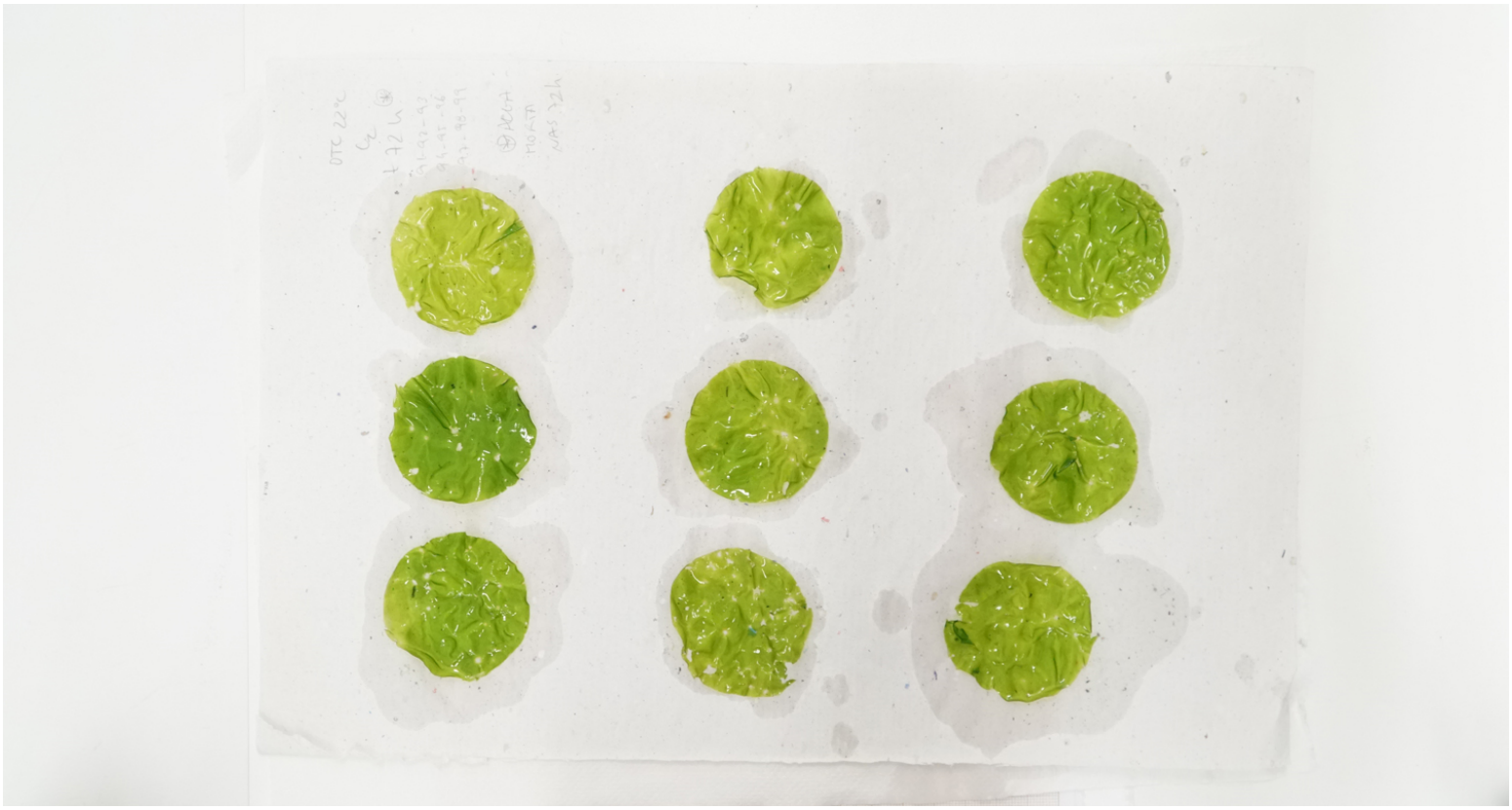
Conclusion

Several works confirm that pharmacokinetics and depletion times vary greatly if we consider different drugs (due to different lipophilicity, hydrophilicity and molecular size for example) and different fish species (differences in digestion and absorption rates). Therefore, this study provides updated and more complete information on the retention of vastly used antibiotics in aquaculture. By using representative conditions, the authors were able to estimate the retention times of OTC, FLU, OXO, SDZ and TRI in European seabass edible tissues, one of the most important species produced in the Mediterranean region aquacultures. The maximum tolerated levels of pharmacologically active substances are set so the consumer is safe upon food consumption. However, even when values are below the MRL established, problems of allergy and resistance to antibiotics may arise in areas such as the Mediterranean and others where fish is a major source of animal protein. We observed that concentrations were retained in muscle samples of *D. labrax* longer than previously reported. Special attention should be given to OTC and TRI and the withdrawal times for species with such economical importance as seabasses must be present in the legislation. Based on the present study, withdrawal times of 0, 2 and 5 days should be set for FLU, OXO and SDZ. The study of several antibiotics for each fish species is vital to determine the retention and elimination times.

CHAPTER IV

2019. Rosa, J., Leston, S., Freitas, A., Vila Pouca, A.S., Barbosa, J., Lemos, M.F.L., Pardal, M.A., Ramos, F. Oxytetracycline accumulation in the macroalgae *Ulva*: potential risks for IMTA systems. *Chemosphere*.

DOI: 10.1016/j.chemosphere.2019.03.112



Experimental sheet containing algal disks ready to photograph in order to measure areas.

Oxytetracycline accumulation in the macroalgae Ulva: potential risks for IMTA systems

Abstract

Oxytetracycline (OTC) is one of the most used antibiotics in aquaculture. With the development of Integrated Multitrophic Aquaculture (IMTA) systems in order to mitigate some aquacultures' adverse effects, attention needs to be shifted to other co-cultured species that can also accumulate such pharmaceuticals and pose a risk to human consumption. Therefore, the present work evaluated the exposure of the seaweed *Ulva* to OTC at two realistic concentrations (0.040 and 0.120 mg L^{-1}). Oxytetracycline degradation rates in seawater were dependent on the initial concentration but were not influenced by the presence of *Ulva*. The macroalgae presented good assimilation rates of OTC, with internal concentrations reaching 40.9934 ng g^{-1} WW for the lowest concentration tested and 108.6787 ng g^{-1} WW for the highest, with a steep decrease after 48 and 24h, respectively. Nonetheless, concentrations were still half of the Maximum Residue Limit set for fish (100 µg kg^{-1}) 48h after C2 treatment. The highest dosage tested stimulated growth 96 h after the beginning of the trial, although some signs of decay could also be found in *Ulva*'s fronds.

Key words: Oxytetracycline; Aquaculture; IMTA; Macroalgae; Accumulation; Legislation.

Introduction

Several reasons have powered the increase in aquaculture production in the last decades. Fish and seafood in general are known to hold high benefits for human health, due to the significant levels of vital components such as vitamin D and B12, selenium, iodine and essential amino acids, but also to the presence of omega-3 fatty acids found in many species (Lund, 2013). Aquaculture has been the major supplier for fish consumption by the global population, which now exceeds the consumption of meat from all terrestrial animals (FAO, 2018). Aquaculture systems have been experiencing unparalleled growth to face the demand of seafood

products that accompanies population progress (Sapkota et al., 2008). But despite the improvement over the years, some issues are still associated with some aquaculture systems. Uneaten or non digested feed will, together with fish excretions, increase the nutrient loading in the surrounding environments of aquaculture sites (Granada et al., 2016; Grigorakis & Rigos, 2011; Read & Fernandes, 2003). A big percentage of phosphorous and nitrogen input as feed in these systems may easily be lost into the environment, with subsequent effects such as eutrophication (Zhou et al., 2006). Goldburg and colleagues (2001) already addressed some of these impacts associated with aquaculture areas (Goldburg et al., 2001), which lead to the development of sustainable approaches to aquacultures in general (Barrington et al., 2009; Bosma & Verdegem, 2011; Zhou et al., 2006).

One possible way to mitigate the release of high amounts of effluent nutrients to the environment is through the establishment of Integrated Multitrophic Aquaculture (IMTA) systems. As can be read in the FAO *Fisheries and Aquaculture Technical Paper* (Barrington et al., 2009), these systems are essentially a group of different organisms belonging to different trophic levels that are co-cultured, and where the by-products from one species become inputs (fertilizers, food, and energy) for another, contrarily to traditional polycultures, where one could have different species but all belonging to the same trophic level. Ideally, all species in the same system have economic value but with the goal of achieving environmental sustainability during production.

Since they present high productivity and can be economically viable, macroalgae, and more particularly seaweeds, can be suitable biofilters for such systems (Granada et al., 2016). The genus *Ulva* is widely distributed in many climate and ecological conditions, being suitable for cultivation practically everywhere, which makes it the ideal candidate for integrating such systems (Ben-Ari et al., 2014; Msuya & Neori, 2008; Nielsen et al., 2011). As biofilters continuously exposed to the aquatic environment, these organisms will also bioaccumulate contaminants that might be present in aquaculture effluents (Lalumera et al., 2004; Leston et al., 2011; 2014), with direct consequences upon its consumption. Although there is a well-established legislation for food products concerning the presence of pharmacologically active substances (EC Regulation 37/2010), namely in fish, this is

virtually inexistent for macroalgae. Considering that a total world production of 23.8 million tons of aquatic algae, from which the majority was seaweed, was already reached in 2012 (FAO, 2014), and that these values will potentially increase if algae are produced in IMTA systems, since they can benefit from the nutrient-enriched waters resulting from fish production (Lahaye et al., 1995), there is a need to establish safe values of contaminants for the consumer.

Oxytetracycline (OTC) is considered one of the most used antibiotics in aquaculture (Alday-Sanz et al., 2012; Rigos & Smith, 2013), being present in 11 out of the top 15 highest aquaculture producing countries (Sapkota et al., 2008). The broad spectrum of activity and effectiveness against diseases caused by both Gram-positive and Gram-negative bacteria, as well as some anaerobic organisms (Burridge et al., 2010; Serrano, 2005) makes it widely prescribed in the treatment of fish, but also pigs, cattle, and poultry worldwide (De Liguoro et al., 2003; Kuhne et al., 2000; Sarmah et al., 2006; Sunderland, 2003; Thurman et al., 2002). Information on elimination rates, hydrolysis factors, and partition between biota and sediments is vastly known, but with the increase in macroalgae consumption for several uses, there is a need for new studies to complement existing knowledge.

Therefore, the present work aims to increase information on the retention of OTC in the macroalgae *Ulva*, if they were to be collected as a co-cultured species from an IMTA system, by presenting experimental data on the exposure of this green macroalgae to OTC, while i) investigating the stability of OTC in natural seawater; ii) assessing the effect of OTC on seaweed's growth, and iii) evaluating the uptake of OTC to *Ulva* tissues at two different concentrations.

Material and methods

Materials and reagents used

Both the internal standard (demethyltetracycline) and the commercial formula of OTC used were purchased from Sigma-Aldrich (Madrid, Spain). Methanol, acetonitrile and formic acid were of analytical grade and supplied by Merck (Darmstadt, Germany). Ethylenediaminetetra acetic acid (EDTA) was purchased from Sigma-Aldrich (Madrid, Spain). A stock solution of OTC (500 mg L^{-1}) was prepared in methanol. From this, two working solutions were prepared in natural filtered

seawater, so that by adding 1 mL to the Erlenmeyer, final concentrations would correspond to 0.040 (treatment C1) and 0.120 mg L^{-1} (treatment C2). Such concentrations were achieved following the recommended dosage of 55 mg of OTC per kg of fish (FDA, 2006; Leal et al., 2018), taking into account the density of fish of semi intensive aquaculture productions, which is the most predominant in the Mediterranean region.

Macroalgae collection

Ulva fronds were collected, thoroughly washed and rinsed with natural seawater to ensure the absence of organisms and debris. Upon careful inspection they were transported to the laboratory in coolers and placed in 25 L glass tanks pre-filled with filtered natural seawater, with Provasoli Enriched Solution (PES), at a final concentration of 20 µL mL^{-1}, with continuous aeration provided by an aquarium air compressor.

The experiment was conducted in a controlled room with the photoperiod set to 14:10 light:dark and 23.2 (± 0.6)° C, under 80 µmol photons m^{-2} s^{-1} of white fluorescent light. Acclimation was kept for two weeks prior to the beginning of the experiments.

Experimental design

Flasks were pre-filled with 244 mL natural filtered seawater and 5 mL of PES and placed on orbital shakers. Immediately before start, algal disks with Ø 5 cm were cut and separated for different treatments. Afterwards, 1 mL of each antibiotic solution was added to Erlenmeyer flasks in order to obtain two final test concentrations (0.040 and 0.120 mg L^{-1}). Each group was composed by three replicates (one flask corresponded to one replicate) per sampling time, each replicate containing three algal disks (sub-replicates). Two control groups were also set under the same conditions. Control A and Control B were established to verify the stability of OTC in seawater through time, and to compare the natural growth of *Ulva* in the absence of OTC, respectively. Each flask was covered with glass lids to prevent evaporation during the experiment, but still allow gas exchange and aeration. Sampling times were as follows: 0, 0.5, 1, 2, 4, 12, 24, 48, 72, and 96h.

Oxytetracycline quantification

At each sampling time, disks of *Ulva* were paper dried in order to remove excessive water, photographed, weighed, and frozen at -80 °C until analysis. The quantification of OTC in algal disks was made following an ultra-high performance liquid chromatography tandem time of flight mass spectrometry (UPLC-ToF-MS) method, based on a previous work from our group ((Leston et al., 2016). Each algal disk (~160 mg) was extracted individually in a 15 mL Falcon tube. Samples were firstly minced, and then 10 µL of internal standard solution (at a concentration of 10 µg L^{-1}) was added. After vortex mixing, samples were left to stand for a minimum of 10 min at room temperature in the dark. The extraction was followed by adding 5 mL of acetonitrile and 1 mL of 0.1 M EDTA to the samples, which were then placed in a Reax shaker for 20 min and centrifuged at 3100 *g* for 15 min. The supernatants were transferred to new glass tubes and evaporated to dryness under a gentle stream of nitrogen at 40 °C. Dry residues were then dissolved with 400 µL of 0.1 % formic acid solution in water (mobile phase A), filtered through 0.45 µm PVDF Mini-Uniprep™ vials and injected into the apparatus under optimized conditions.

An UHPLC Nexera X2 Shimadzu coupled with a triple ToF™ 5600+ (AB Sciex, Framingham, MA, USA) was used to achieve chromatographic separation and MS detection. The system was equipped with a vacuum degasser, autosampler and a binary pump. The column used to achieve separation was the Acquity HSS T3 analytical reverse-phase column (C18, 1.8 µm, 2.1 x 100 mm). Mobile phases were a solution of formic acid 0.1 % in water (A) and acetonitrile (B), at a flow rate of 0.5 mL min^{-1}. The gradient went from 97 % to 40 % (A) in the first 5 min, continuing to decrease to 0 % (A) in the following 4 min, and ramping back up to 97 % (A) for 1 min and held for 2 more minutes. Water samples were diluted in mobile phase A and directly injected under the same conditions as described for *Ulva* analysis. Also, at each sampling time, temperature, salinity, pH and dissolved oxygen were measured and water samples were taken and immediately frozen at -20 °C until further analysis. These samples were then filtered through 0.45 µm PVDF Mini-Uniprep™ vials and injected under the same conditions for direct quantification of OTC in water.

Algae growth

The influence of OTC on *Ulva* growth was determined as variations in disk area through time. Algal disks were photographed at the end of each sampling time, and the images run and analyzed through Adobe Photoshop ® software, with area quantified by pixel count method (Leston et al., 2014).

Statistical analysis

Data was tested for normality and homoscedasticity and a one-way analysis of variance (ANOVA, α=0.05) was used to compare differences in OTC uptakes for both concentrations, as well as their influence in algal growth. ANOVA was run with GraphPad Prism ® 6 software (Graph Pad Software, Inc).

Results

OTC stability in water

Oxytetracycline solutions for C1 and C2 treatments were prepared and analyzed previously to ensure the proposed concentrations were obtained. Samples taken at time 0 contained 0.0474 ± 0.0055 and 0.1249 ± 0.0047 mg L^{-1}, respectively. Degradation rates of OTC in water were significantly different for both initial concentrations (Figure 1; degradation rate constant of 0.0003 and 0.0007h^{-1} for C1 and C2 treatments, respectively). No significant differences were found between OTC concentrations with or without the presence of *Ulva* in the water. Lowest concentrations of OTC were achieved 96h after the beginning of the experiment (0.0174 mg L^{-1} for C1 and 0.0482 mg L^{-1} for C2 treatment).

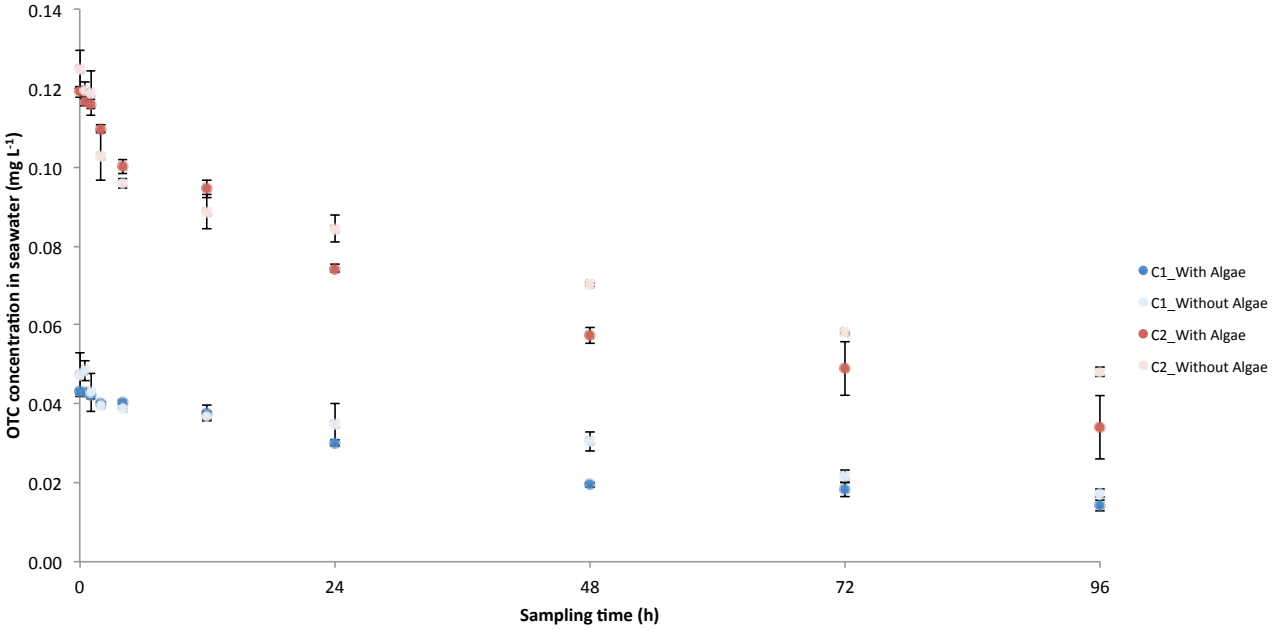


Figure IV.1 – OTC degradation in seawater through time, for both concentrations tested, with and without the presence of *Ulva* disks.

Growth and uptake

Apical endpoints such as growth or mortality were assessed as side effects on the use of OTC. Our data suggest that *Ulva* growth is affected by the presence of OTC in the water (Figure 2). *Ulva* frond size was maintained during the first 12h of exposure. After this, algae areas started to slowly increase in all treatments and continued to increase until the end of the experiment, but higher areas were measured in C2 treatment after 96h of exposure, being significantly different from the Control ($p = 0.0028$). While there was a tendency for *Ulva* fronds' area to increase also in C1 treatment after 96h of exposure, no significant differences in growth were found from the control B treatment.

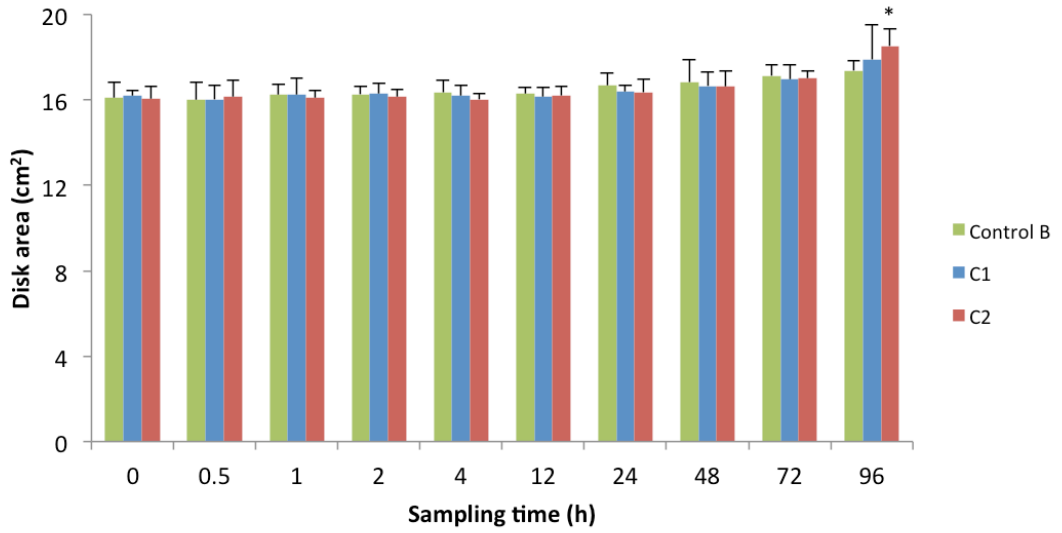


Figure IV.2 – *Ulva* disk area (cm^2) in each sampling point. * indicates significant differences versus area of control B treatment ($p < 0.05$).

To evaluate the risks of *Ulva* consumption after production on IMTA systems or after collection in aquaculture surroundings, OTC uptake at both dosages was determined. Each macroalgal disk was individually analyzed for internal concentration of OTC after exposure. In group C1 (Figure 3A), algae followed a linear increase in OTC internal concentration up to 48h after exposure, reaching its peak at 40.9934 ng g^{-1} WW. From 48 to 72h, concentration dropped 10-fold to 4.5746 ng g^{-1} WW, stabilizing until the end of the experiment (C_{96h} = 5.1176 ng g^{-1} WW). Concentrations for C2 (Figure 3B) treatment peaked after 1h and were maintained for 24h, starting to decrease rapidly afterwards, dropping from 101.5129 to 49.2725 ng g^{-1} WW from 24 to 48h and to 12.5570 ng g^{-1} WW at the final sampling time. Final concentrations were more than the double comparing to C1 treatment. An inverse correlation could be observed between the concentrations measured in the water and in macroalgae, with OTC concentrations slowly decreasing in water samples, while increasing in macroalgae fronds, until the abrupt decrease in OTC concentrations measured in *Ulva* disks.

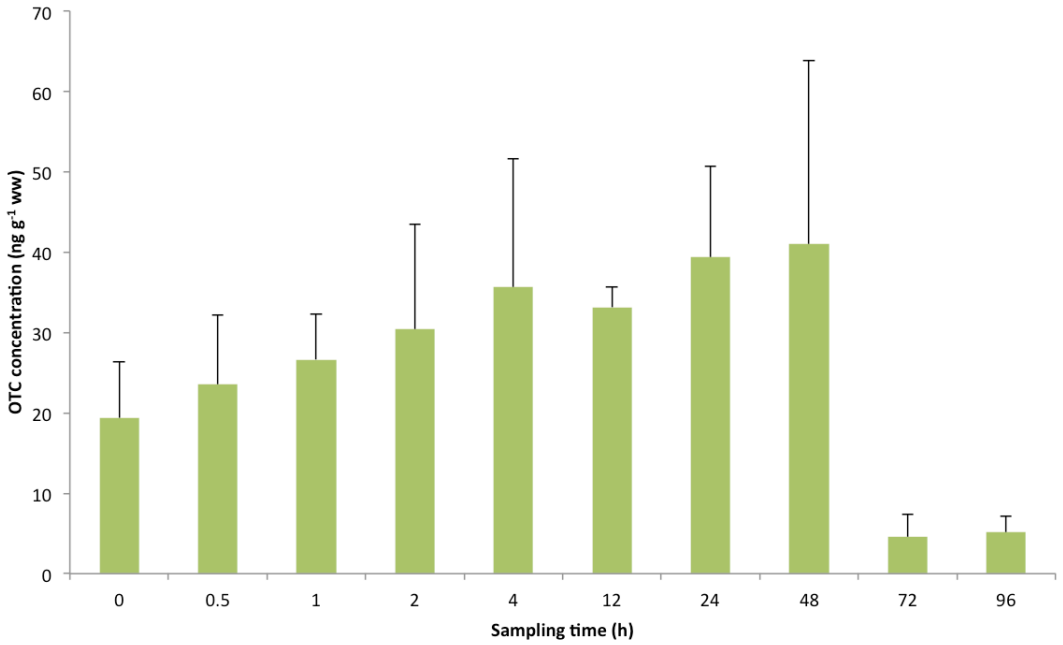


Figure IV.3A – OTC internal concentration in *Ulva* disks for treatment C1.

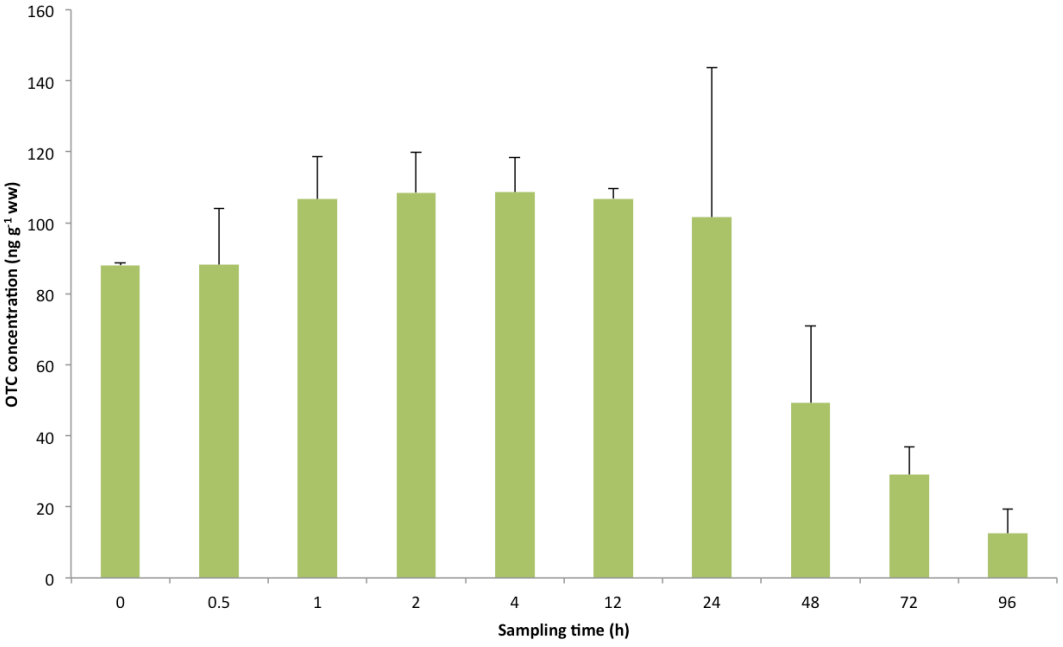


Figure IV.3B – OTC internal concentration in *Ulva* disks for treatment C2.

Discussion

By extrapolating the recommended dosage used for fish to the present trials, recent and relevant data on the accumulation of OTC by the macroalgae *Ulva* was provided. Based on this experiment, OTC showed a relatively high degradation rate

in seawater, both with and without algae, for the concentrations tested. Such results were in accordance with previous studies, where several aspects such as neutral pH, sunlight irradiation and higher temperatures were found to increase OTC hydrolysis (Doi & Stoskopf, 2000; Jin et al., 2017; Samuelsen, 1989; Xuan et al., 2009). Doi and Stoskopf (2000) for example were unable to measure the half life of OTC at 4°C, pH 7.0 (over 77 days), but when they increased the temperature to 43°C, the half life drastically decreased to 0.26 ± 0.11 days. Similarly, Xuan and coworkers (2009) determined an increasing degradation rate with the increase in temperature. Nonetheless, they calculated a degradation rate constant of 0.106 at 25°C, which is much higher than the ones obtained in the present work. This can be due to the presence of Ca^{2+} in seawater, which was already observed to slow down the hydrolysis of OTC in water (Xuan et al., 2009). The authors found it difficult to compare different works and degradation rates where a correct measure of light intensity was not provided, since OTC is known to be susceptible to light degradation (Doi & Stoskopf, 2000; Jin et al., 2017; Samuelsen, 1989; Xuan et al., 2009). Differences in degradation rates were obtained for both initial concentrations, with higher concentrations degrading faster. However, other study obtained similar degradation rates through a range of concentrations (Xuan et al., 2009).

Concentrations of OTC in water at both levels were not influenced by the presence of *Ulva* disks. The adsorption occurring in the first days of experiment was not sufficient to influence OTC concentrations in seawater, which might indicate that *Ulva* will not work efficiently in OTC removal from the water. Also, OTC presents a low partition coefficient (Log K_{ow} = -0.90), meaning this substance is hydrophilic and will tend to stay in the aqueous phase (Walker et al., 2012). Present results were in part in accordance to previous works, where the presence of algae in the trial led to a higher decrease in concentrations measured in the water over time (Leston et al., 2011; 2014). In these works, however, there was a distinct difference between antibiotic concentration with or without the presence of macroalgae from the first hours of exposure, as opposed to the present work. By looking at the degradation pattern presented for OTC for both concentrations, the authors noted that the concentrations obtained in the last sampling point of treatment C2 corresponded to the ones initially obtained after treatment with the lowest concentration. This can

provide information on a longer run, and extrapolations may be made about degradation patterns. If OTC is administered following the highest dosage applied, concentrations around 15 μg L^{-1} could still be found 8 days later.

It is known that macroalgae, and more specifically seaweeds can take up several elements via either adsorption, accumulation, or ionic exchange (Jarvis & Bielmyer-Fraser, 2015). Concentrations detected in the macroalgae disks were much lower than the ones detected in the water at the same sampling times. There was a tendency for internal concentrations to gradually increase in C1 treatment disks, which is in accordance with previous works (Harrison & Hurd, 2001; Wang et al., 2013). It is unclear if the accentuated decrease in internal concentrations noted afterwards was due to passive diffusion of OTC to water or *Ulva* presenting some mechanism to break down OTC into metabolites (Leston et al., 2011). Plants, for example, present the capacity to metabolize xenobiotics following a three-stage process: transformation, conjugation, and compartmentation (Mitsou et al., 2006; Sandermann, 1992; Torres et al., 2008). Such detoxification mechanisms were already described for some macroalgae (Lewis et al., 2001; Mehrtens, 1994) and also for *Ulva* (Pflugmacher & Sandermann, 1998; Pflugmacher et al., 1999), but to the best of knowledge, no studies on OTC have been addressed concerning potential detoxification mechanisms.

It is vastly agreed that macroalgae can have an important bioremediation role, such as in aquaculture sites (e.g. Ben-Ari et al., 2014; Granada et al., 2016), with several works addressing the positive nutrient uptake from such sites (Cahill et al., 2010; Zhou et al., 2006). This capacity will also translate into accumulation of hazardous compounds such as metals (Jarvis & Bielmyer-Fraser, 2015) or antibiotics (Leston et al., 2011; 2014), which may pose a risk to the consumer when macroalgae is cultivated with the purpose of use either in cosmetic or pharmaceutical purposes, or more relevant, as food products (Barrington et al., 2009; FAO, 2014; Neori et al., 2004; Troell et al., 2009).

Presently, Commission Regulation no 37/2010 sets maximum residue limits (MRL) only in foodstuff from animal origin (EC Regulation 37/2010) and for OTC in finfish, this value is set at 100 μg kg^{-1} for muscle tissue. Several other regulations are specific towards seaweeds (EC Regulation 1881/2006 or EC Regulation 396/2005 for

example), but are mostly limited to metals, lacking legislation on the pharmaceutical substances. According to this study results, after exposing *Ulva* to relevant concentrations, seaweeds were able to take up concentrations corresponding to half of the OTC maximum residue limit set for finfish muscle tissues, which is 100 μg kg^{-1}, still for a short period of exposure (96h). It has already been demonstrated that the use of OTC in fish farms increase the frequency of antibiotic resistance to OTC (Romero et al., 2012), and this problem may increase drastically if we add seaweed consumption to the equation. Allergy and resistance problems can be developed even if OTC concentrations are below the MRL. However, there are no legal limits for pharmaceutical compounds in algae or plants since these are intended for animal use. Also, the daily intake of seaweed will not be the same as fish intake. The authors believe the capacity for these organisms to uptake xenobiotics from the seawater needs to be taken into account, and legislations updated accordingly taking into account new estimations of algae daily intake.

Conclusions

The incorporation of seaweed in IMTA systems is seen as beneficial since they can take up really high quantities of nutrients that would be released into the surrounding systems, reducing the burden of the system while benefiting a marketable product. In the present study it was demonstrated that such organisms also accumulate OTC when it is present in the water, and concentrations can be found at levels comparable to the limit established for fish. IMTA systems will most definitely be the future in terms of aquaculture, and also seaweed production. This study considered one of the most common types of aquaculture in the Mediterranean region. But such results should also be considered for other IMTA systems, especially in Asia, where higher concentrations of pharmaceuticals may be used. Also, besides the fact that more macroalgae is consumed in their society, a considerable amount of aquaculture products is still being imported from Asia to Europe. Moreover, these systems will continue to increase worldwide due to the increasing high demand of macroalgae as foodstuff. Contrarily to fish, there is currently no legislation addressing antibiotics in macroalgae, and this will pose a risk to human population since seaweeds are becoming an important source of

nutrients. Not only legal maximum residue limits need to be set for the presence of pharmacologically active substances on seaweed (and other macroalgae), especially for its use as foodstuff, but also updated withdrawal times for the antibiotics allowed for aquaculture, since they can also be present in seaweeds produced in IMTA systems.

CHAPTER V

2019. Rosa, J., Leston, S., Crespo, D., Freitas, A., Vila Pouca, A. S., Barbosa, J., Lemos, M.F.L., Pardal, M.A., Ramos, F. Uptake of enrofloxacin from seawater to the macroalgae *Ulva* and its use in IMTA systems. *Aquaculture.*

DOI: 10.1016/j.aquaculture.2019.734609

Experimental setup used in the exposure trials with selected antibiotics

Uptake of enrofloxacin from seawater to the macroalgae Ulva and its use in IMTA systems

Abstract

Integrated multi-trophic aquaculture systems can minimize the environmental impacts of aquaculture, while delivering economical benefits. However, the use of extractive species such as seaweeds can accumulate pharmaceuticals commonly used in these systems. Therefore, this work evaluated the exposure of the seaweed *Ulva* to enrofloxacin (ENR), a vastly used antibiotic in aquaculture, at two dosages (C1, 7.5 µg L^{-1} and C2, 15 µg L^{-1}), and concentrations in water and in *Ulva* were measured through time. Apical endpoints such as growth and mortality were assessed as ENR effects in the macroalgae. Enrofloxacin presented good stability in seawater, and degradation rates were influenced by the presence of seaweed at the lowest concentration tested. The seaweed was able to assimilate the antibiotic, reaching internal concentrations of 7.76 ± 1.11 ng g^{-1} WW after 30 min of exposure for C1, and 14.51 ± 1.22 ng g^{-1} WW, after 15 min for C2. Lowest concentrations detected at the end of experimental time were 4.08 ± 0.42 ng g^{-1} WW and 5.09 ± 1.57 ng g^{-1} WW for C1 and C2, respectively, which nonetheless, corresponds to ~5% of the maximum residue limit established for fish for ENR by the European regulation. The presence of ENR stimulated *Ulva* growth, with differences observed 96h after the beginning of the trial.

Key words: Enrofloxacin; IMTA; Seaweed; Accumulation; Legislation.

Introduction

With the steep increase in human population and the growing demand for food products, traditional aquacultures, which were characterized by low stock densities and small production sites, are no longer suffice. The average annual increase in consumption of food fish is higher than ever and it even surpassed that of meat produced from bovine, ovine, pig and poultry combined, according to FAO's report on *The State of World Fisheries and Aquaculture* (FAO, 2018). In the last decades, aquaculture has been turning to a much more intensive and bigger industry

in order to face such demand (Goldburg et al., 2001; Henriksson et al., 2018; Sapkota et al., 2008). The expansion of aquaculture areas and the necessary use of pelleted feed bring some environmental concerns. The nutrient loading associated with uneaten or partially digested feed and fish excretions may eventually leak to aquaculture surrounding environments (Granada et al., 2016; Grigorakis and Rigos, 2011; Read and Fernandes, 2003) with subsequent effects, such as eutrophication (Edwards, 2015; Zhou et al., 2006).

To mitigate these impacts, the industry must evolve towards a more environmental responsible system, and Integrated Multi-Trophic Aquaculture (IMTA) systems may present a robust solution for this (FAO, 2014; Granada et al., 2016; Neori et al., 2004; Troell et al., 2003; 2009). Briefly, it consists in farming aquaculture species from different trophic levels in proximity, which take up and remove organic and inorganic compounds from the connected system, nourishing different species while also improving water quality (FAO, 2014; Granada et al., 2016; Troell et al., 2003; 2009).

Seaweeds are largely used in IMTA systems due to their high productivity and high economic value to producers, serving as food source but also for water bioremediation due to their high capacity of extracting inorganic nutrients from the water (Edwards, 2015; Fleurence et al., 2012; Granada et al., 2016; Neori et al., 2004). Neori and colleagues (2000) set up an experimental system where they were able to reduce seawater usage and energy consumption by 75% in *Sparus aurata* production, while also producing 7 kg of *Ulva* per kg of fish. Furthermore, several works already showed *Ulva* capacity to improve water quality in systems producing organisms from other trophic levels (e.g. Brito et al., 2013; Copertino et al., 2008; Elizondo-González et al., 2018; Le Van Khoi and Fotedar, 2011). Due to their role as biofilters, macroalgae have the potential to take up not only nutrients from the water, but also substances like pesticides, disinfectants and pharmaceuticals (Lalumera et al., 2004; Leston et al., 2011; 2014; Rosa et al., 2019), often used to control disease outbreaks and infections in intensive systems (Cabello, 2006; Cabello et al., 2016; Romero et al., 2012; Sapkota et al., 2008). In fact, algae have been widely used as biosorbents for heavy metals pollution (Gavrilescu, 2004; He and Chen, 2014). Several mechanisms such as electrostatic interactions, chelation

adsorption, microprecipitation, ion exchange or complexation can be involved in the uptake of such compounds (Bulgariu and Gavrilescu, 2015; Gavrilescu, 2004; Zeraatkar et al., 2016). Cell walls play a major part on the retention of heavy metals due to the presence of glycogen, starch, cellulose, and polysaccharides that contain several reactive functional groups, which will be involved in the chemical binding of metal ions (Bulgariu and Gavrilescu, 2015), but some works already addressed the incorporation of persistent organic pollutants internally by *Ulva*, not only adsorption to the cell wall (Cheney et al., 2019). Such mechanisms may also be involved in the uptake of antibacterials from surrounding waters, since up to 80% of the used antibacterials may end up in water and sediments close to farm sites (Cabello et al., 2016).

Antibacterials are widely administered pharmaceuticals in the treatment of fish disease (Cabello et al., 2013; Rodgers and Furones, 2009; Romero et al., 2012; Sapkota et al., 2008), with quinolones and tetracyclines heading the most prescribed classes. Quinolones act specifically against gram-negative bacteria, by entering the bacterial cell through passive diffusion via water-filled protein channels (Bermúdez-Almada and Espinosa-Plascencia, 2012; Samuelsen, 2006). Enrofloxacin (ENR) is a synthetic agent from the monofluoride quinolones carboxylic acid derivatives (Figure 1), effective against common bacterial pathogens such as *Vibrio anguillarum*, *Yersinia ruckeri*, *Renibacterium salmoninarum*, and *Aeromonas salmonicida* (Samuelsen, 2006), and is one of the main antibiotics administered in aquaculture systems (FAO, 2005; Quesada et al., 2013). According to Sapkota and coworkers (2008), among the top fifteen aquaculture producing countries, ENR administration was reported in at least three (Liu et al., 2017; Rico et al., 2013; Trouchon and Lefebvre, 2016). Plenty information can be found on ENR pharmacokinetics and degradation rates in fish and shrimp, and which factors can influence it the most. But with changes in aquaculture systems and the increase in macroalgae consumption worldwide, studies focusing on such organisms need to be addressed in order to better understand and predict the effects of new aquaculture practices. And while this can lead to problems of toxicity upon ingestion of contaminated algae, the development of antibiotic resistance can be enhanced, since macroalgae represents a suitable habitat for bacteria colonization, growing dependent on algae exudates

(Florez et al., 2017). Transfer between antibiotic resistance from aquatic to terrestrial bacteria is eminent and this antibiotic resistance can ultimately reach human pathogens, with associated health implications (Cabello et al., 2013; 2016).

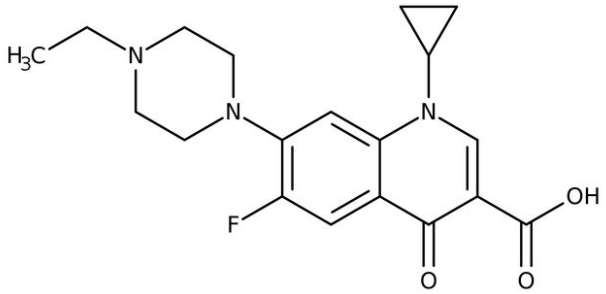


Figure V.1 – Structure of enrofloxacin [1-cyclopropyl-7-(4-ethylpiperazin-1-yl)-6- fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid]

Hence, the objective of the present work is to produce information on the uptake of ENR in the green macroalgae *Ulva*, by presenting experimental data on the exposure to ENR, mimicking a scenario of an IMTA system. The study focused on i) evaluating the stability of ENR in natural seawater; ii) potential effects of ENR in macroalgae growth, and iii) the uptake of ENR at two different concentrations.

Experimental

Materials and reagents used

Both the ENR analytical standard and internal standard (lomefloxacin) were purchased from Sigma-Aldrich (Madrid, Spain). Ethylenediaminetetra acetic acid (EDTA) was also supplied by Sigma-Aldrich (Madrid, Spain), and methanol, acetonitrile and formic acid (of analytical grade) were purchased from Merck (Darmstadt, Germany). A stock solution was prepared by dissolving 15 mg of ENR in 100 mL of methanol. From this, two intermediate working solutions in natural seawater were prepared in the beginning of the trial. By adding 1 mL of these solutions to the correspondent Erlenmeyers, final concentrations of 7.5 µg L^{-1} (treatment C1) and 15.0 µg L^{-1} (treatment C2) were achieved. These concentrations were selected following a dosage of 5 mg kg^{-1} bw, taking into account semi intensive aquacultures and the densities of fish cultured in these systems, which are the most predominant in the Mediterranean region.

Sampling and acclimation

Ulva fronds were collected at a local beach during low tide and thoroughly washed on site with natural seawater. They were carefully inspected to ensure no epibionts or debris were present and then transported to the laboratory in coolers. Once there, algae were placed in 25 L glass tanks already filled with filtered natural seawater and Provasoli Enriched Solution (at 20 mL L^{-1}), continuous aeration and a photoperiod set to 14:10 light:dark – with a set of 3 white fluorescent lights, corresponding to 80 µmol photons m^{-2} s^{-1}. Water temperature was maintained at 22.8 (± 0.8) °C and acclimation was kept for 2 weeks prior to the beginning of the experimental trials.

Experimental design

Exposures were performed as in Rosa et al (2019). Erlenmeyer flasks (250 mL) were pre-filled with natural filtered seawater and PES solution and placed on orbital shakers. Immediately before the start of the experiment, *Ulva* disks with 5 cm diameter were cut and distributed by the different treatments. Then, 1 mL of each prepared antibiotic solution was added to the flasks in order to obtain the test concentrations (7.5 µg L^{-1} and 15.0 µg L^{-1}). Each treatment was composed by three replicates per sampling time, and each replicate comprised three algal disks (sub-replicates). Simultaneously, two control groups were also set under the same conditions to verify the stability of ENR in seawater throughout time (Control A), and to compare the natural growth of *Ulva* with and without ENR (Control B). Each flask was covered with glass lids to prevent evaporation during the experiment, but still allowing gas exchange and aeration. Samples were taken at 0, 0.25, 0.5, 1, 2, 4, 8, 12, 24, 48, 72, 96 and 120h.

Algae growth and antibiotic quantification

At each sampling time, *Ulva* disks were recovered from the glass flask, dried in filter paper to remove excess water, photographed, weighed and frozen at -20 °C until further analysis. Variations in disk area were determined to assess the influence of ENR on *Ulva* growth throughout time. Each image set of 9 disks (sub-replicates)

were run per treatment per sampling time and analyzed through Adobe Photoshop® software, with areas individually quantified by pixel count method. At each sampling time, measurements of temperature, salinity, dissolved oxygen, and pH were taken, with water samples immediately frozen at -20 °C until further analysis.

An ultra-high performance liquid chromatography tandem time of flight mass spectrometry (UPLC-ToF-MS) method, previously developed within this research group, was used to determine ENR concentration on algal disks. Each algal disk (~160 mg) was individually extracted in 15 mL polypropylene tubes. To each sample, 10 µL of internal standard solution were added, followed by vortex mix, and left for a minimum of 10 min in the dark (at room temperature). The extraction proceeded by adding 5 mL of acetonitrile and 1 mL of 0.1 M EDTA to the samples. They were then placed in a Reax shaker for 20 min and centrifuged for 15 min at 3100 *g*. The supernatants were transferred to glass tubes and evaporated to dryness under a gentle stream of nitrogen, at 40 °C. Afterwards, dry residues were redissolved with 400 µL of 0.1 % formic acid solution in water, filtered through 0.45 µm PVDF Mini-UnptripTM vials, and injected into the UHPLC-ToF system. Water samples were diluted in mobile phase A, filtered through 0.45 µm PVDF Mini-Uniprep™ vials and directly injected for direct quantification of ENR in water.

Chromatographic separation and MS detection were performed by an UHPLC Nexera X2 Shimadzu coupled with a triple ToF™ 5600+ (AB 148 Sciex, Framingham, MA, USA) equipped with a vacuum degasser, autosampler and a binary pump. An Acquity HSS T3 analytical reverse phase column (C18, 1.8 µm, 2.1 x 100 mm) was used for separation, and mobile phase consisted in a solution of formic acid 0.1 % in water (A) and acetonitrile (B). Flow rate was 0.5 mL min^{-1} and the gradient was as follows: 0–5 min from 97 % to 40% (A); 5–9 min from 40 % to 0 % (A); 9–10 min from 0 % back to 97 % (A) and held for 2 more minutes at 97 % (A).

Statistics

The effect of ENR on growth for each sampling time, as well as differences in uptakes for both treatments were determined using a one-way analysis of variance (ANOVA), followed by Tukey's test to assess differences among treatments. Data normality was tested following a D'Agostino-Pearson test. Differences in seawater

concentrations through time were assessed through linear regression analyses ($P \leq 0.05$). Results are presented as mean ± SD. Significance level was inferred at $P \leq 0.05$ for all statistical tests. Statistical analysis were run with GraphPad Prism® 6 software (Graph Pad Software, Inc).

Results

ENR stability in seawater

Control A samples taken at sampling time 0 showed ENR concentrations of 7.3 ± 0.1 and 14.4 ± 0.7 µg L^{-1} for C1 and C2 treatments, respectively. Enrofloxacin presented good stability in seawater over time, and at the end of the trial the values of ENR corresponded to 92 and 93 % of the initial concentrations, for C1 and C2 treatments. Degradation rates in seawater were significantly different for both concentrations ($p = 0.0004$), with C2 treatment presenting the highest degradation rate (0.00055 min^{-1} vs 0.00017 min^{-1}).

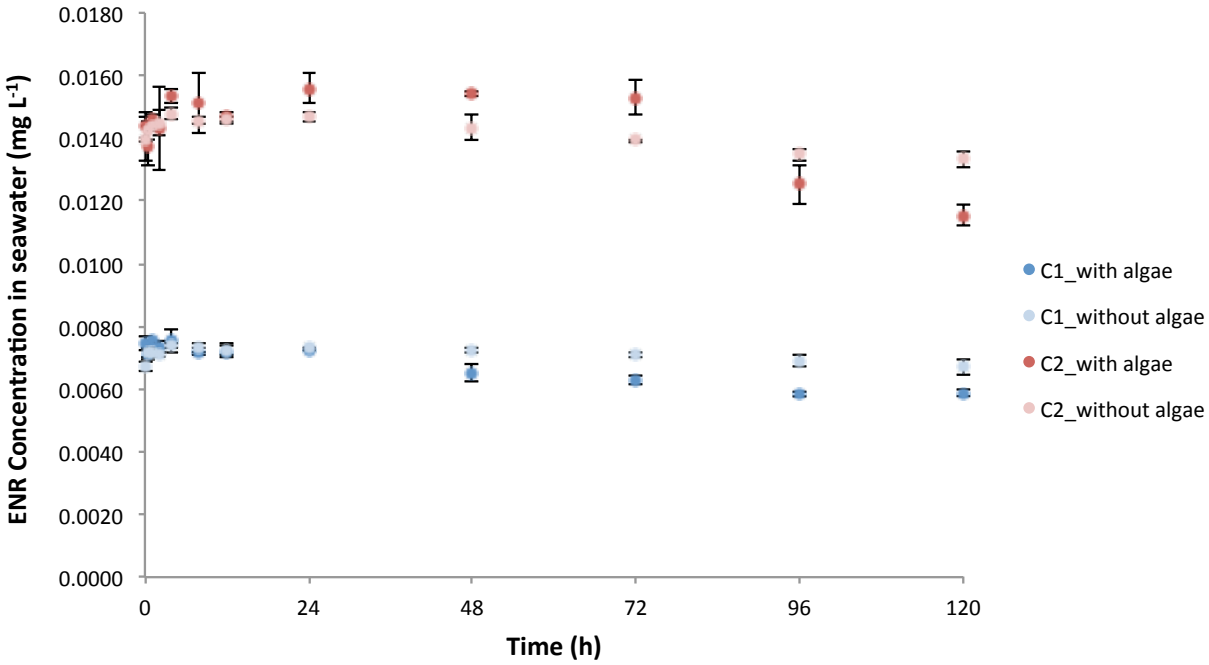


Figure V.2 – Enrofloxacin concentrations in seawater through time, for both concentrations tested, with and without the presence of *Ulva* disks.

Seaweed growth and uptake

In order to assess the effects of *Ulva* from ENR exposure, apical endpoints such as growth and mortality were assessed. Control B evaluated growth under natural conditions without ENR, assessed as differences in algae disks through time (Figure 3). Algae started to increase in size 48h after the beginning of the experimental trial. Disk areas were significantly higher 120h and 96h after the beginning of the experiment, for C1 and C2 treatments, respectively. At the final sampling time, a Specific Growth Rate (SGR) of 3.79 and 5.96% per day was calculated for C1 and C2 treatments, respectively. However, chlorosis started to become visible after 96h of exposure for both treatments with ENR. These pale spots indicate the absence of chlorophyll in the algae, which lead to its death.

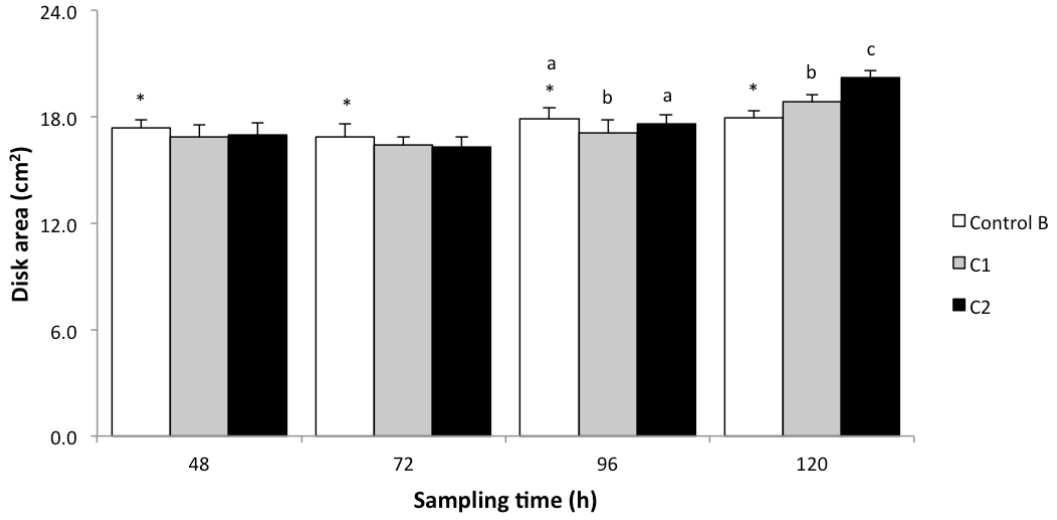
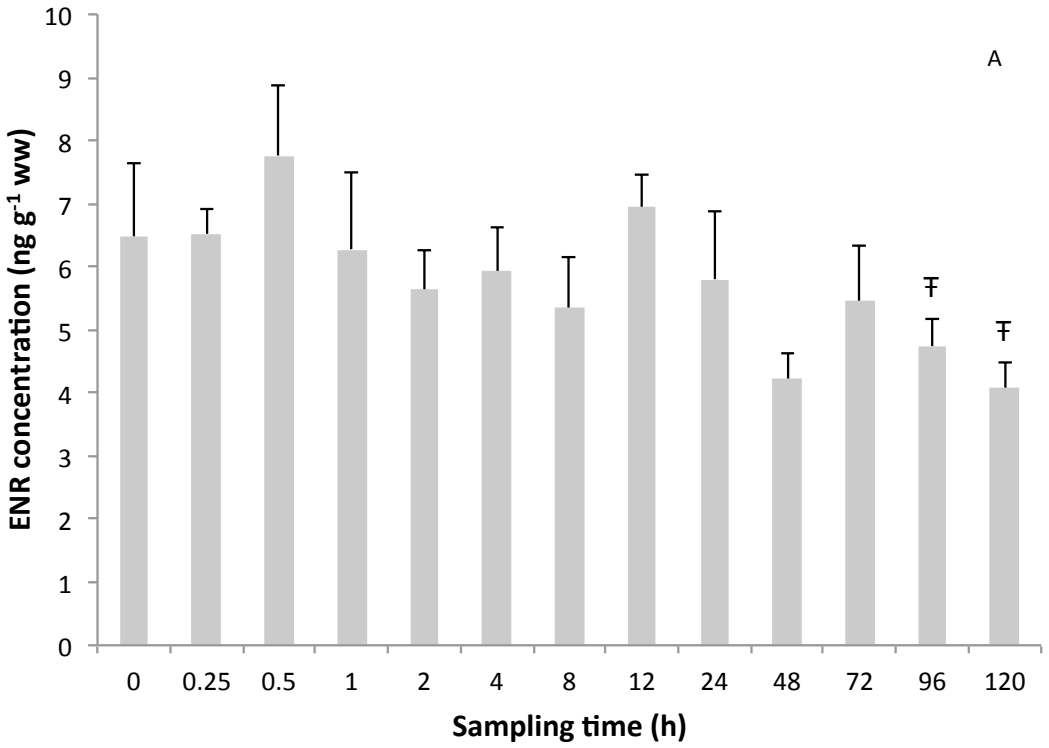


Figure V.3 – Ulva disk area (cm^2) in each sampling point. Data represent mean values of nine independent replicates from 48 h onwards (when algae growth started to be visible). Asterisk (*) indicates significant differences versus initial areas. Different letters indicate differences between treatments ($p < 0.05$).

Ulva capability to take up ENR from the water at both concentrations was assessed to evaluate its potential use as a bioremediator of contaminated waters as well as the risk of consumption. Uptake was measured as the concentration of antibiotic present in each algal disk. A similar pattern was established for both treatments, with concentrations oscillating slightly throughout time. Internal concentration for C1 (Figure 4A) reached its peak after 30 min of exposure (7.76 ± 1.11 ng g^{-1} WW), while it took only 15 min to reach the highest concentration in C2

treatment (14.51 ± 1.22 ng g^{-1} WW; Figure 4B). A gradual decrease in Ulva concentrations was then observed, with concentrations from C2 decreasing significantly faster than in C1 (elimination rate of 7.584 min^{-1} vs 3.048 min^{-1}). Minimum concentrations were measured at the end of experimental time (4.08 ± 0.42 ng g-1 WW for C1 and 5.09 ± 1.57 ng g-1 WW for C2).



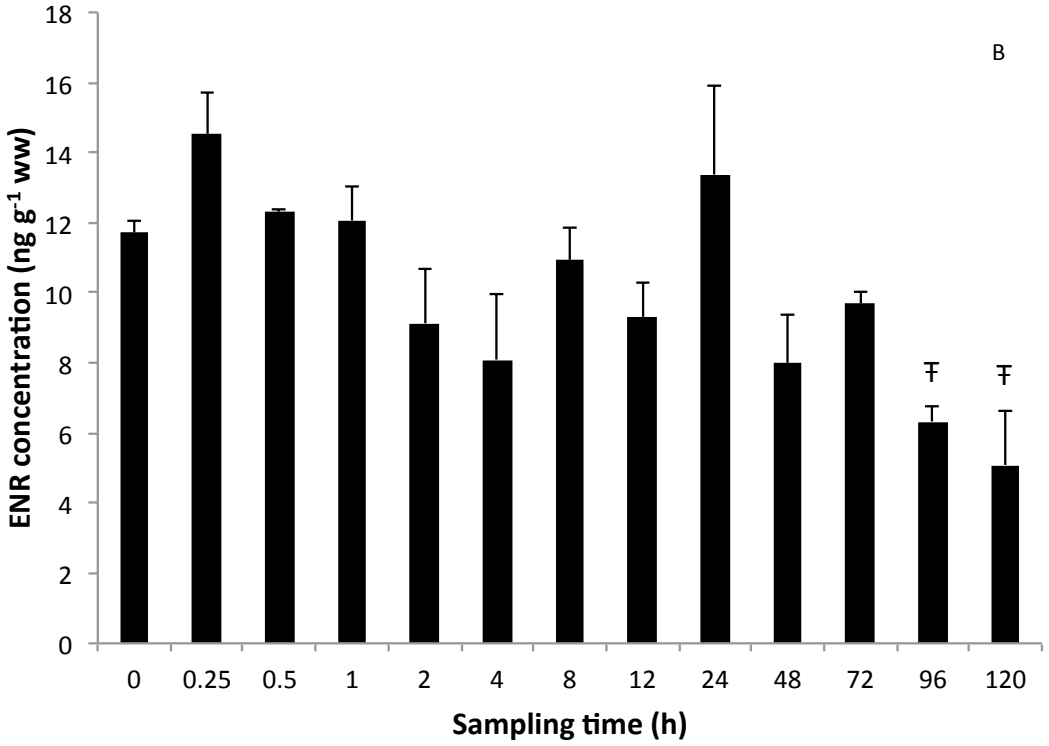


Figure V.4 – Internal concentrations of enrofloxacin (ng g^{-1} ww) for Ulva at each sampling time for treatment C1 (A) and C2 (B). Data represent mean values of three independent replicates. ₸ symbol indicates where samples with chlorosis were observed.

Discussion

With the intensification of aquaculture and the growing importance of IMTA systems, algae consumption may pose a risk if these organisms are assimilating antibiotics used in primary cultures or from surrounding areas. The present study produced experimental data on the accumulation of ENR by the macroalgae *Ulva*, after being exposed to relevant aquaculture concentrations. Enrofloxacin presented a relatively slow degradation rate in seawater, only degrading 20% of its initial concentrations after 5 days. Fluoroquinolones, and more specifically ENR can be of concern in such systems since they are not easily degraded by microorganisms and tend to persist in such sites (Pereira et al., 2015; Robinson et al., 2005; Van Doorslaer et al., 2014). In fact, in a mesocosm study with over 70 pharmaceutical compounds with several veterinary drugs from different classes, ENR presented the highest DT_{50} values, taking longer than 152 days to degrade 50% of the initial concentration (Boxall et al., 2006). On the contrary, Robinson and colleagues (2005) observed relative short half-lives for the quinolones tested, including ENR, which could in that

case be attributed to either photodegradation or sorption to particulate matter. In fact, a correlation with light exposure was already established, and half-lives of 0.8, 3.7 and 72 days were determined for 100%, 28%, and 7.5% of full light exposure (Knapp et al., 2005). Despite ENR can undergo photolysis or oxidation by mineral oxides (Van Doorslaer et al., 2014), they are not susceptible to hydrolysis (Kümmerer, 2009a; Trouchon & Lefebvre, 2016). The present results showed that degradation in seawater is dependent of the initial concentrations, with higher concentrations degrading faster. This difference in degradation rates was already described (Babić et al., 2013; Robinson et al., 2005), but in these works, lower concentrations degraded faster than higher concentrations. However, when *Ulva* disks were present in seawater, such differences in degradation rates were no longer detected.

Concentrations of ENR in seawater decreased much faster with algae than without. This pattern was already expected, since macroalgae can take up several compounds, either via adsorption, ionic exchange or accumulation (Harrison & Hurd, 2001; Jarvis & Bielmyer-Fraser, 2015). The present study suggests that *Ulva* can efficiently remove ENR from the water, supporting its use for bioremediation in IMTA systems but also contaminated sites (Elizondo-González et al., 2018; Fleurence et al., 2012; Neori, 2008; Neori et al., 2004). The octanol-water partition coefficient (K_{ow}) is an important indicator of the behavior of a given contaminant in the environment. With an estimated Log K_{ow} ranging from 2.53 to 4.70 (Lizondo et al., 1997; Sarmah et al., 2006; Van Doorslaer et al., 2014), ENR is a lipophilic molecule indicating it can be efficiently taken by organisms. In a study on the uptake of veterinary medicines from soils into plants, the authors observed that ENR was being incorporated into carrot roots, for example (Boxall et al., 2006). This was already reported for seaweeds for different antibiotics (Leston et al., 2011; 2013; Rosa et al., 2019). In this study, *Ulva* was also capable of take up ENR from the water, with both treatments following a similar pattern. There was an initial uptake of ENR in the first 30 min, followed by a decrease in internal concentrations during the first day of exposure. After this, concentrations tended to decrease for the next days (Figure 4). This suggests that this seaweed is capable of regulating internal concentrations of ENR. Detoxification mechanisms for these organisms have already been identified

for some compounds (Ferrat et al., 2003; Lewis et al., 2001; Mehrtens, 1994; Navarrete et al., 2018; Schweikert & Burritt, 2012; Torres et al., 2008). However, further research needs to be performed to better understand this detoxification capacity at the biochemical level. Nonetheless, this study demonstrated that the macroalgae *Ulva* is capable to take up ENR from the water to levels comparable to the limit established for fish. And even though concentrations addressed were based on the most common type of aquaculture in the Mediterranean region, a considerable amount of aquaculture products, namely seaweeds, are still imported from Asia, where ENR is widely used.

After uptake, enrofloxacin can be dealkylated to ciprofloxacin (CF), a metabolite that also possesses antibacterial activity. This is already described for several species (e.g. Intorre et al., 2000; Migliore et al., 2003; Trouchon & Lefebvre, 2016), but to the authors knowledge, this has not been reported for macroalgae species yet. This metabolite presents higher toxicity to some organisms than enrofloxacin itself. In a work addressing toxicity of fluoroquinolone antibiotics to aquatic organisms, CF showed higher toxicity to *Microcystis aeruginosa*, a cyanobacteria, but lower than ENR to the microalgae *Pseudokirchneriella subcapitata* and the aquatic plant *Lemna minor* (Robinson et al., 2005). Similarly to ENR, CF also presents long persistence in the environment, affecting the activity of several microbial communities (Ebert et al., 2011; Trouchon & Lefebvre, 2016). In 1998, the World Health Organization (World Health Organization, 1998) declared that the antibiotic activity of CF against human intestinal flora could be 4x higher than the activity of ENR, and the maximum residue limit for ENR set by the European Commission is in fact a sum of ENR and CF, which corresponds to 100 µg kg^{-1} for fish (EC Regulation 37/2010). Nonetheless, no CF was detected in *Ulva* fronds in the present work, which may indicate that seaweeds do not metabolize ENR into CF.

Apical endpoints such as growth or mortality were also assessed in order to better understand ENR influence on macroalgae, since discharges of this antibiotic from aquacultures can induce effects in other aquatic organisms (Kümmerer, 2009a). Cyanobacteria are usually the most sensitive organisms (Andrieu et al., 2014; Ebert et al., 2011; Van Doorslaer et al., 2014), but overall toxicity has already been demonstrated in other algae and plants, but with concentration ranges higher than

the ones used in the present study (e.g. Forni et al., 2001; Migliore et al., 2003; Robinson et al., 2005). In the current work, *Ulva* displayed clear symptoms of chlorosis after 96h of exposure, which is in accordance with the study by Migliore and colleagues (2003) with registered chlorosis in *Lemna minor* fronds. Our results suggest the occurrence of hormesis in our experiments (Stebbing, 1998), with low ENR concentrations stimulating algae growth, while higher concentrations assimilated by the algae lead to toxic effects (Calabrese, 2009). This phenomenon was already described for plants and algae, presenting the capacity to allocate resources for growth under stress conditions (Cedergreen et al., 2006).

The safe use of pharmaceuticals to ensure animal and consumers welfare is of vital importance within the European Union and translated into the European legislation. However, to some food products there are still regulative gaps. Commission Regulation no 37/2010 set the maximum residue limits (MRL) of pharmaceuticals in foodstuff from animal origin only (EC Regulation 37/2010). Specific regulations for macroalgae such as EC Regulation 1881/2006 or EC Regulation 396/2005 set MRL for other chemicals, mainly to metals, lacking limits for pharmaceuticals. Maximum residue limits are established based on toxicological values of chemicals used in food products, in order to protect consumers' health, but problems of allergy and resistance to antibiotics may occur even if concentrations are below MRL values (Smith, 2008). Furthermore, human daily intake of seaweeds is not equal to that of fish, meaning that legislations need to be updated accordingly, especially considering the capacity that these organisms have to uptake xenobiotics from the surrounding waters, where, most often, a mixture of several pharmaceuticals is present. This can have cumulative effects, potentiating the problems associated with exposure to such compounds.

Conclusions

Integrated Multitrophic Aquaculture systems are believed to represent the future of aquaculture production, since they can minimize impacts associated with nutrient loading and water use, while also promoting economic added features. Concomitantly, seaweed production will continue to increase in order to face the growing consumption in western societies. The lack of legislation addressing

antibiotics in algae may pose a risk since they are gaining importance as food in wider markets. New MRL need to be set for the presence of pharmacologically active substances on macroalgae, and withdrawal times for antibiotics allowed for aquaculture production updated accordingly, as they can be present in seaweeds produced in IMTA systems for longer periods. Also, *Ulva* may be considered for bioremediation for this compound and others alike.

GENERAL DISCUSSION AND FINAL REMARKS

General discussion and final remarks

With intensive aquacultures being often associated with heavy use of chemicals, knowledge on accumulation of such compounds on several aquaculture products needs to be expanded. Furthermore, the World Health Organization and the Food and Agriculture Organization, together with a vast number of national governments, pointed out the irresponsible use of antibiotics in the fish industry, with associated risks to public health, namely the emergence of antibiotic resistance (Cole et al., 2009; Kümmerer, 2009b; Tuševljak et al., 2012).

Understanding how different compounds will behave after ingestion can be a difficult task dependent on several variables. Factors like temperature, dosage, lipophilicity, molecular size, or mode of administration can influence pharmacokinetic parameters (Hansen & Horsberg, 2000; Rigos & Smith, 2013; Rigos et al , 2013; Samuelsen, 2006). Different fish species also show differences in digestion and absorption rates, and for this reason, the present work aimed to address the most economically important species produced in the Mediterranean region aquacultures (EUMOFA, 2015; 2016; Federation of European Aquaculture Producers, 2016).

Despite being the most effective way of treatment, intravenous administration to individual animals is not practical in terms of aquaculture production (Daniel, 2009). So, by creating experimental feeds with antibiotics, it was possible to have a better estimate on the accumulation of these pharmaceuticals upon administration in aquacultures. However, some problems occurred during the feed manufacturing process: the heat and humidity involved in the pelleting process could possibly have degraded the studied compounds up to a certain degree. In fact, for SDZ and OTC, the highest dosage treatments, concentrations measured in feed were significantly lower than expected, while the lowest concentrations of antibiotics in feed were easily achieved (FLU and OXO dosages were the lowest tested, with 6 and 12 mg kg^{-1} for prophylactic and therapeutic treatment, respectively). As anticipated, residues of these pharmaceuticals were the lowest observed for both experiments, with FLU concentrations always below the MRL established and OXO below the MRL 72 and 36h after the end of the feeding period, for seabream and seabass, respectively. On the contrary, SDZ and OTC presented the

highest AUC values and concentrations at the end of the feeding period for both experiments, which in part could be due to the fact their initial dosages were the highest tested. Trimethoprim is frequently used in combination as potentiated sulfonamides (Sapkota et al., 2008; Suzuki & Hoa, 2012), and for this reason, information on the retention of this isolated compound is scarce. Contrarily to the study on Gilthead seabream, where concentrations dropped below the MRL established (50 μg kg^{-1}) immediately after the medication period was over, concentrations of TRI were above the MRL in seabass muscle samples, up to at least 14 days after the medication period.

The present results reinforced the idea of variability in antibiotic retention from species to species, with European seabass accumulating, in general, higher concentrations in muscle samples than Gilthead seabream. Main differences were observed regarding FLU and OXO, since residues were not detected in seabream 3 and 14 days after medication period, respectively, but were present in seabass muscle tissues until the end of the experimental trial (final concentrations of 2.15 and 2.75 μg kg^{-1} for FLU and 4.45 and 5.00 μg kg^{-1} for OXO, for both concentrations tested). Special attention must be given to OTC and TRI, since antibiotic concentrations were above the maximum residue limits established by the European Commission in the first sampling point for TRI and 7 days after medication period for OTC, for Gilthead seabream. However, concentrations of these pharmaceuticals in seabass remained above the MRL until the end of experimental trial, with potential effects for the consumer. A wider range of concentrations needs to be tested in order to corroborate a dose dependency on antibiotic retention, although few concentrations were tested, TRI and OTC show a dose dependency pattern, with higher dosages leading to higher percentages of retention immediately after the medication period, and these differences were more noticeable for seabass. Specific legislations must be updated for each species, especially in regions like the Mediterranean, where these species play a significant role in economy. Moreover, fish represent a higher dietary contribution for human diet in these countries. Furthermore, both studies showed the presence of residues in edible tissues for longer periods than previously reported, and although below the MRL, antibiotic resistance can be developed due to ingestion of these pharmaceuticals, even at low

dosages (Cole et al., 2009; Kümmerer, 2009b; Tuševljak et al., 2012). In fact, the existence of bacteria resistant to several pharmaceuticals such as oxytetracycline and quinolones has already been found near aquaculture farms even with residual concentrations (Cabello et al., 2013; Dang et al., 2006; Guardabassi et al., 2000; Kümmerer, 2009b). The review made by Sapkota and colleagues (2008) reported the presence of antibacterial resistance in farms of 14 out of the top 15 aquaculture producing countries, but evidence suggests that this resistance can contribute to antibacterial resistance among human populations.

As stated throughout the present thesis, IMTAs offer a sustainable economically viable option to aquaculture. These systems combine production of a main species, which relies on feed input, with one or more extractive species belonging to different trophic levels (Barrington et al., 2009; Neori et al., 2000; Troell et al., 2003; 2017). As extractive species are capable of assimilating high amounts of nutrients, thus reducing the loading associated with intensive productions, the presence of pharmaceuticals in these systems may pose a problem since they can bioconcentrate and bioaccumulate. While crustaceans and/or mollusks, used in IMTAs as extractive species, have been used in human diet for decades, the consumption of seaweeds as food source has been significantly increasing in western societies. For this reason, the present work further addressed antibiotic accumulation in the seaweed *Ulva*.

Antibiotic concentrations were chosen based on the dosage prescribed for treatment in aquacultures, and adapted to concentration in water, taking into account semi-intensive systems, which are the most dominant ones in Portugal and in the Mediterranean region. Around 75 % of the compounds administered can be released into the water via fish feces or uneaten medicated feed, and leaching from the pellets also plays an important role on the concentrations available after administration (Burridge et al., 2010; Halling-Sørensen et al., 1998; Lalumera et al., 2004; Rigos et al., 1999; Sarmah et al., 2006). Assessing the stability of the tested antibiotics was the next objective of the present work, and the patterns were distinctive among the pharmaceuticals tested. While experimental conditions known to alter pharmaceuticals degradation such as pH, temperature, oxygen, and light (Andreozzi et al., 2003; Kümmerer, 2009a) were the same, ENR concentrations in

water were maintained throughout the experiment (92 and 93% of the initial ENR concentrations), while a significant decrease was observed in OTC concentrations (37 and 38 % of the initial OTC concentrations, for C1 and C2 treatments, respectively). Such results were in accordance with previous studies, either reporting a rapid degradation of OTC in water (Doi and Stoskopf, 2000; Jin et al., 2017; Xuan et al., 2009) or slow decrease in ENR concentrations through time (Boxall et al., 2006). In fact, some authors state that ENR is not susceptible to hydrolysis (Kümmerer, 2009a; Trouchon and Lefebvre, 2016), thus increasing the potential risk of this fluoroquinolone since it may persist in aquaculture systems (Pereira et al., 2015; Robinson et al., 2005; Van Doorslaer et al., 2014). However, these degradation patterns are expected in natural clear waters, which is not always the case in aquaculture tanks or ponds. Factors such as light availability or water turbidity are known to interfere with antibiotic degradation (Jin et al., 2017; Van Doorslaer et al., 2014; Xuan et al., 2009).

The accumulation of pharmaceuticals by the seaweed *Ulva* showed two distinct profiles. While concentrations of ENR reached its peak after 30 and 15 min, for C1 and C2, respectively, concentrations of OTC showed a linear increase until 48 and 24 h after the beginning of the trial, for C1 and C2 treatments. Furthermore, while concentrations of ENR showed a tendency for a linear decrease through time, the macroalgae *Ulva* was capable of rapidly decreasing OTC concentrations (more noticeable for C1 treatment, with a 10-fold decrease being determined). The results obtained in the present work suggest that this seaweed is capable of not only regulating internal concentrations of ENR, but also decrease OTC concentrations, showing detoxification mechanisms. Such mechanisms have already been described for these organisms (A. Navarrete et al., 2018; Pflugmacher et al., 1999; Schweikert & Burritt, 2012; Torres et al., 2008), but further research needs to address detoxification processes for OTC and ENR in seaweeds.

The influence of each antibiotic on *Ulva* was also analyzed as variation in disk area in comparison to the control groups, since discharges of such compounds from aquaculture can produce effects in aquatic organisms (Kümmerer, 2009a). Disks were only significantly larger than controls at the last sampling point, for both compounds, but signs of degradation were visible in *Ulva*'s disks. This capacity to

allocate resources for growth when macroalgae is under stress conditions have already been described for plants and some algae (Cedergreen et al., 2006). Furthermore, this can pose an economic risk to IMTA systems where algae are being cultivated in the presence of such pharmaceuticals since their deterioration would imply that biofiltration and mitigation of aquaculture sites were no longer made by these extractive species. Sustainability of such systems might be compromised when one trophic level cease to exist. Nonetheless, concentrations tested throughout the present thesis were different for each pharmaceutical, and direct correlations can be hard to assume. However, the use of real concentrations, following administration dosages for aquaculture, provided a good estimate on the chemicals fate upon administration to fish. While OTC is the most used antibiotic in aquaculture, widely prescribed worldwide (Alday-Sanz et al., 2012; Rigos & Smith, 2013; Sapkota et al., 2008), the fact that ENR is not easily degraded and can persist in aquaculture sites (Pereira et al., 2015) raises the importance of addressing these antibiotics behavior in a possible IMTA scenario. Concentrations of up to 12.55 and 5.09 ng g^{-1} WW were detected in Ulva's fronds, for OTC and ENR C2 treatments, respectively. Taking into account that the MRL for these compounds is 100 μg kg^{-1}, extra caution must be taken upon its consumption. The existing limits in the current legislation are specific to fish, and while a direct comparison must be made with caution due to differences in daily intakes of fish and seaweeds, problems of allergies or resistance problems will always exist since these organisms have the capacity to take up antibiotics from the surrounding waters. Either EC Regulation 37/2010 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin should be updated to incorporate seaweeds as foodstuff, or specific legislations for macroalgae such as the EC Regulation 396/2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin should address MRLs for antibiotics.

FUTURE PERSPECTIVES

Future perspectives

During the course of this work, antibiotic retention in two of the most important fish species in Mediterranean aquaculture were addressed, as well as its accumulation in the seaweed *Ulva*, both commercially available species produced in integrated multitrophic aquaculture systems. Considering human health, and the maximum residue levels allowed by the European legislation, special attention must be given to OTC and TRI, considering that protein intake provided by fish is above the average in these regions. Further research should be made in order to better understand the processes involved in the accumulation of pharmaceutical residues or its metabolites by extractive species such as the seaweed *Ulva*, and there is a need to include such organisms in the current legislations, since antibiotic residues might be present in aquaculture systems during longer periods of time. As shown, accumulation patterns can significantly vary from species to species and among different compounds, and for this reason further investigation on the retention or accumulation of other compounds should also be performed to increase the information available. While IMTAs offer a good alternative to aquaculture intensive production, these aspects need to be taken into account regarding food safety concerns in a world increasingly needing this production method.

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