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ANTIBIOTICS IN THE ENVIRONMENT

Experimental Research on the Effects of Furaltadone, Chloramphenicol and Sulfathiazole on the Non-Target Species *Ulva lactuca* L.

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Antibiotics in the environment: *Experimental Research on the Effects of Furaltadone, Chloramphenicol and Sulfathiazole on the Non-Target Species Ulva lactuca L.*

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CONTENTS

Abbreviations

AMOZ	5-methylmorpholino-3-amino-2-oxazolidinone
BCF	Bioconcentration Factor
CAP	Chloramphenicol
CCα	Decision Limit
CCβ	Detection Capability
CE	Collision Energy
CXP	Cell Exit Potential
DP	Declustering Potential
EC	European Commission
EFSA	European Food Safety Authority
ESI^+	Electrospray Ionization Positive Mode
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FTD	Furaltadone
HPLC-UV	High Performance Liquid Chromatography with Ultraviolet Detection
ICH	International Conference on Harmonization
IS	Internal Standard
K_{ow}	Octanol/Water Partition Coefficient
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification
MRL	Maximum Residue Limits
MRM	Multiple Reactions Monitoring Mode
NFA	Nitrofuraldehyde
NXZ	Nifuroxazide
POP	Persistent Organic Pollutants
RSD	Relative Standard Deviation
SA	Sulfonamides
SPE	Solid Phase Extraction
SRM	Selection Reaction Monitoring
STZ	Sulfathiazole
VMD	Veterinary Medicinal Drugs
WHO	World Health Organization

Abstract

The primary objective of the present thesis was to evaluate the potential threats resulting from environmental contamination by antibiotics to non-target organisms. This evaluation was based on experimental laboratorial studies associated with the development of new analytical methodologies for the assessment of these pharmaceuticals in environmental matrices. The research here described contributes to the overall knowledge on the stability of three distinct classes of antibiotic drugs in seawater and the influence of each compound on *Ulva lactuca* L., a green macroalgae widely distributed in coastal and estuarine ecosystems and commonly found in the vicinities of aquaculture ponds. Therefore, *U. lactuca*'s potential for accumulating such contaminants and the subsequent risk of biomagnification through the trophic web were also assessed while evaluating the suitability of including this species in environmental monitoring programs as a bioindicator tool for their presence. Thus, the present thesis core was divided into three main chapters according to the compounds evaluated: furaltadone (Chapter I), chloramphenicol (Chapter II) and sulfathiazole (Chapter III). Each chapter entails the description of the analytical methodologies developed for the quantification of antibiotics in seawater and in macroalgal tissue, as well as the influence of each pharmaceutical on *U. lactuca*'s growth.

Chapter I focused on the nitrofuran furaltadone (FTD), a broad-spectrum antibiotic banned from use in the European Union but still reported to be illegally administered and therefore should be closely monitored. In this sense two methodologies were described for the quantification of FTD in macroalgae with liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) and in seawater, through high performance liquid chromatography with ultraviolet detection (HPLC-UV). Following validations, the methods were applied to evaluate the effects on exposed *U. lactuca* to two concentrations of FTD for 120h, simulating discharges from aquaculture ponds following prophylactic (25 µg mL^{-1}) and therapeutic (50 µg mL^{-1}) treatments.

Results showed this antibiotic presents low stability in seawater being degraded mainly through photolysis. Also, *U. lactuca* showed an efficient removal of FTD in both treatments evidenced by high internal concentrations. Furthermore, the effect of the nitrofuran on growth indicated macroalgae present different sensitivities to FTD dependent on the concentrations present. At the prophylactic concentration the algae exhibited growth although less than the control group while at the highest concentration, lethal toxicity was registered after 48h. Moreover, *U. lactuca* was able to lower the internal concentration of FTD pointing to the presence of a detoxification mechanism. Finally, since green macroalgae can accumulate FTD should be considered as potential indicator tools for the presence of the antibiotic in natural ecosystems.

Chapter II dealt with research on chloramphenicol (CAP), a highly effective antibiotic whose administration was also banned due to the serious harmful effects induced but, as it happens with FTD, is still reported to remain in use. Therefore, the stability of CAP in environmental seawater and the potential effects its presence might inflict in macroalgae were investigated. To allow an accurate determination of CAP in both matrices a fully validated analytical methodology through the use of HPLC-UV was developed and described. CAP showed an exponential pattern of degradation in both treatments with less than 20% of the initial values recovered after 120h. As for the effects on *U. lactuca*, the same experimental design was applied exposing the algae to the prophylactic and therapeutic concentrations mentioned previously with results indicating that CAP led to a significant increase in growth. Moreover, this group of organisms is a suitable candidate as a tool in biomonitoring programs since it successfully accumulated CAP from the medium while reflecting its concentrations. Nonetheless, this ability pointed to the possibility of bioaccumulation and biomagnifications of CAP along the trophic web through the consumption of *U. lactuca*.

Finally, in chapter III the risks of the presence of sulfathiazole (STZ), a widely prescribed sulfonamide drug were evaluated. As it happened for the previous

compounds it was necessary to develop two methods based on LC-MS/MS and LC-UV for the quantification of the antibiotic in seawater and in *U. lactuca*. The first method showed more sensitivity making it more suitable for samples expected to be in the ng g^{-1} range while LC-UV is more adequate to matrices originated in contaminated locations, (in the µg g^{-1}). Regarding STZ in natural seawater it remained stable for the length of the experimental trial with only 18% of degradation registered for both concentrations tested. Results also demonstrated macroalgae have an efficient uptake of the antibiotic in solution and that the internal concentrations were maintained constant after 24h despite the high values in the medium, which enforced the possibility of a detoxification mechanism. Moreover, STZ induced a slight inhibition in growth after 96h in both treatments although a more pronounced effect was expected since the mechanism of action of this antibiotic causes a disruption in the folate synthetic pathway, which in turn affects cellular division. Additionally, as it was suggested for the previous compounds tested in the present research, *U. lactuca* should be considered as a suitable bioindicator tool in environmental monitoring of pharmaceutical contamination.

Resumo

A presente tese teve como principal objectivo avaliar as potenciais ameaças resultantes da contaminação ambiental por antibióticos sobre organismos não-alvo. Esta avaliação teve como base a realização de estudos laboratoriais experimentais aliados ao desenvolvimento de metodologias analíticas para a determinação de fármacos em matrizes ambientais. A investigação aqui descrita representa uma importante contribuição para ampliar o conhecimento sobre a estabilidade de três antibióticos distintos em água salina bem como a respectiva influência em *Ulva lactuca* L., uma macroalga verde com vasta distribuição em ecossistemas costeiros e estuarinos e encontrada frequentemente junto a instalações de aquacultura. Por este motivo, o potencial de acumulação destes contaminantes e o subsequente risco de biomagnificação ao longo da teia trófica foram incluídos nesta avaliação em conjunção com a possibilidade de inclusão desta espécie em programas de monitorização ambiental como bioindicadora da presença de antibióticos. Assim, a estrutura da presente tese é constituída por três capítulos principais estabelecidos de acordo com os compostos avaliados: furaltadona (capítulo I), cloranfenicol (Capítulo II) e sulfatiazol (Capítulo III). Cada capítulo concentra a descrição das metodologias analíticas desenvolvidas para a quantificação do antibiótico em água salina e em macroalgas bem como a influência do composto estudado no crescimento de *U. lactuca*.

O capítulo I centra-se no estudo de furaltadona (FTD), um antibiótico de largo espectro pertencente à família dos nitrofuranos, cuja utilização está proibida na União Europeia (UE). No entanto, vários relatórios alertaram para o facto de continuar a ser usado ilegalmente e, por conseguinte, a necessidade de monitorizar a sua presença encontra-se plenamente justificada. Neste sentido, foram desenvolvidas duas metodologias para a quantificação de furaltadona em macroalgas através de cromatografia líquida acoplada a detector de massa sequencial (LC-MS/MS) e em água salina com cromatografia líquida de alta resolução com detecção ultravioleta (HPLC-UV).

As metodologias foram posteriormente aplicadas na avaliação dos efeitos da exposição de *U. lactuca* a duas concentrações de FTD durante 120h, simulando descargas de efluentes de tanques de aquacultura após a utilização de doses profilácticas (25 μg mL^{-1}) e terapêuticas (50 μg mL^{-1}). Os resultados obtidos indicaram que este antibiótico apresenta uma estabilidade reduzida em água salina sendo degradado principalmente por fotólise. Quanto à análise de *U. lactuca*, as elevadas concentrações internas de FTD mostraram que esta espécie apresentou eficiência na remoção do composto em solução em ambos tratamentos. Relativamente à avaliação do efeito do nitrofurano no crescimento da macroalga foi demonstrado que a sensibilidade à FTD é dependente da concentração presente. Quando exposta à concentração profiláctica a alga apresentou crescimento, ainda que inferior ao grupo de controlo, enquanto que na concentração mais elevada foi registada toxicidade letal após 48h. Outro aspecto importante deste estudo foi a capacidade de *U. lactuca* reduzir a concentração interna de FTD indicando a possibilidade de existência de um mecanismo de destoxificação. Por último, dado que esta macroalga verde consegue acumular FTD, a sua utilização como espécie indicadora da presença deste antibiótico em ecossistemas naturais deve ser considerada.

O capítulo II concentra a pesquisa relativa a cloranfenicol (CAP), um antibiótico com elevado grau de eficácia, mas cuja administração foi também proibida devido aos efeitos nocivos causados, e que, tal como a FTD, é usado ilegalmente. Por esta razão, a estabilidade de CAP em água salina e os potenciais efeitos que a sua presença no ambiente pode causar em macroalgas foram investigados. De forma a permitir a determinação de CAP nas duas matrizes foi necessário o desenvolvimento de um método analítico adequado com recurso a HPLC-UV. A aplicação desta metodologia permitiu analisar a estabilidade de CAP em água salina, revelando um padrão de degradação onde as concentrações de antibiótico quantificadas após 120h em ambos tratamentos corresponderam a valores inferiores a 20% das quantidades iniciais. Quanto à avaliação dos efeitos da presença deste antibiótico em *U. lactuca*, o mesmo

desenho experimental usado para FTD foi aplicado, expondo a alga às concentrações profiláctica e terapêutica mencionadas tendo os resultados obtidos indicado que a presença de CAP induziu um aumento significativo no crescimento. Relativamente ao uso de macroalgas verdes em programas de biomonitorização, uma vez que foi demonstrada a sua capacidade de acumular CAP a partir do meio reflectindo as concentrações externas a que estão expostos, devem ser considerados como possível ferramenta para a avaliação da presença do antibiótico. No entanto, não pode ser descurado o facto desta capacidade representar o risco de bioacumulação e biomagnificação de CAP ao longo da teia trófica através do consumo da macroalga.

Finalmente, no capítulo III foram avaliados os riscos da presença de sulfatiazol (STZ), um antibiótico pertencente à família das sulfonamidas e como tal, extensivamente prescrito e utilizado. Tal como aconteceu para os compostos anteriores tornou-se necessário desenvolver metodologias adequadas para a quantificação de STZ em água salina e em *U. lactuca* tendo sido descritos dois métodos diferentes baseados em LC-MS/MS e HPLC-UV. O primeiro método mostrou maior sensibilidade tornando-se mais apropriado para a análise de amostras onde são esperadas concentrações na gama de ng g^{-1} enquanto o segundo é mais adequado para matrizes provenientes de locais com elevado grau de contaminação (µg g^{-1}). Quanto ao comportamento de STZ em água salina as concentrações obtidas indicaram que permaneceu estável durante o ensaio laboratorial, registando-se apenas 18% de degradação, independentemente das concentrações iniciais usadas. Relativamente à capacidade de absorção do antibiótico em solução, *U. lactuca* mostrou eficiência no processo tendo mantido concentrações internas de STZ após 24h independentes dos valores externos elevados no meio, reforçando a possibilidade de existência de mecanismos de destoxificação. Quanto ao efeito de STZ, verificou-se a ocorrência de uma ligeira inibição no crescimento em ambos tratamentos, após 96h. No entanto, e uma vez que o mecanismo de acção deste antibiótico leva a alterações na síntese de folatos, que por sua vez afectam a divisão

celular, seria de esperar uma inibição de crescimento mais pronunciada. Tal como sugerido para os compostos acima mencionados, o uso de *U. lactuca* como bioindicador de contaminação por sulfatiazol em estudos de monitorização ambiental deve ser uma hipótese a considerar.

General Introduction

Presently, the aspects related with human nutrition are of significant importance, focusing the attention of scientists and policymakers regarding the safety of food supplies. Furthermore, the world's continuously growing population and the correlated decrease of natural resources have led to the steep increase of animal farming, especially aquaculture. To assure farmed animal's health the resort to chemicals and veterinary drugs is frequently taken. This practice in animal production is well described, whether for prophylaxis or therapeutic needs, which dictated the implementation of regulations regarding a responsible use of drugs and chemicals, to reduce the hazards (defined as "a biological, chemical or physical agent in, or condition of, food with the potential to cause an adverse health effect" according to the European Commission Regulation 178/2002) to consumers. However, the guidelines regarding the hazards of drug use contemplate farmed animals, veterinary drug residues, feeds, withdrawal time and the quality of the water used in aquaculture, neglecting the potential contamination of the surrounding natural ecosystems. Ecologically and economically important species may present similar risks to human health as farmed animals do as they can also accumulate drugs and chemicals, thus representing an increased threat as such species are not subjected to the same safety controls as are farmed animals. Also, the possibility of a given chemical or drug being accumulated along the trophic web (biomagnification) is, in most cases, overlooked.

In this chapter, the risks of food safety and environmental contamination will be addressed.

Nutrition and Food Safety

Nutrition and food safety are two concepts that are becoming every day more intertwined with the development of world's food supplies since it is internationally recognized that each individual has the right to nutritionally adequate and safe food. In fact, according to the Food and Agriculture Organization of the United Nations (FAO) *food security exists when all people, at all times, have physical and economic access to*

sufficient, safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life (World Food Summit, 1996). This statement focuses on supply, safety and nutrition, three fundamental concepts to the existence of security. Each aspect involves many principles and requirements safeguarded in regulations and legislations at international levels, as food is more than ever a global issue. The European Union under the General Food Law Regulation (EC Regulation 178/2002) defined food as *any substance or product, whether processed, partially processed or unprocessed, intended to be, or reasonably expected to be ingested by humans* and established the general principles of food law to assure the protection of human health and consumer interest. This regulation also laid down the ground rules for the European Food Safety Authority (EFSA) which has the responsibility to uphold a scientific and technical support system, constituting an independent scientific point of reference regarding the safety of food and feed supply chains as well as environmental protection as a whole (EC Regulation 178/2002).

According to the Codex Alimentarius (FAO/WHO, 2001) food safety is "the assurance that food will not cause harm to the consumer when it is prepared and/or eaten according to its intended use". Another important aspect included in General Food Law is feed safety, where feed is defined as *any substance or product, including additives, whether processed, partially processed or unprocessed intended to be used as oral feeding to animals*. The ultimate purpose of food and feed safety is to uphold human health but it presents itself as a very intricate task as measuring and securing safety is not easy. On the basis of the whole evaluation of safety is risk analysis which deals with the detection of hazards, defined as *biological, chemical or physical agents in, or condition of, food or feed with potential to cause an adverse health effect* and potential risks which are a *function of the probability of an adverse health effect and the severity of that effect, consequential to a hazard*. In other words, a risk is the negative effect of the occurrence of a hazard. Risk analysis can be broken down in three phases: risk assessment, risk management and risk communication. Risk assessment relates to a scientifically based process consisting of *hazard identification*,

hazard characterization, *exposure assessment* and *risk characterization* (Article 3 of the EC Regulation 178/2002). Scientific studies are a decisive tool in assessing risk as well as in identifying possible emerging risks through the collection, analysis and treatment of relevant data, particularly regarding: food consumption and exposure, biological risks, contaminants in food and feed as well as residues {Articles 33 and 34 of the EC Regulation 178/2002).

The concept *from the farm to the fork* summarizes the new views on food safety. More than ever safety must be addressed along the entire food chain, from primary production to final consumption. With this broaden view, instead of concentrating the responsibility for safe food in the processing sector, all the sectors involved are now held accountable for safety (FAO, 2003a). Instead of focusing on the end product, control points should be placed along the food production process from primary production to the consumer's table. With this holistic food chain perspective, changes will be expected in all the steps involved to guarantee more nutritious and safe food, including more viable environmental and economical practices (FAO, 2003a).

Veterinary Medicinal Drugs

One issue closely related with food safety is the use of chemicals in food animal production, which can constitute potential hazards for human health. The resort to drugs is undeniably necessary to assure the welfare of animals, whether administered to prevent (prophylaxis) or to treat (therapeutics) disease. Nevertheless, the awareness that veterinary medicinal drugs (VMD) can be potentially prejudicial dictated the need to regulate their production, distribution and administration to safeguard public health, uphold in the EU under the Directive 2001/82/EC (EC Regulation 82/2001). This regulation defines VMD as any substance or combination of substances presented as having properties for treating or preventing disease in animals and set the rules for their safe use and marketing (EC Regulation 82/2001). Another important regulation concerning VMD is the Council Regulation 2377/90, which aimed to prevent hazards associated with the consumption of unsafe residues in edible

tissues by establishing their maximum residue limits (MRL) in food of animal origin. This limit refers to the maximum concentration of residue resulting from the use of a veterinary product (expressed in mg/kg or μg/kg on a fresh weight basis) legally permitted without any harm to consumer, including food of environmental origin (EC Regulation 2377/90). The marker residue can be either the original drug or a resulting metabolite that best characterizes the depletion from the edible tissues. This regulation divided VMD according to their pharmacologically active substances, which were grouped into four annexes according to their MRL. Annex I included all the substances to which full MRL have been fixed whereas Annex II incorporated those drugs whose evaluation indicated that no limit is required in order to protect public health. Substances in Annex III had a provisional MRL with a defined period of time not exceeding five years until scientific studies were completed (if necessary to the conclusion of the studies an additional two year period could be granted). Finally, substances included in Annex IV were considered a hazard to consumer's safety as the presence of residues in foodstuff of any origin can be very harmful and no limit could be established. The administration of substances in Annex IV was therefore prohibited in the EU (EC Regulation 2377/90). Another important regulation related with drugs safety is EC Regulation 726/2004, which dictated the procedures for authorization and supervision of medicinal products for human and veterinary use. Moreover, this regulation established the European Medicines Agency improving the previously existing European Agency for the Evaluation of Medicinal Products (EC Regulation 726/2004). Recently as a result of years of research and technical advances in several areas, new regulations were set to improve and in some cases, revoke previously existing regulations. EC Regulation 470/2009, based on the 2377/90, laid down the procedures to establish the residue limits for pharmacologically active substances taking also into account risk assessment and management (EC Regulation 470/2009). Recently, EC Regulation 37/2010 was adopted regarding pharmacologically active substances in foodstuffs of animal origin. Under this decision, substances are classified following the system set by EC Regulation 470/2009, according to their MRL and in the

interest of clarity and simplification are listed alphabetically in only one Annex divided in two tables for allowed and prohibited use incorporating the four annexes set by the now repealed EC Regulation 2377/90 (EC Regulation 37/2010).

Nonetheless, even with all the regulations and legislations set to safeguard human and veterinary health and establish consumers trust, over the last decades several incidents have occurred with chemicals found in food. For instance, clenbuterol, a β_2 adrenergic agonist permitted in the EU as a bronchodilating and tocolytic agent in both human and veterinary medicine has been administered to other ends other than the allowed (Ramos et al., 2003; Barbosa et al., 2005). Since its actions include muscular relaxation it was used as a growth promoter to increase muscular mass and diminish the accumulation of fat making meat more lean and tender. As a result of this illegal application, several cases of intoxication were reported in Portugal (Ramos et al., 2003; Barbosa et al., 2005), Spain (Martínez-Navarro, 1990; Salleras et al., 1995), Italy (Brambilla et al., 2000) and France (Pulce et al., 1991) after ingestion of liver and meat with high residue contents. Other well-known incident occurred with the detection of nitrofuran residues in meat and other food products (Cooper et al., 2004; Barbosa et al., 2007). Nitrofurans are a group of nitroheterocyclic drugs with a broad-spectrum activity against bacterial and protozoan infections, in humans and animals (Ræther and Hänel, 2003; Vass, 2008). However, since studies pointed to the possibility of mutagenic, teratogenic and carcinogenic effects linked to the use of nitrofurans, were included in Annex IV of EC Regulation 2377/90 (EC Regulation 1442/95) and therefore banned from use in livestock production in the EU.

An Overview on Aquaculture

World population doubled during the last 45 years and the latest United Nations projections anticipated world population will rise from 6.8 billion today to 9.1 billion in 2050. The combination of population growth, increasing urbanization and rising incomes has contributed to a greater consumption of animal products. Furthermore, economic development and increasing wealth tend to enhance the

availability and quality of food, better overall nutritional status and the mitigation of food deficiencies. According to the global trends, worldwide fish consumption has increased from 9 kg per capita per year in the early 1960s to 16.7 kg in 2006, and is expected to rise to 17.0 kg by 2020 (Delgado et al., 2003; FAO, 2009). However, the demand for fish increases at a higher rate than wild stocks can support. As stated by FAO, the maximum wild capture fisheries potential from the world's oceans has probably been reached. Overall, 80% of the world fish stocks for which assessment information is available are reported as fully exploited or overexploited (FAO, 2009). With the depletion of wild stocks due to overfishing and failed management practices in capture fisheries, aquaculture offers the only viable alternative to the ever-increasing demand for fishery products. As a result, during the past decades aquaculture has expanded, diversified, intensified and made technological advances. Similar to other animal production systems, such as pork, poultry, and eggs, aquaculture has grown into a highly globalized industry. In fact, aquaculture is growing more rapidly than any other animal food-producing sector in the world. According to FAO statistics, this sector has grown at an average rate of 8.9% per year since 1970, compared with only 1.4% for capture fisheries and 2.8% for terrestrial farmed meat production systems over the same period (FAO, 2003b). In 2006, total fisheries production (capture fisheries and aquaculture production) supplied about 110 million tonnes of food fish, providing the highest per capita supply recorded. After growing steadily in the last four decades, aquaculture contributes for the first time to approximately half of the fish available for human consumption worldwide, while in the 1970s it accounted for only 6% (FAO, 2009).

According to FAO the term aquaculture encompasses all activities associated with the farming of aquatic organisms implying some form of intervention in the rearing process to increase the productivity of these organisms beyond the natural capacity of the environment, such as control of breeding, regular stocking, protection from predators, and supply of artificial feed and medication (FAO, 1997). Fish farming has been practiced since ancient times with traditional aquaculture systems of small

farm size with low stock density and minimal added inputs. However, the early forms of aquaculture differed greatly from those practised today. While until recently there were no reasons for the development of intensive fish farming techniques, the increased demand for farmed products has contributed to an unprecedented growth in this industry over recent decades. Thus, current intensive aquaculture is not only characterized by high stock density and volume, but also by the heavy use of formulated feeds containing antibiotics, antifungals and other pharmaceuticals, and the application of pesticides and disinfectants. The chemicals used in aquacultures enable more control over fish development, i.e. to enhance and control production in hatcheries, increase feeding efficiency, improve survival rates, decrease diseases and reduce transport stress (Huntington et al., 2006). Unfortunately, the intensification of production methods has been accompanied by an increase in potential food safety and health concerns associated with the consumption of farmed products. The majority of intensive aquaculture facilities rely heavily on the input and application of several chemicals. As a result, farmed food products can have elevated levels of VMD residues, antibiotic-resistant bacteria, persistent organic pollutants (POPs), and heavy metals compared to their wild counterparts (Easton et al., 2002; Hites et al., 2004). The potential health effects to the consumers can range from chronic health outcomes associated with chemical exposures to infectious disease related to antibiotic-resistant bacteria (Foran et al., 2005; Samanidou and Evaggelopoulou, 2007).

The dispersion of pharmaceuticals in the environment is another potential key impact of current aquaculture practices that may pose environmental and ecological risks including reductions in water quality (Tovar et al., 2000), pressure on feed resources (Naylor et al., 2000), genetic interaction between wild and escaped aquaculture conspecifics (Youngson et al., 2001), disease transfer to wild fish (Krkosek et al., 2007), eutrophication (Loya et al., 2004) and destruction of natural habitats (Paez-Osuna, 2001). Ecological and economically important wild species may represent the same risks to human health as farmed species through bioaccumulation of chemicals released into the aquatic environment (Katranitsas et al., 2003; Fortt et al.,

2007). Many VMD used are stable chemical compounds and have been shown to persist in water and sediment for several weeks following administration (Björklund et al., 1990). As a result, they may enter the aquatic food web through exposure of non-target biota around aquaculture sites. In fact, wild fish harvested from areas around aquaculture facilities and destined for human consumption, have been reported to present concentrations of antibiotics higher than the MRL (Fortt et al., 2007). The presence of antibacterial agents may also affect the bacterial communities and induce antibiotic resistance (Tendencia and de la Pena, 2002), which in turn may introduce resistant strains to human populations through the consumption of fishery products.

Pharmaceuticals Fate in the Environment

Emerging contaminants include all the chemical compounds which can be found in the environment with the potential to cause adverse effects but that were not until recently monitored in environmental risk assessment studies. However, in the last decades the interest in these substances has grown considerably and with it the need to understand the effects they can produce on biota (Boxall et al., 2004). VMD are part of this emerging class of environmental contaminants, which have existed in the ecosystems for as long as they have been administered. The properties that make drugs effective are also the same that can become a concern when these substances are released in the ecosystems. For instance, drugs are designed to alter specific biochemical pathways in the target species for which their use is intended but when released into the environment non-target organisms are likely to be affected. Moreover, drugs are biologically active and present in many cases low partition coefficients which when discharged in the water confers them higher mobility. For all these reasons, environmental risk assessment of VMD is mandatory in the EU (EC Regulation 81/852 as amended by EC Regulation 92/18) and in other countries, including the USA and Canada.

Between 1999 and 2000 the United States Geological Survey conducted a study that indicated that, out of a network of 139 streams across 30 states, 95 were

contaminated with traceable concentrations of antibiotics (Kolpin et al., 2002). The increasing amount of evidences in the environment made it clear that much research is needed to assess the impact of antibiotics released into the environment, their potential effects on wildlife and ultimately on human health. Although the use of antibiotics in fish farming has fallen over the last years it has been estimated that livestock producers in the United States use 24.6 million pounds of antimicrobials every year for non-therapeutic purposes (Mellon et al., 2001).

A wide variety of pharmaceuticals used in human and veterinary medicine has been found in diverse environmental compartments such as water, soil and sediments. Chemicals reach these compartments through several distinct activities (Figure 1), with significant amounts (up to 75%; Elmund et al., 1971; Feinman and Matheson, 1978) being excreted as active metabolites. Antibiotics are also present in sewages, largely as a result of human excretion. Many active antibiotics are not completely metabolized during therapeutic use and thus enter sewage through excretion in an unchanged form. Veterinary use and the intentional disposal of unused drugs into the sewer also contribute to the quantities of antibiotics found. Discharges from veterinary clinics and runoff from agricultural applications into municipal sewers are also potential sources of veterinary antibiotics in wastewaters (Le-Minh et al., 2010). These compounds can also enter the aquatic environment when medicated feed sinks to the water body bed or is eliminated by fish excretion in aquacultures (Smith and Samuelsen, 1996).

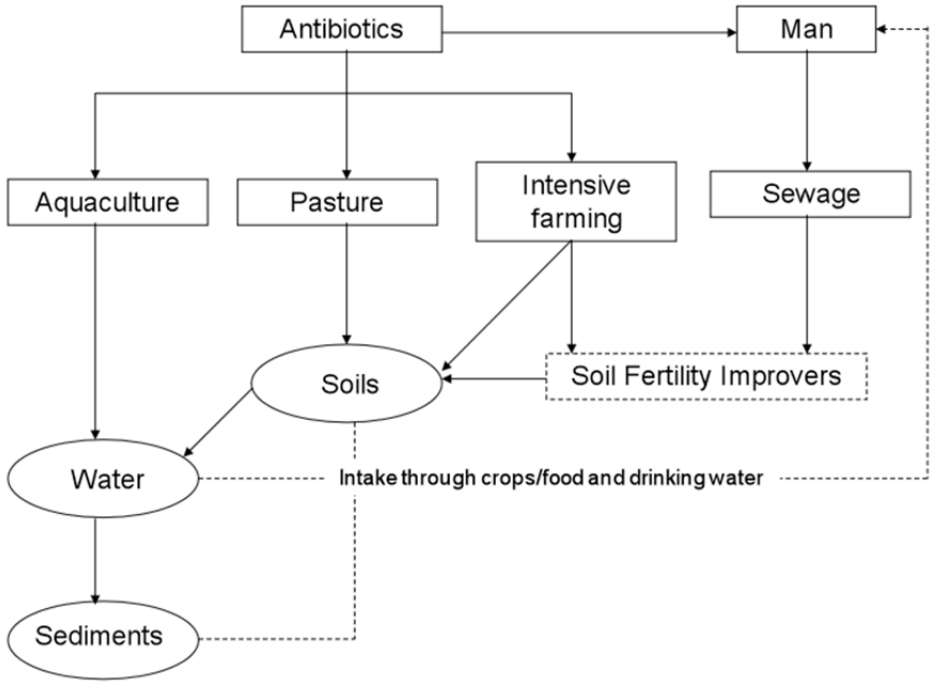

Figure 1. Route and fate of antibiotics in the environment.

Drugs used in fish farms or their sub-products can also be transported directly into surface water or accumulate in the sediment (Björklund et al., 1990; Jacobsen and Berglind, 1988). Several studies reported that substances extensively applied in fish farming present long half-lives in soil and sediment (Jacobsen and Berglind 1988; Samuelsen et al., 1992, 1994; Hektoen et al., 1995; Capone et al., 1996; Marengo et al., 1997). Parameters such as route of drug administration, drug formulation and pharmacokinetics, the dose applied and the frequency of treatment also have influence on the concentration of these chemicals in soils, sediments, surface and ground waters (Kim and Carlson, 2007). Other entry route of these drugs into the environment include land-based forms of livestock production, where in intensive farming the active ingredients or metabolites might reach the soil by leaching from animal slurry and

manure. The excretion ratio will vary depending on the compound but it can be as high as 80-90% of the parent compound, being excreted via urine and faeces (Heberer, 2002; Bound and Voulvoulis, 2004). Measured concentration of veterinary products in animal waste or manure from previous studies has ranged from 11 to 12400 µg/kg (Haller et al., 2002; Schlusener et al., 2003). Farm animals kept in pastures may also contribute as a soil contamination point-source directly through their excrements. McKellar (1997) reported no significant degradation over a 45-day time period for residues of the anti-parasitic ivermectin in faeces of cattle treated with pour-on or subcutaneous preparations while Kolar and Erzen (2006) established a DT_{50} value of 23 and 22 days for dissipation of the also anti-parasitic abamectin and doramectin from sheep faeces under field conditions and in the pasture, respectively (Bound and Voulvoulis, 2004). Research has shown that after passing through wastewater treatment, pharmaceuticals, amongst other compounds, are released directly into the environment (Kümmerer, 2009). Soil may become an entry route for these drugs, when with more or less ease they leach into the aquatic environment of nearby water courses or groundwaters and in turn to the sediments. As a result, antibiotics consumed by humans and animals can be introduced into different environmental compartments depending on their physicochemical properties. Their environmental fate (behavior, persistence in the environment, and toxicity) will depend greatly on their physical/chemical characteristics as well as of the medium they are in (Kim and Carlson, 2007; Kümmerer, 2009).

The octanol/water partition coefficient (K_{ow}) has become a key parameter in organic chemicals environmental fate assessment studies and is defined as the ratio of a chemical's concentration in the n-octanol phase (a surrogate for lipids) to its concentration in the aqueous phase of a two-phase octanol/water system (Tolls, 2001). It can be potentially related to water solubility, partition into lipids and fats, soil/sediment adsorption coefficients and bioconcentration factors for living beings. K_{ow} values per se can be considered meaningful, since they represent the tendency of the chemical to partition itself between the various environmental compartments.

Therefore, chemicals with low K_{ow} values (i.e., lower than 10) may be considered relatively hydrophilic, having high water solubilities, small soil/sediment adsorption coefficients, and small bioconcentration factors. Conversely, chemicals with high K_{ow} values (e.g., greater than 104) are very hydrophobic (for a review in measured sorption coefficients values see Boxall et al., 2004). This parameter, by including water solubility, is of great importance to understand organic chemicals mobility in soils and sediments. Moreover lipophilicity may represent great value to predict bioaccumulation in living tissues.

The fate of the contaminants and their degradation products will depend on these partitioning characteristics and these measures have been considered to be an important tool to understand the fate of a given pharmaceutical in the environment and estimate the probable concentration in different compartments. For instance, sulfonamide antibiotics have a Log K_{ow} of 0.12-1.5 (Le-Minh et al., 2010) and therefore are likely to be mobile and leach from dung and soil to groundwater, posing less risk of soil contamination but on the other hand, with more chance to be found in surface and ground waters, carrying greater risk to the aquatic compartment. Whereas tetracyclines, macrolides, and fluoroquinolone antibiotics will be rather immobile in the soil (Kümmerer et al., 2000), thus are not readily leached into ground water and are accumulated in the soil, which in practice will pose distinct environmental fate and thus different problems. High K_{ow} values cause limited aqueous solubility of these compounds. Therefore, when drug residues reach the environment they tend to be adsorbed on soil or sediment particles.

Nevertheless, there are cases where the K_{ow} might not be sufficient to predict for complex structures behavior, such as pharmaceutical compounds, as they generally possess multiple ionization sites that can represent possible sorption mechanisms. Some antibiotics are large and complex chemical molecules that may even contain acidic and basic groups in the same molecule. Therefore, distribution between the solid and the aqueous phase will also depend greatly on pH, redox potential, the stereo

chemical structure, and the chemical nature of both the sorbent and the sorbed molecule.

The Use of Bioindicators in Environmental Risk Assessment

The rise of an environmental apprehension over the last few decades has spread to the global antibiotic contamination scenario, concerning not only the environment but also human health, since their entry, directly or indirectly into the trophic web is no longer just a remote possibility. Pharmaceuticals have been shown to have effects on reproduction, biological function and survival of non-target aquatic and terrestrial organisms (Lopes et al., 2009; Han et al., 2010; Römbke et al., 2010). Interruption of the trophic web may affect the diversity of a system, which ultimately may disrupt ecological services (Muñoz et al., 2009; Rodriguez-Mozaz and Weinberg, 2010; Zhao et al., 2010). Added to the particular structure of complex pharmaceuticals, the research on the impact and behavior of these compounds in the ecosystems represents great challenges for the future to come as scientists are just beginning to unravel the risks posed by their presence in the environment.

Evaluating the risks of every emerging contaminant to the whole ecosystem is an unrealistic approach as it would be virtually impossible. Because species and ecosystems present a considerable uniformity in response to stressors, here defined as chemical contaminants that may induce adverse effects to a system, a core of indicators of ecological stress (perturbation applied to a system by a stressor) can be identified (Swindol et al., 2000). Therefore, one strategy in risk assessment studies is to single out groups of organisms with key roles in the systems and whose intrinsic characteristics are useful in the prediction of ecosystem response. Ideally, a bioindicator species should provide evidence of exposure to contaminants by their absence or presence, frequency of occurrence and physiological and behavioral variation (Rainbow, 1995; Villares et al., 2002; Melville and Pulkownik, 2006). Also, species should be easily identified, sensitive to contaminants and present a wide geographic significance (Conti and Ceccheti, 2003; Melville and Pulkownik, 2006).

Historically, in aquatic ecosystems, microalgae and invertebrates have been widely used for this role, with a special attention being paid to bivalve molluscs. The research effort around these organisms is related to the following criteria: i) represent a source of food for other organisms in the trophic web, ii) are widespread and easily collected in large amounts, iii) as filter feeders, high volumes of water pass through the organisms thus accumulating contaminants continuously and iv) there is a solid background knowledge on their basic morphology and physiology (Walker, 2006). The same essential characteristics are found in macroalgae, a group of organisms that, so far, has been overlooked. Research on their potential use has only recently begun to be developed and mainly to be applied to heavy metal contamination (Melville and Pulkownik, 2006; Coogan et al., 2007; Turner at al., 2008; Han et al., 2010; Akcali and Kucuksezgin, 2011; Costa et al., 2011). As primary producers, macroalgae represent very high biomasses in estuarine and marine ecosystems in the basis of trophic webs, constituting a very important food supply for consumers in higher levels. Macrolgae are in continuous exposure to water and subsequently to whatever contaminants are present, which is likely to result in uptake and accumulation of these substances in high concentrations. Through consumption, the risk of biomagnification is also increased and expected to be dependent on the compound (Zbikowski et al., 2006; Melville and Pulkownik, 2007). Therefore, the research on the use of macroalgae as bioindicators in environmental risk assessment is essential, with high ecological significance and may contribute greatly to understand the fate of pharmaceuticals in the environment and the influence induced in non-target organisms.

To accomplish a precise understanding of the fate of pharmaceuticals in the environment and the potential use of macroalgae as bioindicators it is essential to have adequate methods to evaluate the concentrations of a given contaminant in new matrices. Currently, there is a lack of validated analytical methods, which together with little information on the fate and effects of these compounds and/or their metabolites and degradation products in the aquatic environment turn accurate risk assessment problematic. The available methods are mostly directed to the determination of

residues in animal food matrices to assure that the MRL are in compliance with the existing legislations to uphold human health. Methodologies for the assessment of residues in sludge, sewage flow, surface and groundwaters and sediments are also available and routinely applied to most classes of antibiotics. However, to quantify antibiotics in environmental matrices such as seawater and macroalgae it becomes necessary to develop and validate adequate methodologies, also taking into account the characteristics of the compound to be quantified. The quantification of antibiotics requires special attention due to their complexity and low levels of occurrence (Fatta-Kassinos et al., 2011). Several steps are involved to assure an accurate determination starting with sample preparation, extraction procedures, clean-up and in some cases derivatization methods that together provide the opportunity to quantify many pharmaceutical compounds and metabolites down to ng L^{-1} levels. Another important aspect in pharmaceutical analysis is the validation of methodologies to assure their suitability for the intended purpose and to have uniformity in the results obtained. At this level, once again the absence of specific legislation for environmental matrices becomes a problem which is commonly overcome by following guidelines directed for animal and food related animal products (EC Regulation 2002/657) as well as regulations regarding human health related methods (ICH 1994, 1996). The parameters regarded as essential and common to these guidelines, include specificity, recovery, stability, precision (measured as reproducibility and repeatability), linearity and limits of detection and quantification. Nonetheless, it is essential that specific regulations for environmental risk assessment be developed since the processes responsible for the presence and concentration of pharmaceuticals in the ecosystems are complex and the exposure of non-target organisms is subjected to a wide mixture of substances.

General Aim and Thesis Outline

Emerging contaminants are now a recognized threat in natural environments representing unknown and potentially harmful effects to non-target organisms. It is therefore paramount to monitor their presence in the ecosystems and to evaluate the extent of their influence. The primary aim of the present research was to contribute to the overall understanding of these issues through the evaluation of the behavior in natural seawater of three pharmaceutical substances and their influence in the chlorophycean macroalgae *Ulva lactuca* L. The compounds selected represent three distinct classes of broad-spectrum antibiotic drugs, with different legal classifications regarding their administration: furaltadone (FTD) and chloramphenicol (CAP) are two banned substances under EU legislations whereas sulfathiazole (STZ) represents one of the most commonly prescribed classes of pharmacological substances. Accordingly, the general aim of the proposed research was assessed through the following specific objectives in agreement with the identified gaps in knowledge:

- Development of suitable and fully validated chromatographic methodologies to the quantification of each antibiotic selected in environmental matrices: natural seawater and *U. lactuca*;
- Investigation on the stability of the pharmaceuticals studied in natural seawater;
- Assessment of the influence of each drug on *U. lactuca* evaluated through differences in growth;
- Ascertain the possibility of bioaccumulation and biomagnification of the listed contaminants through the trophic web;
- Evaluate the suitability of including *U.lactuca* as a bioindicator tool in environmental monitoring programs for the presence of the contaminants tested.

These main objectives will be addressed in the three chapters (Chapters I to III) that constitute the focal point of this thesis. A general discussion and overview of the results is presented at the end, integrating the response to the previous questions, main conclusions and future research interests.

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CHAPTER I

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A LC–MS/MS METHODOLOGY TO DETERMINE FURALTADONE RESIDUES IN THE MACROALGAE *ULVA LACTUCA*

EVALUATION OF FURALTADONE PHOTODEGRADATION IN NATURAL SEAWATER BY HPLC-UV

THE EFFECTS OF THE NITROFURAN FURALTADONE ON *ULVA LACTUCA*

A LC-MS/MS method to determine furaltadone residues in the macroalgae *Ulva lactuca*

Abstract

Presently, the rise of new contaminants in the environment has widened the scope of pharmaceutical analyses as to face the demanding new challenges. An increasing tendency for the interconnection and overlap of research fields, such as ecology and biochemistry, is intensifying the demand for new methodologies to be applied to the survey of drugs in unconventional matrices. Integrated in this group are macrophytes, such as the green macroalgae *Ulva lactuca*, which are under study as to ascertain their ability as indicators of contamination for many substances. Nonetheless, methodologies for extraction and determination of drugs in such matrices are scarce and new studies on the subject are pressing. A new methodology for the determination of the antibiotic furaltadone in *U. lactuca* by liquid chromatography-tandem mass spectrometric (LC-MS/MS) procedure was developed, optimized and validated following the guidelines of the EC Decision 2002/657. The calibration curves showed linearity above 0.99 (R^2). The relative standard deviations obtained for repeatability, expressed as CV, were between 15.3 and 20.5 and for reproducibility 25.3 and 28.2 whereas accuracy was in the interval of 88.9 to 95.5 (%). The limit of decision (CCα) and the detection capability (CCβ) were respectively 5.57 μg kg^{-1} and 10.97 μg kg^{-1}. The method was successfully applied to experimental samples.

1. Introduction

The release of emerging contaminants to the ecosystems is continuously raising concerns with environmental safety as a wide variety of chemicals, including antibiotics, represent potential risks (Heberer 2002; Sarmah et al., 2006; Hansen et al., 2007; Kemper, 2008; Madden et al., 2009; Leston et al., 2011a). Although primarily designed to prevent and treat diseases there is an associated hazard as their active ingredients, present as either the parent compound or metabolites, come in contact with non-target organisms (Sarmah et al., 2006; Hansen et al., 2007; Kemper, 2008; Leston et al., 2006; Kümmerer, 2009). Following exposure there is a risk of accumulation for periods of time, which depends mainly on the degradation rates of the drugs but also on the existing detoxification mechanisms of the species. Moreover, risks depend on the type of agent in question and include bacterial resistance, food residues and toxicity. Legislation regarding the safe use of chemotherapeutants in the food production sectors is very strict and incorporates measures to uphold safety on human consumption but also on the environment (EC Regulation 37/2010). Under this regulation a relatively low number of antibiotics have been approved leading, in some cases, to the misuse of approved substances or to the illegal use of non-approved and/or banned drugs which can be more effective (Vass et al., 2008). These issues demand an evaluation of potential effects in aquatic species to understand to what extent the environment is being impacted by pharmaceutical contamination and how it may affect human populations. Macroalgae are an important group in natural ecosystems in the basis of trophic webs. These primary producers present simple morphologies, with high area to surface:volume ratios, high absorption and growth rates, limited mobility and elevated abundance and distribution (Melville and Pulkownik, 2006). These characteristics make them suitable candidates to be considered as environmental indicators of the presence of pharmaceutical contaminants and also to improve the knowledge on the occurrence, transport and fate of antibiotic residues.

Nitrofurans are synthetic chemotherapeutic drugs with a broad-spectrum activity which act by inhibiting microbial enzymes involved in the carbohydrate metabolism, administered to prevent and treat diseases in livestock and also to promote growth (Vass et al., 2008; Balizs and Hewitt, 2003; Cooper et al. 2005; Barbosa et al., 2007). However, they displayed significant toxicity in human health manifested through carcinogenic, mutagenic and teratogenic effects (Vass et al., 2008; Barbosa et al., 2007; Koten-Vermeulen et al., 1993; Ali, 1999; Bogialli and Di Corcia, 2009) and for this reason the European Union banned the use of nitrofurans, listing them under Annex IV of the EC Regulation 1442/95 (EC Regulation 1442/95) (recently replaced by the EC Regulation 37/2010 (EC Regulation 37/2010). Nonetheless, they are still used due to their high efficiency and low costs (Vass et al. 2008; Bogialli and Di Corcia, 2009, Chadfield and Hinton, 2003). The existing procedures focus on evaluating nitrofurans residues in food samples such as meat, milk, eggs and honey among other complex matrices (Barbosa et al., 2007; Tribalat et al., 2006; McCracken and Kennedy, 2007; Rodziewicz, 2008). The lack of methods to be directly applicable to the quantification of antibiotics in macroalgae dictates the need for advances in environmental residue analyses. Thus, the present work describes a new methodology to detect and quantify the nitrofuran furaltadone (FTD) in the green macroalgae *U. lactuca* by liquid chromatography–tandem mass spectrometry (LC-MS/MS).

2. Experimental

2.1 Reagents and Solvents

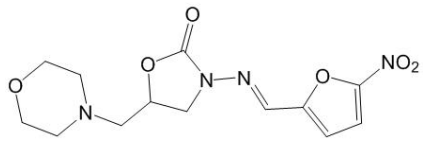
All reagents and chemicals were of analytical grade with the exception of the solvents used in the mobile phase, which were of LC-MS analysis grade. Methanol, ethyl acetate and acetonitrile were purchased from Merck (Darmstadt, Germany) whereas furaltadone and the internal standard nifuroxazide (NXZ) were acquired from Sigma-Aldrich (St. Louis, USA) (Fig 1. A and B). Deionized water was obtained using a Millipore purification system (Millipore, Bedford, USA). HPLC solvents were filtered through 0.45 µm Whatman nylon membrane filters (Whatman, Maidstone, USA).

All solvents were kept in glass recipients to prevent contamination by phthalates.

2.2 Standard Solutions

Individual stock standard solutions of each compound (100 µg mL^{-1}) were prepared in methanol and stored at -20°C. FTD work and calibration standard solutions were prepared individually by adding specific quantities of the stock solution to an appropriate volume of methanol and stored in the dark below 4°C. All solutions were kept in amber glass containers.

A



B

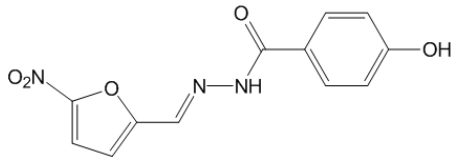


Figure 1. Chemical structures of A) furaltadone and B) nifuroxazide.

2.3 Instrumentation

To perform the procedures involved the following equipment was used: Mettler Toledo PC200 and AE100 balances (Greifensee, Switzerland), Heidolph Reax 2 overhead mixer (Schwabach, Germany), Heraeus Megafuge 1.0 centrifuge (Hanau,

Germany), Turbovap Zymark Evaporator (Hopkinton, MA, USA) and Whatman Mini-Uniprep PVDF 0.45 µm filters (Clifton, NJ, USA).

The chromatographic analysis of furaltadone was performed with an Agilent 1100 Series HPLC system (Agilent Technology, Waldbronn, Germany) coupled to a Triple Quadrupole System Sciex API 2000 tandem mass detector (Applied Biosystem, Foster City, USA) with a TurboIonSpray ion source, operating under the Sciex Analyst 1.4.1 software (Applied Biosystem, Foster City, USA).

The LC columns consisted of a guard column Zorbax Eclipse XDB-C8 (5 µm particle size) 4 mm x 2.1mm and a Zorbax Eclipse XDB-C18 (Sb-aq)(3.5 µm particle size) 12.5 mm x 2.1mm column (Agilent Technology, Palo Alto, USA).

2.4 Procedure

2.4.1 Exposure assay

To verify the ability of *U. lactuca* to take up and accumulate FTD, an experimental design was set with conditions simulating a scenario of discharge from a fish farm. Fronds of the green macroalgae *U. lactuca* were collected during low tide at the Mondego Estuary (Portugal) and were acclimated for two weeks under laboratory conditions. Individual samples weighing 200 mg wet weight were then exposed for 16 h to two hypothetical concentrations, 16 µg mL^{-1} and 32 µg mL^{-1}, respectively simulating prophylactic and therapeutic dosages in bath treatments applied in fish farms. Samples were taken at several time intervals, paper-dried until all water was removed from the surface, weighed and immediately frozen at -20°C until analysis (for further information please see Leston et al., 2011b).

2.4.2 Extraction

Each sample (± 200 mg) was thoroughly minced and placed in 15 mL screw top amber glass centrifuge tubes. 40 µL of internal standard working solution and 10 mL of ethyl acetate were added, vortex mixed and homogenized in an overhead mixer for 10

min. Following centrifugation at 3000 rpm for 10 min, the supernatant containing the organic layer was transferred to clean glassware and evaporated to dryness under gentle nitrogen flux at 50°C. To the resulting dry residue 5 mL of acetonitrile were added, followed by vortex mixing after which 3 mL of n-hexan were added. After shaking, it was let to stand for 10 min. At this time, n-hexan was discarded and the sample was evaporated to dryness under nitrogen stream. The extract was then reconstituted in 500 µL of 50:50 (*v*:*v*) methanol:water, vortexed for 30 s, filtered through a 0.45 µm PVDF Mini-uniprep ^{TM} vial and transferred to the LC autosampler.

2.4.3 LC-MS/MS Analysis

The mobile phase used in the binary gradient elution consisted of ammonium formate solution 1 mM (solvent A) and methanol (solvent B). The gradient developed for the analysis started with 90% (A) for 1 min, linearly decreasing to 55% (A) in 15 min, to 10% (A) in the following 2 min and brought back to 90% (A) in the last 2 min, with a total run time of 20 min. The sample volume injected was 50 µL, at a flow rate of 0.4 mL min^{-1}.

The turbo ion-spray source was used in positive mode (ESI^+) for FTD and NXZ with the following settings: electrospray capillary voltage, 4500 V; ion source block temperature, 400°C; curtain gas (N_2), 25 psi; collision gas (N_2), 4 psi; nebulizer (N_2), 30 psi and heater (N_2) 75psi.

Data was acquired through multiple reactions monitoring mode (MRM), with the following transitions monitored: m/z 325.2 precursor ion to m/z 281.0 and m/z 252.0 product ions for FTD and m/z 276.2 precursor ion to m/z 120.8 product ion for NXZ.

2.5 Validation

Validation for the methodology presented in this work was established according to the EC Regulation 2002/657/EC (EC Regulation 2002/657). Although this legislation is intended to regulate the performance of analytical methods to monitor

substances and residues in live animals and animal products it provides valuable guidelines for the validation of residue determination in environmental samples as legislation for such matrices is lacking.

Specificity was verified with 20 different blank samples of macroalgae obtained from distinct locations with the resulting chromatograms analyzed to exclude the presence of potential interferences in the identification of the analyte at the expected elution time.

Recovery was assessed by fortification of 18 blank samples with the addition of FTD at concentrations of 10, 15 and 20 µg kg^{-1} and IS (10 µg mL^{-1}). After analyzing and determining the concentrations for each sample, a mean recovery value was calculated for each level following the equation:

$$\% \ Recovery = \frac{observed\ concentration}{fortification\ level} \times 100$$

As for the accuracy and stability of FTD and NXZ in solution, two individual stock solutions of each were prepared and immediately analyzed to guarantee the intended initial concentrations were present. From these stock solutions, samples were taken daily for a period of eight weeks to verify the stability. Also, the standard solutions necessary for fortification and calibration curves were also prepared, sampled and analyzed simultaneously with the stock solutions. Temperature was also tested at 25° C to certify that the analytes would not undergo degradation while being analyzed.

Linearity, within-day repeatability, within-laboratory reproducibility, limit of decision (CCα) and detection capability (CCβ) were calculated with data obtained through the analyses of 3 calibration curves performed in 3 different days. Each calibration curve was constructed with 5 concentration levels, namely 0, 10, 15, 20 and 25 µg kg^{-1}, and 6 replicates of levels 10, 15 and 20 µg kg^{-1} were made.

To assess within-day repeatability (intra-day precision) three sets (three concentration levels), each with six fortified samples, were prepared and analyzed. The resulting mean concentration was used to determine the relative standard deviation, expressed as CV, with *n*= 6 x 3. To establish the within-laboratory reproducibility (inter-day precision) the same procedure was repeated for three days, with fresh sets of fortified samples and solutions prepared daily. The CV was calculated from the mean concentration between days.

The decision limit (CCα) was calculated according to the following equation

$$CC\alpha = \frac{2{,}33\ \sigma N}{\varepsilon}$$

where $\sigma_N\sigma\sigma\sigma\sigma$ is the standard deviation of the within-laboratory reproducibility and ε the mean slope of the calibration curve (EC Regulation 2002/657, Antignac et al., 2003).

As for the detection capability (CCβ), the value is given by

$$CC\beta = CC\alpha + 1.64\ (\sigma_N)$$

Standard calibration curves in the same range as the fortified samples were also prepared each day. The coefficient of correlation (R^2) to assess linearity was established by plotting the response of the corresponding FTD/IS peak area ratio against the FTD/IS concentration.

3. Results and Discussion

3.1 Method Development

The development of methods to monitor the presence of nitrofurans in environmental matrices is essential. The metabolism of the parent drugs in animals has been described by several authors but the behavior of these compounds in organisms such as macroalgae is far from being well studied. There is a lack of knowledge on whether there are metabolic routes that act in similar ways as in animals and even the

effect of light in such simple morphologies is of major importance (Edhlund et al., 2006). For this reason it was important to be able to determine FTD instead of its resulting metabolites, the most toxic being AMOZ.

The preparation of samples for analysis routinely requires a clean-up step to remove most interfering agents and improve analyte recovery. Usually this is achieved with solid-phase extraction (SPE) through the use of appropriate cartridges. Moreover, it is accepted that SPE produces the most efficient method of extraction. However, in situations where there are a high number of samples to be analyzed, the balance between efficiency and costs must be taken into account. After comparing the extracts obtained for the present matrix with and without SPE by C_{18} this step was considered unnecessary as clean extracts were obtained in both cases. Thus, without the use of cartridges the costs were significantly reduced.

The composition of the mobile phase was selected based on the least interference with LC-MS/MS analysis (Barbosa et al., 2007). Due to the sensitivity of FTD under acidic conditions, which accelerate its conversion into other metabolites (Edhlund et al., 2006), a neutral volatile salt solution was chosen as additive. Ammonium acetate and ammonium formate dissolved in methanol (w/v) were initially tested but the results favored the use of the latter at a concentration of 1mM. Moreover, the use of this solution with a mildly acidic pH (4) allowed for positive ion mode detection.

NXZ, also a nitrofuran antibiotic, has been selected in a previous study for use as internal standard due to its chromatographic behavior in LC-MS determination (Barbosa et al., 2007).

3.2 Validation

In order to fully validate the methodology presented in this work the necessary performance criteria were established according to the Commission Decision 2002/657/EC since, as mentioned previously, specific legislation for environmental matrices is currently inexistent. This regulation classifies two types of methods according to the performance characteristics analyzed: qualitative and quantitative. In

this work the latter was chosen which requires the determination of the specificity, recovery, stability, precision (here measured as reproducibility and repeatability), linearity, decision limit (CCα) and detection capability (CCβ).

Specificity assays are necessary to clearly and unequivocally identify the analyte among related substances such as metabolites, degradation products and other potential interferences present. Here, this was achieved with the analyses of 20 different blank samples chromatograms where no interfering peaks of eligible size were observed at the retention time windows for the two FTD MRM transitions controlled. Figure 2 presents chromatograms for a spiked (A) and a blank sample (B).

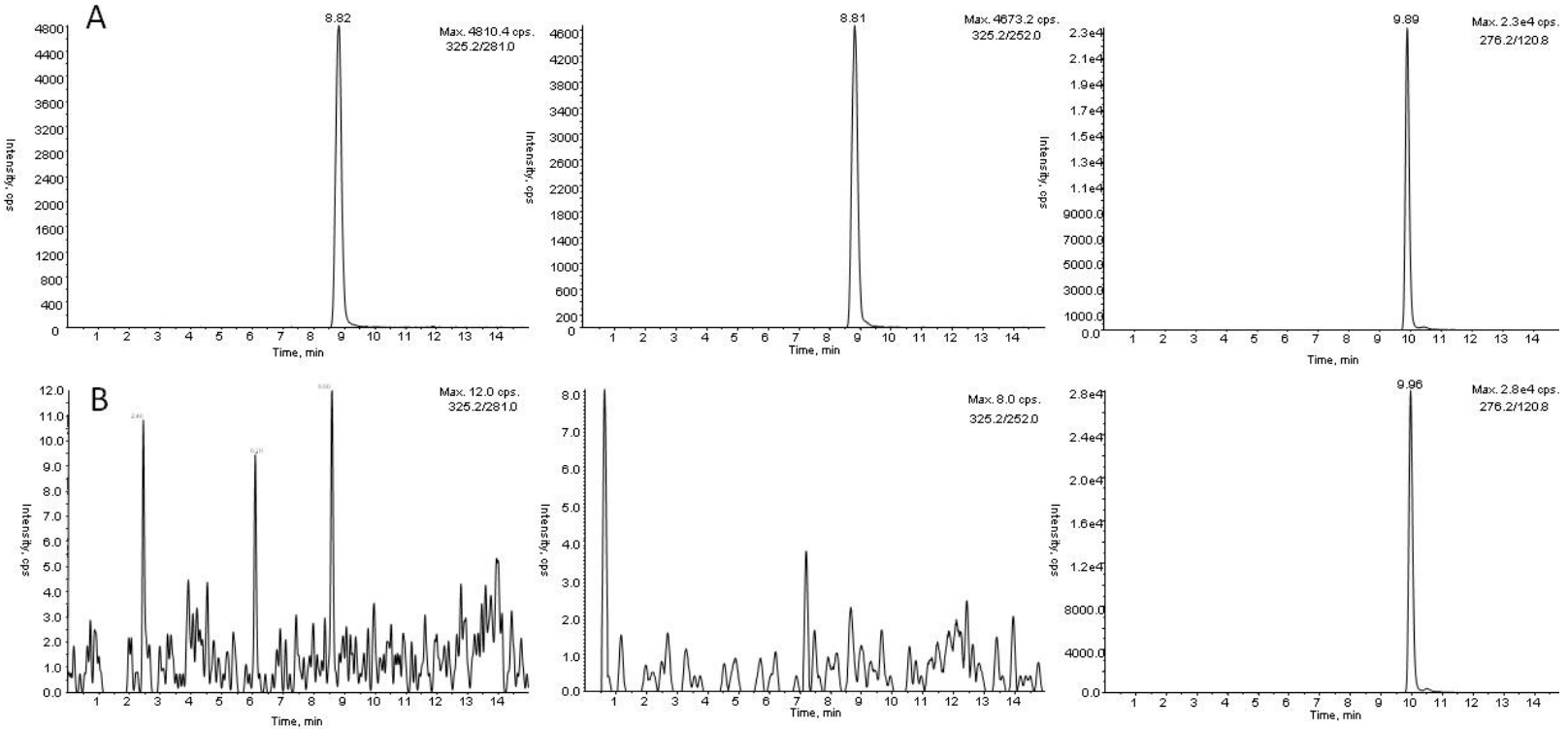


Figure 2. LC-MS/MS chromatograms of furaltadone (325>281 and 325>252) and internal standard nifuroxazide (276>120) for (A) a spiked sample from the calibration curve and (B) a blank sample.

Since currently there is no certified reference material (CRM) for the present matrix required to establish trueness, recovery was measured instead following the Directive guidelines, with 18 samples being fortified at three concentrations levels (10, 25 and 20 μg kg^{-1} and IS (10 μg kg^{-1}), as mentioned previously. After analyzing and determining the concentrations for each sample, mean recovery values were calculated for each level and are listed in Table 1.

Table 1
Results of recovery (%) and precision (% CV)

Concentration (μg/kg)	Recovery	Repeatability (intra-day precision)	Reproducibility (inter-day precision)
10	88.9	17.1	25.3
15	95.5	15.3	28.2
20	90.6	20.5	27.9

As for the stabilities of FTD and NXZ in solution, no differences between measurements were found, with concentrations of the stock solutions showing to be stable for 8 weeks when stored at -20°C and in the case of FTD, in the dark as it is known it suffers photodegradation. The working and calibration standard solutions were stable for at least four weeks, stored at 4°C. Regarding temperature, at 25°C the analyte was not degraded meaning that for analyses running for more than 24 h it was not necessary to adjust the temperature.

Another parameter required is precision, which in this case was determined as within-day repeatability (intra-day precision) and within-laboratory reproducibility (inter-day precision). The values assessed were within the accepted range according to the Horwitz equation meaning the method presented good repeatability and reproducibility. CV values for intra and inter-day precisions are listed in Table 1.

The linearity of the analytical method was proven at five concentration levels with R^2 of the three calibration curves always above 0.99.

Finally, according to the definition of the stated legislation, CCβ refers to the *smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of* β. However, in the case of furaltadone and other compounds to which there are no permitted limits, it is defined by the *lowest concentration at which a method is able to detect truly contaminated samples with a statistical certainty of 1-* β. Following this definition, both CCα and CCβ were calculated based on 18 fortified samples at different concentration levels and the values obtained were respectively 5.57 and 10.97 ug kg^{-1}, which are within the requested range.

3.3 Exposure assay

In addition to the method development with spiked samples, macroalgae from the exposure assay were also tested [Leston et al., 2011b). The chromatogram for the exposed algae is shown in Figure 3. FTD was unambiguously identified and quantified in *U. lactuca*. The results in terms of FTD uptake were however different from what was initially predicted. After 16 h of exposure the internal concentrations taken up from the water reached 9 µg g^{-1} for both conditions tested. It was expected that the concentration sequestered in the therapeutic treatment would be twice the amount taken up in the prophylactic assay. These findings indicate that although the uptake seems not to reflect the amounts of FTD present in the water column, *U. lactuca* is suitable to be used as an indicator for its presence. Since it is known this nitrofuran antibiotic is easily photodegraded its detection on macroalgae shows it has been released into the ecosystems shortly after macrophyte sampling. Moreover, as primary producers, represent an important link in the food web through the consumption by organisms in higher trophic levels, translated in a likely risk of biomagnification.

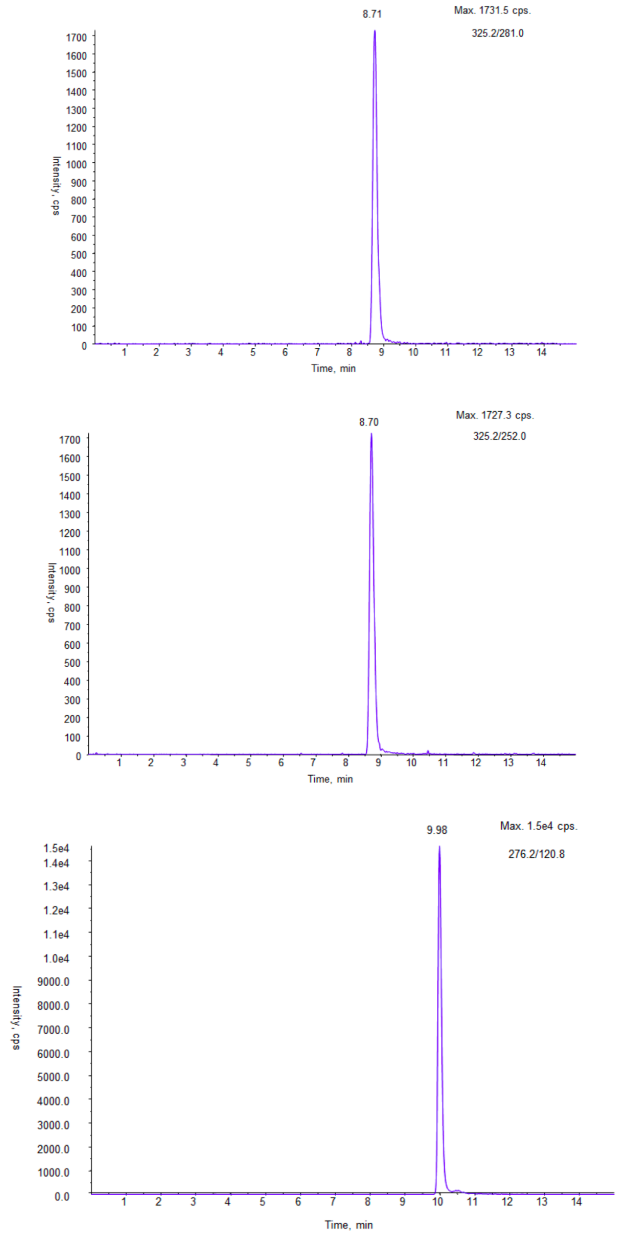


Figure 3 - LC-MS/MS chromatograms of FTD A) 325>281 transition, B) 325>252 transition and the internal standard NXZ C) 276>120 transition, for *Ulva lactuca* exposed to the prophylactic treatment (16 µg kg^{-1}).

4. Conclusion

A LC-MS/MS method was developed to detect and quantify the presence of FTD residues in macroalgae. The optimized procedure possesses a simple and efficient extraction without the need for a SPE step, thus reducing the handling time and costs and increasing the number of samples processed at one time. The recoveries were higher than 88% for all the concentrations tested and the limits of quantification low enough to detect the presence of FTD in environmental samples, where it is expected to be lower than in other matrices. The present method was applied to experimental samples with success and could be considered for use as a tool in environmental contamination assessment of FTD.

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Evaluation of furaltadone photodegradation in natural seawater by HPLC-UV

Abstract

The focus on the presence of emerging contaminants in the environment, has led to the need to develop appropriate and effective methodologies for their determination. Furaltadone (FTD) is a nitrofuran antibiotic banned from use in the European Union but still reported to be illegally used in animal farming. This study presents a simple and direct methodology to determine FTD in natural seawater through HPLC-UV detection. The developed method was fully validated in terms of linearity, accuracy, limits of detection and quantification (LOD and LOQ), repeatability and reproducibility. The mean recoveries ranged between 76-96%. Together with the analytical methodology an experimental trial was established to evaluate the photodegradation of FTD in seawater under natural conditions. Results show that although it degrades continuously it is still present in significant concentrations after 120h, representing a threat to the surrounding aquatic environment.

1. Introduction

In the last decades, environmental pollution has become an increasingly important issue in risk analysis along with a shift in focus from the classic to emerging contaminants, (Hansen, 2007) referring to compounds which "*are not currently covered by existing regulations of water quality, have not been previously studied and are thought to be potential threats to environmental ecosystems and human health and safety*" (Pérez and Barceló, 2007; Kleter et al., 2009). This designation covers several chemicals used in large quantities with a tendency towards increase as a result of the world population's growth. Antibiotics are a relevant group of substances designed to alter specific biochemical pathways, (Fent et al., 2006; Leston et al., 2011) and recently identified as a priority risk group (Hernando, et al., 2006; Watkinson et al., 2009). The properties that make them effective are also the same that can be harmful when released into the different environmental compartments (Ternes, 2001; Crane et al., 2006; Buchberger, 2007) following diverse routes (Boxall et al., 2004; Fent et al., 2006; Kümmerer, 2009) and their fate will be dictated by their physical and chemical characteristics (Fent et al., 2006; Pérez and Barceló, 2007). With the continuous studies on long term effects of antibiotics in human and animal health, together with the development of more sensitive analytical methodologies, a considerable number of substances have been deemed unsafe and their administration regulated and prohibited within the European Union and other countries. Furaltadone (FTD) is a nitrofuran antibiotic with broad spectrum activity included in this category but despite the European Union ban in 1995 (EC Regulation 1442/1995; EC Regulation 37/2010) illegal administrations to food producing animals are still reported, most commonly mixed in feed or in water (Samanidou and Evaggelopoulou, 2007, Vass, 2008). With an octanol/water partition coefficient (K_{ow}) of 0.2 (Moffat et al., 2004) it presents lipophilic characteristics which point to the probability of leaching from soil and sediments to ground and surface waters, constituting a threat to the aquatic compartment (Boxall et al., 2004; Crane et al., 2006; Mompelat et al., 2009; Leston et al., 2011a). For this reason, the monitoring of such compounds must be primarily directed to surface waters

rather than sediments and to the parent compounds itself, since in water metabolic pathways are not expected.

Aquaculture is a growing production sector worldwide (Leston et al., 2011a) where in some cases old salt farms are being transformed into fishponds using tidal fluxes for water renewal. This artisanal way increases the potential risks of contamination to the surrounding aquatic environment, as the purification systems are inexistent. In order to monitor the possible use of illegal substances in these systems, the present study describes a methodology for the quantification of FTD in natural seawater based on high performance liquid chromatography with UV-detection (HPLC-UV) together with an overview on the compound's photodegradation.

2. Experimental

2.1 Reagents

Furaltadone (Fig. 1) analytical standard was acquired from Sigma-Aldrich (St. Louis, USA) while methanol, acetonitrile and glacial acetic acid were obtained from Merck (Darmstadt, Germany). With the exception of the solvents used in the mobile phase, which were HPLC grade, all other reagents were of analytical grade.

Water was purified and obtained daily from a Milli-Q System (Millipore, Bedford, MA, USA).

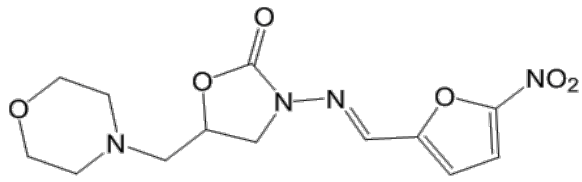

Figure 1. Chemical structure of furaltadone.

2.2 Solutions

A stock solution was prepared by dissolving FTD in methanol to a final concentration of 1 mg mL^{-1}. Calibration standards (ranging from 1 to 50 µg mL^{-1}) were assembled from the stock solution by adding specific volumes to purified water and kept in solvent washed amber glass vials. Blanks were used to detect potential effects of field and laboratory handling, consisting respectively of sampled field water and purified water.

2.3 Sampling

Seawater was collected during low tide at a local beach in the Portuguese central region (Buarcos, Portugal) in 1 L solvent washed amber glass bottles. Before collection, bottles were rinsed with site water after which samples were taken and placed in refrigerated coolers for transportation. Salinity and temperature were measured on location and again upon arrival to the laboratory.

Water was filtered through 0.45 µm glass fiber filters (Millipore, Bedford, MA, USA) for the experimental setup to establish the natural degradation of FTD in seawater and through 0.22 µm filters for HPLC determination.

2.3 FTD Degradation Assay

To assess the degradation of FTD in seawater under simulated natural conditions, acid washed Erlenmeyers were filled with 250 mL of filtered seawater (salinity 35) to which was added the appropriate volume of FTD solution to obtain concentrations of 16 µg mL^{-1} and 32 µg mL^{-1}, simulating hypothetical prophylactic and therapeutic treatments (selected based on indication of veterinary experts). The containers were then placed in an orbital shaker, under UV lights with intensity of 80 µmol photons m^{-2} s^{-1}, temperature set at 25°C and a photoperiod of 14:10h LD. Water samples were collected with sterile syringes at the following times: 0, 1, 2, 5, 8, 12, 16, 24h and afterwards daily, for a week. Before injection samples were filtered through 0.22 µm filters. Three replicates were used for both concentrations at each time.

2.4 Instrumental Analysis

Samples were analyzed with a Dionex HPLC system equipped with degasser, quaternary pump (P580), autosampler (ASI-100) and column thermostat coupled with a diode array detector (UVD340U). The separation of the target compound was achieved with a 250-4 LiChrospher 100 RP-18 column (Merck, Darmstadt, Germany) and a NewGuard C18 pre-column (PerkinElmer, Norwalk, USA), both with 5 µm Ø size. An isocratic gradient was applied with a vacuum-filtered mobile phase (14 mM) consisting of ammonium acetate (70%) and acetonitrile (30%), with pH set at 4.6 with addition of glacial acetic acid. 20 µL of sample were injected, flowing at a rate of 1.2 mL min^{-1} for a total run time of 10 min.

The most suitable detection wavelength for elution monitoring was selected by scanning a solution of FTD in methanol from 200 to 400 nm. The maximum absorption was achieved at 356 nm.

For quantification, a first-order calibration curve was determined and the concentrations of the target analyte were calculated using the commercial software Chromeleon Dionex 6.70 (Chromatography Data System Chromeleon® PA, Dionex)

2.5 Validation

For the validation of the method presented a quantitative approach following the guidelines of the Commission Regulation 2002/657 was preferred to establish the performance parameters. The limit of detection (LOD) and the limit of quantification (LOQ) together with recovery, repeatability and within laboratory reproducibility were set in a simultaneous experiment. Blank samples were fortified in triplicate at 1, 1.5 and 2 times the concentration. For within-lab reproducibility and repeatability the procedure was repeated twice over time and with different operators. The results (*Yi*) were plotted against the concentration (Xi) and given by a linear regression equation ($Yi = a + bXi$).

3. Results and Discussion

3.1 Method development and validation

The existing analytical methodologies to the survey of furaltadone and related nitrofurans are directed to food-related matrices with higher complexity such as meat (Cooper et al., 2005), eggs (McCracken et al., 2007), milk (Galeano-Díaz et al., 1997) and honey (Tribalat et al., 2006) among others. Specific procedures to determine FTD in seawater are inexistent and in order to achieve the objectives of the present work it was necessary to develop a suitable and efficient method, which also allowed processing a large number of samples.

Most analytical methods applied to the quantification of antibiotics in environmental waters include a pre-concentration step since the levels normally found are usually very low. Solid phase extraction (SPE) is the most commonly used due to its efficiency and resulting cleaner extracts. However, when dealing with a large number of samples it can become time-consuming and for this reason a direct injection method was tested with fortified water samples. Nonetheless, SPE was also applied to the same samples through C_{18} cartridges and then compared with direct injection. Although the chromatograms resulting from SPE extracts were in fact cleaner, were not significantly different from the chromatographic separation presented in this study, which showed enough resolution to isolate the analyte. The parameters calculated for validation showed that under the developed method accurate and repeatable measurements were obtained, with LOD and LOQ values of 0.284 µg mL^{-1} and 0.562 µg mL^{-1} respectively. Linearity was assessed by plotting the FTD peak areas against calibration samples (ranging from 1 to 50 µg mL^{-1}), with a resulting coefficient of correlation of 0.999. Repeatability, reproducibility and recovery values listed in Table 1.

Table 1
Results of recovery (%) and precision (% RSD)

Concentration (µg/kg)	Recovery	Repeatability (intra-day precision)	Reproducibility (inter-day precision)
1	76.6	9.0	11.5
1.5	89.8	3.7	5.7
2	96.1	12.4	21.0

Previous studies by Edhlund and colleagues (2006) on the photochemistry of nitrofurans in water showed that this class of antibiotics undergoes photodegradation, with direct photolysis being the dominant process. This reaction leads to the formation of a stationary state between *syn* and *anti* isomers during the first minutes. Figure 2 shows the chromatograms of FTD in the dark (A) and under natural light (B), extracted with HPLC-UV. By comparing both situations it became clear that in the presence of light, degradation was observed resulting in the presence of a smaller peak near the parent peak. When the quantification was performed in the dark, the analyte remained unaltered. To ensure the correct quantifications of the photodegradation, all the procedures were performed in the dark and standards were prepared freshly right before analyses.

To assert the effect of temperature on the behavior of FTD in natural seawater, two solutions were kept for a week in the dark, at room temperature (25°C) and refrigerated (4°C) with daily analyses showing no degradation during this period. Thus, for the conditions tested temperature does not influence the concentration of FTD.

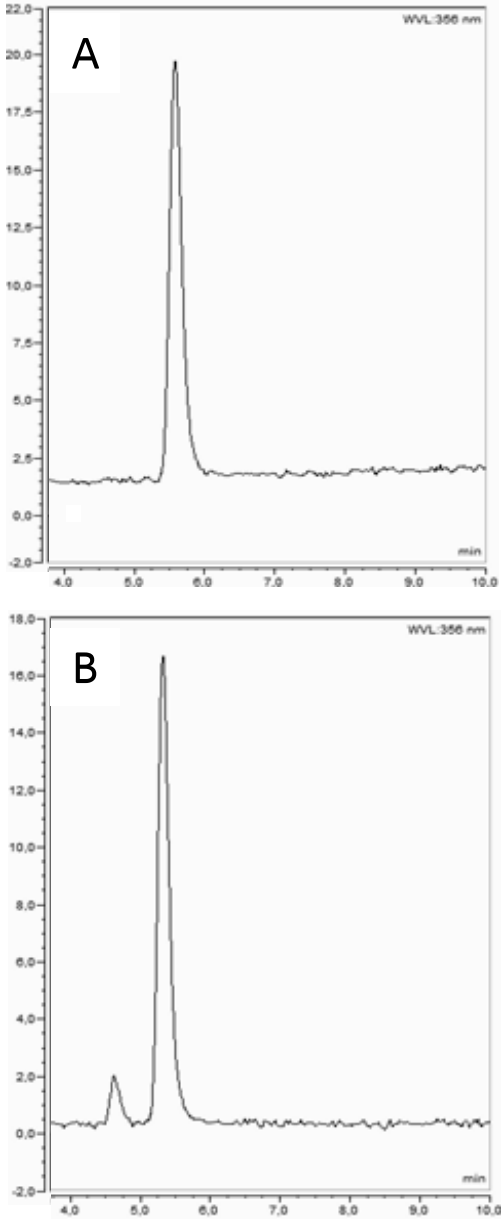

Figure 2. Chromatograms of FTD from HPLC-UV analyses A) in the dark and B) under natural light.

3.2 Photodegradation assay

To guarantee a precise evaluation of the degradation in seawater all samples were kept from light after collection thus inhibiting further reactions. The quantification was also light protected and conducted in amber glass vials. Since this assay was carried under light the quantification of FTD was done taking into account the second peak on the chromatograms. Figure 3 depicts the variation in concentration of FTD in seawater (expressed in $\mu g\ mL^{-1}$) as a function of time for both concentrations. From the graphical analysis it is possible to see that the starting concentration influences greatly the degradation of FTD under the same conditions, shown by the time required for the concentration to reach half the initial values. At the highest concentration it took 72h to attain this level whereas at the lowest only 8 hours were necessary. Nonetheless, for both situations results show that FTD in seawater degrades continuously but it is still present in significant concentrations after 120h. In cases of illegal use, effluent outfalls released in this timeline will still carry large quantities of FTD into the environment, increasing the risk of being accumulated in the trophic webs. Based on the present study, a bioaccumulation experiment with the green macroalgae *U. lactuca* was conducted with results showing FTD can be taken up by primary producers and at the highest concentration induces algal decay (for further information see Leston et al., 2011b).

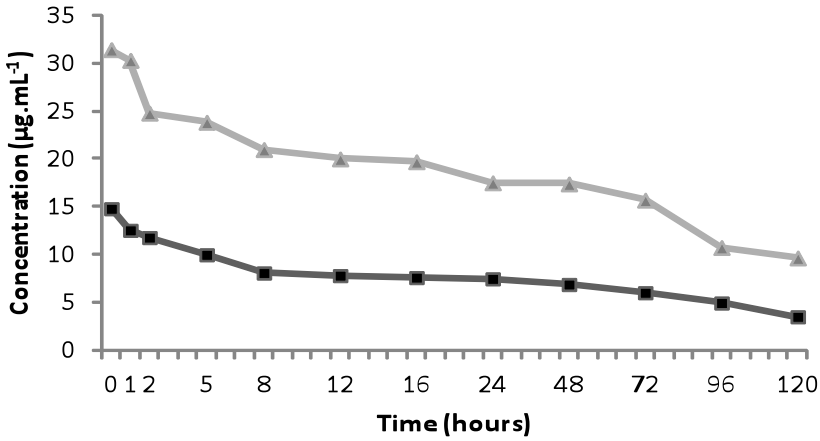

Figure 3. Degradation of FTD in seawater under simulated natural conditions, where (■) depicts an initial concentration of 16 µg mL^{-1} and (▲) a concentration of 32 µg mL^{-1}. Data represent mean values + SE of three independent replicates.

4. Conclusions

In this study, a simple and direct methodology to determine the antibiotic FTD in seawater is presented, allowing for a large number of samples to be handled at the same time. Moreover it can be successfully applied to the survey of illegal administration in aquaculture systems, both in pond and effluent water samples. Also, the degradation of FTD does not seem to be influenced by temperature and degradation through photolysis is a process that occurs continuously but not as fast as to prevent environmental contamination and its potential impact in the ecosystem.

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The effects of the nitrofuran furaltadone on *Ulva lactuca*

Abstract

The use of pharmaceuticals in the food production industry as prophylatic and therapeutic agents is necessary to promote animal health, but may entail significant consequences to natural ecosystems, especially in the cases of overdosing and use of banned pharmaceuticals. The vast effects that antibiotics released into the environment have on non-target organisms are already under the scope of researchers but little attention has been given to primary producers such as macroalgae. The present study assessed furaltadone's, an antibacterial agent illegally used for veterinary purposes, uptake capacity by *Ulva lactuca* and its effect in the growth of this cosmopolitan macroalgae. Differences in macroalgal growth were shown when submitted to prophylactic and therapeutic concentrations of furaltadone in the water (16 and 32 µg mL^{-1}, respectively). The therapeutic concentration caused higher growth impairment than the prophylactic treatment did, with 87.5 and 58% reductions respectively. Furthermore, together with data collected from the accumulation assays, with values of internal concentrations as high as 18.84 µg g^{-1} WW, suggest that the macroalgae *U. lactuca* should be included in field surveys as a biomonitor for the detection of nitrofurans.

1. Introduction

Environmental chemical contamination can occur as a result of human activities, carrying with it potential adverse ecological impacts (Olette et al., 2008). To assess such effects it becomes necessary to understand which chemical stressors are present and their likelihood to impact exposed receptors. A vast array of veterinary medicinal drugs (VMD) is used in animal production serving several purposes from growth-promotion to the treatment of diseases (Stolker et al. 2007). In the last decades the avian industry grew considerably and even though there have been significant improvements and a higher sanitary control, there is still a concern with the leakage of VMD to the environment (Boyd, 2003; Halling-Sørensen et al., 1998; Lalumera et al., 2004; Wollenberger et al., 2000). Antibiotics are among these potentially harmful substances since they are especially tailored to be biologically active (Edhlund et al., 2006; Halling-Sørensen et al., 1998; Kümmerer, 2009; Wollenberger et al., 2000). Among these is furaltadone (FTD), a nitrofuran derivative and a highly effective chemotherapeutic drug, used as an antibacterial agent to fight common bacterial and protozoan infections, which acts by inhibiting microbial enzymes involved in the carbohydrate metabolism (Balizs and Hewitt, 2003; Vass et al., 2008). It is rapidly metabolized resulting in very stable metabolites that have been linked with carcinogenic, mutagenic and teratogenic effects in humans (Barbosa et al., 2007; Bartel et al., 2009; Chadfield and Hinton, 2003; Jager et al., 1997). For this reason the use of furaltadone together with 3 other nitrofurans was banned from use in livestock in the EU in 2004 (Barbosa et al., 2007; EC Regulation 37/2010; Vass et al., 2008; Verdon et al., 2007). In spite of this, since it is very inexpensive and has a high success rate, in many countries it is illegally used as it can be found on the blackmarket (Report of a Joint FAO/OIE/WHO Expert Consultation on Antimicrobial Use in Aquaculture and Antimicrobial Resistance, 2006; Vass et al., 2008). According to data predicted by a model (unpublished), it will likely be found in an eventual discharge up to 50 $\mu g\ mL^{-1}$, corresponding to the commonly used therapeutic dosage (prophylactic concentration: 25 $\mu g\ mL^{-1}$).

The prediction of distribution patterns of a given chemical through the different compartments of an ecosystem is often difficult, thus the pharmacokinetics and effects on individual groups of organisms constituting the system is often preferred (McCarty et al., 2004; Walker et al., 2006), despite the importance of assessing indirect and mixture effects (Geiszinger et al., 2009). Macroalgae 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 and a source of food and habitat for aquatic biota (Conti and Cecchetti, 2003; Melville and Pulkownik, 2006; Torres et al., 2008).

In aquatic systems macroalgae are continuously exposed to the contaminants present and as they represent a very large biomass they can act as a sink for xenobiotics (Pflugmacher et al., 1999; Sandermann, 1992; Walker et al., 2006) becoming a gateway for higher trophic levels (Walker et al., 2006). They also possess characteristics that allow them to be considered as bioindicators as they are relatively sedentary, easy to identify and with a wide geographic distribution (Melville and Pulkownik, 2006, Torres et al., 2008). Nevertheless, little information is available on bioconcentration and effects of xenobiotics (including antibiotics) on macroalgae (Bierkens et al., 1998; Carafa et al., 2007; La Barre et al., 2004; Macrì and Sbardella, 1984; Pflugmacher et al., 1999; Lai et al., 2008; Torres et al., 2008). In order to improve the knowledge on the consequences of the presence of antibiotics in estuarine macroalgae, *Ulva lactuca* was selected. The aims of this research were (i) to investigate the behavior of furaltadone in seawater, (ii) to ascertain the capacity of the macroalgae to uptake nitrofuran from the water, (iii) to assess the influence on growth of *U. lactuca* at two concentrations simulating the prophylactic and therapeutic use, (iv) to investigate the probability of the bioaccumulation in the trophic web and (v) to evaluate the suitability of using macroalgae as a bioindicator for the presence of nitrofurans.

2. Experimental

2.1 Reagents

Furaltadone (Fig. 1) analytical standard and the internal standard nifuroxazide were obtained from Sigma-Aldrich (St. Louis, USA). Methanol, ethyl acetate, acetonitrile and N-hexan were purchased from Merck (Darmstadt, Germany). All chemicals were of analytical-reagent grade except solvents used in mobile phase that were HPLC grade.

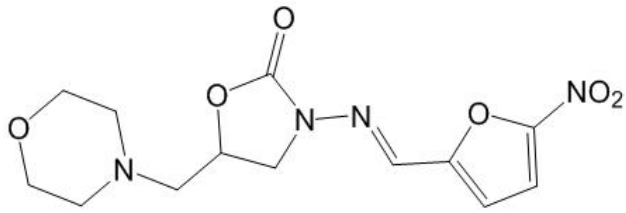

Figure 1. Structure of furaltadone [5-morpholinomethyl-3-(5-nitrofurfurylidereamino)-2-oxazolidinone].

2.2 Macroalgae

The green macroalgae *Ulva lactuca* was collected during low tide in the Mondego estuary (Portugal, 40°80`N, 8°50`W). After being washed and rinsed to remove possible epibionts present on the surface, macroalgae were placed in a refrigerated container along with seawater and transported to the laboratory. After a thorough inspection of the fronds to assure the absence of organisms, 2 kg WW of *U. lactuca* were placed in 40 L glass fiber tanks, which had been previously filled with filtered natural seawater (pore Ø 0.45 µm) and Provasoli's Enriched Medium (PES; L. Provasoli, 1963) modified by Bold and Wynne, 1978 and acclimated for 48h under 80 µmol photons m^{-2} s^{-1} of white fluorescent light, 14:10-h LD photoperiod, 25°C and 35 psu. Macroalgae were maintained in the same conditions for 4 weeks prior to the beginning of the experiment. Natural seawater was collected 1 week before, filtered through sterile Ø 0.45 µm filters and stored at 4°C before use.

2.3 Experimental design

The same conditions set during acclimation were kept during all experiments but aeration was replaced by constant and gentle horizontal stir. The day prior to the start of the trial, glass containers were filled with 250 mL of filtered seawater and 5 mL of PES, placed on orbital shakers and left to acclimate for 24h. Just before beginning, healthy algal disks with Ø 5 cm (aprox 20 cm^2) were cut and a stock solution of FTD was prepared in methanol. The concentrations were then adjusted from the stock solution and added to the flasks to obtain two test concentrations: 16 µg mL^{-1} (hypothetical prophylactic concentration) and 32 µg mL^{-1} (hypothetical therapeutic concentration) designated as groups P and T respectively. These concentrations correspond to 60% of the concentrations used for prophylaxis (25 µg mL^{-1}) and therapy (50 µg mL^{-1}). Each group had 3 replicates for each sampling time and each replicate contained 3 algal disks. Additionally 4 control groups designated as A, B, C and D were prepared. Control A followed the same preparation as for P and T without addition of FTD, to establish the natural growth of *U. lactuca*. Control B was used to verify the natural degradation of the nitrofuran and consisted of seawater with antibiotic for both concentrations P and T. Control C was set to exclude the possible effects of methanol on the macroalgae and the same volumes used in P and T were added to the respective flasks [1.6 and 3.2% (v/v) respectively]. Finally, since the solution has a very strong yellow color that could theoretically interfere with growth, two solutions with yellow dye were prepared with the same wavelengths, corresponding to Control D. To avoid evaporation but still allow gas exchange, all containers were loosely covered with glass lids.

Sampling times were as follows: 0, 1, 2, 5, 8, 12, 16, 24, 48, 72, 96 and 120h.

2.3.1 Water

At each sampling time, after algae had been removed, temperature, salinity and pH were measured, water was filtered through glass fiber filters (Ø 0.22 mm) and immediately frozen at -20°C until extraction and kept from light to prevent photodegradation.

To determine the concentration of FTD present in the water, samples were filtered through 0.22 µm pore filters and transferred into amber vials equipped with inserts for high-performance liquid chromatography with UV detection (HPLC-UV) analysis. The equipment consisted of a Dionex HPLC-system with a degasser, quaternary pump (P580), autosampler (ASI-100), column thermostat and a diode array detector (UVD340U). The columns used were a New Guard Perkin Elmer C18 pre-column and a Merck RT 250-4 Lichrospher 100 RP-18 column, both with 5 µm particle size. The mobile phase consisted of ammonium acetate and acetonitrile delivered isocratically. Analyses were performed in the dark to prevent possible degradation due to light.

2.3.2 Furaltadone Uptake

To determine the concentration of FTD taken up from the water column, macroalgae were analyzed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) using a method adapted from McCracken and Kennedy (2007). After removal from the water, algae were paper-dried, weighed and stored at -20°C, until further analysis. To extract furaltadone's residues, samples were minced individually and placed in centrifuge tubes to which 40 µL of nifuroxazide and 10 mL of ethyl acetate were added. After centrifugation, the supernatant was collected and evaporated, followed by addition of 10 mL of acetonitrile and 3 mL of N-hexan. Samples were then left to stand and N-hexan was discarded. The sample was again evaporated to dryness under a nitrogen stream and the resulting residue reconstituted in 500 µL of methanol: water solution. Samples were transferred to amber vials and placed in the LC autosampler. The equipment was composed of an Agilent 1100 Series HPLC system coupled to a Triple Quadrupole System Sciex API 2000 tandem mass detector with a

TurboIonSpray ion source, operating under the Sciex Analyst 1.4.1 software. The LC columns consisted of a guard column Zorbax Eclipse XDB-C8 and a Zorbax Eclipse XDB-C18 column, with 5 µm particle size.

2.3.3 Growth

To determine the effects of FTD on macroalgal growth, disks were photographed at the beginning and end of each time point. Disk areas were determined using computer-assisted software (Photoshop CS3-extended) and variations calculated. Macroalgal death was considered whenever there were clear signs of decay.

2.4 Statistics

All data were checked for normality and homoscedascity. The effect of the concentration of furaltadone on growth and the differences in uptake were assessed using the *t* test to determine significant differences between the control and the treatments. When applicable, results are presented as mean ± SE. The significance level was inferred at $p \leq 0.05$ for all statistical tests. All calculations were performed using GraphPad Prism® 5 software (Graph Pad Software, Inc.)

3. Results

3.1 Water

The nominal concentrations used in the trial were 16 µg mL^{-1} and 32 µg mL^{-1}, which were confirmed in Control B (Fig. 2).

The stability of FTD in saline aqueous solution for both concentrations was determined with control group B, so that it could be compared with the degradation in the presence of *U. lactuca* (Fig. 2). The patterns were similar with and without the algae according to the findings of Edhlund and colleagues (2006) for the natural photolysis of the compound. Nonetheless the concentrations in the water were much lower in the presence of the plant. For group P the minimum concentration at 120h was 0.22 µg mL^{-}

[1], whereas the correspondent Control B was much higher with a concentration of 3.48 µg mL^{-1}. Group T followed the same pattern decreasing to 2.11 µg mL^{-1} while the lowest control concentration was 9.64 µg mL^{-1}.

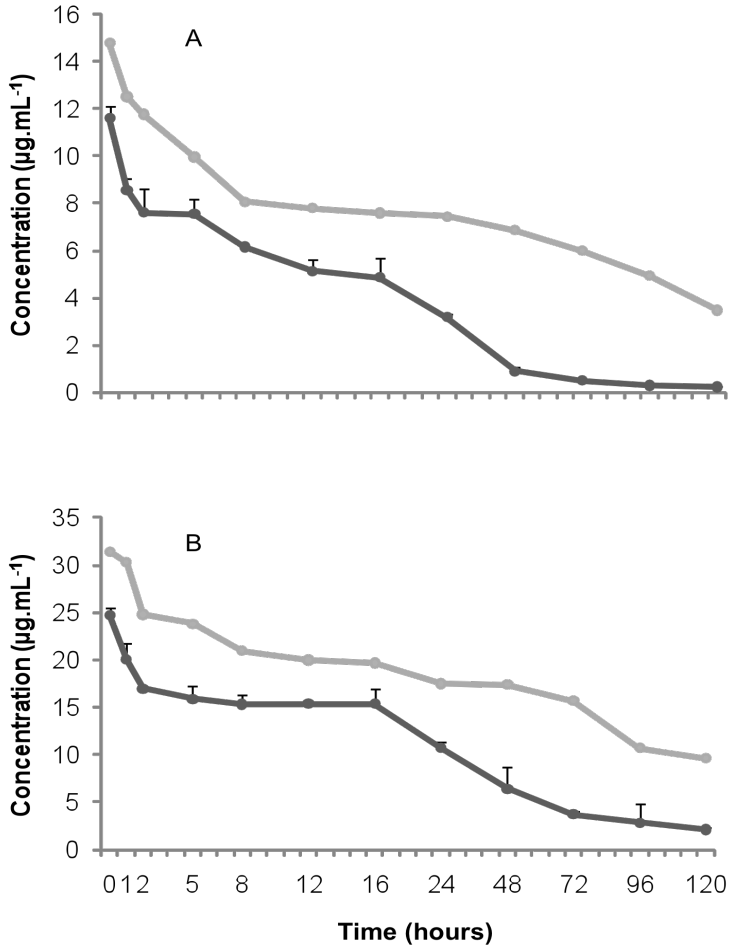

Figure 2. (A) Stability test of furaltadone in aqueous solution with (dark line) and without (grey line) the presence of *U. lactuca* at 16 µg mL^{-1}, during the course of the experiment. Data represent mean values of three independent replicates. (B) Stability test of furaltadone in aqueous solution with (dark line) and without (grey line) the presence of U. lactuca at 32 µg mL^{-1} during the course of the experiment. Data represent mean values of three independent replicates.

3.2 Furaltadone Uptake

In group P, removal of FTD from the water column was relatively slow during the first 2h after which it peaked attaining a maximum value of 18.84 μg g^{-1} WW within the first 5h. Subsequently, the uptake decreased gradually until it reached the minimum value of 0.03 μg g^{-1} WW after 72h, remaining constant thereafter (Fig. 3A).

In group T the maximum internal concentration of 12.18 μg g^{-1} WW was reached within the first hour (Fig. 4A). In the proceeding hours there was a slight decrease to concentrations around 9 μg g^{-1} WW which were constant for 12h. Following this time, concentrations decreased again to 3.68 μg g^{-1} WW after 48h which was still much higher than the values found for the same interval of time in group P. Although algal death was determined at 48h, macroalgae were still left in the water and analyzed to assess their ability to maintain internal amounts of FTD, which was confirmed. Concentrations were higher than those presented by group P until the end of the trial.

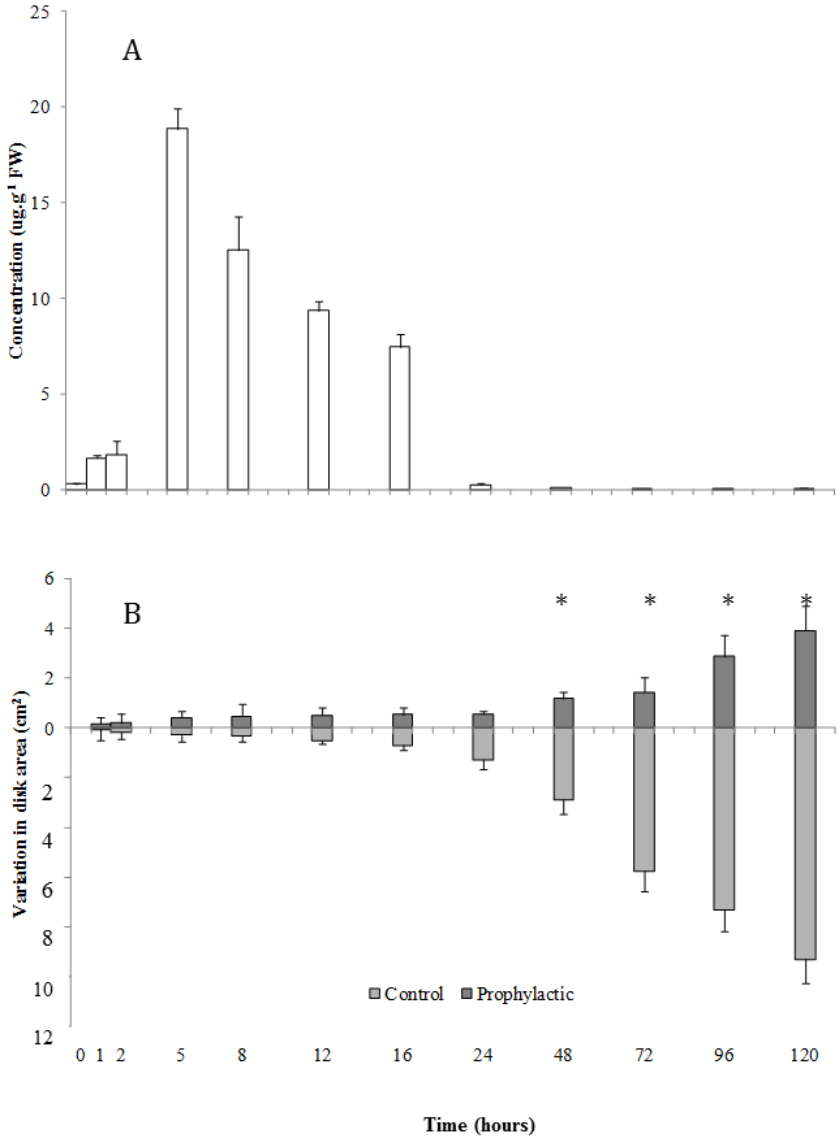

Figure 3. (A) Internal concentrations of furaltadone (µg g^{-1} FW) for *U. lactuca* at each sampling time for the prophylactic group P. Data represent mean values + SE of three independent replicates. (B) Growth measured as variation in disk area (cm^2) at each sampling time for group P, plotted against the control. Data represent mean values + SE of three independent replicates. Asterisk (*) indicates results significantly different from the control.

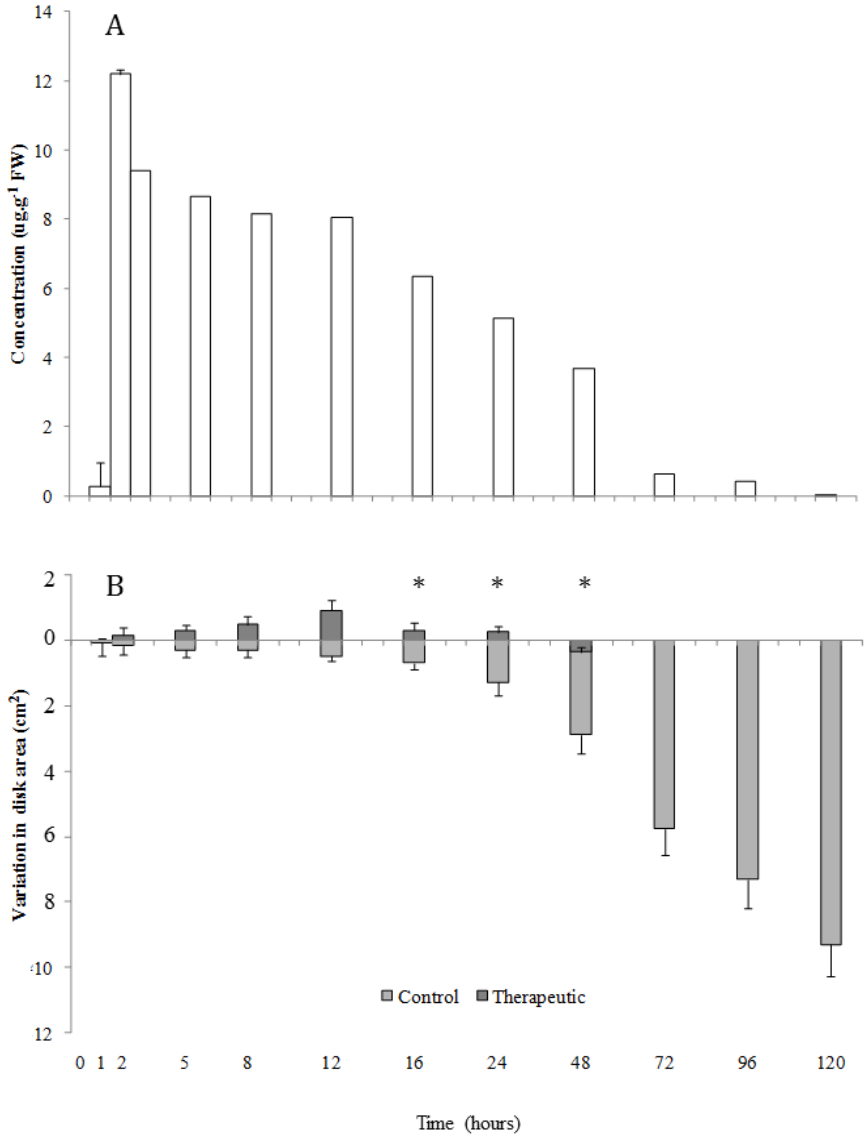

Figure 4. (A) Internal concentrations of furaltadone (μg g^{-1} FW) for *U. lactuca* at each sampling time for the therapeutic group T. Data represent mean values + SE of three independent replicates. (B) Growth measured as variation in disk area (cm^2) at each sampling time for group T, plotted against the control. Data represent mean values + SE of three independent replicates. Asterisk (*) indicates results significantly different from the control.

3.3 Growth

The results provide evidence that the growth of *U. lactuca* was significantly affected by the presence of FTD in the water. Figures 3B and 4B present the variations in growth during the 5-day exposure to the antibiotic at both concentrations and in comparison with the control. In the prophylactic treatment the variation followed the same increasing pattern as the control for the first 24h ($P>0.05$) and significantly different thereafter ($P<0.05$). Growth still continued however, although less pronounced than the control group, suggesting that the presence of FTD inhibited growth (Fig. 2A). On the contrary, group T exhibited a very distinct growth pattern. During the first 12h it showed a variation in disk area slightly higher than the control however not statistically different ($P>0.05$). After 16h a decrease in growth rates was observed, coinciding with the first signs of algal decay, becoming statistically different from the control group ($P<0.05$). Regarding growth, the test was terminated after 48h but macroalgae were still maintained for the length of the experiment to determine water and internal concentrations.

4. Discussion

Antibiotics can easily enter aquatic environments mainly through wastewater runoffs, representing a potential threat to aquatic biota. The present study focuses on the behavior of the nitrofuran furaltadone in seawater and its effects on the green macroalgae *U. lactuca*.

Based on the results from this experiment, furaltadone has a very low-persistency in seawater when compared to other drugs that can remain for weeks and even years (Lai et al., 2009). This was expected since other studies have referred the fast metabolism of the molecule itself (Edhlund et al., 2006; Pimpitak et al., 2009; Verdon et al., 2007; Zuidema et al., 2005). Moreover, Edhlund and colleagues (2006) found that FTD, as well as other nitrofuran antibiotics, is photochemically degraded to nitrofuraldehyde (NFA) in aqueous solution, predominantly via photolysis, which is due to the overlap of the absorption spectrum with the solar spectral output (Edhlund et al.,

2006). Also, according to these authors there is a pH dependence with higher degradation occurring at acidic values (Edhlund et al., 2006). However, the pH values for the experiment were in the alkaline range to ensure the optimum conditions for growth and to be in accordance with the environmental range, which means that in natural ecosystems FTD photodegradation rate is probably slower than in other mediums. In this scenario, this nitrofuran could be available for longer periods and be absorbed in higher concentrations.

The ability of the macroalgae to remove furaltadone from the water was tested at two different concentrations, 16 and 32 μg mL^{-1}. FTD is a lipophilic compound with an additional morpholino-methyl ring that enhances its water solubility (Stammati et al., 1997), presenting a K_{ow} of 0.2 (Moffat et al., 2004) indicating that it may be efficiently taken up by plants, as is the case with many other xenobiotics (Coleman et al., 1997; Walker et al., 2006). The tested green macroalgae showed an efficient removal of nitrofuran, involving absorption and/or adsorption processes. *U. lactuca* was able to take up FTD in both treatments and this uptake would account for the differences in concentration of the antibiotic in solution found between the controls and groups P and T. One important aspect to be discussed is the fact that both groups reflected the concentration present in solution. The continuous decline of FTD in both solutions was accompanied by a gradual decrease in the internal concentration. It is unclear however whether this reduction in the amount taken up is due to passive diffusion to the water or if there is a mechanism by which the nitrofuran is broken down into its metabolites, 5-methylmorfolino-3-amino-2-oxazolidinone (AMOZ) being the primary metabolite and the most toxic. According to the concept of the “green liver” introduced by Sandermann (1992), plants possess a set of enzymes very similar to those found in animals that are able to metabolize xenobiotics (Coleman et al, 1997; Sandermann, 1992, 1994; Suresh and Ravishankar, 2004; Torres et al., 2008). The process consists of three phases divided in transformation (I) mostly in cytochrome P-450 monooxygenases, conjugation (II) under the activity of glutathione S-transferases and glucosyltransferases, and compartmentation (III) either in vacuoles or in the cell

wall fraction (Coleman et al, 1997; Mitsou et al., 2006; Sandermann, 1992, 1994; Suresh and Ravishankar, 2004;). Although there are currently few studies, this detoxification mechanism was already confirmed for other xenobiotics in marine macroalgae including *U. lactuca* (Lewis et al., 2001; Mehrtens, 1994; Mitsou et al., 2006; Pflugmacher and Sandermann, 1998 a,b; Pflugmacher et al., 1999). To establish which mechanism is involved, further studies focusing on enzymatic variation are required since data on the metabolism of nitrofurans in plants are not actually available. Also, the determination of AMOZ should be conducted for both macroalgae and water.

The effects of FTD on growth were also analyzed in this study. Based on the results, *U. lactuca* showed different sensitivities to furaltadone dependent on the concentrations present. At the lowest concentration, macroalgae were able to grow although less than the control which indicates inhibition. As for the higher concentration, macroalgae died after 48h. The outcome of the trial points to severe inhibition of growth and also to lethal toxicity. Growth inhibition and toxicity are therefore dependent on the concentrations of FTD present with values in the range of 25 µg mL^{-1} being tolerated by *U. lactuca* whereas higher concentrations proved to be lethal. Similar findings were reported for the microalgae *Selenastrum capricornutum* which presented a value of EC_{90} after 96h for concentrations of furaltadone higher than 34 µg mL^{-1} (Macrì and Sbardella, 1984). Lai and colleagues used three different phenicol antibiotics to test the effects on growth of three algae and the results indicate different sensitivities dependent on the concentrations present (Lai et al., 2009). In the same study the growth rate during the first 24h was similar to the control but after that, it decreased as the concentrations increased (Lai et al., 2009), a situation very similar to what was reported in this experiment.

Antibiotics in general are considered as potential micropollutants as the concentrations in which they may occur in natural environments are very low, in the ppb range (Le Bris and Pouliquen, 2004; Halling-Sørensen et al., 1998). The concentrations used in this study were much higher (ppm range) and may occur only in exceptional conditions in the environment as a result of inadequate treatment of

effluents or neglect of the safety rules regarding wastewater disposal (Boyd, 2003; Hektoen et al., 1995; Radjenović et al., 2007; Wollenberger et al., 2000). Still, the possibility of the presence of nitrofurans in the ecosystems is very high. Since *U. lactuca* can sustain growth at nominal concentrations of 25 μg mL^{-1} it is not anticipated that it will be severely affected in natural ecosystems (Walker et al., 2006). Furthemore, since *U. lactuca* is able to lower the internal concentration of FTD it is not likely that it will be passed along the food chain in high amounts. However, if the compound is being metabolized by the macroalgae then the resulting metabolites (predominantly AMOZ) can be bioaccumulated into higher trophic levels, as their stability is much higher (Barbosa et al., 2007; Leitner et al., 2001; Verdon et al., 2007).

The potential accumulation of FTD by green macroalgae could represent a potential risk of biomagnification of nitrofurans through the trophic web, but in the present case a decrease in internal concentration in *U. lactuca* was observed. Nevertheless, future studies about the mechanism of depuration and metabolism of FTD could give a clearer picture of the environmental risk associated to this compound. As a primary producer, it represents an important link in the food web as it is consumed by organisms in higher trophic levels that in many cases represent economically important species. Moreover, the probability of metabolism of FTD to AMOZ increases with the higher levels, which represents an additional and serious risk to the trophic web. Furthermore, the fact that *U. lactuca* is a free-floating species may play an important role in transport of these substances from contaminated to non-contaminated sites.

5. Conclusions

Since green macroalgae can reflect the concentrations present in the water they can be considered as potential indicator tools for the presence of FTD in natural ecosystems. Nevertheless, the use of macroalgae as bioindicator and/or biomonitor of FTD contamination will entail more work. The mechanism of depuration and metabolism are still not clarified and the coupling of laboratory and field experiment

will be required to validate green macroalgae as a prospective tool in environmental risk assessment.

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CHAPTER II

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HPLC-UV ANALYSIS OF CHLORAMPHENICOL IN ENVIRONMENTAL MATRICES: SEAWATER AND THE MACROALGAE *ULVA LACTUCA*

THE EFFECTS OF CHLORAMPHENICOL ON THE GREEN MACROALGAE *ULVA LACTUCA*

HPLC-UV Analysis of Chloramphenicol in Environmental Matrices: Seawater and the Macroalgae *Ulva lactuca*

Abstract

Within the steadily developing food production industry to supply the needs of the world population, aquaculture represents a major sector contributing with almost half the fish consumed. Antibiotic use is well-described practice to promote animal health whether for prevention or treatment. Nonetheless, it can also cause a number of potentially harmful effects, which dictate the need to implement regulations to assure a reduction of hazards to the consumers and the environment. In the light of these regulations some drugs have been excluded from use in animal food production since the risks to human health were too high. Chloramphenicol (CAP) is a broad spectrum antibacterial included in this list but despite this, reports of illegal use still persist. More recently, the awareness that the surrounding natural ecosystems can potentially be contaminated by pharmaceuticals has risen and the extent of their effects in non-target organisms is already under the scope of researchers.

To face the demanding new challenges a methodology for the determination of CAP in seawater and in the green macroalgae *Ulva lactuca* by high performance liquid chromatography coupled to UV detection (HPLC–UV) was developed, optimized and fully validated following the guidelines of the EC Decision 2002/657.

1. Introduction

To ensure sufficient food supplies to a steadily growing world population the food production industry is continuously increasing with aquaculture playing a crucial role, representing the most rapidly developing sector. Fish farms currently contribute with almost half the total amount of fish available for consumption, relieving the pressure on wild capture in a time where estimates point to the overexploitation of natural stocks (FAO, 2010; Leston et al., 2011a). Nonetheless, although undeniably necessary, this industry is responsible for several potentially harmful effects to the surrounding environment, including water pollution, eutrophication, chemical contamination and bacterial resistance (Fernandes et al., 2001; Primavera, 2006; FAO, 2006, 2009; Baquero et al., 2009; Martínez, 2009; Leston et al., 2011a).

Veterinary medicinal drugs (VMD) are widely administered to guarantee and increase production efficiency by preventing and treating diseases and assure overall animal welfare in aquaculture (Ashley, 2007; Cañada-Cañada et al., 2009; Park and Choi, 2009). Despite the benefits, however, their use is linked to undesirable ramifications comprising the uncertainty of the effects in non-target organisms, the above mentioned bacterial resistance and the presence of residues in food products, to name a few (Holmström et al., 2003; Boxall et al., 2004; Cabello, 2006; Martínez, 2009; Park and Choi, 2009; Leston et al., 2011a). This last concern is well incorporated on the regulations on the use of VMD which reflect the issues with food security and safety by setting limits to the maximum concentration of residues legally permitted in edible tissues to prevent any harm to consumers, safeguarding also food products of environmental origins [EC Regulation 470/2009; EC Regulation 37/2010; FAO, 2004, 2005]. The risk of such residues being present is regularly monitored and if proven to be harmful to human health can ultimately result in the restriction or even revoke the authorization for administration. Consequently, very few VMD are currently approved for the prevention and treatment of diseases in aquaculture and this low availability may lead to unwanted situations of misuse of substances approved for other species and also to the clandestine administration of banned drugs (Vass et al., 2008; FAO,

2001, 2010). Chloramphenicol (CAP) is a very effective broad-spectrum antibiotic that blocks bacterial protein synthesis by binding to the 50S subunit of the 70S ribosome. However, it can also bind to mitochondrial ribosomes of mammalian cells with several studies linking its administration to blood dyscrasia, aplastic anemia and even leukemia (Rang et al., 1994; AlKhouri and Ericson, 1999; Rueff et al., 2000). For this reason, the use of CAP was prohibited within the EU since 1994, but is still reported to be illegally used (FAO, 2001; Wongtavatchai et al., 2004; Santos et al., 2005).

Monitoring aquaculture facilities for control and prevention is paramount but it is unlikely that farmers disrespecting the guidelines for safe use of VMD will be cooperative and in such cases alternative methods should be applied. The green macroalgae *Ulva lactuca* is a species commonly found at and near effluent discharge points in many fish farms, presenting a simple morphology with a thin layer cell wall, high absorption and growth rates and a wide distribution (Melville and Pulkownik, 2006). Due to these characteristics it is being studied as an indicator for the presence of several contaminants from heavy metals (Ho, 1990; Villares et al., 2001; Han et al., 2008) to antibiotics (Leston et al., 2011b, c).

A variety of analytical methods are available for the quantification of CAP in various food matrices mainly in bovine, porcine and poultry tissue samples, shrimp, fish, animal tissues in dry powdered form, honey and milk (e.g. Impens et al., 2003; Van de Riet et al., 2003; Shen and Jiang, 2005) but none to be directly applied to macroalgae. Within this view, the present work describes a methodology to determine the concentration of CAP in natural seawater and in *Ulva lactuca*, through High Pressure Liquid Chromatography with UV detection (HPLC-UV).

2. Experimental

2.1 Samples

Seawater was collected in acid washed amber glass bottles at a local beach (Buarcos, Portugal) during low tide and transported in refrigerated coolers to the

laboratory. Water was then filtered through 0.22 µm Whatman filters (Whatman GmbH, Dassel, Germany) and kept at 4°C until use.

Fronds of the green macroalgae *U. lactuca* were also assembled at the same location, acclimated for two weeks under laboratory conditions, after which time individual samples weighing 200 mg wet weight were taken and stored at -20°C.

2.2 Reagents and equipment

Chloramphenicol (Fig.1) with 99% purity was purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Methanol and acetonitrile were acquired from Panreac Química (Barcelona, Spain) whereas glacial acetic acid, ethyl acetate and N-hexane were obtained from Merck (Darmstadt, Germany). All solvents were of HPLC analytical grade and were filtered through 0.45 m Whatman nylon membrane filters (Whatman, Maidstone, USA). Water was deionized daily with a Milli-Q apparatus (Millipore, Bedford, MA, USA).

To perform the several procedures involved the following equipment was used: Agimatic-S Selecta magnetic stirrer (Barcelona, Spain), Retsh vortex mixer (Haan, Germany), Mettler Toledo PB-303-S/Fact balance (Zurich, Switzerland), Sonorex RK 100 ultrasonic bath (Berlin, Germany), Selecta Meditronic centrifuge (Barcelona, Spain), Liebisch Labortechnik evaporator (Bielefeld, Germany), Hitachi U-3900 spectrophotometer (Tokyo, Japan) and Gilson micropipettes (Villiers-le-Bel, France).

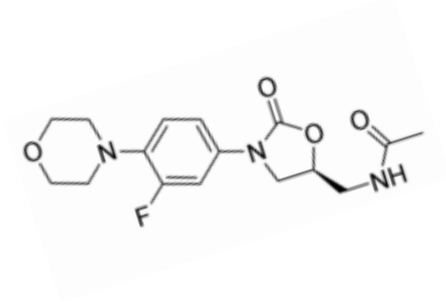

Figure 1. Chemical structure of chloramphenicol.

2.3 Standard Solutions

A stock solution (1 mg mL^{-1}) was prepared by dissolving 50.0 mg of CAP in 50.0 mL of methanol and stored in an amber flask at -20°C, protected from light. An intermediate standard solution of 50 µg mL^{-1} was prepared by dilution of the previous in methanol. Work standard solutions were prepared individually by adding specific quantities of the intermediate solution to filtered seawater. Calibration curves were established by spiking 10 mL water samples with the previous standard aqueous solutions to obtain 1, 5, 10, 20, 30, 40 and 50 µg mL^{-1}. Quality controls were also established by preparing a 50 µg mL^{-1} aqueous solution from another stock standard solution.

2.4 Experimental

2.4.1 Extraction, Clean-up and Optimization

After filtration, water samples were spiked with CAP solution to a final concentration of 25 and 50 µg mL^{-1} representing hypothetical prophylactic and therapeutic dosages respectively. Afterwards samples were vortex mixed for 1 min and then transferred to HPLC autosampler vials. Together with the samples and the calibration curve solutions, quality controls were also injected.

Macroalgae samples (200 mg) spiked with 1 mL of a 50 µg mL^{-1} CAP standard solution were thoroughly minced and placed in 15 mL amber glass centrifuge tubes. To optimize the extraction three procedures were tested from this step forward, with three solvent combinations: a) 5 mL of acetonitrile and 3 mL of N-hexane, b) 5 mL of N-hexane and c) 10 mL of ethyl acetate. After adding the solvent, samples were vortex mixed for 1 min and let to stand for 10 min. Samples were centrifuged for 10 min at 3000 rpm, the N-hexane discarded and the supernatant transferred to clean tubes followed by evaporation to dryness under a gentle nitrogen stream, at 50°C. To reconstitute the dry residues 500 µL of ultrapure water were added, vortex mixed for 30s and transferred to autosampler vials.

2.4.2 HPLC analysis

High performance liquid chromatography analyses were performed on a Gilson modular system (Gilson, Middleton, WI, USA) equipped with a pump (Gilson 321) and automatic injector (Gilson 234) coupled to a UV/Vis detector (Gilson 155). Separation was obtained with a LiChrospher 100 RP-18 column (Merck, Darmstadt, Germany) and a NewGuard C18 pre-column (PerkinElmer, Norwalk, USA) with 5 µm Ø size and equilibrated at 25°C.

To determine the appropriate wavelength for elution monitoring a 10 µg mL^{-1} CAP solution in methanol was scanned between 200 and 400 nm, with the maximum absorption recorded at 278 nm.

To establish the most suitable elution conditions, water and macroalgae samples were run both in isocratic and gradient conditions. The isocratic analysis was conducted with a mobile phase consisting of ultrapure water acidified with 0.1% glacial acetic acid (solvent A) and ultrapure water/acetonitrile (30/70, v/v) acidified with 0.1% glacial acetic acid (solvent B), both filtered and degassed, with 37.5% A and 62.5% of B. The gradient selected consisted of 65% (A) for 3 min, linearly decreasing to 25% (A) in 9 min and brought back to 65% (A) in the last 4 min, with a total run time of 12 min. The sample volume injected was 50 µL, flowing at a rate of 1.2 mL min^{-1} for a run time of 7 min with 2 min equilibration between runs.

2.5. Validation

Since legislation specific for macroalgae and other environmental matrices is currently inexistent, validation for the present method was based on the guidelines described by the EC Regulation 2002/657, which set the criteria regarding the performance of analytical methods and the interpretation of results for the monitoring of substances on live animals and animal products. Accordingly, recovery, repeatability, within-laboratory reproducibility, decision limit (CCα) and detection capability (CCβ) were determined. Calibration curves were obtained by plotting the ratios of the analyte

peak areas against the analyte concentrations, with the results being calculated by linear regression equations (*Yi*= a+ b*Xi*).

The procedure was repeated in three distinct days and with different operators for repeatability and within-lab reproducibility, expressed as coefficients of variation (CV). As for recovery, since there is no certified reference material available, it was determined by analysis of 18 blank samples fortified at 1, 1.5 and 2 μg mL^{-1}. The CCα and CCβ were also calculated from the spiked samples since for banned substances there are no permitted limits established. To guarantee the stability and accuracy of CAP in solution, two individual stock solutions were prepared and analyzed to verify the intended initial concentrations with samples collected daily for six weeks. The same routine was applied to the standard solutions used for fortification and calibration curves. Specificity for seawater and macroalgae was demonstrated by analyzing 20 blank samples of each matrix collected at different locations to exclude the presence of possible interferences in the identification of CAP at the expected elution time.

3. Results and Discussion

3.1 Water

In aquaculture facilities, the main vehicle by which antibiotics enter the surrounding aquatic environments is through effluent discharges and leakage from the ponds. Therefore it is paramount to monitor the water in the tanks, whenever access is permitted and in cases it is not possible, sampling should be done at and near the discharge points. With this purpose, a method for the quantification of CAP in natural seawater was developed.

Usually, Solid Phase Extraction (SPE) is applied as a pre-concentration step of the analytes in matrices where it is expected their presence will be very low and also to obtain cleaner extracts due to the high efficiency of this procedure. However, to reduce the time effort and the costs associated with the analysis of a large number of samples, a direct injection method was tested. The resulting chromatograms presented enough resolution to isolate the antibiotic and the selection of the most suitable elution

method became the next step, with gradient and isocratic conditions being tested. The chromatograms from gradient analysis showed a smaller peak just before the eluted analyte whereas in isocratic conditions the peak was inexistent (Fig. 2A and B). Moreover, isocratic analysis provided more reproducible results, a better peak integration and lower elution time and for these reasons it was selected as the elution method for validation (Fig. 2B).

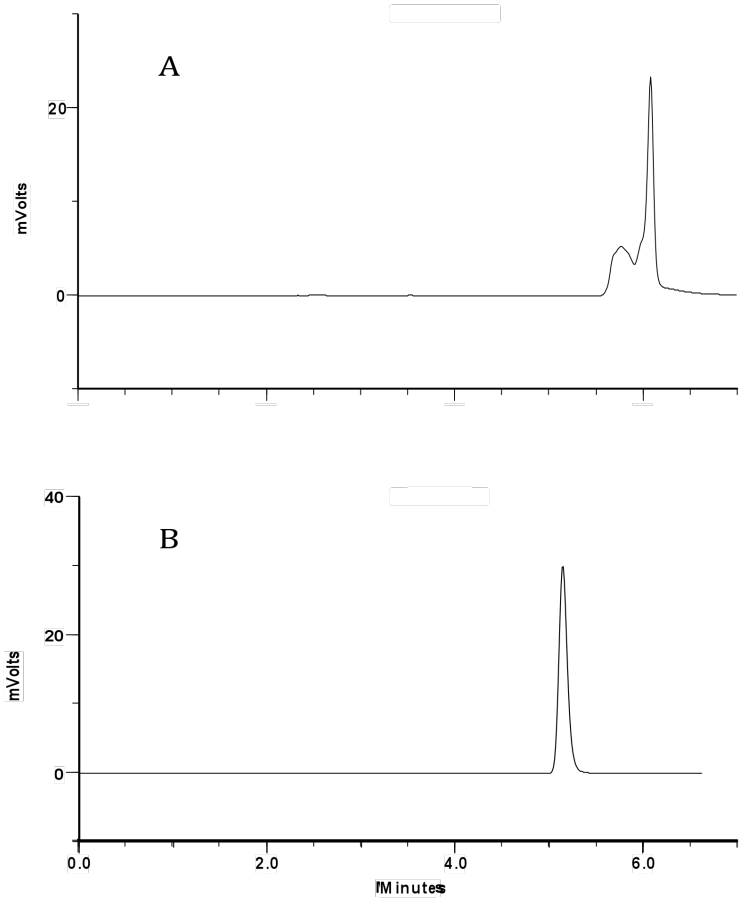

Figure 2. HPLC-UV chromatograms of a 50 µg mL^{-1} standard CAP solution run with A) gradient elution and B) isocratic elution.

As mentioned previously validation followed the guidelines of the EC Regulation 2002/657 with the establishment of the quantitative performance criteria. Recovery values were determined for each of the three fortification levels of the 18 blank samples spiked with a mean value of 84.8%. As for CCα and CCβ, the directive states that for banned substances to which no permitted limit can be set, the CCβ *is the lowest concentration at which a method is able to detect truly contaminated samples with a statistical certainty of 1−β*. The obtained values of 0.116 and 0.352 μg mL^{-1} respectively, are within the requested range. Precision was measured as within-day repeatability (intra-precision) and within-laboratory reproducibility (inter-day precision) with mean values of 1.27% and 5.61% respectively, which are in accordance with the Horowitz equation. Linearity was determined at five concentrations levels with a mean R^2 of 0,998 (EC Regulation 657/2002). Specificity was confirmed with the analysis of 20 blank water samples where the analyte was unequivocally identified, with no significant interfering peaks observed at the retention time (Fig. 3). Regarding the stability of the solutions, the concentrations of the stock solutions showed to be stable for 8 weeks when stored at -20°C whereas the work standard solutions were stable for one month stored at 4°C.

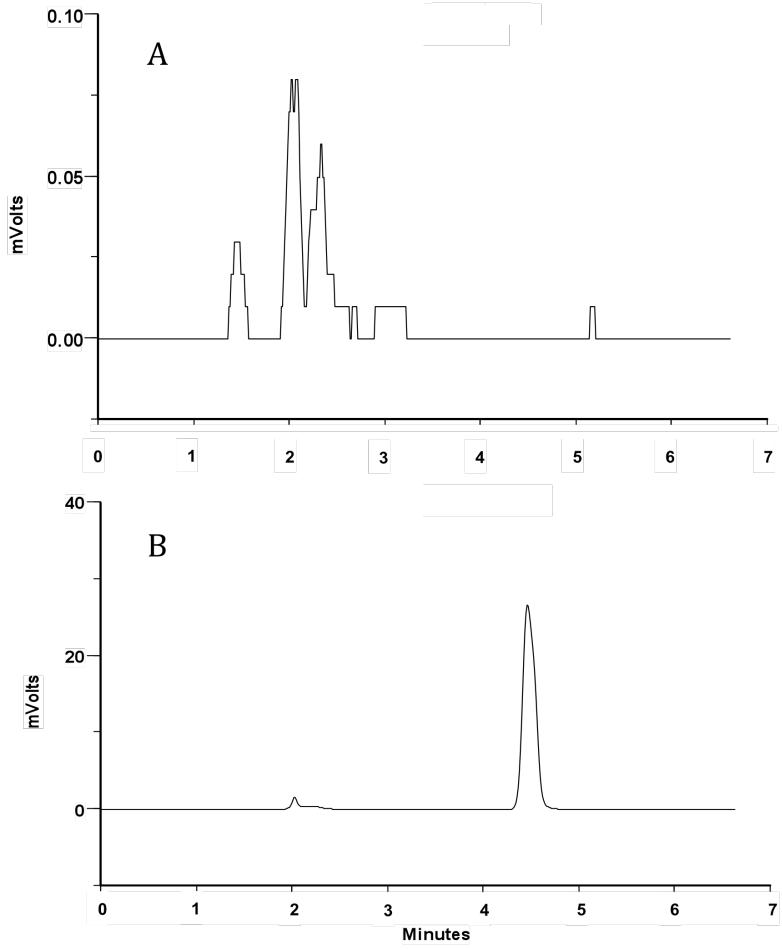

Figure 3. HPLC-UV chromatograms of A) blank seawater sample B) fortified sample from the calibration curve.

3.2 Macroalgae

The development of the methodology for the quantification of CAP in *U. lactuca* was based on a previous work by the authors establishing a procedure for the determination of furaltadone in the same macroalgae (Leston et al., 2011c). In the referred method the comparison between chromatograms with and without SPE

revealed that both produced clean extracts and for that reason the latter was chosen allowing for a significant cost reduction.

In order to select the best solvent for extraction, fortified macroalgae were submitted to three solvent combinations to be analyzed on a qualitative and quantitative basis: a) acetonitrile and N-hexane, b) N-hexane and c) ethyl acetate. The comparison of the chromatograms revealed that acetonitrile and N-hexane produced higher background noise and lower absolute areas that translated into a more difficult integration of peaks. On the other hand, the chromatogram resulting from ethyl acetate extraction presented lower interferences and higher absolute areas (Fig. 4). For this reason, ethyl acetate was chosen as the elution solvent to be validated. After extraction, the chromatographic conditions followed the method optimized and described above for determination of CAP in seawater.

Validation followed the same quantitative performance criteria described for seawater. Recovery determination from the 18 fortified blank samples resulted in a mean value of 76.4% whereas the mean values for CCα and CCβ were 2.845 and 8.621 μg mL^{-1}, respectively. Within-day repeatability and within-laboratory reproducibility were also in accordance with the Horowitz equation with mean values of 2.81% and 13.99%. As for linearity, the R^2 resulting from the mean values of the calibration curves was 0.992. Finally, specificity was ascertained with the analysis of 20 samples from different locations where CAP was clearly identified.

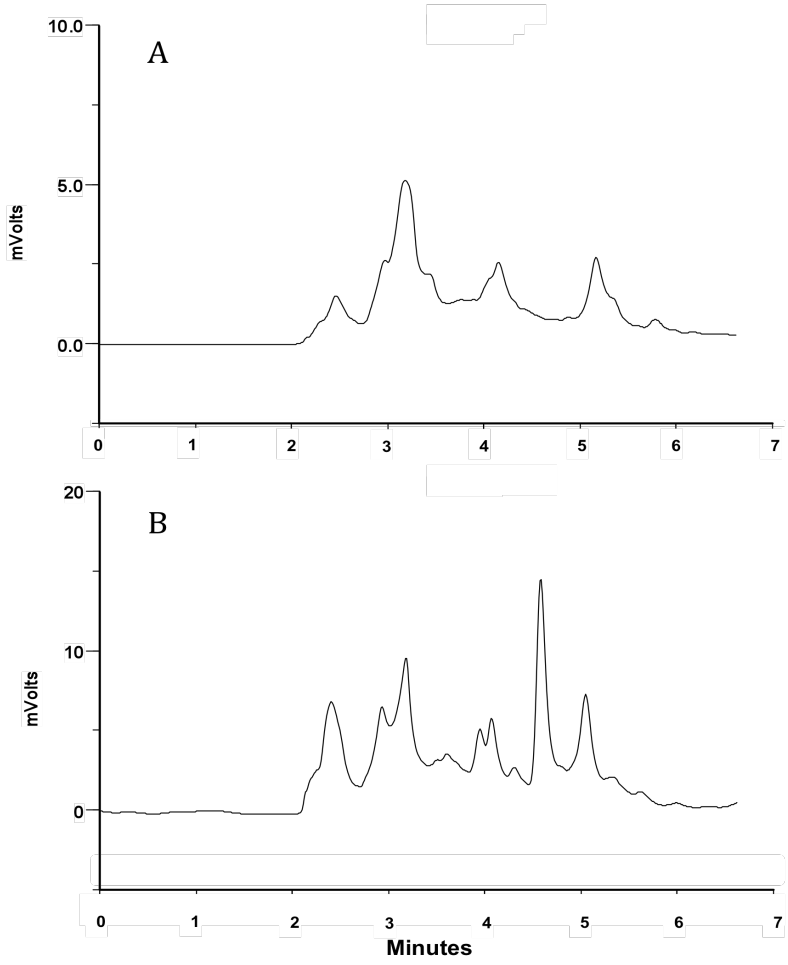

Figure 4. HPLC-UV chromatograms of A) blank macroalgae sample B) fortified sample from the calibration curve.

4. Conclusion

The methodology presented was developed as a tool in monitoring for the presence of CAP in and near aquaculture facilities, through the use of environmental matrices. The method is based on a simple extraction without the need for a clean-up step. For seawater, after filtration the extracts can be directly analyzed whereas for *U.*

lactuca, ethyl acetate extraction precedes injection. Sample preparation for both matrices is thus simple and effective allowing for a high number of samples to be processed at the same time, at lower costs. The performance criteria established according to EU guidelines have demonstrated it was fully validated. The method will also be an important tool in further research on the extent of environmental contamination and the effects it can produce on non-target organisms, including macroalgae.

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The effects of chloramphenicol on the green macroalgae *Ulva lactuca*

Abstract

The administration of pharmacological substances in the food producing industry is a crucial and long established practice in ensuring animal welfare. However, a very high percentage of the drugs used will directly or indirectly be present in the various compartments of natural ecosystems therefore constituting a source of pollution. The reactions that these active compounds may impose on non-target organisms are still widely unknown and further research is essential. Also, new approaches on monitoring are necessary and in this sense, the present work aimed to assess the persistence of chloramphenicol (CAP) (a banned but illegally used antibiotic) in seawater, together with its effects on the growth of the green macroalgae *Ulva lactuca*. Moreover, the potential use of this species as a bioindicator and/or biomonitor along with the risk of bioaccumulation and biomagnification of CAP through the trophic web were assessed. Results showed CAP presented an exponential degradation pattern in seawater with concentrations decreasing faster than expected. As for the effects on *U. lactuca* it acted as a growth promoter also contradicting the initial assumptions. Regarding the possible role of this species in biomonitoring since it successfully took up CAP in solution while reflecting the external concentrations it can be considered for use as a biomonitor. On the other hand, this ability points to a high probability of CAP being bioaccumulated and transferred along the trophic web through the consumption of *U. lactuca* by marine organisms in higher levels.

1. Introduction

Antibiotics are an extensive family of pharmaceuticals that play a significant role as agents of environmental pollution (Heberer, 2002; Veach and Bernot, 2011). Several sources contribute to their presence in the ecosystems, mainly through human and veterinary administration (Heberer, 2002; Boxall et al., 2004; Martínez, 2009; Leston et al., 2011a), where thousands of compounds from distinct classes have been introduced and commercialized, mostly to the prevention and treatment of diseases and as growth-promoting agents (Sarmah et al., 2006; Martínez, 2009). In aquaculture antibiotics are commonly incorporated in feeds and bath treatments, resulting in large quantities of the parent drugs and their metabolites being present in wastewaters and effluents that will eventually leach into the various environmental compartments (Park and Choi, 2008; Martínez, 2009; Leston et al., 2011a). The major concern surrounding their presence rests on the fact that they are developed to cause biological effects and the probability of the same effects being exerted in non-target organisms is very high (Halling-Sorensen et al., 1998). Moreover, antibiotics are designed to easily permeate cell barriers increasing the risks of bioaccumulation and biomagnification (Gerofke et al., 2005; Torres et al., 2008).

Primary producers are paramount in natural ecosystems constituting the basis of the trophic web with the responsibility to convert inorganic forms of energy into biomass, which will then be transferred to higher levels (Conti and Cecchetti, 2003; Melville and Pulkownik, 2006; Torres et al., 2008). In coastal and estuarine environments macroalgae are among the most productive species, taking advantage of the ability to tolerate fluctuating conditions and to rapidly sequester nutrients from the surroundings and contributing with large quantities of organic matter (Godbold et al., 2009; Raffaelli, 2000) through leaching, decomposition and direct animal consumption. Due to the crucial role of macroalgae in the ecosystems their use as indicators of contamination is a valuable tool in environmental risk assessment. These primary producers are continuously exposed to contamination in the aquatic environment reacting more quickly to the presence of pollutants than the higher trophic levels

(Ferrat et al., 2003) and may act as a sink and gateway of xenobiotics to the trophic web (Sandermann et al., 1992 Pflugmacher et al., 1998; Walker, 2006). Aiming to increase the knowledge on how antibiotics affect estuarine macroalgae and based on a previous research on the effects of the antibiotic furaltadone (Leston et al., 2011b), an experimental setup was developed exposing the Chlorophycean *Ulva lactuca* L. to chloramphenicol (CAP). CAP is a well-known antibacterial agent that acts by binding to the 50S subunit of the 70S ribosome in the prokaryotic cell causing the inhibition of protein synthesis (Ashwin et al., 2005). Nonetheless, the alarming potential risks linked to its use, namely teratogenic, mutagenic and carcinogenic effects (Biancotto et al., 2009) have led to its prohibition under strict legislations (Commission Regulation (EC) 37/2010; Leston et al., 2011a). Unfortunately, this ban has been repeatedly ignored in favor of low costs and high efficacy, with reports of clandestine administration still persisting (Wongtavatchai et al., 2004; FAO 2008).

Hence, the present research focused on the following objectives: (1) to assess the stability of CAP when dissolved in seawater, (2) to investigate the uptake capacity of *U. lactuca* and the potential for bioaccumulation, (3) to ascertain the possibility of *U. lactuca* to be used as an indicator for the presence of CAP following illegal use and (4) to evaluate the effect of CAP on *U. lactuca*'s growth at two concentrations.

2. Experimental Methods

2.1 Chemicals and solutions

Chloramphenicol (Fig.1) analytical standard with 99% purity was supplied by Sigma-Aldrich (Steinheim, Germany) while methanol, acetonitrile, glacial acetic acid, ethyl acetate and N-hexane were acquired from Merck (Darmstadt, Germany). All solvents were of HPLC analytical grade and were filtered and degassed before use. Ultrapure water was obtained daily with a Milli-Q apparatus (Millipore, Bedford, MA, USA).

A standard stock solution of 1 mg mL^{-1} was prepared by dissolving 50 mg of CAP in 50 mL of methanol and stored at -20°C protected from light. Intermediate

standard solutions were prepared just before use by diluting the appropriate volume of the stock solution in methanol to achieve two final concentrations of 25 µg mL^{-1}, representing an hypothetical prophylactic bath treatment and 50 µg mL^{-1} for a therapeutic dosage, both simulating scenarios of discharges in coastal waters after treatments had been administered (e.g. fish farms).

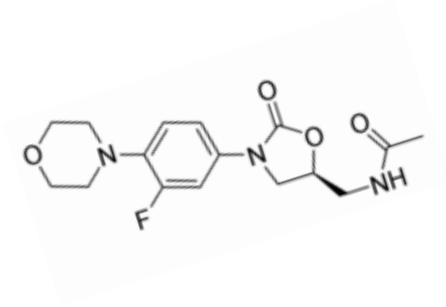

Figure 1. Chemical structure of chloramphenicol.

2.2 Acclimation

Healthy fronds of *U. lactuca* were collected during low tide in the Mondego estuary (Portugal, 40° 80' N, 8° 50' W). At the site, macroalgae were washed and rinsed to remove debris and epibionts and placed in refrigerated containers for transportation. Fresh natural seawater was also collected in acid washed amber bottles, immediately filtered (0.45 µm) upon arrival and stored at 4°C.

To guarantee the removal of potential organisms, fronds were carefully inspected before being placed in 40 L glass aerated tanks filled with filtered seawater (35 psu) to which was added Provasoli Enriched Medium to a final concentration of 20 mL L^{-1} (PES; Provasoli, 1963 and modified by Bold and Wynne, 1978). The tanks were kept in a controlled room with 14:10h LD photoperiod, 25° C and under 80 µmol photons m^{-2} s^{-1} of white fluorescent light, for 48h before macroalgae were submerged. Acclimation lasted for three weeks prior to the beginning of the experiment.

2.3 Experimental Design

Before the beginning of the experiment acid washed Erlenmeyer flasks were filled with 250 mL of seawater and 5 mL of PES, placed on orbital shakers and left to acclimate for 24h in the same conditions but with aeration replaced with constant horizontal stir at 100 rpm. On the starting day, algal disks were cut with Ø 5 cm (approx. 20 cm^2) and the antibiotic solutions prepared. Two groups were established (P and T) with each group composed of three replicates per sampling time and each replicate containing three algal disks (one flask constituted one replicate). Group P simulated a prophylactic concentration (25 µg mL^{-1}) whereas group T corresponded to the therapeutic dosage (50 µg mL^{-1}). Additionally, two control groups were set with control A verifying the behavior of CAP in seawater for both concentrations P and T without the presence of macroalgae. Control B followed the same conditions as P and T but without the antibiotic in order to determine the natural growth. The experimental design was based on a previous work by the authors on the effects of furaltadone in *U. lactuca,* where the effects of methanol were tested and for that reason not replicated here (for further information see Leston et al., 2011a). To prevent losses due to evaporation but still allowing gas exchange every flask was covered with glass lids. Water samples and photographs to register growth variations were taken at the following times: 0, 1, 2, 5, 8, 12,16, 24, 48, 72, 96 and 120h.

2.4 Antibiotic Quantification

2.4.1 Water

Temperature, salinity and pH were measured at each sampling time and water samples were taken, filtered through Ø 22 µm glass fiber filters and frozen at -20°C until analysis.

To quantify CAP in seawater, samples were transferred to vials and automatically injected in a Gilson modular system (Gilson, Middleton, WI, USA) equipped with a Merck 250-4 LiChrospher 100 RP-18 column and a PerkinElmer NewGuard C18 pre-column for high pressure liquid chromatography with UV detection

(HPLC-UV) analysis. Elution was conducted in isocratic mode with a mobile phase consisting of ultrapure water acidified with 0.1% glacial acetic acid (solvent A) and ultrapure water/acetonitrile (30/70, v/v), acidified with 0.1% glacial acetic acid (solvent B), both filtered and degassed, with 37.5% A and 62.5% of B.

2.4.2 Macroalgae

To establish the capacity of *U. lactuca* to take up and accumulate CAP from the water column the internal concentrations were determined with an HPLC-UV method adapted from Leston and colleagues (2011b). To remove the excess water, macroalgae disks were paper-dried, weighed and stored frozen at -20°C. Upon analysis disks (± 200 mg) were thoroughly minced and placed in 15 mL amber glass centrifuge tubes. After adding 10 mL of ethyl acetate and vortex mixing for 1 min, tubes were let to stand for 10 min. Samples were then centrifuged for 10 min at 3000 rpm and the supernatant transferred to clean tubes followed by evaporation to dryness under a gentle nitrogen stream, at 50°C. To reconstitute the dry residues 500 µL of ultrapure water were added, vortex mixed for 30s and transferred to autosampler vials. The chromatographic separation followed the same conditions described above for water analysis.

2.5 Growth

The effect of CAP on *U. lactuca* growth was evaluated as variations in disk area. For that purpose macroalgae disks were photographed at the beginning and end of each sampling time and then analyzed with a computer-assisted software (Adobe® Photoshop® CS5 extended) to establish areas.

2.6 Statistics

Data were tested for normality and homoscedasticity. Analysis of variance (ANOVA) was carried out to assess the effects of both concentrations of CAP in growth and the differences in uptake for groups P and T. The significance level was inferred at *p*

< 0.05 for all statistical tests. Analyses were performed with GraphPad Prism® 5 software (Graph Pad Software, Inc.).

3. Results and Discussion

3.1 Water

Prior to the beginning of the trial both standard solutions were analyzed along with seawater samples taken immediately after the addition of CAP to guarantee that the intended concentrations were in fact reached. Control A samples taken at time point 0 also confirmed the concentrations for the prophylactic and therapeutic dosages, respectively 24.83 (± 0.09) and 49.75 (± 0.7) μg mL^{-1} (Fig.2).

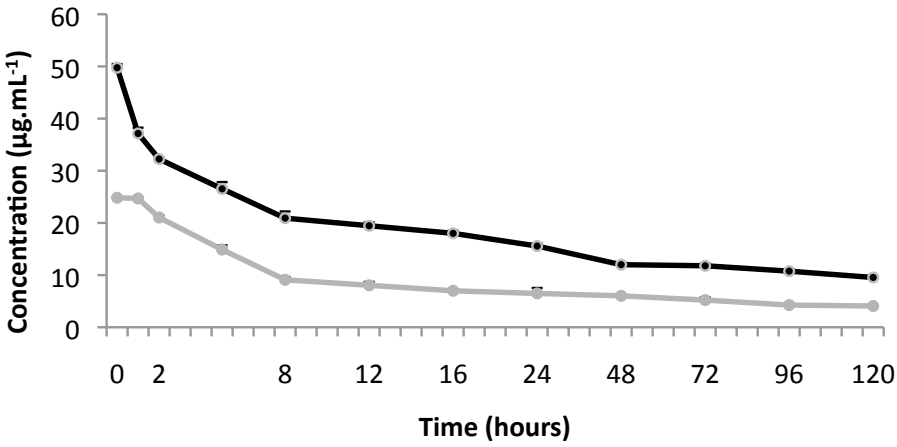

Figure 2. Degradation pattern of CAP in natural seawater resulting from data established in control A. The grey line represents a starting concentration of 25 μg mL^{-1} (prophylactic dosage) and the dark line a concentration of 50 μg mL^{-1} (therapeutic dosage). Data represent mean values ± SE of three independent replicates for each sampling time point

As for the stability of CAP in natural seawater results show it follows an exponential pattern of degradation with concentrations decreasing rapidly in the first 5h to approximately half the initial values and decelerating subsequently. After 120h

the levels recovered corresponded to less than 20% of the initially supplied. Under the given experimental conditions light was the most likely factor responsible for the decrease in concentration, which is supported by previous researches that demonstrated CAP undergoes photodegradation in aqueous solution through photolysis (Shih, 1971; Chien et al., 1999; Wongtavatchai et al., 2004; Chatzitakis et al., 2008; Zhou et al., 2010). However, a higher persistency of the antibiotic was expected as studies show other factors including salinity and oxygen greatly influence the rate of light-induced decomposition. More specifically, salinity values over 35 will slow the process due to the concentration of sodium chloride, which is likely to affect the microbial communities involved in the process (Chien et al., 1999; Zhou et al., 2010). As for oxygen, when testing the degradation of CAP in sediments under aerobic and anaerobic conditions a significantly higher rate was attained in the absence of oxygen, with depletion reached in over 8 days against 65 days in its presence (Chien et al., 1999). One possible explanation for the difference observed in the present work is the presence of ferric ions ($FeCl_3.6H_2O$) resulting from the addition of PES to the medium. Iron, which is also present in natural waters, is know to act as a catalyst in photolysis by oxidizing CAP with $OH^{\bullet}$ radicals (Zhou et al., 2010) resulting in higher degradation rates. Nevertheless, the concentrations found at the end of the experiment are still very high and cause for concern when considering the adverse effects it can induce.

Antibiotics have been considered as microcontaminants since the concentrations in which they are usually found are very low in most cases constant. The dosages tested in this study are, however, very high and only likely to occur at and near effluent points and/or leakage following prophylactic and therapeuthic bath administrations. Still, in artisanal aquaculture ponds without sewage treatment and dependent on tidal water renovation the scenarios tested are possible and commonly found in southern European countries.

3.2 CAP Uptake

The capacity of *U. lactuca* to take up CAP from the water phase at both dosages was assessed as the internal concentration of antibiotic present in each algal disk. The pattern presented in both cases was very similar but the values reported for the therapeutic group were a two-fold reason higher than the prophylactic group (Fig. 3A and 4A). For both groups the highest values reported were reached at time 0, respectively 19.10 ± 1.80 and 29.49 ± 2.20 μg g^{-1}, decreasing quickly within the hour and remaining relatively constant for the proceeding 24 hours. After this time a gradual decrease was observed until minimum values were reached at the end of the trial (4.69 ± 0.16 and 6.41 ± 0.54 μg g^{-1}). Through the comparison between the internal concentrations taken up and the remaining in the surrounding medium it is clear that both groups reflected the concentrations of CAP present in solution (Fig. 3A and 4A), with no significant statistical differences at $p > 0.05$. Also, the differences in water concentration found between control A and the samples taken in groups P and T may be explained by macroalgae uptake.

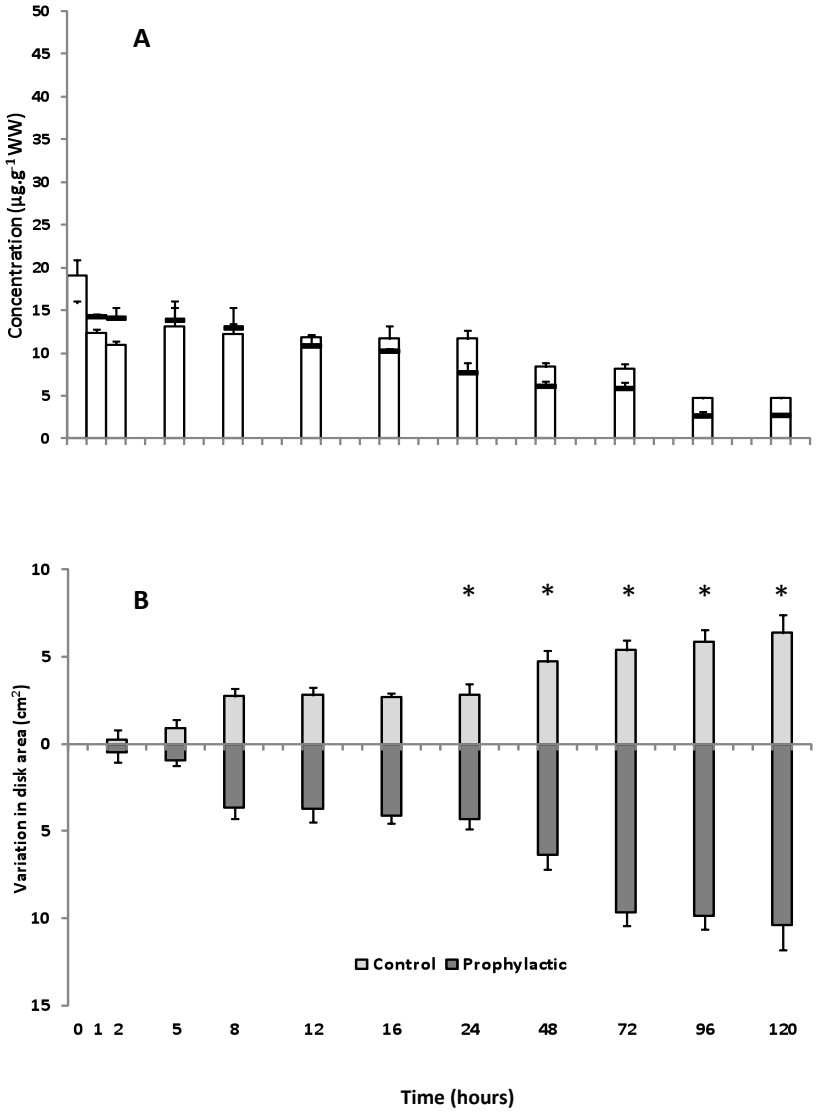

Figure 3. (A) Uptake of CAP determined as internal concentrations (µg g^{-1} WW) in *U. lactuca* for each sampling time (bars) in comparison with the concentration in aqueous solution for the correspondent time point (-), for the prophylactic group P. Data represent mean values ± SE of three independent replicates for each point. (B) Growth measured as variation in disk area (cm^2) at each sampling point for group P, plotted against the control. Data represent mean values ± SE of three independent replicates for each point. Asterisk (*) indicates results significantly different from the control.

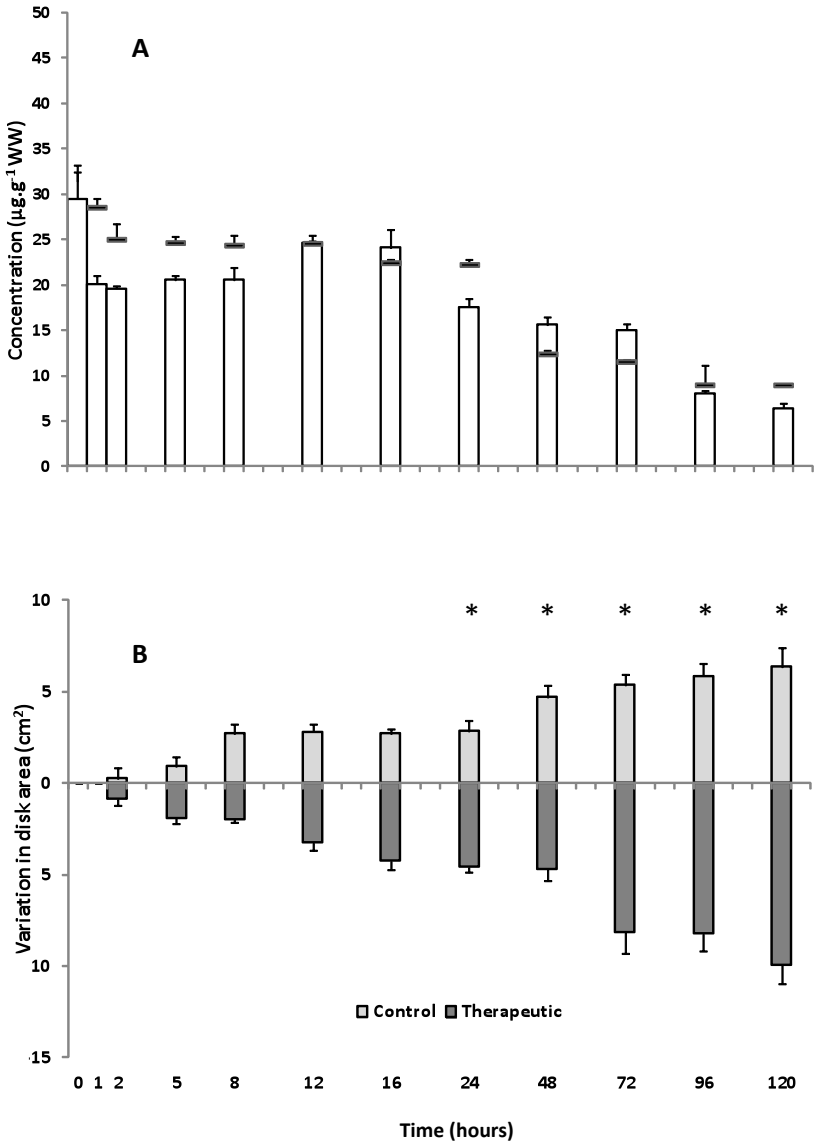

Figure 4. (A) Uptake of CAP determined as internal concentrations (µg g^{-1} WW) in *U. lactuca* for each sampling time (bars) in comparison with the concentration in aqueous solution for the correspondent time point (-), for the therapeutic group T. Data represent mean values ± SE of three independent replicates for each point. (B) Growth measured as variation in disk area (cm^2) at each sampling point for group T, plotted against the control. Data represent mean values ± SE of three independent replicates for each point. Asterisk (*) indicates results significantly different from the control.

The octanol-water partition coefficient (K_{ow}) is one of the most important descriptors of chemical behavior in the environment providing a good estimate if a given contaminant will be efficiently taken up and also to predict its bioaccumulation potential. Octanol is a lipophilic solvent that is immiscible with water and thus is used as a representative of lipids in organisms due to the similar carbon to oxygen ratio (Walker et al., 2006). The Log K_{ow} estimated for CAP is 1.14 (Wongtavatchai et al., 2004) indicating it is highly hydrophilic and may be easily absorbed by simple passive diffusion across the cell membrane, which was confirmed by the high internal concentrations observed (Walker et al., 2006). Data obtained in this study also indicates that uptake kinetics was rapid and that equilibrium between the macroalgal and water phase was reached within hours. Another important descriptor is the bioconcentration factor (BCF) defined as the ratio for the concentration of a given chemical in an organism and the concentration in the environmental media to which it is exposed. However, in the present work it could not be established since the concentration of the dissolved antibiotic was not constant throughout, which is a requirement for BCF determination (Gerofke et al., 2005).

An important issue to be further investigated is the mechanism through which *U. lactuca* is able to lower the internal concentration of this xenobiotic. One possibility is through simple passive diffusion from the cells to the water column without energetic costs associated. Another hypothesis to consider and already discussed in a previous work by the authors (Leston et al., 2011b) is the possibility of a detoxification system. Such system has already been identified in *U. lactuca* and other macroalgae species and consists of three phases: phase I (transformation), phase II (conjugation) and phase III (compartmention) (Sandermann, 1992; Coleman et al., 1997; Pflugmacher et al., 1999: Torres et al., 2008). Furthermore, degradation of antibiotics resorting to the use of enzymes involved in the detoxification mechanism (e.g. glutathione-S-transferases) has proven highly effective (Park and Choung, 2007).

3.3 Growth

The effect of CAP on *U. lactuca*'s growth was assessed as variations in disk area. Control B established growth under natural conditions and therefore setting the pattern for comparison with groups P and T (Figs 3B and 4B). For both concentrations tested variation followed the same pattern as the control but at 24h it became significantly higher ($p < 0.05$). Growth continued to increase thereafter, reaching the highest values at the end of the trial (10.41 ± 0.75 and 9.23 ± 0.90 cm^2 for P and T against 6.35 ± 1.00 for control B). When comparing groups P and T, the prophylactic dosage had a more pronounced effect becoming statistically different after 48h ($p < 0.05$) (Figs 3B and 4B).

The response of *U. lactuca* to CAP was contrary to what was expected since it has been classified as "Toxic to Aquatic Organisms" under the European Community Directive 2006/121 (European Parliament Directive 2006/121/EC). Also, research by Lai and co-authors (2009) on the effects of CAP and other related drugs on growth of microalgae reported the antibiotics to be toxic causing a significant inhibition in the range of concentrations tested (from 2.5 to 50 mg L^{-1}). Furthermore, CAP has been extensively applied in studies on photosynthesis as an inhibitor of chloroplast-encoded protein synthesis resulting in pronounced inhibition of photosystem II (Greer et al., 1986; Demmig-Adams and Adams III, 1993; Franklin, 1994). The mode of action of CAP molecules can be direct by preventing protein synthesis at the ribosomal level through binding to the histidine in the L16 protein responsible for attaching the tRNA to the active site of peptidyl-transferase, which in turn will prevent transpeptidation (Okada et al., 1991). CAP can also act indirectly by affecting other processes such as energy supply via prevention of ion uptake, oxidative phosphorylation and photophosphorylation (Smith, 1977). Nonetheless, although protein synthesis is prevented in ribosomal chloroplasts it is not affected in cytoplasmatic ribosomes. Moreover, CAP does not inhibit cell division and new chloroplasts can still be produced however smaller and with a lesser number of internal lamellae (Bishop et al., 1973).

One other important issue that may help explain the results obtained resides in the photodecomposition process. For nitroaromatic organic compounds like CAP, the nitro- group will be converted to nitrite (NO_2^-) in the final oxidation state by electrophilic substitution of the $OH^\bullet$ radicals at the *para* position in the aromatic ring. NO_2^- can be further oxidized to nitrate (NO_3^-) that will then be reduced to ammonium (NH_4^+) (Low et al., 1991; Wongtavatchai et al., 2004; Chatzitakis et al., 2008). Another reaction includes the direct release of NH_4^+ by reduction of the nitro- group to an amine group in the ring (Mahdavi et al., 1993). *U. lactuca* presents a high affinity for both these ions however with different uptake rates since NO_3^- is a negatively charged molecule and requires an energy-dependent uptake and assimilation (Runcie et al., 2003; Cohen and Fong, 2004). The high concentrations of both these forms in natural environments have been linked to eutrophication scenarios represented by blooms of primary producers, with a high prevalence of green macroalgae including the genus *Ulva* (Pedersen and Borum, 1997; Valiela et al., 1997; Lartigue and Sherman, 2005; Lillebø et al., 2005). This species is characterized by a simple thallus with high surface area to volume ratio adapting rapidly to ambient conditions in transient environments translated in high uptake and growth rates (Pedersen and Borum, 1997; Naldi and Wheeler, 1999; Pérez-Mayorga et al., 2011). Thus, the increase in availability of NO_3^- and NH_4^+, especially the latter, in addition to the nutrients supplied with PES, may have acted as a growth promoter for *U. lactuca*.

The processes of degradation of antibiotics are in the majority of cases complex and involve a number of intermediates that can potentially be more harmful than the parent compound itself. However, in the present case the effects observed were beneficial for *U. lactuca*, unlike expected. Nonetheless, a considerable increase in biomass of green macroalgae is known to be, in many cases, prejudicial to ecosystems as it can lead to the presence of unwanted blooms in natural environments that in turn will have serious consequences in the underlying communities. In this sense, CAP can be considered a source of pollution in aquatic ecosystems.

4. Conclusions

Howbeit the uncertainty regarding the underlying mechanisms involved, *U. lactuca* can efficiently remove CAP from natural seawater while influencing its concentration in solution. Also, macroalgae can reflect satisfactorily the amounts present therefore representing a good candidate as a CAP bioindicator in environmental risk assessment. Nonetheless, ecologically such capacity can potentially be prejudicial since it represents a significant entry point of this xenobiotic into estuarine and marine trophic webs, where it can suffer biomagnification. Moreover, there is a risk of eutrophication associated with the presence of CAP since it enhanced growth in this known bloom-forming green macroalgae.

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CHAPTER III

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HPLC-UV ANALYSIS OF CHLORAMPHENICOL IN ENVIRONMENTAL MATRICES: SEAWATER AND THE MACROALGAE *ULVA LACTUCA*

THE EFFECTS OF CHLORAMPHENICOL ON THE GREEN MACROALGAE *ULVA LACTUCA*

Liquid Chromatographic Determination of Sulfathiazole in Seawater and Macroalgae by MS/MS and UV detections

Abstract

The awareness of the interconnection between pharmaceutical residues, human health and environment has highlighted the concern with the potential harmful effects it can induce. Furthermore, to better understand the consequences more research is needed and to achieve that new methodologies on the detection and quantification of pharmaceuticals are necessary.

Antibiotics are a major class of drugs included in the designation of emerging contaminants, representing a high risk to natural ecosystems. Among the most prescribed are sulfonamides, with sulfathiazole being the selected compound to be investigated in this study. In the environment, macroalgae are an important group of producers with a significant role in the trophic web and that are continuously exposed to contaminants. Due to these characteristics are already under scope for the possibility of being used as bioindicators.

The present work describes two new methods based on liquid chromatography for the determination of sulfathiazole in seawater and in the green macroalgae *Ulva lactuca*. Results show both methods were validated according to international standards, with MS/MS detection showing more sensitivity as expected with LODs of 2.79 ng g^{-1} and 1.40 ng mL^{-1} for algae and seawater. As for UV detection the values presented were respectively 2.83 µg g^{-1} and 2.88 µg mL^{-1}, making it more suitable for samples originated in more contaminated sites. The methods were also applied to experimental data with success with results showing macroalgae have potential use as indicators of contamination.

1. Introduction

Macroalgae represent an important group of primary producers with several fundamental ecological functions in the ecosystems. However, their constant exposure to contaminants in the water body may have serious implications and can also represent an entryway of contamination to the food web through their direct consumption by organisms of higher trophic levels (Melville and Pulkownik, 2006; Walker et al., 2006; Torres et al., 2008). Due to these characteristics some species are currently under the scope of researchers to establish to what extent are being impacted from exposure to antibiotics and their potential use as indicators of contamination (Pflugmacher et al., 1999; La Barre et al., 2004; Carafa et al., 2007; Torres et al., 2008; Leston et al., 2011 a, b). The intensification of animal production to meet the dietary demands of a continuously growing world population is increasing the anthropogenic pressure on natural ecosystems. The administration of antibiotics to prevent and/or treat disease outbreaks in animal husbandries is a recognized and undeniably necessary practice, which has become more intense as the food sector expands (Boxall et al., 2004; Arikan et al., 2008; Kemper, 2008). In order to increase their effectiveness, antibiotics are designed to be highly soluble and with low biodegradability which potentiates persistence in the ecosystems (Wollenberger et al., 2000) and their bioaccumulation in several organisms in the trophic web (Halling-Sørensen et al., 1998; Kümmerer, 2001). Nonetheless, despite the efforts and ongoing research to remove and neutralize such compounds in effluents and wastewaters resulting from this industry, their fate is ultimately the environment (Radjenović et al., 2007). The recognition of this threat has rendered the classification of human and veterinary pharmaceuticals as emerging contaminants due to their potential to cause adverse effects in human and environmental health which may be irreversible (Boreen et al., 2004; Arikan et al., 2008; Radjenović et al., 2007; Leston et al., 2011b).

Sulfonamides (SA) are among the main classes of antibiotics administered presently, which also include β–lactams, quinolones, tetracyclines, macrolides (FAO, 2005; Arikan et al., 2008; Kemper, 2008; Kümmerer, 2009, Hruska and Franek, 2012).

This group of synthetic bacteriostatic agents acts as competitive inhibitors of p-aminobenzoate in folate biosynthesis, interfering with folic acid synthesis and ultimately preventing cell growth (Boreen et al., 2004; García-Galán et al., 2008; Braschi et al., 2010). Sulfathiazole (STZ) is a commonly prescribed SA, extensively used in livestock production and aquaculture to treat bacterial, protozoal and fungal infections. Along with other SA, studies point to its presence in the environment which is in most part due to the inability to fully eliminate these compounds during sewage treatment (García-Galán et al., 2008; Braschi et al., 2010; Garoma et al., 2010; Hruska and Franek, 2012). The potential ecological significance of the effects of STZ in non-target organisms is scarcely unknown and the need for further research is pressing. *Ulva lactuca* is a cosmopolitan green macroalgae with a wide distribution and simple morphology (Melville and Pulkownik, 2006; Torres et al., 2008) commonly found in the surrounding areas of aquaculture ponds thus representing a good candidate to evaluate the effects of exposure to STZ. However, currently there are no methods available for its quantification in macroalgae, which are indispensable tools to assess their potential as bioindicators of contamination.

In light of these issues the objective for the present work was to develop an accurate, reproducible and sensitive method for the determination of STZ in *U. lactuca* through LC-MS/MS. Moreover, considering the fact that MS instrumentation is not present in all laboratories a LC-UV methodology is also presented.

2. Experimental

2.1. Chemicals and Reagents

Sulfathiazole (purity > 98%) and the internal standard (IS) sulfadimethoxine-d6 were acquired from Sigma-Aldrich (Steinheim, Germany) (Fig. 1). Formic acid (purity >99%) and ethyl acetate were obtained from Merck (Darmstadt, Germany) while methanol and acetonitrile were purchased at Scharlau Chemie (Barcelona, Spain). All solvents used were LC grade and were filtered through 0.45 µm Whatman nylon membrane filters (Whatman, Maidstone, USA) prior to degassing in ultrasonic bath.

Ultrapure water was obtained daily from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Nitrogen was generated in-house with a nitrogen generator from Peak Scientific Instruments Ltd. (Chicago, IL, USA).

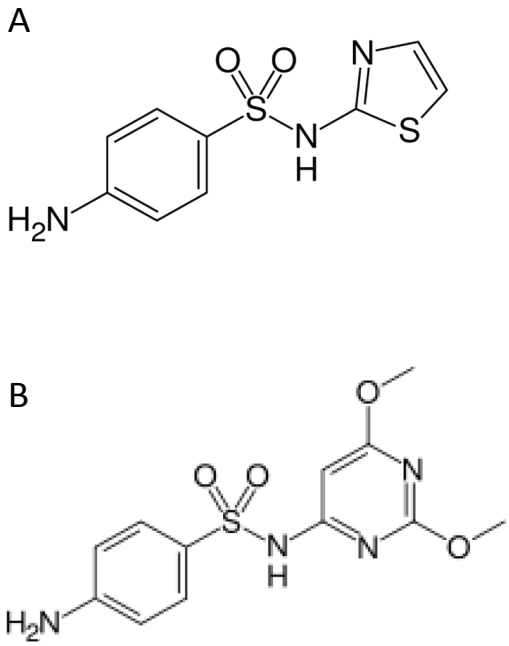

Figure 1. Chemical structures of sulfathiazole (A) and the internal standard sulfadimethoxine-d6 (B).

2.2. Solutions

For LC-MS/MS analyses, individual standard stock solutions of STZ and IS (0.6 mg mL^{-1}) were prepared by accurately weighing 30 mg of each compound into 50 mL of methanol. Work and calibration standard solutions were then obtained by diluting appropriate volumes of each stock in 0.1 % of formic acid in acetonitrile.

For LC-UV analyses, an individual stock standard solution (1 mg mL^{-1}) was prepared by dissolving 50 mg of STZ accurately weighed into 50 mL of methanol. From

this, an intermediate solution (50 µg mL^{-1}) was obtained by dilution in appropriate volume of methanol. Work and calibration standard solutions at various concentration levels were prepared individually by spiking 10 mL of filtered seawater with specific quantities of the stock solution.

Quality controls were also established by preparing new batches of solutions from independent stock solutions.

All solutions were kept in amber glass containers and stored in the dark at 4°C.

2.3. Sampling and Exposure assay

U. lactuca fronds were collected during low tide at a local beach, transported in refrigerated containers after rinsing with seawater to remove eventual epibionts and debris on the surface. Macroalgae were acclimated for a period of two weeks in filtered natural seawater (0.45 µm) collected at the site. After this time, sets of discs weighing 200 mg were cut from the algae, spiked with standard solutions and immediately frozen at -20°C, until analysis.

Since currently there is no information on the ability of *U. lactuca* to take up and accumulate STZ from natural seawaters, an experimental trial was set following the design described by the authors to the analyses of the nitrofuran antibiotic furaltadone in the same species (Leston et al., 2011a, b). As such, after acclimation macroalgae discs were exposed for a period of 24 h to two concentrations simulating prophylactic (25 ug mL^{-1}) and therapeutic (50 ug mL^{-1}) bath treatments discharged in effluents from aquaculture ponds. After sampling, seawater was filtered again and macroalgae discs were paper-dried to remove the excess water, weighted and frozen at -20°C.

2.3.1. LC-MS/MS

Seawater samples were diluted 100 times with 0.1% of formic acid in acetonitrile prior to direct injection.

As for macroalgae samples, an extraction step was required. Ethyl acetate, acetonitrile, 0.1% of formic acid in acetonitrile, dichloromethane and 0.1% of formic

acid in dichloromethane were investigated to determine the best extraction solvent The test was conducted with six blank samples spiked with STZ at 1.2, 2.5 and 5 mg Kg^{-1}. Sample size was also tested with 100 mg, 200 mg and 300 mg investigated. The final extraction protocol was performed with 100 mg of macroalgae accurately weighed, minced and placed in a 2 mL eppendorf tube to which were added 1.5 mL of acetronitrile and 15 µL of IS working solution (1 µg mL^{-1}). The mixture was vortex mixed, sonicated for 10 min and centrifuged at 1509 g for 10 min. The supernatant was transferred into a clean 10 mL Pyrex® conical centrifuge glass tube and the procedure repeated with 1.5 mL of acetonitrile. The resulting supernatant was then combined with the previous and the mixture evaporated to dryness under a gentle stream of nitrogen at 40°C. The dry extract was reconstituted in 0.2 mL of 0.1% of formic acid in acetonitrile and transferred into an amber glass HPLC vial with an integrated 0.3 mL insert vial. The extracts were stored at −20°C (maximum of three weeks) prior to sample analysis.

The chromatographic separation of the analytes was performed using a Gemini 3µ C18 110A (50 mm × 4.60 mm) analytical column in conjunction with a Security Guard Cartridge Gemini C18 (4 x 3.0 mm) from Phenomenex (Macclesfield, UK). The mobile phase consisted of 0.1% formic acid in water (phase A) and 0.1% formic acid in methanol (phase B), running in gradient mode with the following profile: 0-1 min, 95% A; 1-8 min, 0% A; 8-10 min, 95% A; 10-15 min, 95% A. The mobile phase was pumped at 500 µL min^{-1} for the whole run. Analyte detection was optimized by direct flow infusion of individual standard solutions of both compounds at 1 µg mL^{-1}, to select the most representative precursor and product ions for each analyte. STZ determination was performed with electrospray ion source operating in positive mode with source temperature set at 650°C and vacuum gauge at $3.5x10^5$ Pa. The transition conditions together with declustering potential (DP), collision energy (CE) and cell exit potential (CXP) specific for the detection of STZ and IS are summarized in Table 1.

Table I
Optimized HPLC-MS/MS detection parameters

Analyte	Precursor $[m/z]^+$	Product $[m/z]^+$	t_R (min)	DP	CE	CXP
STZ	256	156 [a]	13.8	66	21	14
		92		66	37	6
		108		66	33	8
IS	314	156 [a]	11.8	71	23	12
		92		71	45	6
		108		71	37	6

2.3.2. LC-UV

Water samples were injected directly after filtration through 0.22 µm filters.

Each individual macroalgae disc was thoroughly minced and placed in 15 mL conical centrifuge glass tubes. 5 mL of ethyl acetate were added and vortex mixed for 1 min (Retsh vortex mixer, Haan, Germany). Samples were let to stand for 10 min followed by vortex mixing and centrifuged at 3000 rpm for 10 min. The supernatants were then transferred to new tubes and evaporated to dryness under gentle nitrogen stream at 50º C. Dry residues were reconstituted with 500 µL of ultrapure water and placed in vials before injection.

LC-UV determination was conducted with a Gilson modular system (Gilson, Middleton, WI, USA) equipped with a pump (Gilson 321) and an automatic injector (Gilson 234) coupled to an UV/Vis detector (Gilson 155). The chromatographic column used for separation was a 250-4 LiChrospher 100 RP-18 column (Merck, Darmstadt, Germany) and a NewGuard C18 pre-column (PerkinElmer, Norwalk, USA) equilibrated at 25°C. The selection of the most accurate wavelength for elution monitoring was

performed by scanning a solution of STZ from 200 to 400 nm, with maximum absorption reached at 288 nm. STZ was analyzed in isocratic mode with a mobile phase constituted by: A) 0.1% formic acid in water (70%) and B) methanol (30%). 20 µL of sample were injected, flowing at a rate of 1.2 mL min^{-1} for a total run time of 10 min.

2.4. Validation

The validation procedures for both methods followed the guidelines established by the ICH on the performance of analytical methods to register pharmaceuticals for human use (ICH 1994, 1996), since currently there is still a lack of legislation specifically directed to environmental matrices including macroalgae.

Specificity was assessed with the analyses of 20 blank samples collected at different sites to exclude the possible existence of endogenous interferences in the identification of the analyte at the expected elution time.

Precision, determined as within-day repeatability (intra-day precision) and within-laboratory reproducibility (inter-day precision), linearity, limits of detection (LOD) and quantification (LOQ) were calculated through the analyses of three calibration curves performed at three distinct days. Each calibration curve was constructed with five concentration levels (5-5000 µg kg^{-1}) and with 6 replicates. The within-day repeatability was calculated with three sets, each with six fortified samples where the resulting mean concentration was used to determine the relative standard deviation (RSD), expressed as CV, with n = 6×3. To establish the within-laboratory reproducibility the same procedure was repeated for three days, with fresh sets of fortified samples and solutions prepared daily. The CV was calculated from the mean concentration between days.

Standard calibration curves in the same range as the fortified samples were also prepared each day. The coefficient of correlation (R^2) to assess linearity was established by plotting the response of the corresponding STZ/IS peak area ratio against the STZ/IS concentration for the MS/MS detection and peak area *versus* respective STZ concentration for UV detection.

3. Results and Discussion

3.1 Optimization of the MS/MS methodology

Several methods have been described previously for the determination of STZ residues in matrices such as liver, kidney, milk and honey which include characterization studies of the mass spectra of the analyte (Bogalli et al., 2003; Serra and Lacalle, 2009; Gao et al., 2010). Based on these works, electrospray ionization was chosen and performed in positive mode, which was more favorable. After scanning the representative ions with direct infusion of standard solutions, the most characteristic ion *m/z* was 156.0 (Table 1). The MS settings were then tuned to achieve the maximum signal response for each ion and data acquired through selected reaction monitoring (SRM).

As for elution, the first gradient tested was based on a method for the simultaneous determination of nine sulfonamides through LC-MS/MS (Nebot et al., 2010), where the antibiotics were separated with a Synergi 4 μ Polar-RP 80A (50 mm × 2.00 mm) column and a mobile phase consisting of 0.1% of formic acid in water and in acetonitrile. However, for the present method results showed that STZ was more retained by the column than the IS and for that reason different gradients were tested but no satisfactory results were obtained. Therefore a Gemini C18 column more suitable for less polar compounds was evaluated, resulting in good peak shapes for STZ. Repetitive injections of standard solutions containing a mixture of STZ (5 - 50 μg L^{-1}) and IS at different concentrations showed no variability in the retention time (t_R) of the compounds (RSD < 0.1 %). The experiment was repeated with seawater and macroalgae extract and the t_R of the analytes remained stable (RSD < 0.1%). The analytes were identified by their t_R and two SRM transitions.

As for the most suitable extraction solvent, recoveries of STZ extracted from *U. lactuca* were higher with ethyl acetate (73%) and acetonitrile (70%) than with dichloromethane (41%), acidified dichloromethane (18%) and acidified acetonitrile (40%). However, acetonitrile was selected as the most suitable solvent as it presents a lower RSD (4%) than the one calculated for ethyl acetate (15%).

Regarding sample size, interferences increased with weight and no improvements were obtained in the determination of LOD and LOQ. The best results were achieved with 100 mg of macroalgae, which set a LOD of 2.79 ng g^{-1} and a LOQ of 9.98 ng g^{-1} (Table 2).

3.2 Optimization of the UV methodology

A previous work developed for HPLC-UV determination of FTD in *U. lactuca* tested the extraction of the antibiotic through solid phase extraction (SPE) and liquid-liquid extraction (LLE) (Leston et al., 2011b) with both techniques producing similar results. This was expected since *U. lactuca* presents a low level of complexity when compared with more structured matrices such as meat, liver or eggs, with higher levels of interferents that require removal. Based on these findings, LLE was also applied in the present study to reduce time and costs associated with SPE. As for the choice of the extraction solvent, recoveries were higher for ethyl acetate (> 70 %).

For the selection of the most adequate mobile phase, the zwitterionic character of STZ had to be considered. This characteristic indicates it can easily capture a proton from the medium and to prevent this, an acidic eluent should be used. In the present work, the best results were obtained with ultrapure water acidified with 0.1% formic acid in combination with methanol, avoiding the use of buffers.

3.3 Validation

Through the analyses of blank samples both methods described were proven to be specific for seawater and macroalgae. The comparison with chromatograms of fortified samples showed no interfering peaks at the retention time (Figs 2-5).

STZ and IS stock solutions were stable for at least eight weeks when kept at -20°C and protected from light whereas standard and working solutions were stable for four weeks at 4°C. Standard and working solutions for UV detection were, however, stable for only 2 weeks which is related to the dilution in seawater.

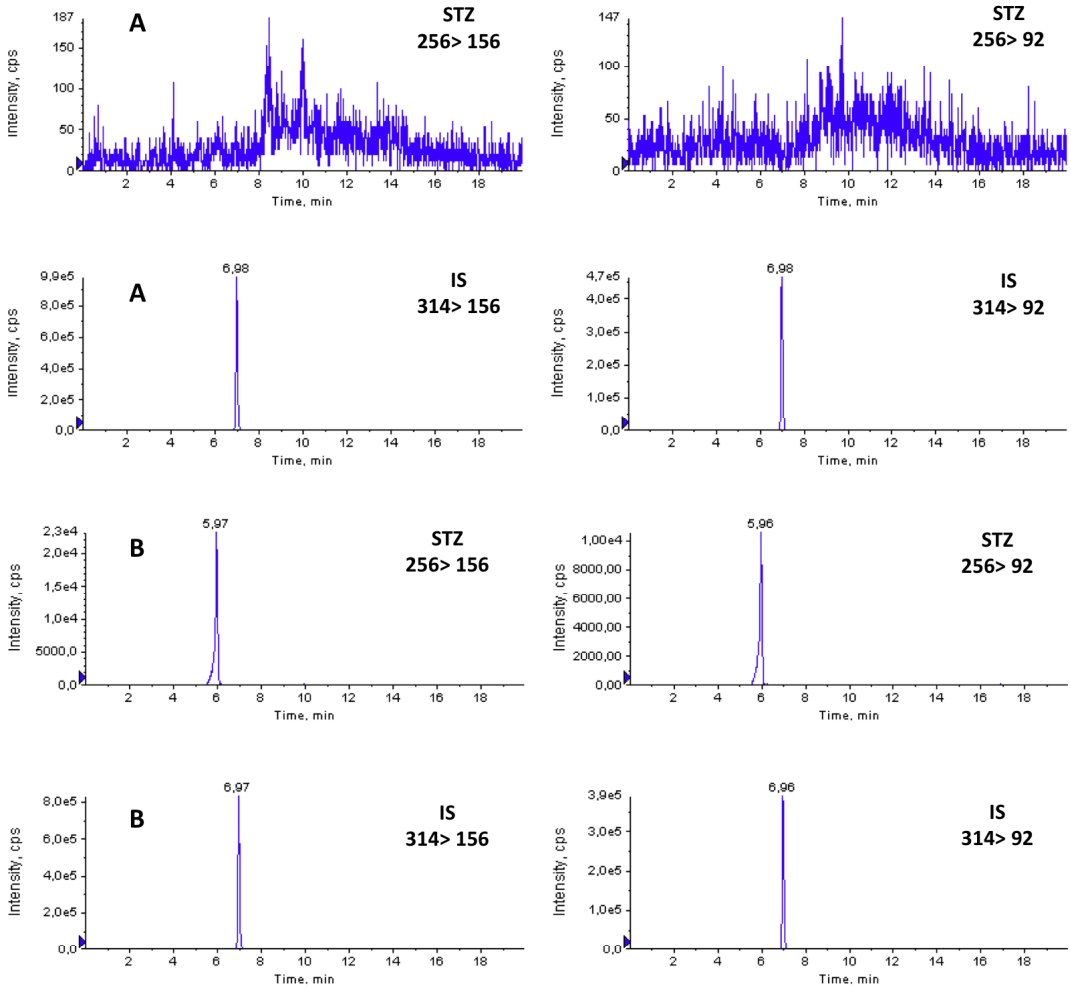

Figure 2. SRM chromatograms of sulfathiazole and the internal standard (IS) for (A) blank seawater sample and (B) a fortified seawater sample at 10 µg L^{-1} of STZ.

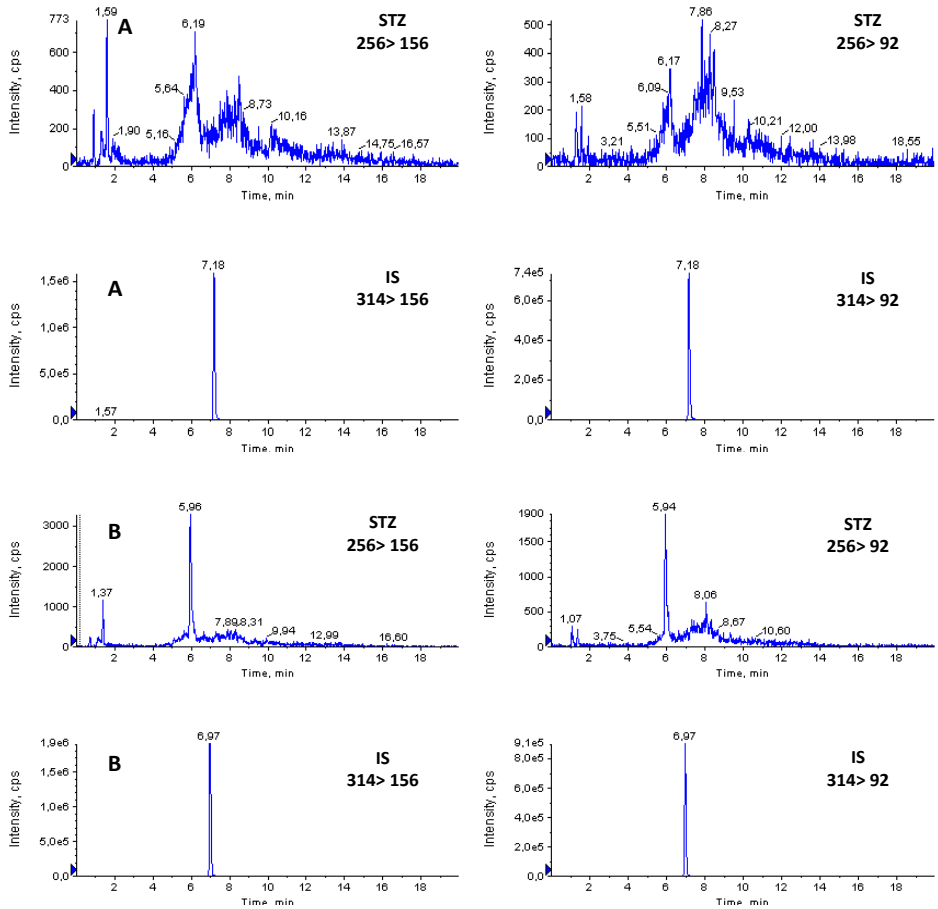

Figure 3. SRM chromatograms of sulfathiazole and the internal standard (IS) for (A) blank macroalgae sample and (B) a fortified macroalgae sample at 10 µg L^{-1} of STZ.

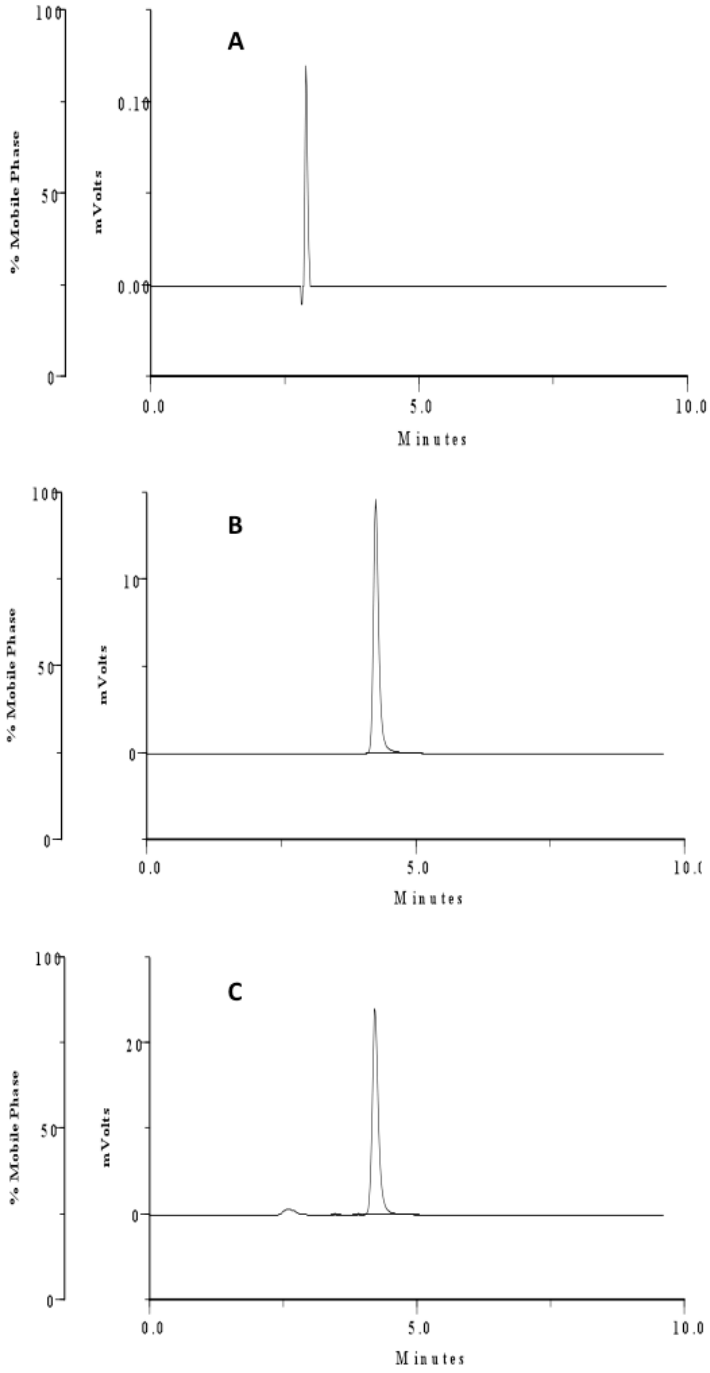

Figure 4. LC–UV chromatograms of STZ (A) blank seawater sample, B) STZ standard solution at 10 µg mL^{-1} and C) a fortified seawater sample at 10 µg L^{-1}.

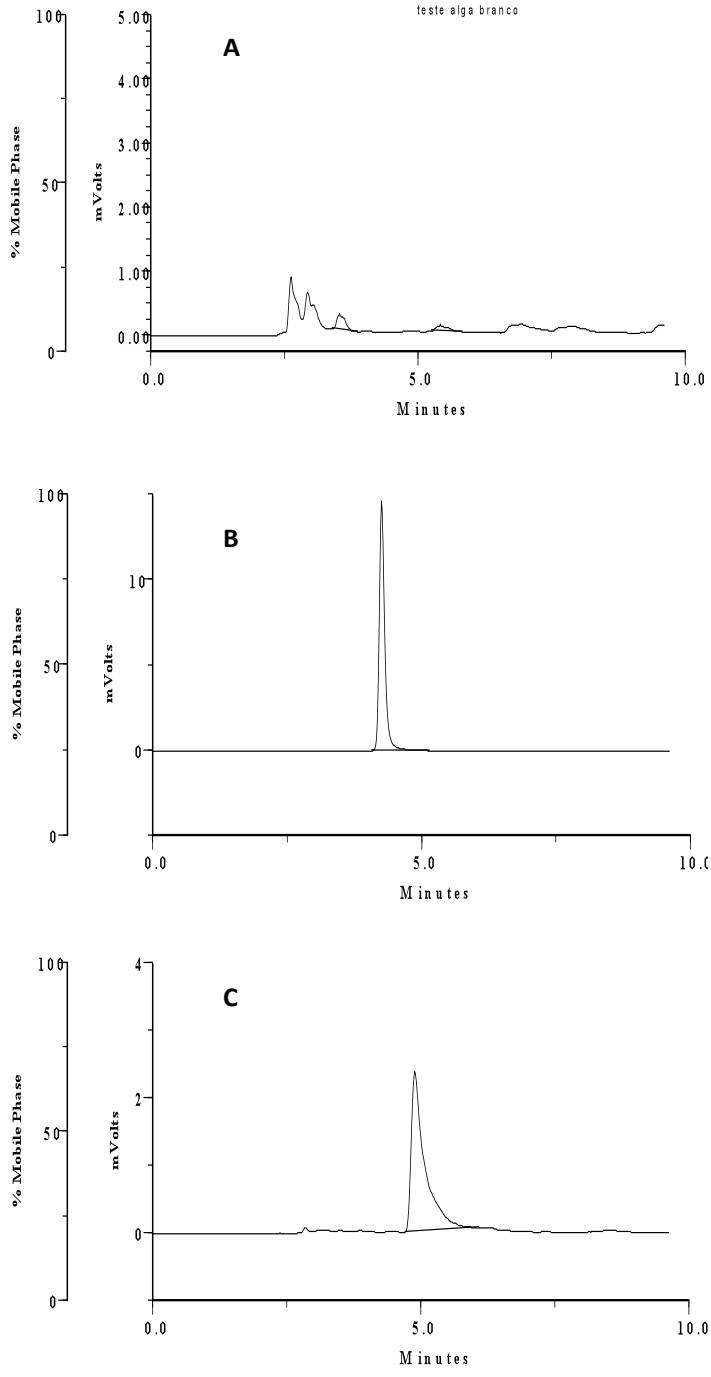

Figure 5. LC–UV chromatograms of STZ (A) blank macroalgae sample, B) STZ standard solution at 10 µg mL^{-1} and C) a fortified macroalgae sample at 10 µg L^{-1}.

Linearity was confirmed with the high correlation coefficients (R^2) obtained from the calibration curves, with values always above 0.99 (Table 2). These results show that both methods can be applied to determine STZ in a wide contamination range.

As for within-day repeatability and within-laboratory reproducibility MS/MS presented higher CV values than UV detection with 13.88 and 18.33% against 4.64 and 9.27% respectively (Table 2).

Relatively to the LOD and LOQ, the values obtained were very different for both methods. With MS/MS detection, the values determined were one order of magnitude lower (Table 2). The comparison with the values obtained with UV detection indicate that mass detection is, as expected, a more sensitive method which is essential for the application of the method to the analysis of waters at the threshold concentration levels of antibiotics.

Table II

Validation parameters for MS/MS and UV

Method	Matrix	Linearity (R^2)	Within-day repeatability (CV)	Within-laboratory reproducibility (CV)	LOD (ng g^{-1}/ µg g^{-1})	LOQ (ng g^{-1}/ µg g^{-1})
MS/MS	Seawater	0.9994	2.53	9.05	1.4	3.3
	Macroalgae	0.9933	13.88	18.33	2.79	9.98
UV	Seawater	0.9983	7.47	11.80	2.88	8.72
	Macroalgae	0.9968	4.64	9.27	2.83	8.56

3.4 STZ in Seawater and Macroalgae

The main purpose for the present work was to develop two methods that could be applied to the quantification of STZ in environmental matrices, more specifically seawater and the green macroalgae *U. lactuca*. Samples taken during the 24h exposure assay were analyzed with both methods. The mean concentration values determined by MS/MS and by UV in the water samples at the end of the prophylactic

trial were not significantly different and were respectively 20.15 and 20. 10 µg mL^{-1}. As for the therapeutic treatment the values were also similar, more specifically 45.12 and 45.19 µg mL^{-1}. These results represent a 20% reduction in concentration for the prophylactic treatment and only 10% for the therapeutic trial, which was not expected since studies indicate this compound is readily photodegraded in natural waters (Boreen et al., 2004). The potential slow degradation of STZ in natural seawater may have significant environmental consequences, especially in the vicinities of discharge points of aquacultures and other animal husbandries, but a more detailed research should be conducted.

Macroalgae samples were also analyzed to establish the amount of STZ taken up from the water in the same period of time, under both methods described. In the prophylactic trial the mean concentration quantified by MS/MS detection was 5.62 whereas with UV detection 5.53 µg g^{-1} and the highest concentration trial values were respectively 5.02 and 4.98 µg g^{-1}. These results indicate that both methods can efficiently quantify concentrations of STZ in this matrix and that the values do not differ significantly. Moreover, results also indicate that *U. lactuca* seems to reach a maximum internal concentration of STZ since the concentrations measured were in the same range, regardless of the amount present in the water.

As primary producers, macroalgae represent an important link in the trophic web and since it was shown STZ can be taken up and accumulated, the risk of biomagnification of this antibiotic and of other sulfonamides is very high and should be further investigated.

4. Conclusion

The present study described two methods for the determination of sulfathiazole in the green macroalgae *U. lactuca* through MS/MS and UV. Both methods were validated following the ICH guidelines. MS/MS prove to be more sensitive, with lower LOD and LOQ, indicated for routine use and for samples where residual concentrations are expected in the µg Kg^{-1} range. UV detection also produced good

results and can also be routinely applied in samples from environments with higher amounts of STZ (mg Kg^{-1} range) such as fish farms and other animal husbandries.

The 24h exposure assessment of *U. lactuca* at two concentrations of STZ in seawater showed it can take up and accumulate the antibiotic, representing a good candidate to be used as an indicator of contamination. However, these findings must be further confirmed.

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The Influence of Sulfathiazole on the Macroalgae *Ulva lactuca*

Abstract

The occurrence of pharmaceuticals in natural ecosystems has only recently been acknowledged as an important issue that must be addressed. The presence of these emerging contaminants is primarily a result of human activities from their administration in human and veterinary medicine for prophylaxis and therapeutic treatments and also to improve feed efficiency. Sulfonamides (SA) are a class of antibiotics routinely found in environmental matrices and therefore their role as contaminants should be investigated in non-target organisms. The present experimental work has evaluated the exposure of the chlorophycean *Ulva lactuca* L. to sulfathiazole (STZ), a SA drug commonly used in aquaculture, at two concentrations representing prophylactic (25 μg mL^{-1}) and therapeutic (50 μg mL^{-1}) administrations. Results showed that STZ presents high stability in seawater with only 18% degradation over the 5 days assay at both dosages tested. Also, macroalgae demonstrated an efficient uptake capacity with constant internal concentrations after 24h regardless of the external solutions and thus should be considered as a bioindicator species in risk assessment. As for the influence of STZ on growth, it induced a slight inhibition after 96h at both concentrations, which is likely related to mechanism of action of the drug disrupting the folate synthetic pathway.

Introduction

Natural ecosystems are continuously challenged by change whether gradually and at smaller dimensions or at a massive and abrupt pace, shifting the systems away from optimum conditions. Chemical contamination of anthropogenic origin represents a significant stressor to the environment since it can be responsible for adverse ecological effects in the systems (Swindoll et al., 2000). Environmental contaminants comprise various classes of substances with different chemical properties from metals to organic compounds. Within this category, pharmaceuticals represent a particular group of substances to which special attention must be given since they are designed to cross biological barriers and induce specific responses (Halling-Sorensen et al., 1998). The potential harm resulting from their administration in human and veterinary medicine is well reflected in current legislations, which set maximum levels of veterinary drug residues in food products to assure human health protection (EC Regulation 37/2010). Limits in environmental matrices, however, are more difficult to establish due to the very high number of drugs available and the uncertainty of responses in non-target organisms. Due to the impossibility of studying the whole ecosystem to determine the effects of a given drug, several species have become increasingly used as bioindicators and biomonitors of ecological distress (Villares et al., 2002; Melville and Pulkownik, 2007). In this sense a bioindicator will indicate exposure to a given contaminant by its presence or absence whereas a biomonitor must be able to accumulate the compound in its tissues and provide a detectable and time-integrated estimate of the available concentration (Rainbow and Phillips, 1993; Melville and Pulkownik, 2007). When considering a particular group of organisms for both functions attention to its characteristics is very important and should include wide geographic distribution, sedentarity and unequivocal identification (Rainbow and Phillips, 1993; Melville and Pulkownik, 2007). Usually, animal species are selected but primary producers are also being researched for these roles, including macroalgae (Coogan et al., 2007; Melville and Pulkownik, 2007; Akcali and Kucuksezgin, 2011; Costa et al., 2011; Leston et al., 2011). Macroalgae contribute with high biomass production in aquatic ecosystems and

also represent an important link in the trophic webs, which can lead to the biomagnification of contaminants.

Sulfonamides (SA) are extensively used pharmaceuticals with antibacterial activity against a large range of infectious agents by competitively inhibiting the use of *p*-aminobenzoate in the biosynthesis of folic acid (Braschi et al., 2010; Michelini et al., 2012). SA are administered in the treatment of respiratory and urinary infections and in veterinary medicine are used for prophylaxis and therapy and also to increase food conversion efficiency in the animal production sector (Boreen et al., 2004; Sarmah et al., 2006). Sulfathiazole (STZ) is a routinely prescribed drug that, together with other SA, has repeatedly been detected in the environment, reaching the water bodies discharged in wastewaters from hospitals, sewage treatment plants and even aquacultures facilities, escaping soil filtering and depuration actions due to their anionic character (Halling-Sorensen et al., 1998; García-Galán et al., 2008; Braschi et al., 2010; Hruska and Franek, 20120).

The ecological consequences of the presence of STZ in the environment and the possible effects induced in non-target organisms require further research. To enhance the knowledge on these effects the present study describes experimental data on the exposure of the green macroalgae *Ulva lactuca* L. to STZ focusing on the following points: (i) to investigate the stability of STZ in natural seawater, (ii) to assess the influence of STZ on *U. lactuca*'s growth at two concentrations simulating prophylactic and therapeutic dosages (iii) to investigate the possibility of bioaccumulation and biomagnification of the contaminant through the trophic web and (iv) to evaluate the potential use of *U. lactuca* as a bioindicator and biomonitor for the presence of STZ.

2.Experimental

2.1 Chemicals and Solutions

The STZ analytical standard (Fig. 1) and the internal standard sulfadimethoxine-d6 were purchased from Sigma-Aldrich (Steinheim, Germany). Formic acid and ethyl acetate were acquired from Merck (Darmstadt, Germany) while methanol and

acetonitrile were from Scharlau Chemie (Barcelona, Spain). All chemicals were filtered and degassed and of LC analytical grade. Ultrapure water was obtained daily from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

An individual STZ standard stock solution of 1 mg mL^{-1} was prepared by dissolving 50 mg of the compound in 50 mL of methanol and kept at -20°C in an amber glass bottle. Just before beginning the experiment two intermediate standard solutions were adjusted from the stock by dilution in filtered natural seawater to obtain two final concentrations of 25 µg mL^{-1} and 50 µg mL^{-1}**.** Both solutions intend to simulate discharges from aquaculture tanks after the administration of bath treatments with concentrations representing hypothetical dosages for prophylaxis (25 µg mL^{-1}) and therapy (50 µg mL^{-1}).

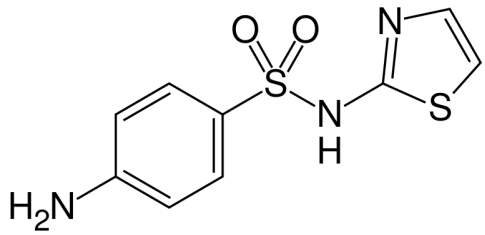

Figure 1. Chemical structure of sulfathiazole.

2.2 Sampling and Acclimation

Ulva lactuca fronds were collected at the Mondego Estuary (Portugal, 40° 80' N, 8° 50' W) during low tide. Macroalgae were thoroughly washed and rinsed to assure the absence of organisms and debris and then placed in refrigerated coolers for transportation to the laboratory. Fresh natural seawater was also collected at the site in acid washed amber bottles and upon arrival, immediately filtered (0.45 µm) and stored at 4°C until use.

To simulate the optimum conditions for *U. lactuca* the experiment was conducted in a controlled room with the photoperiod set to 14:10h LD (light:dark), 25°C and under 80 µmol photons m^{-2} s^{-1} of white fluorescent light. Tanks with 40 L capacity were filled with filtered seawater (35 psu) to which was added Provasoli Enriched Medium to a final concentration of 20 mL L^{-1} (PES; Provasoli, 1963 and modified by Bold and Wynne, 1978) and aerated for 48h. After this time fronds were once more carefully inspected before placement in the tanks. Acclimation was kept for three weeks prior to the beginning of the experiment.

2.3 Experimental Design

In the preceding 24h acid washed Erlemeyer flasks were filled with 250 mL of seawater and 5 mL of PES, placed on orbital shakers and acclimated during that period in the same conditions described above but with aeration replaced by a constant horizontal stir at 100 rpm. Right before start algal disks with Ø 5 cm (approx. 20 cm^2) were cut, separated in the different groups, weighed and photographed. STZ solutions were prepared and added to the corresponding flasks. Based on the concentrations, two groups, designated P and T, were established with group P testing the prophylactic concentration (25 µg mL^{-1}) whereas group T corresponded to the therapeutic dosage (50 µg mL^{-1}). Each group was composed of three replicates per sampling time (one flask constituted one replicate), with each replicate containing three algal disks. Two control groups were also set under the same conditions. Control A was established to verify the behavior of STZ in seawater for both concentrations P and T without the presence of macroalgae whereas Control B was prepared to assess the natural growth of *U. lactuca* in the absence of the antibiotic. The effect of methanol present in the solutions was assessed in a previous work following the same experimental design and for that reason was not replicated here (for further reading see Leston et al., 2011a). To prevent losses due to evaporation but still allowing gas exchange every flask was covered with glass

lids. Water samples and photographs to register growth variations were taken at the following times: 0, 1, 2, 5, 8, 12,16, 24, 48, 72, 96 and 120h.

2.4 Sulfathiazole Quantification

2.4.1 Water analysis

At each sampling time, temperature, salinity and pH were measured and water samples filtered through glass fiber filters (Ø 0.22 mm) and immediately frozen at -20°C until extraction.

Water samples for STZ quantification were transferred to appropriate LC-UV vials and placed in a Gilson modular system (Gilson, Middleton, WI, USA) equipped with a pump (Gilson 321) and an automatic injector (Gilson 234) coupled to a UV/Vis detector (Gilson 155). The chromatographic column used for separation was a 250-4 LiChrospher 100 RP-18 column (Merck, Darmstadt, Germany) and a NewGuard C18 pre-column (PerkinElmer, Norwalk, USA) equilibrated at 25°C. Elution of STZ was conducted in isocratic mode with a mobile phase constituted by: A) 0.1% formic acid in ultrapure water (70%) and B) methanol (30%). 20 µL of sample were injected, flowing at a rate of 1.2 mL min^{-1} for a total run time of 10 min.

2.4.2 Macroalgae

To establish the uptake and accumulation capacity of *U. lactuca* of STZ in the water, internal concentrations were quantified with a LC-MS/MS methodology. After removal from the water, algal disks were paper-dried to remove the excess water, weighed and frozen at -20°C. For extraction of STZ in *U. lactuca*, 100 mg of sample were thoroughly weighed, minced and placed in 2 mL Eppendorf tubes followed by addition of 1.5 mL of acetonitrile and 15 µL of IS working solution (1 µg mL^{-1}). After vortex mixing and sonication, samples were centrifuged for 10 min and the supernatant collected to a clean 10 mL conical centrifuge glass tube. The procedure was repeated with another 1.5 mL of acetonitrile added to the remnant sample. The supernatant resulting from the second extraction was then combined with the previous and the mixture evaporated to

dryness under gentle nitrogen stream at 40°C. The dry residue was reconstituted with 0.2 mL of 0.1% of formic acid in acetonitrile and placed in an amber glass vial. Chromatographic analysis was carried out with Gemini 3µ C18 110A (50 mm × 4.60 mm) analytical column in conjunction with a Security Guard Cartridge Gemini C18 (4 x 3.0 mm) , both from Phenomenex (Macclesfield, UK). Elution was run in gradient mode with a mobile phase consisting of 0.1% formic acid in water (solvent A) and 0.1% formic acid in methanol (solvent B), pumped at 500 µL min^{-1}.

2.5 Growth

The influence of STZ on *U. lactuca*'s growth was assessed by analysis of the variations in disk area. Photographs of each individual disk were taken at the beginning and end of each sampling time and analyzed with a computer-assisted software (Adobe® Photoshop® CS5 extended).

2.6 Statistics

After testing data for normality and homoscedasticity a one-way analysis of variance (ANOVA) was used to compare the differences in STZ uptake at both concentrations and to assess its influence in growth. The level of statistical significance for all analyses was inferred at $p < 0.05$. ANOVA was run with GraphPad Prism® 5 software (Graph Pad Software, Inc.).

3. Results and Discussion

3.1 Water

Solutions of STZ in seawater for groups P and T were prepared and analyzed previously to assure the proposed concentrations were obtained (Fig. 2). Control A samples taken at time 0 confirmed both concentrations, respectively 24.81 ± 0.1 and 48.96 ± 0.7 µg mL^{-1}. Through the analysis of the subsequent results it was possible to estimate a pattern of STZ degradation and assess its the stability under the given experimental conditions and in the absence of macroalgae. During the first 24h, the

prophylactic control group presented a 19% decrease in concentration whereas in the same time frame the therapeutic group suffered only an 8% reduction. In the following days the decline in concentrations decelerated and at the end of the trial the values of STZ recovered corresponded to 72% of the initial amount supplied for both dosages tested.

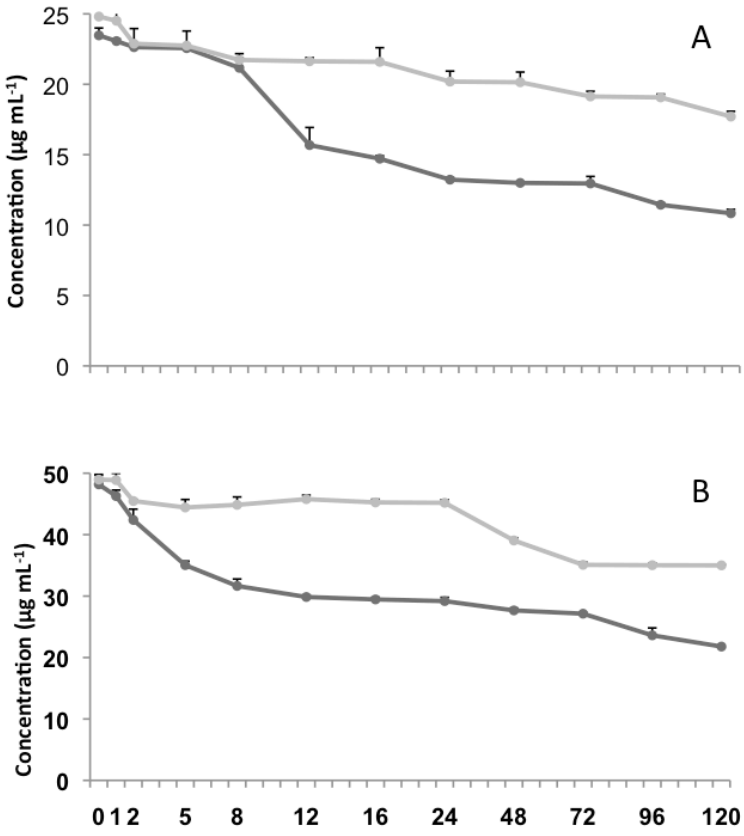

Figure 2. (A) Stability test of furaltadone in aqueous solution without (grey line) and with (dark line) the presence of *U. lactuca* at 25 µg mL^{-1}. (B) Stability test of furaltadone in aqueous solution without (grey line) and with (dark line) the presence of *U. lactuca* at 50 µg mL^{-1} during the course of the experiment. Data represent mean values of three independent replicates.

The degradation of pharmaceuticals in surface waters occurs mainly through two important abiotic processes, hydrolysis and photolysis (Park and Choi, 2008; Andreozzi et al., 2003). However, many compounds, including SA drugs are designed to resist hydrolysis after oral administration to maintain its biological activity thus making photolysis the main degradation mechanism (Andreozzi et al., 2003; Boreen et al., 2004; Kümmerer, 2009). Previous studies have reported that SA drugs are sensitive to light but not readily photodegradable (Lunestad et al., 1995; Kümmerer, 2009), presenting long half-life times ($t_{1/2}$) dependent on latitude and seasonality with higher persistence at higher latitudes and during winter (Andreozzi et al., 2003; Boreen et al., 2004). It was also found that SA photodegradation is pH dependent and for STZ, values in the basic range would increase the rate. In the present work, the temperature simulated (25°C) is usually found during summer and according to Boreen and colleagues (2004) at pH 7, STZ should have presented a $t_{1/2}$ of 3h resulting from direct photolysis, which was not verified. The pH values for seawater in the current research were always between 7-8. On the other hand, it has been suggested that the presence of catalysts naturally occurring in environmental waters such as nitrate (NO_3^-) can speed the degradation rate (Andreozzi et al., 2003). In order to assure an optimum growth nutrients, including NO_3^-, were supplied to the medium but nevertheless concentrations of STZ still remained very high. Therefore, under the present experimental conditions the high stability of STZ in seawater is in agreement with other works that stated SA drugs are resistant to degradation and can persist for long time periods in the environment (Halling-Sørensen et al., 1998; Kümmerer, 2001, 2009; Baran et al., 2006; García-Galán et al., 2008).

3.2 STZ Uptake

To evaluate the potential role of *U. lactuca* as a bioindicator and/or biomonitor for the presence of STZ in the aquatic environment it was essential to assess its capacity for uptake at both dosages tested. Each macroalgal disk was analyzed to determine the internal concentration present and to establish a comparison between these values and the remainder concentrations in the surrounding water. The pattern of uptake was

similar for both groups P and T with the highest amounts reached at time 0, respectively 6.81 ± 0.35 and 7.32 ± 0.15 μg g^{-1} (Fig.3 A and 4A). From the initial values, concentrations suffered a slight decrease in the following 24h and again at 48h remaining constant thereafter, with minimum values of 5.21 ± 0.15 and 4.78 ± 0.16 μg g^{-1} for P and T respectively. No statistical differences were found between both groups at $p > 0.05$. Considering the concentrations of STZ in the water, the pattern was very different from control A, for both groups. After 24h, group P presented a reduction of 44% of the initial concentration (13.23 ± 0.15 μg g^{-1}) whereas concentrations in group T decreased to 40% of the initial value (29.18 ± 0.61 μg g^{-1}) during the same time (Fig. 2). At the end of the experiment STZ present in the water was less than 46% in both groups, a much lower percentage compared to the control groups where 72% of the starting values were still detected which may be attributed to the presence of the macrophytic biomass.

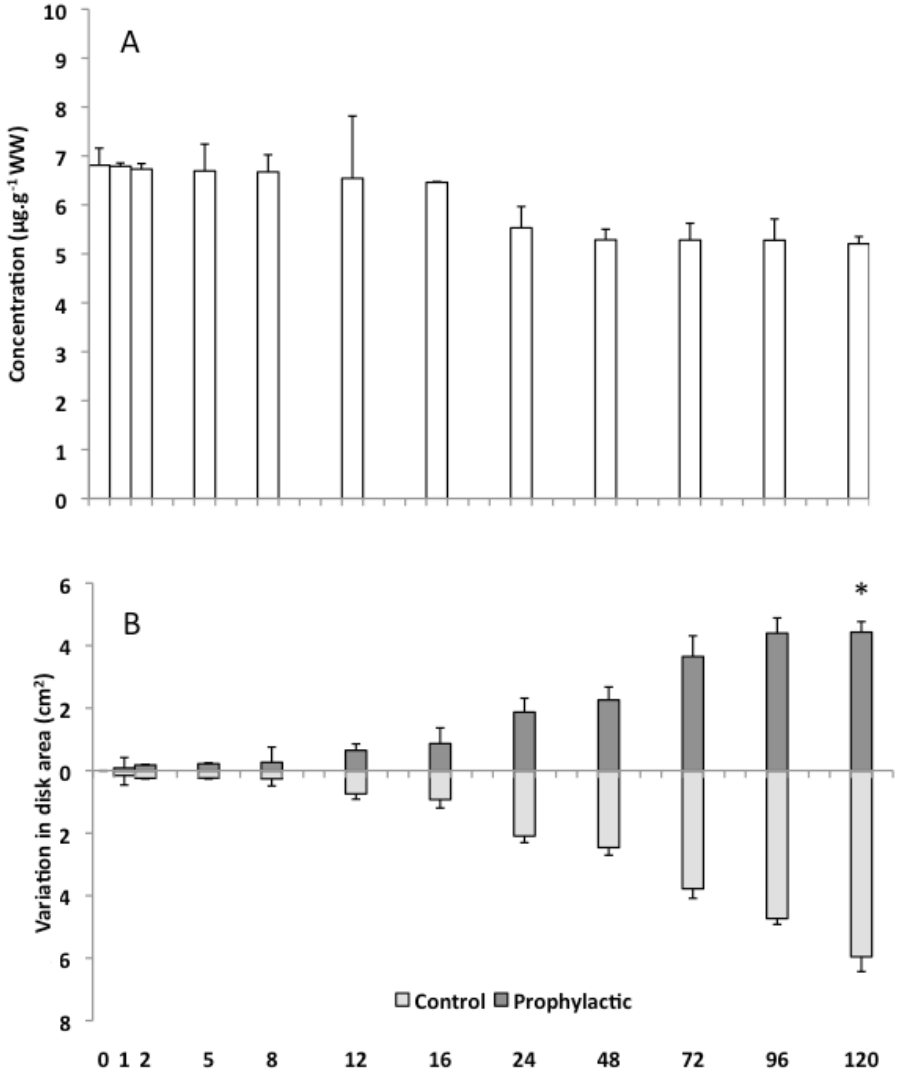

Figure 3. (A) Uptake of STZ determined as internal concentrations (µg g^{-1} WW) in *U. lactuca* for each sampling time (bars) in comparison with the concentration in aqueous solution for the correspondent time point (-), for the prophylactic group P. Data represent mean values ± SE of three independent replicates for each point. (B) Growth measured as variation in disk area (cm^2) at each sampling point for group P, plotted against the control. Data represent mean values ± SE of three independent replicates for each point. Asterisk (*) indicates results significantly different from the control.

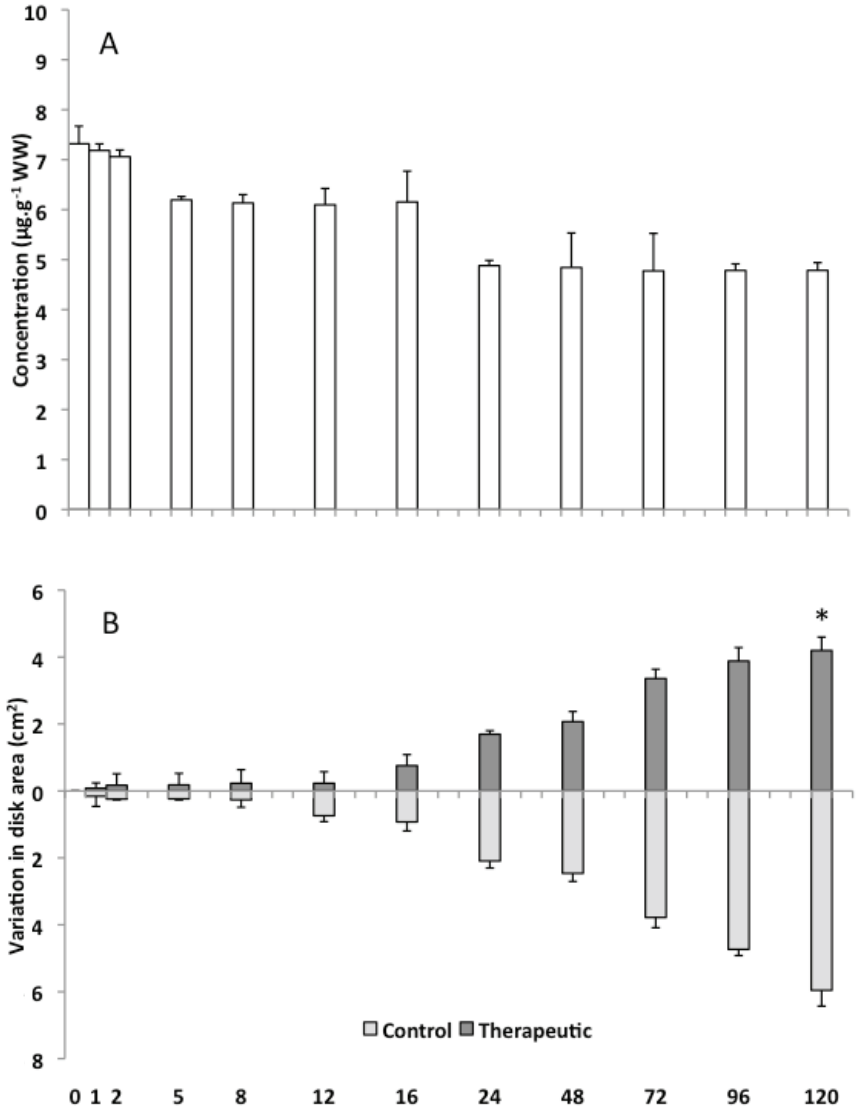

Figure 4. (A) Uptake of STZ determined as internal concentrations (µg g^{-1} WW) in *U. lactuca* for each sampling time (bars) in comparison with the concentration in aqueous solution for the correspondent time point (-), for the therapeutic group T. Data represent mean values ± SE of three independent replicates for each point. (B) Growth measured as variation in disk area (cm^2) at each sampling point for group T, plotted against the control. Data represent mean values ± SE of three independent replicates for each point. Asterisk (*) indicates results significantly different from the control.

One important descriptor in drug development is the octanol/water partition coefficient (K_{ow}) that provides a good estimate of the hydrophobicity/hidrophilicity of a given substance and thus is used to predict membrane permeability. The same coefficient is applied in environmental risk assessment to anticipate the uptake efficiency of contaminants by non-target organisms and their subsequent bioaccumulation potential (Walker et al., 2006; Fatta-Kassinos et al., 2011). With a log K_{ow} of 0.05 (Pérez et al., 2005) STZ is a highly hydrophilic compound that can easily penetrate cell membranes through simple passive diffusion. However, despite the high levels of STZ available in the water the internal concentrations remained low and constant. In a previous work to test the effects of furaltadone under the same experimental conditions, *U. lactuca* was able to take up almost 19 µg g^{-1} within the first hours of exposure to the prophylactic dosage (Leston et al., 2011). This nitrofuran antibiotic presents a log K_{ow} of 0.2, which is higher than STZ and for this reason high internal concentrations were also expected in the present study. One likely explanation for the low concentrations found may reside in the protective detoxification mechanisms present in *U. lactuca* that are activated in the presence of xenobiotics to reduce the potential harmful effects (Sandermann, 1992; Coleman et al., 1997; Pflugmacher et al., 1999; Torres et al., 2008). Glutathione-S-Transferases (GST) represent an important enzymatic complex in these systems with an active role in the degradation of STZ. It is possible that GST's could have been involved in maintaining constant internal concentrations of the antibiotic while contributing to its reduction in the water (Park and Choung, 2007).

One important objective for the present work was to assess the possibility to use *U. lactuca* as a bioindicator and/or biomonitor species for the presence of STZ in the environment. Since the internal concentrations resulting from uptake were constant and unable to reflect the concentrations in the water the role of *U. lactuca* as a biomonitor is not in agreement with the necessary requirements. However, it can be used successfully as an indicator for the presence of STZ. Moreover, since *U. lactuca* can

accumulate this contaminant and represents very high biomasses in natural ecosystems the risk of biomagnification along the trophic web is a serious concern.

3.3 Growth

One other crucial goal for the current research was to assess the influence of STZ on *U. lactuca* growth determined as variations in disk area. The results obtained with groups P and T were compared with control B, which set the pattern of growth in the absence of antibiotic (Figs. 3B and 4B). Both dosages tested followed the increasing pattern of the control group but became statistically different after 120h ($p < 0.05$). At this time growth variations for P and T were lower than the control, respectively 4.43 ± 0.34 cm^2 (Group P) and 4.20 ± 0.40 cm^2 (Group T) against 5.26 ± 0.47 cm^2 (control B), indicating STZ had an inhibitory effect on macroalgal growth although not pronounced since severe effects such as chlorosis were not observed. The influence of STZ on macroalgal growth demonstrated in this work is in agreement with findings on the exposure of *Lemna gibba* to SA drugs (Brain et al., 2004) where growth inhibition was detected after 96h for all the compounds tested. Another study by Baran and colleagues (2006) reported that, along with other substances tested, STZ caused inhibition to the green algae *Chlorella vulgaris*.

The research on the potential effects of STZ to non-target aquatic organisms is a key factor in determining its role as an environmental contaminant. The phytotoxicity demonstrated in macroalgae is not surprising since photosynthetic organisms have similar folate pathways as bacteria. Folate, also known as Vitamin B9, is involved in the transfer of carbon units (C1) in metabolic pathways crucial to cellular division and growth, including the synthesis of nucleic acids and aminoacids, pantothenate (Vitamin B5, necessary for coenzyme-A formation) and chlorophyll. In primary producers, it also has an important role in photorespiration. SA drugs are structural analogues of *p*-aminobenzoate (folate precursor) and through competition for the enzyme dihydropteroate synthase inhibit the formation of dihydropteroate, which is ultimately transformed to tetrahydrofolate (Hanson and Gregory, 2002; Basset et al., 2005; Brain

et al., 2008). The resulting deficiency in folates will have severe consequences on cell division that in turn will be translated in diminished growth. Nonetheless, studies have demonstrated that STZ-induced inhibition is concentration and time dependent (Brain et al., 2004; Baran et al., 2006; Park and Choi, 2008). The dosages tested in the present work are very high and only likely to be found in natural ecosystems in areas at and very close to discharge points from animal husbandries and therefore it is not expected that STZ will have a severe impact on macroalgal growth in the concentrations usually reported for environmental waters, which are in the ng L^{-1} to µg L^{-1} range.

4. Conclusions

The environmental presence of a given substance and its potential to cause adverse effects in non-target organisms will depend greatly on its ability to resist degradation. In the present study it was found that STZ presents high stability in seawater at the concentrations tested thus representing a source of chemical pollution in natural waters. Nonetheless, the presence of *U. lactuca* greatly influenced the concentrations of STZ in solution by efficiently taking up the compound from the water while maintaining the internal values constant at both dosages tested, most likely through a detoxification mechanism. In environmental risk assessment this ability makes it a suitable choice as a bioindicator species but ecologically it represents the bioaccumulation potential of STZ which given the role of macroalgae as primary producers in trophic webs poses the risk of biomagnification.

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General Discussion

The following section constitutes an overview of the several topics discussed in the previous chapters while linking the main outcomes to the specific aims of the present thesis. According to the European Community Regulation on chemicals and their safe use, compounds within the high tonnage range, as is the case of pharmaceuticals, require a short-term toxicity evaluation for aquatic invertebrates and growth inhibition studies for aquatic plants together with bioconcentration and bioaccumulation assessment (Smith and König, 2010). Until now there were no reports of comparable studies on exposure effects of green macroalgae to antibiotics. This research has furthered the knowledge on these issues while providing adequate and validated methodologies for the determination of antibiotics in macroalgae.

To assure an accurate assessment of antibiotics in the environment, the compound under evaluation must be unmistakably identified, which can be a difficult task when adequate methodologies are not available. The methods described in the previous chapters were developed to fulfill this requirement for the specific analysis of FTD, CAP and STZ in seawater and *U. lactuca*. The fact these antibiotics present low log K_{ow} (< 2) and are therefore considered hydrophilic drugs, provided an indication that in the aquatic environment they are most likely concentrated in the water compartment rather than adsorbed to sediment (Boxall et al., 2004; Walker, 2006). Together with the lack of knowledge on whether the metabolic processes already described in animals were likely to be found in the water phase it was concluded that the analyses should focus on the parent compounds rather than on the resulting metabolites.

Taking a closer look at the methodologies developed for seawater samples, consistency in the procedures was evident as the quantification of antibiotics was achieved through direct injection of filtered samples without the need for preconcentration and clean-up steps, reducing the volume necessary as well as time and costs when handling a high number of samples.

Unlike seawater, however, the preparation of macroalgal extracts involved further steps before being injected. The optimization of the analytical procedures for the quantification of each pharmaceutical was initiated with the selection of the most appropriate conditions for chromatographic separation. When choosing the analytical components to be tested the properties of each substance were taken under close consideration (Pavlovic et al. 2007). All three antibiotics are polar compounds requiring the use of organic solvents to which they can be extracted. Consequently, ethyl acetate was used in the first step of extraction followed by the addition of acetonitrile for FTD analysis in macroalgal samples. As for CAP, the extraction was achieved solely with ethyl acetate and in the case of STZ, depending on the chromatographic equipment used, extraction was achieved with ethyl acetate (LC-UV) or with acetonitrile (LC-MS/MS). The fact that, for STZ, the most suitable extraction solvent was determined by the sensitivity of the equipment shows the complexity of developing accurate analytical methodologies. Another important step is the reduction of interfering agents intrinsic to matrices to improve analyte recovery and identification. These matrix effects are usually removed through clean-up steps such as solid-phase extraction (SPE), which is currently the most commonly used technique. However, clean extracts were produced with and without SPE and therefore, in order to reduce time and costs, filtration through Ø 0.45 µm filters demonstrated to be sufficient. The selection of appropriate mobile phases also reflected the properties of each antibiotic to induce the least interference in analysis. FTD, for instances, demonstrated sensitivity to acidic pH values and required a neutral volatile salt solution as an additive for stability whereas for CAP and STZ acidified mobile phases were preferred.

Another challenge in developing new methodologies was the inexistence of specific validation parameters for environmental samples and as a result the guidelines followed were based on regulations set for approval of pharmaceuticals in human medicine (ICH 1994, 1996) and to the quality assurance of analytical procedures directed to substances and residues in live animals and animal products (EC Regulation 2002/657). When considering these guidelines the methods described were fully

validated for all the parameters required. However, since the presence of antibiotics in the environment involves a complex web of processes that ultimately dictate their availability and consequently their effects (Kim and Carlson, 2007; Kümmerer et al., 2009), suited regulations should be developed and implemented. Moreover, the MRL allowed in non-target organisms should take into account the continuous exposure of low concentrations of antibiotics and the influence on the trophic webs (Ternes et al., 2001; Crane et al., 2006; Kümmerer et al., 2009). Overall, the comparison of the limits of detection and quantification obtained with UV detection and MS/MS detection demonstrated, as expected, that the latter is a more sensitive method which is essential for the analysis of waters at the threshold concentration levels of antibiotics. Nonetheless, to monitor water and algae at and near discharge points of effluents from animal husbandries, the use of HPLC-UV methodologies is suitable and reduces costs in routine analyses.

The stability of the three antibiotics tested was the next objective of the present work, which was achieved through the analyses of experimental samples with the specific methodologies developed for each compound. The patterns obtained enforced the notion that despite the abiotic mechanisms associated with the degradation of pharmaceuticals are essentially the same, the patterns presented differed greatly. Photolysis and hydrolysis are considered the main processes under which antibiotic drugs are degraded but most substances are designed to resist hydrolytic decomposition to assure successful oral administrations (Boreen et al., 2004; Kümmerer et al., 2009). Therefore, in the present experimental designs light was expected to have a significant role in degradation. According to existing literature FTD, CAP and STZ are light-sensitive and it was anticipated that all compounds would photo-degradate (Boreen et al., 2004; Edlhund et al., 2006; Kümmerer et al., 2009). This was verified for FTD where concentrations reached 50% of the initial amounts supplied after 24h and also for CAP, where higher degradation rates were registered with less than 20% of the initial concentrations recovered after 120h. However, STZ remained very stable in seawater with only 28% of the compound degraded regardless of the

concentration. Also, the influence of other factors such as environmental parameters as pH, temperature, oxygen, the presence of calcium and sodium chloride or the existence of naturally occurring catalysers may slow or speed up the process (Andreozzi et al., 2003; Kümmerer et al., 2009; Zhou et al., 2010). Moreover, the degradation patterns reported here would be expected in natural clear waters which is not always the fact and external factors influencing light availability such as water turbidity or shadow may also interfere with degradation. Nonetheless, due to the impossibility of testing antibiotic stability in the field under natural conditions, the results obtained represent a valuable estimate of the behavior expected and an important basis in exposure assessment research.

With an increased knowledge on the stability of the three antibiotics in seawater the next step was to determine the ability for uptake and accumulation of each drug in solution while simultaneously establishing their influence on the growth of *U. lactuca*. The scenarios tested were based on the observation of great biomasses of this floating macroalgae at and in the vicinities of effluent discharge points of aquaculture facilities. Therefore, the concentrations to which *U. lactuca* was exposed were based on hypothetic dosages of prophylactic administration (25 µg mL^{-1}) and therapeutic bath treatments (50 µg mL^{-1}), which would ultimately be discharged into the ecosystem with tidal water renewal of the tanks. The experimental parameters were based on the optimum growth conditions of the macroalgae and special attention was also given in selecting healthy organisms. The log K_{ow} of the FTD, CAP and STZ were respectively 0.2, 1.14 and 0.05, which are very low values indicating the three antibiotics are highly hydrophilic and therefore predicted to easily cross the cell membranes (Halling-Sørensen et al., 1998; Tolls et al., 2001). This prediction was confirmed with the high internal concentrations obtained at the beginning of the experiments for all the compounds. However, the highest values were registered for CAP that presented the higher log K_{ow} value when it was expected to have the lowest concentrations. Moreover, FTD's highest concentration registered was attained after 5h while for the remaining drugs it was reached immediately. These results indicate that

although this descriptor gives a good estimate of how compounds will behave, uptake and accumulation are still dependent on other factors. This is notorious when taking a closer look at the accumulation patterns of each antibiotic. FTD was present in relatively high concentrations for the first 16h in the prophylactic group but after 24h it dropped to almost being inexistent and remained constant for the rest of the experiment. In the CAP trial the behavior was different with the pattern of accumulation for both groups being very similar, with the concentrations reflecting the values in the water. STZ, on the other hand, presented constantly low internal concentrations with values similar for both dosages tested regardless of the concentrations in the water. Based on the results, *U. lactuca* should be included in risk assessment programs, as a bioindicator for the environmental presence of the three antibiotics tested. However, its use in biomonitoring, can only be considered for CAP as it was the only antibiotic where the internal concentrations were a reflection of the values present in the water column. Finally, the fact that *U. lactuca* bioaccumulate antibiotics and maintain internal concentrations represents a serious risk of biomagnification in the trophic web through consumption by organisms in higher trophic levels.

The different patterns demonstrated the weight of the properties of each antibiotic on the responses of *U. lactuca* in the bioaccumulation process. The fact that the macroalgae was able to lower the internal concentrations also pointed to the possible presence of detoxification mechanisms. This was most evident in the FTD experiment when the macroalgae exposed to the prophylatic dosage was able to lower the concentrations and maintaining them constant while concentrations in the therapeutic group remained higher and constant after algal death. This observation indicates that the mechanism through which it regulated the antibiotic concentration was ceased and FTD accumulated remained. This possibility is also supported by the results obtained with STZ where regardless of the external values *U. lactuca* was able to maintain constant internal concentrations. These findings are in conformity with the concept of “green liver” introduced by Sandermann (1992), proposing a system by which plants are able to metabolize xenobiotics through endogenous enzymatic

biotransformation and elimination processes (Torres et al., 2008). Such system would consist of three phases very similar to what is found in animals divided in transformation (I) mostly in cytochrome P-450 monooxygenases, conjugation (II) under the activity of glutathione S-transferases (GST'S) and glucosyltransferases, and compartmentation (III) either in vacuoles or in the cell wall fraction (Sandermann, 1992, 1994; Coleman et al., 1997; Suresh and Ravishankar, 2004). Further research has demonstrated the existence of this system for detoxification of several contaminants (Mehrtens, 1994; Pflugmacher and Sandermann, 1998; Pflugmacher et al., 1999; Lewis et al., 2001; Mitsou et al., 2006; Torres et al., 2008). For instance, the use of GST's has been studied for the degradation of sulfonamides in effluent discharges which reinforces the possibility of the same enzymatic complex being responsible for the control of internal STZ exhibited in *U. lactuca* (Park and Choung, 2007).

Bioaccumulation is a result of the net accumulation of a given contaminant in living organisms (Torres et al., 2008). The consequences can be revealed at several levels from physiological responses in single organisms to influences in entire trophic webs. In the present research, the influence of each antibiotic on *U. lactuca* was analyzed as effects on growth, measured as variations in disk areas followed by a comparison to the control group. FTD caused an inhibition on growth at the lower concentration while at the therapeutic dosage it was proven lethal after 48h. CAP, unlike expected, induced growth at both concentrations, being more pronounced at the prophylactic dosage. Finally, STZ had little influence in growth during the length of the experimental trial inducing a light inhibition only at the end. The different effects in growth described here are most likely due to the mechanisms of action of each antibiotic in combination with their degradation metabolites. Most antibacterial agents are able to exploit differences in the structure or metabolism of bacterial and mammalian cells in order to achieve their selective effect, acting in different cell compartments and intervening in specific processes. FTD acts by inhibiting enzymes involved in the carbohydrate metabolism in bacteria. The exposure of *U. lactuca* to this nitrofuran antibiotic indicated that the effects on growth were dependent on the

concentration present with severe inhibition at lower concentrations and lethal toxicity at the highest. Also, the action of a detoxification mechanism would be responsible for the presence of the toxic metabolite AMOZ that in turn would act simultaneously with FTD causing increased toxicity to the macroalgae. CAP, on the other hand, was expected to lead to a strong inhibition in growth since it prevents protein synthesis at the ribosomal level by binding to the protein responsible for attaching the tRNA to the active site of peptidyl-transferase thus preventing transpeptidation. Nonetheless, it induced growth, which is most likely related to its photodegradation in water with the subsequent release of nitrate (NO_3^-) and ammonium (NH_4^+). *U. lactuca* presents a high affinity for both these ions which are present naturally in the environment and have been linked to eutrophication with the formation of algal blooms (Pedersen and Borum, 1997; Valiela et al., 1997; Lartigue and Sherman, 2005; Lillebø et al., 2005; Leston et al., 2008). As for STZ, it acts in the folate pathway by filling as a structural analogue of p-aminobenzoate, a precursor for folate, and by competition for the enzyme responsible for its conversion ultimately disrupts folate synthesis with consequences in cell division and growth (Braschi et al., 2010). The inhibition in growth evidenced by *U. lactuca* could therefore be explained by its presence but a more accentuated effect was expected. The fact that the macroalgae was able to maintain constant concentrations of STZ could help explain the mild effects described.

The results obtained for the influence of antibiotics confirm the notion that the basic effects of contaminants in photosynthetic organisms are reflected in growth. Moreover, toxicity tests based on algae are useful to assess associated environmental effects of contaminants and ecosystems integrity.

Future Perspectives

The work developed in the present thesis has contributed greatly to increase the knowledge on the behavior of antibiotics in natural ecosystems while revealing the so far unknown consequences of their action in the green macroalgae *U. lactuca*. It also demonstrated the potential use of these photosynthesizing organisms as bioindicators of pharmaceutical pollution while giving a useful overview of the ecosystem response to exposure to xenobiotcs. Nonetheless, to have a full view on the potential of macroalgae as tools in risk assessment and the mechanisms underlying the physiological responses here demonstrated, efforts towards the investigation of detoxification systems should be taken. Moreover, antibiotics do not occur individually in the environment but rather in mixture and consequently the effect of mixtures should be studied thoroughly as it is difficult to predict the possible outcome. Furthermore, multi-residue analytical methodologies applicable to the environmental matrices here presented should be developed to facilitate routine monitoring of ecosystems.

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"Would you tell me, please, which way I ought to go from here?"

"That depends a good deal on where you want to get to"

Lewis Carroll *in* "Alice's Adventures in Wonderland"