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An optimized sample treatment method for the determination of antibiotics in seawater, marine sediments and biological samples using LC-TOF/MS

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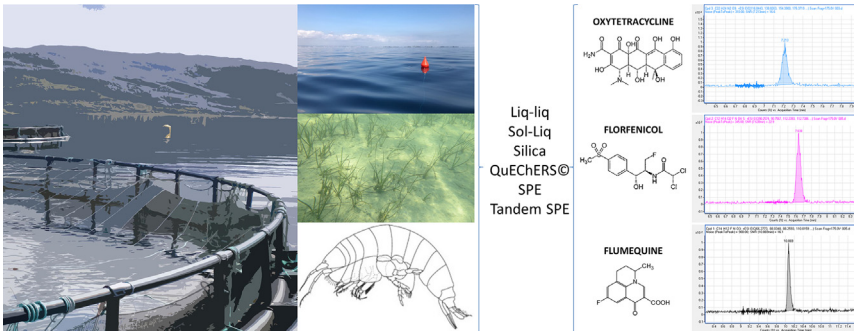
HIGHLIGHTS

- LC-TOF MS determination of antibiotics in several marine samples
- Antibiotic extraction from environmental samples is based on SPE tandem purification.
- The methods show a high sensitivity and reproducibility.
- MQLs of 0.1–0.5 μg L^{-1} in seawater; 0.3–50 μg/kg in sediments and biota samples
- Measurable concentrations of oxytetracycline and flumequine were found.

GRAPHICAL ABSTRACT



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ABSTRACT

Antibiotics used in marine aquaculture have been reported to accumulate in sediments and non-target aquatic organisms, modifying the biodiversity and the environmental conditions in areas close to the fish farms. Improved analytical methods are required to assess the spread and the impacts of aquaculture antibiotics in the marine environment, as well as to estimate resistance development risks. In this study, we have optimized a method for simultaneous quantitative determination of oxytetracycline, florfenicol and flumequine in marine samples using liquid chromatography coupled to time-of-flight high resolution mass spectrometry (LC-TOF/MS). The method optimization was carried out for seawater, sediment and biological samples (biofilm and two benthic invertebrate species: *Gammarus aequicauda* and *Monodonta articulata*). Special attention was paid to the optimization of the extraction and purification steps, testing: liquid-liquid and solid-liquid extractions, the use of silica and other commercial sorbents' clean-up, and single and tandem solid phase extraction procedures. The limits of quantification (MQLs) achieved with the developed method are 0.1–0.5 μg L^{-1} in seawater; 1–5 μg kg^{-1} in marine sediments; 5–25 μg kg^{-1} in biofilm; and 100–500 μg kg^{-1} in invertebrates, with good accuracy and precision. Method recoveries in spiked samples are 65–120% in seawater and sediment samples, and 63–110% in the biological samples. The method has been successfully implemented for the determination of antibiotic concentrations in sediment and invertebrate samples collected from a Mediterranean bay in south-east Spain. These represent significant advances in the analysis of antibiotics in environmental samples, especially for wild marine taxa, and attend for a proper assessment of the environmental fate and side effects of aquaculture antibiotics in the marine environment.

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1. Introduction

Assessing the occurrence and risks of anthropogenic organic pollutants in aquatic ecosystems has been a key research topic in environmental studies during the last decades. The more persistent, bioaccumulative and toxic contaminants have been traditionally regulated (Hengstler et al., 2006; UNEP, 2009), whereas the more degradable polar compounds (i.e., pharmaceuticals and home and personal care products) have been usually less controlled in the open environment (Boxall et al., 2012). Only recently, particular efforts have been dedicated towards the development of appropriate techniques to assess their environmental occurrence, fate and risks. In particular, antibiotics are of high concern, not only because of their chemical properties and the threat they may pose to wild aquatic organisms (Rico et al., 2014), but also because of their contribution to the development and spread of bacterial resistance in the environment (Martínez, 2008). The transfer of resistance genes from the environment to humans is expected to trigger the human sanitary system, and thus the presence of antibiotics in the environment should be further controlled and regulated (Felipe C. Cabello, 2006; Martínez et al., 2014).

Intensive aquaculture has been described as one of the highest local, continuous and direct sources of antibiotics into the aquatic environment globally (Felipe C. Cabello, 2006; Henriksson et al., 2017; Kümmerer, 2009a, 2009b). Antibiotics administered to caged fish may be spread into marine ecosystems (1) by leaching out from pelleted feeds, (2) by the deposition of uneaten fish feeds, and (3) by the spread of fish faeces containing antibiotic residues, and may be accumulated in sediments and in aquatic biota (Capone et al., 1996; Rico et al., 2014; Samuelsen et al., 1992). Therefore, advanced analytical methods are needed to assess the fate and spread of aquaculture antibiotics across different environmental matrices and to evaluate their bioaccumulation and risks to non-target aquatic organisms.

The low stability of most antibiotics and the low concentrations at which they can exert ecotoxicological effects and increase resistance levels makes the analytical determination of these compounds rather challenging (Boxall et al., 2012; Obimakinde et al., 2017; Seifrtová et al., 2009). Moreover, most of the available analytical methods are compromised by the complexity of the matrices in which they are analyzed (i.e., sewage waters, sediments, biological tissues and organs), and the particular physicochemical properties of the target compounds (i.e., ionisable at environmentally relevant pHs, susceptible to chelation and degradable) (Kümmerer, 2009a, 2009b; Seifrtová et al., 2009). It is common to find studies using rough methodologies leading to very high detection limits, which results on inaccurate environmental assessments. One of the practices commonly found in the literature is the use of matrix-matched calibration curves, which attempts for the elimination of large matrix effects during the analysis and quantification process (Ben et al., 2008; Miller et al., 2015; Pfeifer et al., 2002). However, this may encounter limitations as the chromatographic signal of the selected blank matrix of the calibration curve can be enhanced or supressed in the detector on a different extent than that on the real sample. Such method is particularly misguided for environmental samples that have a significantly different composition and origin than the blank matrix, e.g. due to the high variability in organic matter across monitoring sites (Hernández et al., 2007). In some studies, matrix interferences have been circumvented by diluting the environmental sample, however this approach usually affects negatively the sensitivity of the analytical method (Gómez et al., 2006). Conversely, the use of internal standards or standard addition quantification, which is highly recommended to account for matrix effects, is often neglected due to unavailability of isotopically labelled standards for many target analytes, their expensive prices, and/or the difficulty in finding surrogates that match the properties of the array of antibiotics that is being analyzed (Göbel et al., 2004; Hilton and Thomas, 2003).

Some antibiotics are applied together in animal production (e.g. sulfadiazines-trimethoprim) and may form complex mixtures in the environment due to their simultaneous application and discharge (Norambuena et al., 2013; Rico et al., 2014). The simultaneous determination of antibiotics can have constraints during the sample processing. For instance, the pH of the extraction solvent can favour the extraction of some compounds while degrading others (e.g. enhanced recoveries of quinolones in acidic samples favoring the degradation of other antibiotics such as penicillin) (Pozo et al., 2006; Sørensen and Elbaek, 2004). Also, qualitative assessments or semi-quantitative approaches are frequent for the detection of antibiotics in environmental samples, as they are generally faster and provide a higher amount of studied compounds with a non-directed analysis, even though they do not provide sufficiently precise measurements (Hernández et al., 2007). Due to the limitations described above, many of the current analytical methods described in literature can only be used to perform measurements at very high concentrations and/or to provide semi-quantitative risk estimates. Therefore, the development of refined analytical techniques is needed to assess the spread and accumulation of antibiotics in different environmental matrices and to screen for exposure-related responses in aquatic populations and communities.

The objective of this work was to develop and validate an analytical method for the determination of some of the most commonly used antibiotics in aquaculture in several marine matrices. The selected compounds were oxytetracycline, flumequine and florfenicol, which have been reported to be applied in Atlantic fish production as well as in Mediterranean aquaculture (AEMPS, 2016; Burridge et al., 2010; VMD, 2016). The environmental matrices selected included natural and artificial seawater (as surrogate for natural seawater as explained in Section 2.2), sediments and biological samples (marine biofilms and two species of invertebrates, a crustacean and a gastropod mollusk). They were selected with the intention of covering several exposure routes (water vs sediment) and trophic levels (primary producers vs primary consumers) thus allowing the further elaboration of studies that quantify antibiotic dispersal and trophic transfer and accumulation in marine ecosystems. This method has been successfully applied to the study of antibiotic accumulation in environmental samples collected from an old aquaculture emplacement in the south-east Spanish Mediterranean coast.

2. Material and methods

2.1. Chemicals, reagents, working solutions and materials

All antibiotic standards of oxytetracycline hydrocloride, flumequine and florfenicol and the isotope-labelled internal standard (IS) flumequine-(1,2-carboxy-$13C^3$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock standard solutions were prepared in methanol at 100 mg L^{-1} and stored in vials at −20 °C. Working and spiking standard solutions were prepared frequently by volume dilution in methanol before use. Solvents were supplied by VWR (Radnor, Pennsylvania) and Scharlab (Madrid, Spain): formic acid ≥98%; methanol and acetonitrile, both LC-MS grade; hexane for residue analysis; acetic acid, ACS reagent ≥99.7%. Oxalic acid dihydrate reagent grade, ethylenediaminetetraacetic acid disodium salt dihydrate extra pure (EDTA), were obtained from Scharlab (Madrid, Spain). Prodac International provided the Ocean Fish artificial salt used to create the artificial seawater.

For the treatment of the samples several cartridges were tested; silica cartridges, DSC-18Lt, 100 mg, from Supelco (Darmstand, Germany); QuEChERS enhanced matrix removal – lipid Kit-1 g, from Agilent (Palo Alto, CA, USA); Oasis Solid Phase Extraction (SPE) cartridges HLB 60 mg and 200 mg, HLB prime 60 mg and MAX 150 mg, from Waters (Milford, MA, USA); supelclean™ envi-florisil® SPE tubes 60 mg and 150 mg, from Sigma-Aldrich (St. Louis, MO, USA); Phree phospholipid removal plates 1 mL and strata-XL-A strong anion mixed mode cartridges 150 mg, from Phenomenex (Torrance, CA, USA); and Bond elut-SAX cartridges 100 mg, from Varian (Palo Alto, CA, USA). Also carbon filters Sep-Pak C18 plus cartridges 360 mg from Waters (Milford,