Combining an effect-based methodology with chemical analysis for antibiotics determination in wastewater and receiving freshwater and marine environment

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Abstract

Two different methodologies were combined to evaluate the risks that antibiotics can pose in the environment; i) an effect-based methodology based on microbial growth inhibition and ii) an analytical method based on liquid-chromatography coupled to mass spectrometry (LC-MS). The first approach was adapted and validated for the screening of four antibiotic families, specifically macrolides/β-lactams, quinolones, sulfonamides and tetracyclines. The LC-MS method was applied for the identification and quantification of target antibiotics; then, the obtained results were combined with ecotoxicological data from literature to determine the environmental risk. The two methodologies were used for the analysis of antibiotics in water samples (wastewater, river water and seawater) and biofluids (fish plasma and mollusk hemolymph) in two monitoring campaigns undertaken in the Ebro Delta and Mar Menor Lagoon (both in the Mediterranean coast of Spain). Both approaches highlighted macrolides (azithromycin) and quinolones (ciprofloxacin and ofloxacin) as the main antibiotics in wastewater treatment plant (WWTP) effluents with potential risk for the environment. However, no risk for the aquatic life was identified in the river, lagoon and seawater as antibiotic levels were much lower than those in WWTP effluents. Fish from Ebro River were the organisms presenting the highest antibiotic concentration when compared with bivalves (mussels) from the Mediterranean Sea and gastropods (marine snails) from the Mar Menor Lagoon. The effect-based methodology successfully determined antibiotic risk in wastewater, but its applicability was less clear in environmental waters such as seawater, due to its high detection limits. Improving sample preconcentration could increase the method sensibility. Overall, combination of both methodologies provides comprehensive insights in antibiotic occurrence and risk associated in areas under study.

Capsule: The combination of two methodologies allowed to comprehensively evaluate antibiotic risk in two areas of ecological interest

Keywords: Antibiotics; effect-based methodology; wastewater; surface water; biota

1. Introduction

The presence of antibiotics in the aquatic environment is an issue of increasing concern. The highest concentrations are usually detected in wastewater, up to few μg/L, (Manzetti and Ghisi, 2014), whereas lower levels, below 0.001 μg/L, are commonly measured in surface and groundwater (Manzetti and Ghisi, 2014). Natural attenuation processes such as dilution, sorption to sediment or to suspended solids, chemical and biological degradation, contribute to the reduction of antibiotics concentrations from Waste Water Treatment Plants (WWTP) effluents to the receiving water bodies (Celic et al. 2019; Manzetti and Ghisi, 2014). However, the continuous discharge of these contaminants makes them pseudo-persistent in the aquatic environment (Carvalho and Santos, 2016). As a result, some of the most consumed antibiotics for human or veterinary purposes like tetracyclines, quinolones, β-lactams, macrolides and lincosamides, among others, have been detected in several water bodies worldwide ranging from ng/L up to several μg/L (Chen et al., 2014; Kümmerer, 2009; Rodriguez-Mozaz et al., 2017).

Since antibiotics are used to kill or inhibit pathogenic bacteria, their presence in natural environments may pose a risk for the aquatic communities (Kümmerer, 2009), including non-targeted organisms. Primary producers and decomposers may be vulnerable to these contaminants, compromising the essential ecological functions that these organisms perform in the natural ecosystem, such as the biogeochemical cycling and organic contaminant degradation(Grenni et al., 2018). In addition, the continuous exposure to antibiotics allows them to bioaccumulate, as well as, provoke ecotoxicological effects, altering organisms functions and metabolism in invertebrates or fish (Le Bris et al. 2004; Serra-Compte et al., 2019a). Antibiotics can also promote the spread of antibiotic resistant genes (ARGs) in the different aquatic environments, including rivers, lakes and coastal areas (Martínez, 2008). Besides, some studies have described the increase of ARGs copies in the bacteria located in gastrointestinal tracts of

86 shrimp (Su et al., 2017), and mussel (Serra-Compte et al., 2019b) as a result of their exposure to
87 antibiotics.

88 In order to evaluate the risk that antibiotics pose to the environment, several studies have
89 determined antibiotics concentration threshold i.e. predicted non effect concentration (PNEC)
90 based on ecotoxicological parameters, such as survival or reproduction impairment (Park and
91 Choi, 2008; Santos et al., 2013). Recently, a PNEC was developed considering the capacity of
92 antibiotics to promote antimicrobial resistance spread (Bengtsson-Palme and Larsson, 2016; Tell
93 et al., 2019). This approach determined the lowest concentration of an antibiotic in the
94 environment capable to promote antibiotic resistance dissemination. The combination of both,
95 ecotoxicological PNEC and PNEC related to antibiotic resistance promotion was postulated as a
96 comprehensive approach to establish a final PNEC for antibiotics in the environment (Tell et al.,
97 2019).

98 In addition to the effects that antibiotic pollution may provoke to the exposed organisms, it may
99 be of concern in terms of human health. The presence of antibiotics in seafood may pose a risk
100 for consumers such as allergy and toxicity (Cabello, 2006). To reduce this risk, authorities have
101 established measures to control the occurrence of these contaminants in the natural
102 environment and in the foodstuff from animal origin. For instance, the use of antibiotics as
103 growth promoters in livestock has been forbidden in the European Union since 2006 (Carvalho
104 and Santos, 2016). Besides, Maximum Residue Limits (MRLs) have been established by the
105 authorities for some antibiotics in foodstuff from animal origin (European Commission, 2010).
106 Recently, the European Union (EU) included four antibiotics in the latest watch list revision (EU,
107 2018) highlighting the increasing concern of antibiotic occurrence in the environment.

108 Monitoring antibiotic occurrence in the water bodies and organisms is the first step to evaluate
109 the risk of these contaminants for the environment and human health. In this regard, effect-
110 based techniques for screening chemical pollution in the environment have gained importance

111 as they provide a powerful tool for water quality monitoring without the necessity of analyzing
112 hundreds of chemical contaminants potentially present in the sample (Doyle et al., 2015). Effect-
113 based methodologies for antibiotics screening, like microbial growth inhibition tests (Pikkemaat
114 et al., 2008), can provide a wide view of antibiotic pollution in a given sample, as not only the
115 antibiotics, but also their active transformation products and metabolites can be detected.
116 Besides, microbial growth inhibition are cost-effective tests when compared with immunological
117 or receptor-based assays but they do not provide single compound identification nor
118 quantification, also the required analysis time is usually longer than immunoassays. (Cháfer-
119 Pericás et al., 2010; Pikkemaat, 2009). Few methodologies based on microbial growth inhibition
120 have been developed, they were applied to food control in livestock production (Gondová et al.,
121 2014; Pikkemaat et al., 2008), in seafood like shrimps (Dang et al., 2010) and in trout (Barker,
122 1994). The use of biota biofluids (such as mussel hemolymph) instead of organism's tissues (like
123 mussels soft tissue) extract also allows simplifying the extraction protocol and reducing the
124 potential loss of antibiotics during the extraction procedure. Furthermore, matrix complexity
125 which may interfere with their detection with the microbial inhibition test is lower in biofluids
126 than in biota extracts (Serra-Compte et al., 2017). The microbial growth inhibition test has been
127 applied to screen antibiotics in environmental samples such as sediment and water (Huerta et
128 al., 2011). However, it has not yet been used for monitoring of biota samples in natural aquatic
129 ecosystems, nor to the monitoring of wastewater samples.

130 In this work, a screening method based on microbial growth inhibition was adapted for the
131 detection of a broad range of antibiotics in biota biofluids (mollusks hemolymph and fish plasma)
132 and in water sample extracts; namely WWTP influents and effluents, freshwater and seawater.
133 The screening method was applied for the screening of antibiotics in biological and water
134 samples from two monitoring campaigns undertaken in two areas of ecological and human
135 interest located in the Mediterranean coast of Spain: river Ebro delta and Mar Menor Lagoon.

In addition, a chemical analysis based on liquid-chromatography coupled to mass-spectrometry (LC-MS) was used for the identification and quantification of the target antibiotics.

2. Material and methods

2.1 Chemicals and reagents

Antibiotic standards were of high purity grade (>90 %), purchased from Sigma- Aldrich (St Louis, MO, USA) (table S1, list of antibiotics). Stock standards were prepared in methanol at a concentration of 1000 mg/L and stored at -20 °C. The cartridges OASIS HLB (200 mg, 6 mL) were used for solid phase extraction. HPLC grade methanol, water and acetonitrile were purchased from Merck (Darmstadt, Germany), EDTA 0.01 mol/ L, was obtained from Scharlab (Barcelona, Spain).

2.2 Study areas and sample collection

The Ebro delta is located in NE Spain and has a surface area of approximately 320 Km^2. Most of its surface is used for agriculture, mainly rice culture. The Ebro delta is composed of a wide variety of environments such as natural Lagoons, wetlands, marshes and it includes two coastal bays (Alfacs and Fangar). Further information regarding the Ebro delta area can be found elsewhere e.g. (Čelic et al., 2019). A sampling campaign of water and biota samples was performed in June 2018 in dry weather conditions. Twenty-four hours composite water samples were obtained from wastewater, whereas grab samples were collected from freshwater and marine environments. For freshwater analysis, water samples were taken from three different sampling sites at the Ebro river (FW1, FW2, FW3), figure 1A. Wastewater Influent and effluent samples were obtained from two different wastewater treatment plants, WWTP1, WWTP2, figure 1A. WWTP1 has a primary and secondary treatment with activated sludge, with a capacity of 27.500 inhabitant equivalent, and it discharges directly into the Ebro river. WWTP2 has a primary, secondary and tertiary treatment, consisting in activated sludge followed by a sand

filter. Its maximum capacity is 28.921 inhabitant equivalents, and it discharges into the Mediterranean Sea (Alfacs Bay). Seawater samples were collected from eight different sampling sites, four of them located in Fangar bay (SW1, SW2, SW3, SW4), and the other four in Alfacs bay (SW5, SW6, SW7, SW8) at locations ranging between 4 and 10 Km approximately from the WWTP2 facility (figure 1A). Fish and mussels were sampled for biofluid extraction in sampling sites located close to those selected for water. Freshwater fish were taken from 2 sampling sites located at the Ebro river, marine fish and mussels were sampled from the Mediterranean sea concretely, fish from 2 sites located at Alfacs bay (figure 1A) and mussels from aquaculture structures at 2 sampling sites at Alfacs bay and another 2 at the Fangar bay (figure 1A).

Mar Menor Lagoon is located in the South East of Spain. It is a hypersaline restricted Lagoon, covering an area of 135 km^2. Water was collected from the Lagoon in nine sampling sites, (LW1, LW2, LW3, LW4, LW5, LW6, LW7, LW8, LW9), (Figure 1B), whereas biota, gastropod (*Hexaplex trunculus*), was taken in three of them (BG1, BG2, BG3), (figure 1B).

2.3 Sample pre-treatment

Sample pre-treatment for the different matrices and for the two methodologies applied (microbial and chemical analysis) are summarized in figure S1. For water analysis, 1 L of seawater or freshwater was pre-concentrated using solid phase extraction (SPE) following the methodology developed by Gros et al. (Gros et al., 2013) (except for WWTP influent and effluent where 300 mL were used). Briefly, water samples were filtered through 1 µm glass fiber filters and 0.45 µm nylon membrane filter prior SPE extraction. SPE cartridges were conditioned with 6 mL of methanol, followed by 6 mL of HPLC water at pH 2.5. Then, the pH of water samples was adjusted at 2.5 and passed through the cartridges, prior addition of an appropriate amount of EDTA. Then, cartridges were rinsed with 6 mL of water at pH 2.5 and dried under air for 5 min. Samples were eluted with 6 mL of methanol, dried down under nitrogen and reconstituted in 1

185 mL of methanol:water (30:70) before their analysis with the microbial growth inhibition test. For
186 chemical analysis an aliquot (50 µL) of the same extract was further dried down and
187 reconstituted with 100 µL methanol:water 50:50 (dilution 1:2), to reduce matrix interferences.
188 Acceptable extraction recoveries were obtained for most of the tested antibiotics. Despite lower
189 recoveries were achieved in biota samples compared to water; they were similar than previously
190 reported values for pharmaceuticals extraction in biota matrices (Fernandez-Torres et al., 2011;
191 Huerta et al., 2013). The obtained recoveries were used for correction of contaminants
192 concentration in the different matrices (table S2).

193 Mussels (*Mytilus galloprovincialis*) collected in the study sites from the Mediterranean Sea were
194 transported under refrigerated conditions to the laboratory. The same day of mussel sampling,
195 hemolymph was extracted from the mussel's adductor muscle, and collected in vials containing
196 heparin. Then, samples were centrifuged at 3000 rpm during 10 min and immediately frozen. A
197 similar protocol was followed for gastropod hemolymph extraction from the Mar Menor Lagoon.
198 Hemolymph was extracted from the foot muscle and collected in vials without heparin. Samples
199 were centrifuged at 1000 g for 10 min, then, the supernatant was collected and frozen until
200 analysis. Fish blood extracted (at each sampling site) was transferred to vials containing heparin,
201 immediately centrifuged at 3000 rpm during 10 min, plasma (≈3 mL) was collected and frozen
202 until analysis. Both, mollusk hemolymph and fish plasma were kept at -70°C until their analysis.
203 Biota biofluids extracts were analyzed in the microbial growth inhibition test whereas a dilution
204 with methanol (1:2) followed by centrifugation (10 min at 5000 rpm) was necessary previously
205 to their analysis in LC-MS.

206

207 2.4 Chemical analysis – LC-MS

208 The obtained extracts from water and biota biofluids samples (as explained in section 2.3) were
209 analyzed in triplicate by liquid chromatography coupled to mass spectrometry using ultra high-

pressure liquid chromatography coupled to a quadrupole linear ion trap tandem mass spectrometry (UHPLC-QqLIT) following the method of Gros et al. (Gros et al., 2013) for the target analysis of 27 antibiotics. Chromatographic separation was done with an Acquity HSS T3 column 5 (50 mm × 2.1 mm i.d., 1.8 µm particle size), solvent (A) Acetonitrile, solvent (B) HPLC grade water acidified with 0.1% of formic acid. Further details of the method can be found elsewhere (Gros et al., 2013). Further information regarding chemical analysis, limits of quantification and detection can be found in table S2.

2.5 Microbial growth inhibition test

The test comprises four plates for the specific analysis of each of the four antibiotic families namely, sulfonamides, tetracyclines, fluoro(quinolones) and macrolides/β-lactams. The microorganisms used: Kocuria rhizophila (formerly known as Micrococcus luteus) ATCC 9341 (macrolides/β-lactams); *Bacillus cereus* ATCC 17788 (tetracyclines); *Yersinia ruckeri* NCIM 13282 (quinolones); *Bacillus pumilus* CN 607 (sulfonamides), were kept at -70 °C, until the analysis. The culture media were, plate count agar from Difco, BD diagnostic systems (Breda, Netherlands) and DST-agar and Iso-sensitest agar purchased from Oxoid (Basingstoke, UK). The characteristics of the test plates are specified in table 1. Plates preparation was adapted from (Pikkemaat et al., 2008). Briefly, after sterilization, media were cooled down and the synergistic antibiotics to increase method sensitivity were added to the corresponding plate namely, tylosine (macrolides/β-lactams), chloramphenicol (tetracyclines), cloxacilline (quinolones) and trimethoprim (sulfonamides) (table 1). When agar temperature was below 48 °C, bacteria were inoculated into the liquid agar which was poured to form a 2.5 mm thick layer except for sulfonamides that was 3 mm. Fourteen-millimeter diameter holes were made in the agar after its solidification. Two hundred fifty microliters of sample extract (sample extraction explanation can be found in section 2.3) was applied into the punched holes in the agar and 50 µL of the

235 corresponding buffer were added prior incubation at 30-37 °C for 16/18 hours. After overnight
236 incubation, plates were observed. A positive result consists of a bacterial growth inhibition area
237 around the punched hole. An example of the developed plate can be seen in figure S2. The
238 diameter of the inhibition areas was measured with a precision of 0.1 mm using a Vernier caliper.

239

240 2.6 Microbial growth inhibition test adaptation

241 Microbial method optimization was carried out with blank sample extracts (for sample
242 extraction, see section 2.3) (seawater, freshwater, mollusk hemolymph and fish plasma) spiked
243 with known concentrations of the tested antibiotics (ranging from 1 to 200 µg/L). Prior spiking,
244 blank samples were analyzed with a method based on liquid chromatography coupled to tandem
245 mass spectrometry (LC-MS) (Gros et al. 2013; Serra-Compte et al., 2017) showing no presence
246 of antibiotics. The screening biological method was adapted for the detection of the 17
247 antibiotics presented in table S1. These antibiotics were selected according to their reported
248 presence and potential impact to the aquatic ecosystem and human health based on their MRL
249 in foodstuff from animal origin (European Commission, 2010; Rodriguez-Mozaz et al., 2017,
250 2015; Santos et al., 2013). The detection limit, defined as the minimum concentration of each
251 antibiotic showing a clear inhibition area (> 1 mm around the punched hole), was established
252 for the different matrices tested and for each of the 17 antibiotics considered. The detection
253 limit was calculated by correcting the lowest spiked concentration showing a clear inhibition
254 area with the percentage of recovery, as well as by the total sample volume preconcentrated
255 (1L freshwater and seawater, 300 mL wastewater and 1 mL biota biofluids). Besides, a positive
256 control of spiked water (100 µg/L) with oxytetracycline, enrofloxacin, erythromycin and
257 sulfamethoxazole was applied in a hole of each of the corresponding plates: tetracycline,
258 fluoro(quinolones), macrolides/β-lactams and sulfonamides, respectively; and a negative

control by analyzing a blank sample (seawater, freshwater, mollusk hemolymph and/or fish plasma depending on the analysis undertaken) without antibiotic presence.

Once the method was optimized it was validated in terms of accuracy, sensitivity and specificity according to Dang et al. 2010 (Dang et al., 2010). Sets of 20 blank samples and 20 spiked samples were analyzed for the different matrix types and the 17 antibiotics reported in table S1. Spiking was done for each antibiotic at its corresponding detection limit. Accuracy was defined as the number of correct results (when no false positive or negatives results were reported) given by the methodology considering the total number of analyzed samples and expressed as percentage. Sensitivity was defined as the number of positive samples correctly given by the methodology considering the total number of positive samples (also expressed in percentage). Specificity was defined as the number of negative samples correctly given by the methodology taking into account the total number of negative samples analyzed (Dang et al., 2010). Furthermore, method ruggedness was evaluated through its implementation in two different laboratories (namely, Wageningen Food Safety Research, Netherlands, and ICRA, Spain), hence, different batches of tests, different days, and spikes from different standard solutions, as well as, different instrumentation were applied (Pikkemaat, 2009). Due to the low availability of fish plasma and the difficulty to obtain wastewater without antibiotics, the method was validated for freshwater, seawater and mollusk hemolymph.

2.7 Antibiotics risk assessment

Antibiotics risk was evaluated by calculating a hazard quotient (HQ) for each compound according to the European Community (EC) guidelines (European Commission, 2003). HQs were calculated as follows:

HQ = Antibiotic concentration/Predicted No Effect Concentration (PNEC).

283 Antibiotic concentration refers to the measured concentration of antibiotics in the environment
284 (LC-MS methodology). PNECs were calculated for each antibiotic following the approach of Tell
285 et al. (Tell et al. 2019), which combines ecotoxicological PNEC and MIC-PNEC (related to
286 antimicrobial resistance spread). Ecotoxicological PNECs were obtained from the reported
287 literature (when information was not available from literature the ECOSAR software was used),
288 presented as the lowest EC50 or LC50 and applying an assessment factor of 1000 (European
289 Commission, 2003). MIC-PNECs were also obtained from the literature (Bengston-Palme et al.
290 2016). The final PNEC was determined for each antibiotic as the lowest one reported when
291 comparing ecotoxicological PNEC and MIC-PNEC (ecotoxicological, MIC and final PNECs for the
292 tested antibiotics are reported at table S1). Antibiotics with a HQ above 1 are considered a
293 potential risk for the environment, (European Commission, 2003). In order to assess the
294 environmental risk of antibiotics mixtures, the sum of calculated HQ was performed per each
295 water sample, as previously reported in the literature (Backhaus, 2016).

296

297 **3. Results and Discussion**

298 3.1 Microbial growth inhibition test performance

299 The microbial growth inhibition test conditions indicated in table 1 were used to screen
300 antibiotics in all the matrices tested; the only difference was the buffer used in the macrolides/β-
301 lactams plate. Therefore, in the macrolides/β-lactams plate, a buffer without tylosine and with
302 a slightly lower pH (which reduced the sensitivity of the analysis in the macrolides/β-lactams
303 plate) allowed avoiding false positive in water analysis.

304 The detection limits of the plates were established by using the final method conditions and
305 analyzing different sets of blank samples (freshwater, seawater, wastewater, mussel
306 hemolymph and fish plasma). The detection limits in the plates (table 2) were similar for

307 freshwater and seawater ranging between 0.01 µg/L and 0.29 µg/L. Overall, for water samples
308 the analysis of tetracyclines, quinolones and macrolides/β-lactams allowed lower detection
309 limits when compared to sulfonamides, (table 2). Regarding the biota biofluids, mollusk
310 hemolymph and fish plasma, similar results were obtained for both matrices, ranging from 10
311 µg/L up to 100 µg/L. Despite the high differences even within the same antibiotic family,
312 tetracyclines were detected with the lowest detection limits whereas sulfonamides the highest
313 (table 2).

314 Microbial growth inhibition test showed good performance in terms of accuracy and sensitivity
315 being higher than 95% for all the tested antibiotics, results are presented at supporting
316 information, table S3. Specificity was 100% for all the antibiotics as no false positive were
317 detected in any analysis (data not shown). Besides, no differences in methodology results were
318 obtained when performed in different laboratories. Consequently, the method was validated in
319 terms of accuracy, sensitivity and specificity as the error was 5% or lower in all cases
320 (Commission Decision, 2002; Dang et al., 2010), and showed robust results

321

322 3.2 Antibiotic occurrence and risk assessment in wastewater

323 Wastewater samples can contain high concentrations of antibiotics coming from different urban
324 or farming activities. In this study, two WWTPS were considered in the area of the Ebro Delta,
325 receiving effluents from the surrounding towns. Results of antibiotics determination in
326 wastewater are shown in figure 2 (figure 2A microbial test results; figure 2B wastewater
327 characterization with LC-MS analysis) and table 3 and at supporting information, table S4
328 microbial test inhibition areas and table S5 quantification of antibiotics with LC-MS. Both
329 methodologies (chemical and microbial analysis) showed the occurrence of quinolones,
330 macrolides and sulfonamides antibiotics in WWTP influent samples. The antibiotic detected with
331 the highest concentration, determined with LC-MS analysis, was ciprofloxacin, at 2.1 and 5.9

µg/L in the influent of WWTP1 and WWTP2, respectively. The only mismatch between both methodologies in influent samples was found for tetracyclines because they showed an inhibition area in the microbial test, but tetracyclines were not detected with LCMS analysis. The inhibition observed in the tetracycline plates test can be attributed to other substances, such as soaps or disinfectants, which occur in WWTP influents and able to inhibit the growth of *B. cereus* (Monarca et al., 2000). The occurrence of these substances with bactericidal properties in untreated wastewater may also provoke the irregular inhibition zone observed in macrolides plates, despite macrolide antibiotics occurred in WWTP influent samples.

WWTP significantly reduced antibiotic concentrations and antibiotic activity when comparing influent and effluent samples (figure 2). However, in few cases higher concentrations of antibiotics were found in the effluent when compared with influent, as it was observed for azithromycin antibiotic. Previous studies reported this behavior for some contaminants, including macrolide antibiotics (Gros et al., 2010), which was attributed to the conversion of glucuronide metabolites to the parent compound. Effluent samples of the two analyzed WWTPs were dominated by quinolones and macrolides families according to both methodologies (figure 2). Sulfonamides were present in both effluents according to LC-MS analysis but in higher concentration in WWTP2. However, the microbial growth inhibition test only showed inhibition in the sulfonamides plate at the effluent of WWTP1. This can be explained by the presence of other antibiotics in the WWTP1 effluent that inhibited the activity of this plate, such as, trimethoprim (not occurring in the effluent of WWTP2). These results indicated that the interaction between sulfonamides (sulfamethoxazole) and trimethoprim provoked a higher antibacterial activity when compared with the activity of sulfonamides alone (WHO, 2019). This demonstrated the potential of the microbial test in identifying synergistic activity between antibiotics.

356 The occurrence of antibiotics in WWTP effluents can pose a risk for the receiving environments.
357 Effluent samples from WWTP1 and WWTP2 presented HQ > 1 for individual antibiotics, such as
358 azithromycin, ciprofloxacin and ofloxacin (figure 3) and showed inhibition in the corresponding
359 plates of the microbial test (macrolides and quinolones) (figure 2). In previous studies that
360 targeted several WWTPs located at the Ebro River area, macrolides (azithromycin), sulfonamides
361 (sulfamethoxazole), quinolones (ofloxacin and ciprofloxacin) and trimethoprim were the main
362 antibiotics discharged by the WWTPs effluents to the receiving environment (Celic et al. 2019;
363 Gros et al., 2007). Garcia-Galán (García-Galán et al., 2011) also reported a HQ value higher than
364 1 for sulfamethoxazole in the effluent of another WWTP located in the area of Ebro Delta.

365

366 3.3 Antibiotic occurrence and risk assessment in freshwater

367 Freshwater samples were characterized from the lower reach of the Ebro River. Results of water
368 samples from the Ebro River are shown in figure 4 (4A microbial test; 4B LC-MS analysis) and
369 table 3 and at supporting information, table S4 shows the measured inhibition area values with
370 microbial test and table S5 quantification of antibiotics with LC-MS analysis. Both methodologies
371 pointed out the sites FW1 and FW3 as the most antibiotic polluted ones in the Ebro River (figure
372 4); whereas, FW2 site presented lower concentration of antibiotics according to LC-MS and no
373 inhibition in the test plates. Inhibition in tetracyclines plate in sites FW1 and FW3 could be
374 attributed to doxycycline occurrence quantified with LC-MS method at levels of 0.07 and 0.08
375 µg/L in FW1 and FW3 samples, respectively. Inhibition in sulfonamides plate in a sample from
376 FW1 could be due to simultaneous occurrence of sulfonamides and trimethoprim antibiotics, as
377 it was observed for WWTP samples the synergistic interaction between these two antibiotics
378 was shown in the plates. Lincosamides were also quantified with LC-MS analysis in all river
379 samples (FW1, FW2 and FW3) but at lower concentrations compared to tetracyclines, figure 4B.

Samples taken in the river water FW1 showed some of the highest antibiotic's concentrations, despite it is located upstream of the discharge of both WWTPs. The same was observed in previous studies in this area and was attributed to the anthropogenic and agricultural activities from towns located near to this sampling site (Čelic et al., 2019). Furthermore, the antibiotics with the highest concentrations in FW1 were tetracyclines, not found in the effluent of the WWTP (figure 4). Therefore, non-point sources or WWTP discharges located upstream but not considered in the present work may explain the occurrence of these compounds in this sampling site of Ebro river. Lower concentration of antibiotics was observed in the FW2 sampling site, probably due to dilution effects from upstream site (FW1) and the absence of WWTP discharge in this river section (figure 4). FW3 sampling site, located downstream of the WWTP1 presented a higher amount of antibiotics compared to the FW2. FW3 showed antibiotic occurrence mainly for sulfonamides and lincosamides, also present in WWTP1 effluent, so these antibiotics may be related to the input of WWTP effluents. The contribution of WWTP to pharmaceuticals including antibiotics occurrence in the area of Ebro River was previously observed, mainly for macrolides and sulfonamide antibiotics (Silva et al., 2011). However, the antibiotics detected at the highest concentration in FW3 site where tetracyclines, not occurring in WWTP1 effluent. Therefore, as the case of FW1 site, other sources of antibiotics such as livestock production should be considered. Despite tetracyclines were the antibiotics detected at the highest concentration in river water, they posed no risk for the ecosystem according to the calculated HQ (figure 3), and no risk was determined for the rest of the antibiotics quantified in river water nor for the sum of HQ per sample (figure 3).

3.4 Antibiotic occurrence and risk assessment in seawater

Two different types of marine environments were considered in the study. The Mediterranean Sea area located in the Ebro Delta, receiving the Ebro River discharge (figure 1), and the Mar

Menor Lagoon, a costal saltwater Lagoon located in the south-east of Spain near the Mediterranean Sea (figure 1).

Regarding seawater in the Mediterranean Sea area, the microbial growth inhibition test showed no inhibition in any of the analyzed samples (figure 4A, table 3, table S4), whereas, chemical analysis with LC-MS reported antibiotic concentration (mainly for sulfonamides, macrolides and lincosamides) in all the samples at low concentrations (all of them were detected at concentrations of few ng/L) (figure 4B, table 3, table S5). These differences between the outcome of the two methodologies can be attributed to higher sensitivity of LC-MS when compared with the microbial inhibition test. Sulfonamides were the most widespread antibiotics in seawater present in all samples except for site SW1 (figure 4). They were found at concentrations ranging from 3 to 6 ng/L and no differences were observed between the different locations, probably due to dilution effects. The reported antibiotic concentrations in sea water presented no risk for the ecosystem according to the calculated individual antibiotic HQ and the sum of HQ per sample, figure 3; similar concentrations in Mediterranean Sea water (low ng/L levels) were observed for emerging contaminants including some antibiotic (Brumovsky et al., 2017). Despite the lack of reported risk, the chronic exposure of wildlife to biological active substances needs further research to discard any potential negative implications.

Similar results to Mediterranean Sea water were obtained when characterizing the Mar Menor Lagoon. The microbial growth inhibition test did not report inhibition in any of the test samples (figure 4A). Chemical analysis showed occurrence of antibiotics in 7 out of the 9 samples analyzed (figure 4B). Sulfonamides were the most widespread antibiotic family in the Mar Menor Lagoon, although macrolides were detected in four out of the nine samples analyzed. Previous studies determined the main antibiotic inputs to Mar Menor Lagoon including the presence of sulfamethoxazole and clarithromycin (Moreno-González et al., 2014), two of the main antibiotics determined in the present work. However, the concentrations determined in the

429 present work, ranging from 6 to 16 ng/L, were lower than the ones obtained in previous studies
430 (Moreno-González et al., 2014) which can be related with the improvement of this environment
431 through the reduction of WWTP discharges. Furthermore, the studied area is strongly affected
432 by tourism, which may provoke seasonal variations on the impact of emerging contaminants, as
433 previously observed in other environments (Mandaric et al., 2017). The low concentrations of
434 antibiotics presented no risk for the ecosystem according to the individual antibiotic HQ. Only
435 one sample (LW6) showed a HQ higher than 1 when summing the individual antibiotic risks of
436 sulfamethoxazole and clarithromycin.

437 3.5 Antibiotic occurrence in biota biofluids

438 In this study, different biota classes were characterized, namely, fish samples from the Ebro
439 River and the Mediterranean Sea, marine mussels from the Mediterranean Sea and gastropods
440 from the Mar Menor Lagoon. Analysis was performed in the organisms biofluids (fish plasma
441 and mollusk hemolymph). The microbial test showed inhibition in the sulfonamide's plates in
442 two plasma samples from Ebro fish (figure 5A, table S6). Chemical analysis reported antibiotic
443 concentration of tetracyclines, macrolides, lincosamides and trimethoprim in four fish samples
444 (Ebro River) and quinolones in one mussel sample from Mediterranean Sea (figure 5B, table S7).
445 No antibiotic occurrence was detected in gastropod from the Mar Menor Lagoon, neither with
446 chemical analysis nor with the microbial test.

447 The two applied methodologies reported different results in biota biofluids analysis. None of the
448 antibiotic concentrations quantified with LC-MS was high enough to provoke inhibition to the
449 test plates. Namely, the sensitivity of the microbial test (LODs between 10 and 150 µg/L) was
450 not enough to detect the presence of these compounds in the biological samples
451 (concentrations between 0.1 and 5.8 µg/L). Besides, the two fish plasma samples that showed
452 inhibition with the microbial inhibition test presented low or no quantifiable levels of antibiotics,
453 figure 5. No matrix interferences would be expected as no inhibition was seen in the other

454 characterized fish plasma samples. The occurrence of other antibiotics in fish plasma not
455 targeted with the LC-MS methodology or the presence of antibiotic active metabolites, may
456 explain the observed inhibition.

457 The reported concentrations of antibiotics in biota fluids measured by LC-MS, could be related
458 with the antibiotic occurrence in water samples. Tetracyclines, lincosamides and trimethoprim
459 detected in fish plasma samples from the Ebro River were also detected in the water samples
460 closest to the fish sampling point. However, other antibiotics like macrolides and quinolones
461 found in biota biofluids were not detected in environmental water samples, although they were
462 highly detected in WWTP effluents. Quinolones persistence time in surface water is low due to
463 its rapid photodegradation, hence, they are more frequently detected in sediment and biota,
464 rather than in water, which may explain its detection in biota tissues but not in surrounding
465 water (Li et al., 2012). Besides, thebioaccumulation measured of macrolides and quinolones
466 may correspond to other time frame, as bioaccumulation of contaminants in aquatic organisms
467 represent long time series rather than an occasional sampling time.

468 3.6 Combining chemical and microbial methodologies

469 The combination of different methodologies for the determination of antibiotics in
470 environmental samples can facilitate the implementation of antibiotics monitoring in the
471 environment. Besides, further insights regarding the risks posed by antibiotics may be
472 spotlighted.

473 All water samples that showed a potential antibiotic risk based on their HQ calculated with LC-
474 MS results also exhibited inhibition with the microbial growth inhibition test. Therefore, the
475 method can be used to screen those water samples with potential antibiotic risk. Then, antibiotic
476 identification and quantification can be carried out with chemical analysis only in those samples
477 with potential risk. This combination could provide a significant decrease of analytical costs and
478 facilitate its implementation and application to a broader range of institutions and/or companies

for routine analysis of antibiotics risk such as WWTPs, hospital and livestock production effluents. In fact, the microbial inhibition test is routinely applied for the screening of antibiotics in livestock samples for food quality control (Pikkemaat et al., 2008). Besides, the application of both methodologies provided further insights regarding antibiotic risk in the aquatic environment, allowing to determine antibiotic occurrence (with LC-MS) and potential antibiotic synergistic effects (microbial test), However, the environmental water samples presenting low levels of antibiotics concentrations were not highlighted as positive with the microbial inhibition test. Other approaches used to evaluate antibiotic risk based on LC-MS/MS analysis followed by antibiotic risk calculation, can provide lower limits of detection but they lack on identifying synergies between compounds (Yan et al., 2013). Recently applied methods such as suspect screening or non-target analysis for environmental contaminants prioritization allow the identification of a broader range of contaminants in a single run including compounds of different classes (pharmaceuticals, pesticides, herbicides, etc.), and they are not limited by compounds with analytical standards availability (Čelic et al., 2021). Therefore, comprehensive risk assessment can be obtained with these methodologies, but requiring complex instrumentation and exhaustive data treatment.

4.- Conclusions

In this work an effect-based methodology based on microbial growth inhibition test was adapted for its application in different environmental matrices (water and biota biofluids). The optimized screening method was combined with LC-MS for antibiotics risk assessment in the Ebro Delta area and the Mar Menor Lagoon. According to the reported antibiotic occurrence, the different water samples characterized can be ordered as follows (decreasing order) WWTP influent >

503 WWTP effluent > river water > Lagoon water > seawater mainly related to dilution effects. Biota
504 samples (fish) from the Ebro river showed significant higher concentrations compared with
505 mussels (Mediterranean Sea) and gastropods (Mar Menor Lagoon). The combination of
506 screening methods followed by chemical analysis can provide a reduction of antibiotics analysis
507 costs, facilitating its implementation for environmental monitoring. Besides, the antibiotics
508 identification and quantification capacity of LC-MS can be complemented with the potential of
509 the microbial test to determine synergistic effects between antibiotics. However, the high
510 effect-based methodology detection limits difficulted its applicability in surface waters, such as
511 seawater. Further improvement of water preconcentration step could increase the effect-based
512 methodology sensibility to screen antibiotics when occur at low concentrations. The application
513 of combined approaches such as this would be beneficial in order better understand and
514 evaluate the risk of antibiotics in the environment and the potential hazard consequences for
515 the environment and the human health.

516

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528

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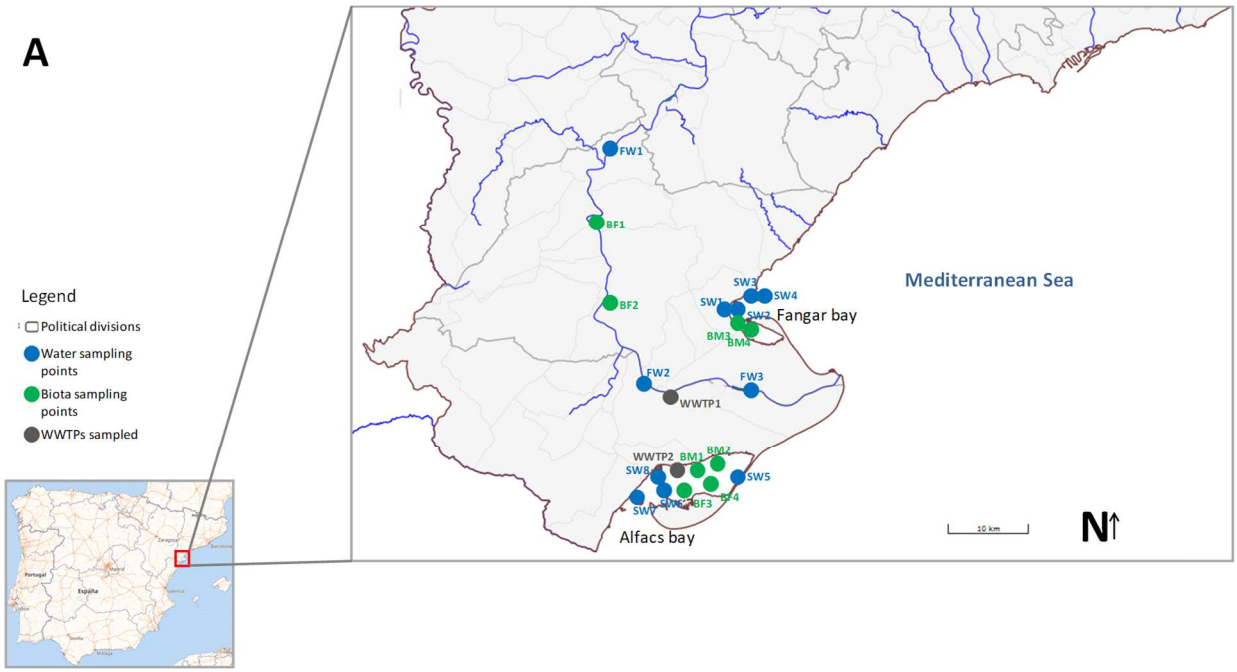
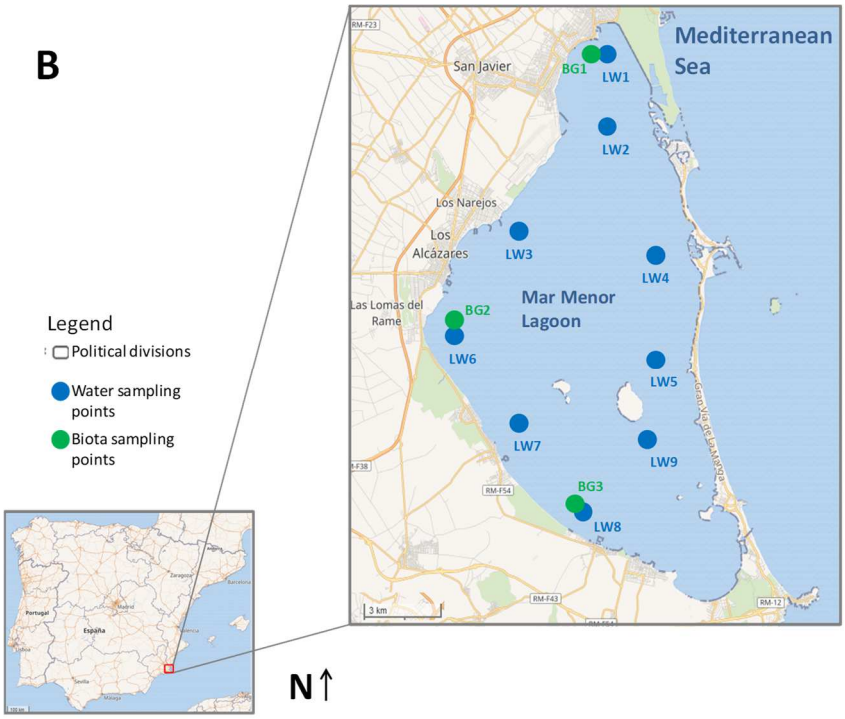
Figures and Tables

Table 1. Microbial growth inhibition test parameters.

Antibiotic family	Agar medium	pH	Synergistic antibiotic	Bacteria	Supplement buffer	Incubation conditions
Macrolides / β-lactams	Iso-sensitest agar	8.0	7.5 µg/L tylosine	*M. luteus ATCC 9341*	1M phosphate buffer pH 8.0 + 0.01 µg/mL tylosine / 0.5 M phosphate pH 7.5*	30 °C / 16-18 h
Tetracyclines	Iso-sensitest agar	6.0	625 µg/L chloramphenicol	*B. cereus ATCC 17788*	1M phosphate buffer pH 6.0	30 °C / 16-18 h
Quinolones	2/3 PCA + 1 M 5% fosfat buffer pH 6.5	6.5	8000 µg/L cloxicilline	*Y. ruckeri NCIM 13282*	1M phosphate buffer pH 6.5	30 °C / 16-18 h
Sulphonamides	DST agar	7.0	7 µg/L trimethoprim	*B. pumilus CN 607*	1.5M phosphate buffer pH 8 + 0.01 µg/mL TMP	37 °C / 16-18 h

*0.5 M phosphate pH 7.5 phosphate buffer was used in water samples





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713 Figure 1. Sampling sites in A) the Ebro Delta area and B) Mar Menor Lagoon.

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Table 2. Antibiotic list with predicted non effect concentration and microbial growth inhibition test detection limits in different matrices.

Antibiotic family	Compound	PNEC (µg/L)	Detection limits (µg/L)				
			Freshwater	Seawater	Wastewater	Fish plasma	Mussel hemolymph
Tetracyclines	Oxytetracycline	0.31	0.08	0.12	0.27	100	100
	Chlortetracycline	5.00	0.25	0.02	0.83	10	10
	Tetracycline	1.00	0.06	0.08	0.20	50	50
	Doxycycline	0.30	0.02	0.02	0.07	10	10
Quinolones	Ofloxacin	0.02	0.11	0.10	0.37	100	100
	Enrofloxacin	0.06	0.05	0.04	0.17	25	25
	Ciprofloxacin	0.05	0.04	0.04	0.13	10	50
	Norfloxacin	0.50	0.07	0.11	0.23	100	150
Macrolides	Tylosine	1.00	0.11	0.29	0.37	100	100
	Tilmicosin	0.52	0.11	0.06	0.37	100	50
	Erythromycin	0.20	0.06	0.06	0.20	50	25
	Azithromycin	0.01	0.01	0.01	0.03	25	25
	Spiramycin	0.50	0.13	0.18	0.43	100	100
Sulfonamides	Sulfamethazine	4.00	0.16	0.25	0.53	100	100
	Sulfadiazine	10.33	0.24	0.29	0.80	150	50
	Sulfamethoxazole	0.03	0.16	0.10	0.53	100	50
	Sulfapyridine	6.20	0.17	0.16	0.57	100	100

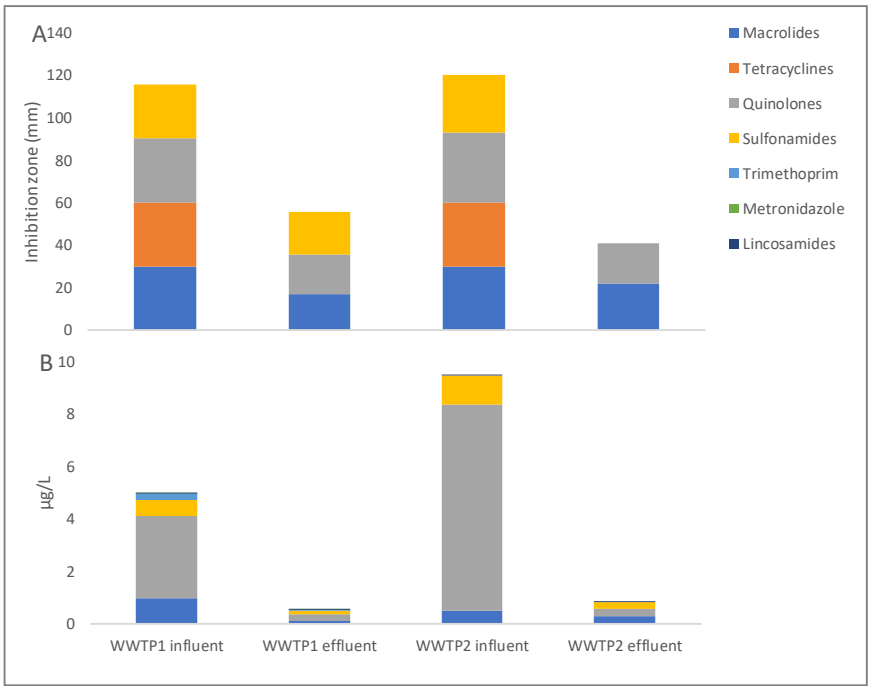

Figure 2. Antibiotics occurrence in wastewater (influent and effluent). A) Antibiotic families detected with the microbial growth inhibition test (macrolides and tetracyclines area in both influent samples are approximate inhibition area); B) antibiotic families quantified with LC-MS/MS methodology.

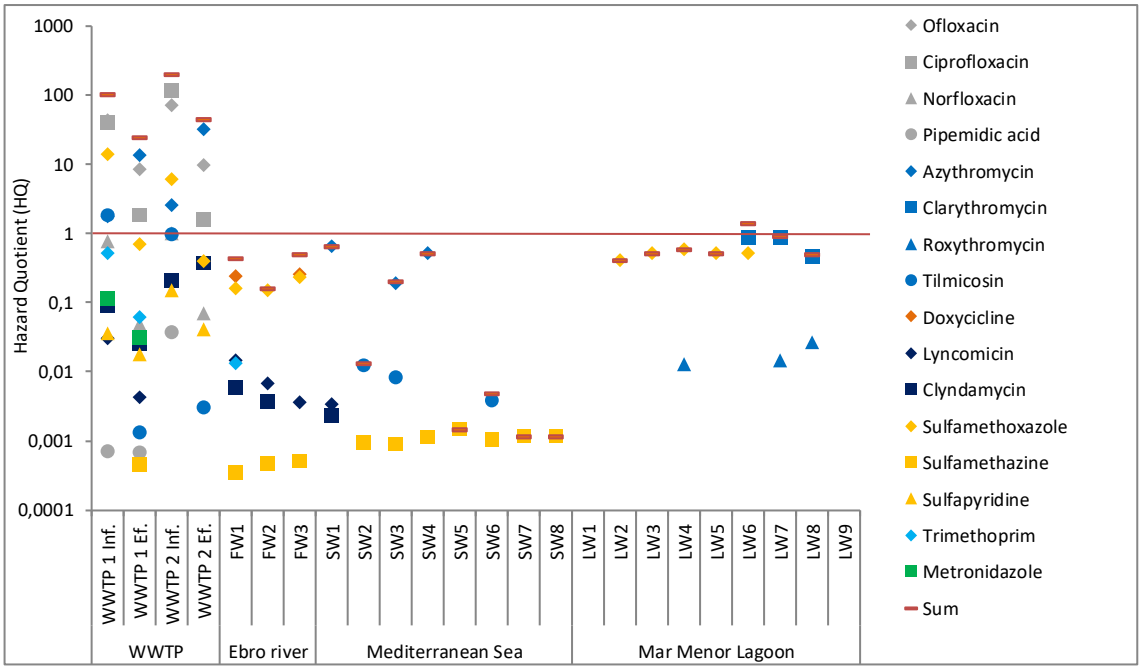

Figure 3. Hazard quotients (HQ) representation for the antibiotic quantified in water samples with LC-MS. Individual antibiotic HQ and the sum per water sample is presented.

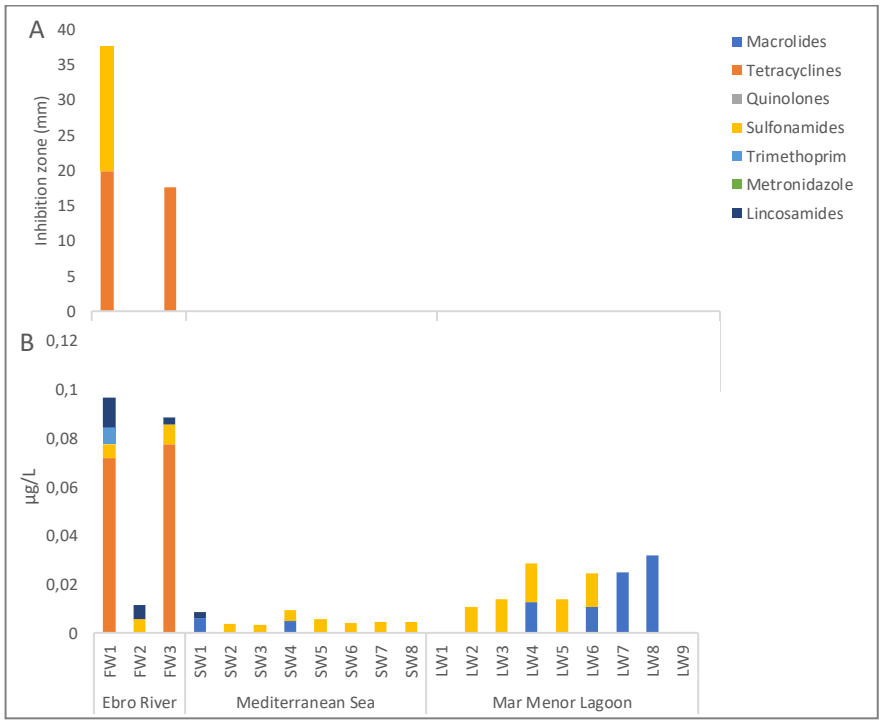

Figure 4. Antibiotics occurrence in surface water (freshwater, Ebro River; seawater, (Mediterranean Sea and Mar Menor Lagoon). A) antibiotic families detected with SPE+microbial growth inhibition test; B) antibiotic families quantified with SPE+LC-MS methodology.

Table 3. Summary of antibiotic concentration and antibiotic risk from the different water matrices analyzed. Antibiotic concentration refers to the sum of individual antibiotics measured from a same antibiotic family; the highest concentration of the different sites is presented. **+** refers that antibiotic risk was identified. – no antibiotic risk identified.

Antibiotic family	Wastewater effluent[a]			Freshwater[b]			Seawater[c]		
	Antibiotic concentration (µg/L)	Antibiotic risk (LC-MS)	Microbial inhibition	Antibiotic concentration (µg/L)	Antibiotic risk (LC-MS)	Microbial inhibition	Antibiotic concentration (µg/L)	Antibiotic risk (LC-MS)	Microbial inhibition
Macrolides	0,30	+	+	0,00	-	-	0,03	-	-
Tetracyclines	0,00	-	+	0,08	-	+	0,00	-	-
Quinolones	0,27	+	+	0,00	-	-	0,00	-	-
Sulfonamides	0,27	-	+	0,01	-	+	0,02	-	-
Trimethoprim	0,03	-	n.p.	0,01	-	n.p.	0,00	-	n.p.
Metronidazole	0,00	-	n.p.	0,00	-	n.p.	0,00	-	n.p.
Lincosamides	0,04	-	n.p.	0,01	-	n.p.	0,00	-	n.p.

n.p. = no specific microbial inhibition plate

[a]Highest antibiotc concentration from the two WWTP effluents measured

[b]Highest antibiotic concentration from the three freshwater sites monitored

[c]Highest antibiotic concentration from the 16 seawater and lagoon sites monitored

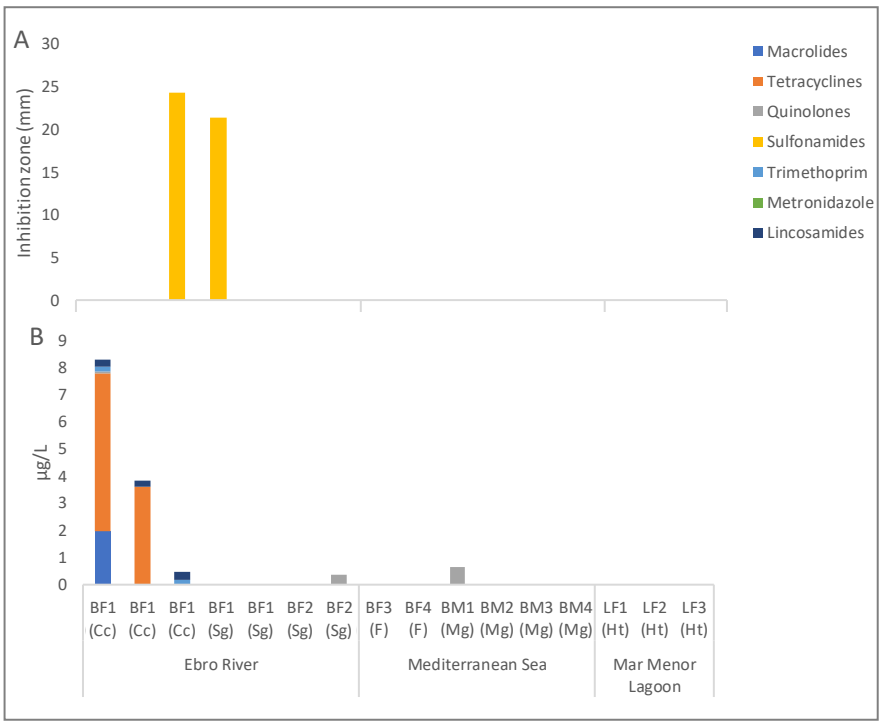

Figure 5. Antibiotics occurrence in biota biofluids for each sampling site (localization codes according to figure 1). A) Antibiotic families detected with the microbial growth inhibition test; B) antibiotic families quantified with LC-MS methodology. In brackets letters indicate organism species, Cc, *Cyprinus carpio; Sg, Silurus glanis; Mg, Mytilus galloprovincialis; Ht, Hexaplex trunculus*.