Occurrence of antibiotics and bacterial resistance in wastewater and sea water from the Antarctic

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Abstract

The potential presence of introduced antibiotics in the aquatic environment is a hot topic of concern, particularly in the Antarctic, a highly vulnerable area protected under the Madrid protocol. The increasing presence of human population, especially during summer, might led to the appearance of pharmaceuticals in wastewater. The previous discovery of *Escherichia coli* strains resistant to antibiotics in sea water and wastewater collected in King George Island motivated our investigation on antibiotics occurrence in these samples. The application of a multi-residue LC-MS/MS method for 20 antibiotics, revealed the presence of 8 compounds in treated wastewater, mainly the quinolones ciprofloxacin and norfloxacin (92% and 54% of the samples analyzed, average concentrations 0.89 µg/L and 0.75 µg/L, respectively) and the macrolides azithromycin and clarithromycin (15% positive samples, and average concentrations near 0.4 µg/L), and erythromycin (38% positive samples, average concentration 0.003 µg/L). Metronidazole and clindamycin were found in one sample, at 0.17 and 0.1 µg/L, respectively; and trimethoprim in two samples, at 0.001 µg/L. Analysis of sea water collected near the outfall of the wastewater discharges also showed the sporadic presence of 3 antibiotics (ciprofloxacin, clindamycin, trimethoprim) at low ng/L level, illustrating the impact of pharmaceuticals consumption and the poor removal of these compounds in conventional WWTPs. The most widespread antibiotic in sea water was ciprofloxacin, which was found in 15 out of 34 sea water samples analyzed, at concentrations ranging from 4 to 218 ng/L. Bacteria resistance was observed for some antibiotics identified in the samples (e.g. trimetropim and nalidixic acid –a first generation quinolone). However, resistance to some groups of antibiotics could not be correlated to their presence in the water samples due to analytical limitations (penicillins, tetraciclines). On the contrary, for some groups of antibiotics detected in samples (macrolides), the antibacterial activity against *E. Coli* was not investigated because these antibiotics do not include this bacterial species in their spectrum of activity.

Our preliminary data demonstrate that antibiotics occurrence in the Antarctic aquatic environment is an issue that needs to be properly addressed. Periodical monitoring of water samples and the implementation of additional treatments in the WWTPs are recommended as a first step to prevent potential problems related to the presence of antibiotics and other emerging contaminants in the near future in Antarctica.

Keywords: Antarctic; antibiotics; bacterial resistance; wastewater; sea water; liquid chromatography-tandem mass spectrometry.

1. Introduction

Antarctica is a vulnerable and important global continent. Because the Antarctic is the coldest place on Earth, is critical in the global climate system, acting as the largest heat sink from southern hemisphere [1]. The importance of preserving Antarctica is based on its influence on the global balance and on the fact that the ecosystems are particularly sensitive to global changes, spite of its distance from the rest of the continents [2,3].

King George Island is a part of the archipelago of the South Shetland Islands, considered one of the areas with high anthropogenic presence. Human activities in King George Island, located about 900 km from Cabo de Hornos, began in the XIX century with the arrival of the sealers and whalers. Today is one of the favorite tourist Antarctic destinations and it is considered as one of the areas with the highest concentration of international scientific stations in the world [4,5]. Both tourism and research are the main types of activities in the Antarctic and potential sources of pollution through the excreta, mainly urine and faeces, where high concentrations of microorganisms, pharmaceuticals and recreational drugs and their metabolites are commonly present.

During the last decades, a large number of organic micro pollutants have been released into the environment as a result of anthropogenic activities [6]. Among them, human and veterinary pharmaceuticals are of justified concern due to the possible impact of these pharmacologically active compounds on the aquatic environment, especially over the long-term toxicological effects on living organisms and the combined effect of exposure to multiple compounds, particularly antibiotics. They are continuously excreted or disposed into the sewer systems as the unaltered parent compound or as metabolites [7]. Subsequently, they often end up in environmental waters, as a consequence of incomplete elimination by wastewater treatment plants (WWTPs). The presence of pharmaceuticals in the aquatic environment has been widely reported around the world, e.g. [6,8–14], and is considered a problem of concern over the world. However, until now few data exist on this issue on Antarctic environment. The article by Esteban el at (2016) reported the presence of endocrine disruptors (natural and synthetic estrogens) in freshwater in the northern Antarctic Peninsula region [15]. Very recently, a wide range of pharmaceuticals and psychotropic drugs have been investigated in the Antarctic Peninsula region [16], with the result that high concentrations of acetaminophen, diclofenac and ibuprofen were found at the µg/L level in wastewaters discharged directly into the ocean. The antibiotics sulfamethoxazole and clarithromycin were present in all water samples analyzed, which was of concern due to the highest risk of these substances to the environment associated to the development of resistant bacteria [10,17]. The presence of pharmaceuticals and other emerging contaminants (e.g. personal care products) in the environment is expected to rise due to the increase in visitors to the

Antarctic continent [16]. The Protocol on Environmental Protection to the Antarctic Treaty requires the application of responsible waste management principles and the development of waste management plans to minimize the impact of human activities on the Antarctic environment. However, numerous direct effluents into the sea from Antarctic scientific stations have been reported, as well as operational problems and malfunctions in the treatment systems [18,19]. Although most studies have been focused on microbial contamination, it can be assumed that emerging contaminants associated with urban effluents are not efficiently eliminated in the treatment processes applied [16], a fact that also occurs in most of WWTPs around the world, as reported in numerous studies that show low removal efficiencies in conventional WWTPs.

Several studies have reported the presence of total coliforms, as well as *Escherichia coli*, near sewage outfalls in different locations in Antarctica [20–22]. The *E. coli* strains derived from humans and birds usually differ in their antibiotic-resistance patterns and in their abundance. This bacterial species is virtually absent in animals or birds without direct contact with humans, and in these cases has low or no resistance to antibiotics [23]. The opposite case are the human-associated *E. coli* which showed higher antibiotic resistance [24].

The first study of antibiotic resistance among *E. coli* isolates in the Fildes Peninsula was carried out in January 2011 [25]. Five years later, similar resistance patterns were reported in strains isolated from the same area [26]. The significance of human-associated bacteria or the mobilization of antibiotic resistance genes in the Antarctic wildlife and environment is still an open question, but the influence of human activities on this growing concern issue seems obvious [27], and treated wastewater discharged from treatment plants is the most likely origin of bacterial antibiotic resistance found in sea water on the Fildes Peninsula [26].

In this work, wastewater and sea water samples collected from different points in Antarctica in 2016 and 2017 have been examined for the presence of pharmaceuticals, with special emphasis on antibiotics, trying to relate their presence with possible resistance to antibiotics. Analysis were performed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) with triple quadrupole analyser (QqQ), which is considered the technique of choice for the determination of these compounds due to its excellent performance in terms of sensitivity, selectivity and robustness [15,28,29]. Numerous quality control samples were prepared in the field, when the samples were collected, as well as in the laboratory, for appropriate quality control of analyses, an essential issue when measuring low analyte concentrations and when the samples/extracts have to be shipped to another continent.

2. Study area and samples collection

2.1. Sampling sites for chemical analysis and preparation of samples in the Antarctic laboratory

Samples of wastewater and sea water were collected in 2016 and 2017 in King George Island (archipelago of the South Shetland Islands) (**Figure 1**). In this area, there are numerous scientific and logistic facilities, including several permanent stations from different countries (Chile, Argentina, Poland, Korea, Uruguay, Brazil, China, Peru and Russia) as well as some settlements of summer occupation. There is also an increased tourism presence in summer. As a consequence, this is one of the most densely populated areas of Antarctica, with a notable number of man-made infrastructures, including an airfield, thus generating a significant amount of waste [5,15].

Thirteen wastewater samples were collected along January 2017 in 8 stations directly from the discharge of the treatment systems into the sea. The majority of stations applied conventional treatments in a small treatment plant placed in the surrounding of the station. The different systems applied consisted on mechanical, biological, physico-chemical and/or disinfection processes (www.comnap.aq). **Table 1** summarizes the treatment systems applied in some of the stations located in Fildes Bay, where some of the samples were collected to perform the present study. Composite wastewater samples (400 mL) were obtained by pooling four individual samples (100 mL each) collected every 20-30 minutes. When possible, the sampling was performed in three consecutive days. However, in most of the stations the collection of samples was made just in one day due to logistic limitations, leading a total of 13 composite samples. Once in the Antarctic laboratory, a 5-mL aliquot was spiked with the corresponding isotope-labeled internal standards (ILIS) at final concentration of 500 ng/L, and stored at -20°C until analysis.

Insert Table 1

Sea water samples were also collected (around 5L) from the sea surface at distances of 0, 10-15 and 20-25 meters from the sewage outfalls. In some cases, due to the inaccessibility, the three samples were taken at the described distances, but in the on-shore of the beach (on the left and right of the discharge). Once in the Antarctic laboratory, 1L-aliquot of the sea water sample was spiked with the corresponding ILIS (final conc. 2.5 ng/L), filtered (GFB filter, 1.0 µm Whatman) and passed through SPE cartridges (OASIS HLB, 500 mg, Waters). The cartridges were dried for 2 minutes under vacuum and kept and temperatures of 4 ºC.

All wastewater samples (5 mL) and SPE cartridges were shipped to Spain in a cooler, at 4 ºC, and were received in the Spanish laboratory, in a maximum of 48 h.

2.2. Sampling sites for microbiological analysis

The samples were collected in January 2016 and 2017 from sites evenly distributed around the sewage outfalls of three Antarctic stations on Fildes (Maxwell) Bay, King George Island (**Figure 2**). Sea water samples were collected from the sea surface, on the coastline and the open sea, at distances of 0, 10, 15, 25, 50, 150, 350, and 650 m from the sewage outfalls of two Chilean Stations: Fuerza Aérea de Chile (FACH; 62º 12'1.65"S, 58º 57'36.96"W); and Instituto Antártico Chileno (INACH) and Estación Marítima Bahia Fildes (62º 12'5.07"S, 58º 57'39.58"W). Samples were also collected in the same manner from the Kitiesh River mouth (62º 11'59.37"S, 58 º57'31.16"W), where the Russian Bellinghausen station discharges its treated wastewater. In addition, wastewater samples were taken from the WWTP of the Chilean stations. Control samples were collected from pristine sites.

2.3. Physico-chemical parameteres

The following physico-chemical parameters were measured: temperature, electrical conductivity, pH, dissolved oxygen, salinity and Oxidation-Reduction Potential (ORP).

Insert Figure 1

Insert Figure 2

3. Methods

3.1. Analytical methodology for the determination of antibiotics

Upon receipt at the analytical laboratory, wastewater samples were centrifuged, and then directly injected in the LC-MS system. Additionally, a 10-fold pre-concentration was carried out, taking 2-mL of sample which were passed through OASIS HLB cartridges (60 mg, Waters), previously conditioned with methanol and HPLC water. After drying for 30 minutes, the cartridges were eluted with 2 mL of methanol. The eluates were dried until dryness under N_2 stream and reconstituted with 200 µL H_2O: MeOH (90:10).

In the case of sea waters, Oasis HLB cartridges received in the lab were eluted with 5 mL MeOH. The eluates were dried until dryness under N_2 stream and reconstituted with 1 mL H_2O:MeOH (90:10).

3.1.1. Analysis by UHPLC—MS/MS

50 µL of the effluent wastewater sample, after centrifugation, were directly injected in the UHPLC–MS/MS system (Xevo TQS, Waters). The 10-fold SPE pre-concentrated extract was also injected for confirmation of positives and to test for additional compounds present in the samples as well as to evaluate the effect of SPE step on matrix effects.

In brief, a column Acquity UPLC C18 1.8 µm, 100 mm x 2.1 mm, was used for chromatographic separation, using water/methanol gradient (both 0.1 mM NH_4Ac) as mobile phase at 0.3 ml/min. Three MS/MS transitions were acquired per each compound, using the most sensitive (Q) for quantification and the other two (q) for confirmation. The agreement in retention time (± 0.1 min) and at least one q/Q ion ratio (±30%) compared with the reference standard, was used as general criterion for confirmation of the identity of the compound detected in the sample. Ten isotope-labeled internal standards (ILIS) were used as surrogates (i.e. directly added to the samples) for correction of potential errors associated to sample treatment, transport and matrix effects in LC-MS/MS analysis. The analytical method was previously validated for surface water and wastewater [28], and was recently tested in wastewaters from Colombia with satisfactory quality control data [30]. Analysis of sea water extracts was made in a similar manner, injecting 50 µL of the SPE extract into the UHPLC-MS/MS.

The selection of the analytes was based on our own experience on the trace level determination of pharmaceuticals in waters, as well as on previous data on their occurrence in wastewaters analyzed in other parts of the world [28,30]. A total of 29 compounds were investigated: 20 antibiotics (amoxicillin, ampicillin, cefotaxime, ceftriaxone, cloxacillin, oxacillin, imipenem, meropenem, azithromycin, clarithromycin, erythromycin, ciprofloxacin, norfloxacin, clindamycin, tetracycline, oxytetracycline, doxycycline, metronidazole, sulfamethoxazole, trimethoprim), 1 antidepressant (Venlafaxine), 3 nonsteroidal anti-inflammatory drugs (NSAID) (diclofenac, naproxen, ketoprofen), 3 angiotensin II receptor antagonists (losartan, irbesartan, valsartan), 1 analgesic (acetaminophen), and 1 anticonvulsant (carbamazepine). Twelve analytes ILIS were available for these compounds, and 6 of which corresponded to antibiotics (azithromycin, erythromycin, ciprofloxacin, norfloxacin, sulfamethoxazole, trimethoprim).

3.1.2. Preparation of Quality Control samples

Special attention was made on the quality control of the analytical process including sampling, sample pre-treatment, storage and shipping to the IUPA laboratory at Spain. To this aim two sets of Quality Control (QC) samples were prepared: QCs prepared in the Antarctic laboratory at the INACH scientific station, and QCs prepared at the IUPA laboratory to be analyzed together with each batch of samples.

Antarctic QCs were prepared in order to check the overall analytical procedure, and test for stability of analytes during shipment. For this purpose, selected wastewater (5 mL) and sea water (1 L) samples were spiked with the ILIS mix at 500 ng/L and 2.5 ng/L, respectively, together with a mix of reference standards of pharmaceuticals at the same concentration level. The "blank" samples used for preparation of QCs, but without spiking, were also processed and analyzed in order to subtract the analyte concentration obtained in the analyses from that resulting for the spiked sample. The analytical procedure applied to QCs was exactly the same as for the samples.

Laboratory QCs were prepared with the same wastewaters used to prepare Antarctic QCs. These samples were also spiked at the same level than Antarctic QCs (500 ng/L) and analyzed together with the set of samples, with the objective to control the analytical methodology applied at our laboratory. Two laboratory QCs were prepared using sea water collected from the Mediterranean Sea, near Castellon, Spain. The samples were spiked at 2.5 and 10 ng/L before being subjected to SPE.

3.2. Bacterial resistance methodology

3.2.1. Bacterial strains

Total coliforms and *E. coli* were counted by filtering 1 mL, 10 mL, and 134 mL sea water samples and 100 µL of wastewater samples. The membrane filters were placed on chromogenic selective agar (ChromoCult® Coliform Agar ES, Merck) and incubated at 37 ºC for 24 h. The filters with countable salmon-red colonies (coliforms) and dark blue to violet colonies (*E. coli*) were selected. The bacterial isolates were purified by streaking a single colony on Levine agar (Merck) and incubating it at 37 ºC for 18-24 h. Trypticase soy broth (Oxoid Ltd.) was then inoculated with the presumptive *E. coli* strains and incubated at 37 ºC overnight. Aliquots were stored at -80 ^{o}C in 50% (v/v) glycerol in Trypticase soy broth.

3.2.2. Antibiotic susceptibility testing

A total of 115 strains of *E. Coli* isolated from different water samples were selected to determine antibiotic susceptibility by a disk diffusion method [31] using different groups of antibiotics: penicillins (ampicillin), cephalosporins (cefuroxime, cefoxitin, cefotaxime, ceftazidime, cefepime), carbapenems (ertapenem, meropenem), aminoglycosides (streptomycin, kanamycin, gentamicin, amikacin), quinolones (nalidixic acid, ciprofloxacin), tetracycline, chloramphenicol, sulfonamide, and trimethoprim. *Escherichia coli* ATCC 25922 was used as the control for the susceptibility tests. The results were interpreted according to the CLSI document M100 [32].

4. Results and discussion

4.1. Bacterial counts

Two hundred and forty six coastal and open sea samples were taken in the summer of years 2016 and 2017. Their analysis showed the presence of total coliforms and *E. coli* in 76.4 % and 67.5 % of the samples, respectively. Furthermore, 84.8% of the coastal samples and 47.4% of the open sea samples also had *E. coli*.

The highest bacterial counts were in sea water surrounding the sewage outfalls (total coliforms were in the range 21-5600 CFU/100mL and *E coli* in the range of 3-1900 CFU/100mL). The bacterial counts decreased rapidly with increasing distance from the point of discharge, possibly as a result of dilution by currents passing through the bay. *E. coli* were absent in all samples collected beyond 150 m from the outfall. Fecal coliforms and *E. coli* were not detected at the control sample.

There has been relatively little work looking at the environmental impacts of WWTP outfalls in Antarctica but several studies have detected wastewater tracers at 50 m to 2 Km distance from the point of discharge [21]. There have been reports of similar decreasing microbiological contamination patterns with increasing distance from the outfall, with *E. coli* always present in its direct vicinity [20,22].

The counts of total coliforms and *E coli* in the WWTP samples were in the range 210-120,000 CFU/100mL and 20-50,000 CFU/100mL, respectively. The wide variation of the counts probably has to do with WWTPs difficulties to respond to sudden population increases occurring in the summer season.

4.2. Antibiotic susceptibility testing

A total of 115 strains isolated from sea water were selected to determine antibiotic susceptibility. The strains showed resistance to 8 out of 18 antibiotics tested. Ampicillin, showed the most frequent resistance and was found in 46 strains, followed by tetracycline (32 strains), trimethoprim (25 strains), sulfonamide (21 strains), cefoxitin (13 strains), streptomycin (9 strains), nalidixic acid (5 strains) and cefotaxime (2 strains).

Fifty-one strains (44.5 %) were resistant to at least one antibiotic, and 9 strains (8 %) were multidrug resistant (MDR), according to the criteria of Magiorakos et al. [33]. Likewise, 51 strains (44.5 %) showed susceptibility to every compound tested and 13 strains (11%) were classified as intermediate.

With respect to 20 strains isolated from the WWTPs, we observed resistance to 7 out of 18 antibiotics tested (nalidixic acid, ampicillin, sulfonamide, trimethoprim, streptomycin, cefoxitin and

chloramphenicol). Seven strains showed resistance to at least one antibiotic, and six were resistant to antibiotics from at least three different groups and could therefore be classified as MDR strains. No resistance to any of the compounds tested was observed in 13 strains.

The presence of antibiotic resistance in *E. coli* strains isolated from the sea water around Fildes (Maxwell) Bay could be associated with the treated wastewater discharged in the area, because coliforms were only present in the outfall vicinity and the antibiotic-resistance patterns are similar in strains isolated from sea water and in those isolate from the WWTP. This situation has been previously reported by Rabbia et al (2016) [26]. The biological WWTPs creates an environment potentially suitable for antibiotic resistance development and spread, as they combine several favorable factors, namely a high cell density sustained by a nutrient rich environment, and a recurrent contamination with both antibiotics and resistant bacteria [34]. However, not long ago, the current knowledge on the prevalence and types of antibiotic resistance in the environment was barely sufficient and the information regarding resistance of environmental bacteria was still very fragmented [25,34].

Similar resistance patterns to those obtained in this study have been reported in *E. coli* strains isolated from Fildes (Maxwell) Bay in 2011 and 2013 [25,26]. Therefore, the results of this study demonstrate the continuous presence of antibiotic resistance in Fildes (Maxwell) Bay sea water attributable to the uninterrupted wastewater discharges.

The presence of bacteria with antimicrobial resistance in the Antarctic environment is indicative of how widespread the global antibiotic resistance situation has become. The antibiotic-resistance profiles of *E. coli* isolated from sea water around Fildes (Maxwell) Bay are a cause of concern, especially considering the possibility of horizontal transfer of genetic resistance determinants to autochthonous Antarctic bacteria [26]. Currently the efforts to suppress further development and dispersal of resistance are focused on reducing the consumption of antibiotics. However, the factors that maintain and perhaps facilitate further resistance dispersal in natural environments (e.g., sewage treatment) are given less attention [25,26].

4.3. Determination of antibiotics

4.3.1. Quality Control samples

In this work, we put especial emphasis on the use of quality control samples to support the reliability of the analytical data. The logistic problems in the Antarctic, and the special conditions for sampling, sample treatment, storage, and shipping the samples/extracts to our laboratory in Castellon, Spain, make highly recommendable to extreme precautions along the overall analytical process. In addition,

the very low analyte concentrations and the complexity of the samples (wastewater and sea water) is also a key issue from the analytical point of view.

A set of quality control samples were prepared in the Antarctica (see section 3.1.2) as well as in the Spanish laboratory, in order to test stability of samples/extracts along the overall analytical process and shipping. In addition to the preparation of spiked samples, used as QCs, the use of ILIS allowed additional testing of stability and the correction of matrix effects, rather common in LC-MS/MS based methods.

In the case of wastewater samples, a total of 16 QCs were analyzed. Eight QCs were prepared in the Antarctic using eight different wastewater samples, and another eight QCs were prepared in the Spanish laboratory using the same samples, spiking them at the same concentration level than Antarctic QCs. From the 20 antibiotics investigated, only 11 could be further analyzed (see **Table 2**), as the analysis was not successful for the remaining 9 compounds due to instability issues, chromatographic limitations and/or lack of sensitivity. The results obtained for the analysis of QCs (expressed as recovery percentage) for the 11 antibiotics finally determined in the samples are summarized in **Table 2**. QCs' data give information on two important issues: 1) the stability of the analytes in Antarctic spiked samples (by comparison to the samples prepared in the laboratory the same day of the analysis); 2) the influence of the matrix components on quantification (matrix effects) and their potential correction by the ILIS used. The highest method sensitivity was observed for the macrolides clarithromycin and erythromycin, as well as for clindamycin, trimethoprim and sulfamethoxazole. For these compounds, it was possible to reach limits of quantification around 0.001 µg/L. For metronidazole, the limit quantification was near 0.01 µg/L, and for the remaining analytes around 0.05 µg/L.

The use of eight different wastewaters for preparation of QCs allowed us to observe differences in the behavior of the analytes depending on the sample. The behavior was in general rather coherent, although two wastewater samples seemed to be particularly problematic, leading to outliers in the recovery data. We considered as acceptable QC recoveries between 60-140%, which is in the line of some guidelines such as for pesticide residue analysis [35].

As shown in **Table 2**, the Antarctic QC recoveries for erythromycin and trimethoprim were excellent, with an average of 111% and 90%, respectively, and without outliers. Satisfactory data were also obtained for ciprofloxacin and norfloxacin, although some individual QCs could not be used for the average calculation due to the presence of the analyte in the "blank" sample used for preparation of QCs. In those cases, the number of individual QCs used for the calculation decreased down to 5 (ciprofloxacin) and 6 (norfloxacin). For another five antibiotics (azithromycin, clarithromycin, cloxacillin,

metronidazole, sulfamethoxazole), average QCs including the eight individual data were within the tolerance range, but some outliers occurred. Obviously, average recovery improved when eliminating the outliers, which in general were 2 out of 8 analyzed. The worst results were observed for clindamycin (4 outliers) and especially for cefotaxime (8 outliers). For the later, all QCs values were below 50%, preventing the interpretation of data for this compound, which in fact was not found in any sample.

In addition to the Antarctic QCs, eight Laboratory QCs were also prepared for wastewater. The results are also summarized in **Table 2**. The comparison between top data (Antarctic) and bottom data (laboratory) allows a rapid evaluation of the stability of analytes in the wastewater samples. The results of laboratory QCs are in general agreement with the Antarctic QCs, with satisfactory data for erythromycin, trimethoprim, ciprofloxacin and azithromycin. The main difference observed was for cefotaxime, which in the laboratory QCs presented only 2 outliers in comparison with all outliers obtained in the Antarctic QCs (all below 50%). This was interpreted as degradation of this compound in the Antarctic samples. For clindamycin and cloxacillin the low recoveries observed in some individual QCs were surely due to matrix effects suppression, as no ILIS was used for these compounds.

Insert Table 2

It is worth to noticing that a notable degradation was observed for ciprofloxacin and norfloxacin in the wastewater samples received in the laboratory. This was estimated by comparison of peak intensities between the Antarctic QC and the recently prepared Laboratory QC, both at the same spiking level. In addition, matrix effect enhancement was observed for both compounds. In some way, these opposite aspects might contribute to compensate for the results finally reported, but without an appropriate control of the process. Fortunately, the use of analyte LIS for both compounds allowed to correct both degradation and matrix effects, as illustrated by the satisfactory recoveries obtained for Antarctic and Laboratory QCs.

As a summary, for the eleven antibiotics finally analyzed in wastewaters, the results could be considered as questionable for cefotaxime, and in a minor extent for clindamycin, in both cases with a trend to be reported by default. The fact that cefotaxime was not found in any sample might be also due to degradation of this compound along the overall analytical process and shipment of samples.

Quality control samples were also prepared for sea water (see section 3.1.2), by spiking five different sea waters with the analytes and adding the available ILIS. The fact that no samples, but the SPE cartridges, were shipped to the Spanish laboratory made unfeasible the preparation of Laboratory QCs with the same samples used for Antarctic QCs. Instead, water collected from the Mediterranean Sea in the area of Castellon was used for Laboratory QCs preparation. The results for seawater QCs are shown

in **Table 3**. It can be seen that cefotaxime and clindamycin were again the most problematic compounds with unsatisfactory recoveries. Cloxacillin was poorly recovered, with most recoveries around 40%. Surprisingly, norfloxacin was not recovered, a fact that could not be properly explained. This might be due to the degradation of this compound in the seawater and/or SPE cartridge. This hypothesis was supported by the satisfactory recoveries observed in the recently prepared Laboratory QCs. For the remaining compounds, the recoveries were satisfactory, with only one or none outliers.

Insert Table 3

4.3.2. Results from Antarctic samples

Our results illustrate the presence of several pharmaceuticals in wastewater, including 8 antibiotics. The most frequent antibiotics were the quinolones ciprofloxacin and norfloxacin, which were present in 92% and 54% of the samples analyzed, at average concentrations of 0.89 μg/L and 0.75 μg/L, respectively. Ciprofloxacin concentrations ranged from 0.37 to 1.86 μg/L, and exceeded 1 μg/L in 5 out of 13 samples analyzed. A similar trend was observed for norfloxacin, which concentrations varied from 0.58 to 1.23 μg/L, but only one sample above 1 μg/L **(Figure 3**).

Three macrolides were also identified: azithromycin and clarithromycin (both with 15% positive samples, and average concentrations close to 0.4 μg/L) and erythromycin (38% positive samples, average concentration 0.003 μg/L). Metronidazole and clindamycin were found in just one sample, at 0.17 and 0.1 μg/L, respectively; and trimethoprim was present in two samples, at 0.001 μg/L. In a recent study, Gonzalez-Alonso and coworkers found 4 out of 6 antibiotics analyzed in the Antarctic Peninsula region, with clarithromycin and sulfamethoxazole being the most widespread. The concentrations found in our work for clarithromycin in the two positive samples (0.41 and 0.55 μg/L) were quite similar to that reported by Gonzalez-Alonso, who analyzed just one wastewater discharge (0.14 μg/L).

Insert Figure 3

The results found are in agreement with other studies around the world, in which antibiotics are commonly found in wastewater, both raw and treated. The concentrations do not much differ from other studies, which illustrate that consumption of pharmaceuticals in the Antarctic is rather similar to other parts of the world. It has been reported that anti-inflammatory drugs, analgesics and antibiotics are the most widely consumed pharmaceuticals in the Antarctic, which is consistent with the climate conditions and characteristics of the activities performed in that area [16].

Other pharmaceuticals such as acetaminophen, carbamazepine, venlafaxine, losartan, valsartan, irbesartan, diclofenac and naproxen were also found; supporting again that consumption of pharmaceuticals in the Antarctic is comparable to other worldwide localities studied. The results and discussion on these other pharmaceuticals will be object of a separate publication

Our data illustrate that treatment systems applied in the majority of Antarctic stations do not efficiently eliminate pharmaceuticals, which is not surprising as it also occurs in the wide majority of WWTPs around the world, as reported in the scientific literature [6,36–39].

The Protocol on Environmental Protection to the Antarctic Treaty was signed in Madrid on October 4, 1991 and entered into force in 1998. It designates Antarctica as a "natural reserve, devoted to peace and science". The Annex III to this protocol, dealing with waste disposal and waste management, establishes that "the amount of wastes produced or disposed of in the Antarctic Treaty area shall be reduced as far as practicable so as to minimize impact on the Antarctic environment and to minimize interferences with the natural values of Antarctica". In addition, "sewage and domestic liquid wastes, shall, to the maximum extent practicable, be removed from the Antarctic Treaty area by the generator of such wastes". Interestingly, Article 5, dealing with disposal of waste in the sea, establishes that "sewage and domestic liquid wastes may be discharged directly into the sea, taking into account the assimilative capacity of the receiving marine environment and provided that: a) such discharge is located, wherever practicable, where conditions exist for initial dilution and rapid dispersal; and b) large quantities of such wastes (generated in a station where the average weekly occupancy over the austral summer is approximately 30 individuals or more) shall be treated at least by maceration".

The Protocol on Environmental Protection also establishes a waste disposal classification system as a basis for recording wastes and to facilitate the evaluation of environmental impacts of scientific activity and associated logistic support. Wastes produced are classified into five groups, where sewage and domestic liquid wastes constitute Group 1. In order to reduce the impact of waste on the Antarctic environment, each Party must "prepare and annually review and update its waste management plans".

Despite the above considerations neither the Madrid Protocol nor current monitoring campaigns establish normalized parameters for the quality of wastewater discharged into the sea, and therefore each party can define its own requirements for the discharges.

According to Tarasenko et al [40], 63% of the stations had wastewater treatment systems, a figure that fits with the fact that 62% of Antarctic stations operate the whole year, while 38% operate only in summer. Most of treatment systems when that study was made applied biological treatments, while 7% applied physico-chemical processes, and 13% only mechanical systems. In the Antarctic, wastewater

treatment is limited by logistic, energetic and maintenance issues rather than economic reasons. The difficulties to implement appropriate systems for removal of emerging contaminants in the Antarctic stations are easy to understand. However, in the light of our data and other recently reported in the scientific literature on the presence of emerging contaminants (pharmaceuticals, personal care products, hormones) in the Antarctic area [15,16,18], it seems necessary to improve the treatments applied in the most populated stations in order to efficiently remove antibiotics and other pharmaceuticals, at least in those facilities where the average weekly occupancy over the austral summer is 30 individuals or more, accordingly to the Madrid Protocol. There is no doubt of the huge dilution suffered by wastewater discharges into the sea, but the effect of high concentrations of antibiotics in the outfall of the discharge may favor the selection of bacterial resistance as suggested in this and other works.

The importance of QC prepared in the Antarctic must be emphasized as some compounds seemed to be notably degraded along sample treatment and storage (e.g. norfloxacin, ciprofloxacin). This fact could be corrected by the use of the analyte-ILIS. In addition, ILIS, applied in Antarctic as surrogated, also allowed the correction of matrix effects, which was corroborated by the use of QCs prepared at the analytical laboratory. Unfortunately, for other compounds, such as cefotaxime, Antarctic QCs showed that the compound was degraded along the overall analytical process and/or shipment of samples, a fact that could not be corrected as no ILIS was available for this compound.

The results obtained for the wastewater samples collected in 2017 are in the line of the 2016 campaign. In the first year, two tetracyclines (doxycycline, tetracycline), two macrolides (azithromycin, clarithromycin), two quinolones (norfloxacin, ciprofloxacin), the nitroimidazol metronidazole, and the lincosamide clindamycin were found. However, some β-lactams (e.g. ampicilline, amoxicillin), found at least in one of the four studied stations of King George Island in 2016, could not be confirmed in the samples from 2017.

Regarding seawater, the pre-concentration factor in the SPE step (x 1000) allowed us to perform quantification in the samples at concentrations as low as 0.01-0.1 ng/L for all analytes. Only three antibiotics (ciprofloxacin, clindamycin, trimethoprim) were present in some of the samples. These compounds were also found in wastewater, illustrating that wastewater discharges lead to contamination of sea water, at least in the surrounding of the discharges. The most widespread antibiotic in seawater was ciprofloxacin, which was found in 15 out of 34 seawater samples analyzed, at concentrations ranging from 4 to 218 ng/L (average of positive samples, 48 ng/L). Clindamycin and trimethoprim were found in 3 and 2 out of 34 samples, respectively, both at very low concentrations (below 0.1 ng/L). Ii is worth to noticing that all detections were in the sea samples collected in the

surroundings of the sewage outfalls. However, ciprofloxacin was also found (90 ng/L) in three sea water samples collected from Ardley Island, one of the largest breeding colonies for the penguins. This issue should be investigated in more detail performing additional analysis. It is not discarded that tourists visiting this Island and boats in the nearby area are potential sources of pollution by antibiotics.

Our data on bacteria resistance for some antibiotics are in agreement with the presence of such compounds in the samples. This was the case for trimethoprim (21.7% of resistant strains). In addition, resistance was observed for the quinolone nalidixic acid (4.3% of resistant strains). Unfortunately, nalidixic acid was not included in the analytical method, but our data showed the quinolones ciprofloxacin and norfloxacin were among the most frequent antibiotics detected in the wastewater. A relationship between the compounds identified in the samples and resistance could not be established for penicillins and tetracyclines. For these families, a high number of resistance cases were observed among the strains studied, but no data were available on their presence in samples due to analytical limitations (low sensitivity, and/or low stability in samples, and/or poor chromatographic separation). The choice of *E. Coli* as an antibiotic resistance test microorganism, although is adequate to determine the impact of water discharges, has limitations in the study of resistance profiles for some antibiotics. This is the case of macrolides, which were frequently found in wastewater but their spectrum of activity did not include *E. Coli*. This made troublesome to link the occurrence of these antibiotics in the water samples with the presence of resistant bacterial strains.

Although it is reasonable to assume that antibiotics present in the seawater collected close to the scientific stations come from the wastewater discharges, no clear correlation was found between composition of the discharges and the sea water. The impact of wastewater discharges into the receiving ocean water can be clearly observed in terms of fecal microorganisms, .e.g *E. coli*. However, to evaluate such impact on the antibiotic presence and concentrations in the ocean, more detailed studies are required, increasing the frequency of sampling and widening the list of target compounds included in the analytical method.

5. Conclusions

Data obtained in this research confirm the occurrence of several antibiotics in treated wastewater from all the stations (scientific and military) monitored. As expected for conventional treatments applied in most WWTPs, pharmaceuticals- specifically antibiotics- are not completely removed in WWTPs and therefore are released into the sea water nearby the stations. Our data also show that a few antibiotics are also present at the low ng/L level in the sea water collected close to the outfall of the wastewater

discharges. Parallel studies made on strains isolated from sea water and wastewater reveled resistance to the same antibiotics found in the samples (e.g. trimethoprim and the group of quinolones), a fact that illustrates the human anthropogenic impact derived from pharmaceutical consumption in the area. Future research is required to widen the knowledge on the presence of antibiotics and the existence of bacteria resistant to them, and to evaluate the potential impact of antibiotics in the Antarctic environment.

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