

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 resistance to antibiotics in seawater 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 several compounds, mainly quinolones and macrolids, in treated wastewater. Analysis of seawater collected near the exit of the human wastes also showed the sporadic presence of a few compounds at low ng/L levels, illustrating the impact of pharmaceuticals consumption and the poor removal of these compounds in conventional WWTPs. 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 in the near future in Antarctica.

Keywords: Antarctic; antibiotics; bacterial resistance; wastewater and 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 (Bargagli, 2005). 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 (Croxall, 2005; Smetacek, 2005).

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 whales. 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 (Kennicutt, 2009; IAATO, 2015).

During the last decades, a large number of organic micro pollutants have been released into the environment as a result of anthropogenic activities (Gracia-Lo, 2011). Among them, human and veterinarian 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 [Richardson, 2012]. Subsequently, they often end up in environmental waters, as a consequence of incomplete elimination by WWTPs. The presence of pharmaceuticals in the aquatic environment has been widely reported (Gracia-Lor, 2011), and is considered a problem of concern over the world. However, until now very few data exist on this issue on Antarctic environment.

Several studies have reported the presence of fecal coliforms, as well as *Escherichia coli*, near sewage outfalls in different locations in Antarctica (Delille, 2003). The *E. coli* strains derived from humans and birds usually differ in their antibiotic-resistance patterns and in their abundance. *E. coli* is virtually absent in animals or birds without direct contact with animals, and in these cases has low or no resistance to antibiotics. (Bonnedahl, 2008). The opposite case are the human-associated *E. coli* strains which showed higher antibiotic resistance (Davies, 2010).

Previous studies have reported the presence of endocrine disruptors (natural and synthetic estrogens) in freshwater in the northern Antarctic Peninsula region (Esteban, 2016). Although no information has been found on the concentration levels of pharmaceuticals in the Antarctic

environment, different studies have demonstrated antibiotic resistance in this region. The first study of antibiotic resistance among *Escherichia coli* isolates in the Fildes Peninsula was carried out in January 2011 (Hernández, 2012). Five years later, Rabbia (2016) reported similar resistance patterns in strains isolated from the same area.

In this work, wastewater and sea samples collected from different points of the Antarctic in 2016 and 2017 have been searched 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 (Boix, 2015; Hernández, 2014; Esteban, 2016). 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 and location for chemical analysis

The analysis consisted of wastewater and sea water samples 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 has 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 (Esteban, 2016; IAATO 2015).

13 samples were collected along January 2017 directly from the discharge of the treatment systems into the sea. Composite wastewater samples (400 mL) were obtained by pooling four individual samples (100 mL each) collected every 20-30 minutes. Once in the Antarctic laboratory, a 5-mL aliquot was spiked with the corresponding isotope-labelled internal standards (ILIS), at final concentration of 500 ng/L, and stored at -20°C until analysis.

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°C.

Several QC samples 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 seawater (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.

All wastewater samples (5 mL) and SPE cartridges were shipped to Spain in a cooler, at 4°C, and were received in the Spanish laboratory, in a maximum of 36 h.

2.2. Sampling sites and location 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 King George Island (**Figure 2**). Seawater 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; 62° 12'1.65"S, 58° 57'36.96"W); and Instituto Antártico Chileno (INACH) and Estación Marítima Bahía Fildes (62° 12'5.07"S, 58° 57'39.58"W). Samples were also collected in the same manner from the Kitiash River mouth (62° 11'59.37"S, 58 °57'31.16"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 site.

2.3. Physico-chemical parameteres

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

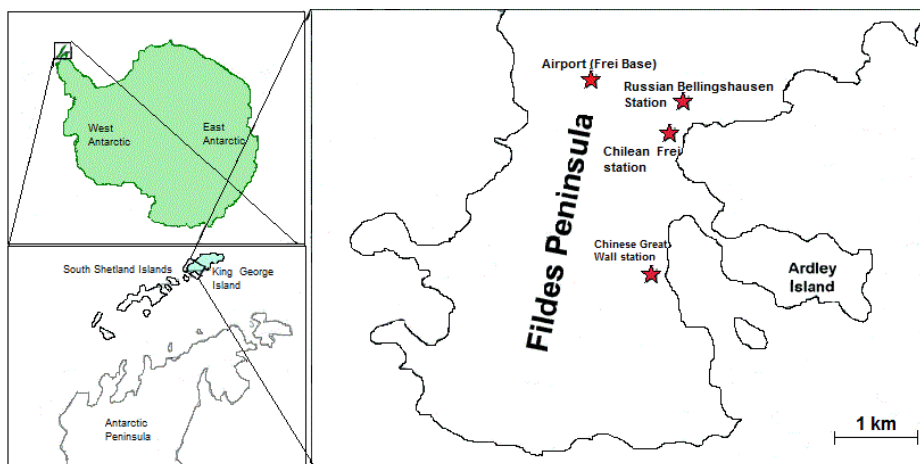


Figure 1. Location map of the areas sampled.

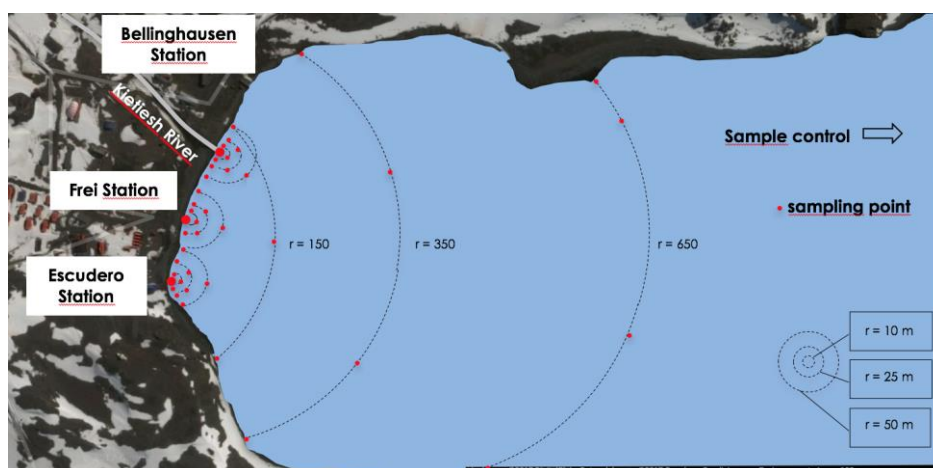


Figure 2. Detailed map of the areas sampled for microbiological analyses

3. Methods

3.1. Analytical methodology for the determination of antibiotics

Upon receipt at the analytical laboratory, wastewater samples were 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 N₂ stream and reconstituted with 200 µL H₂O: MeOH (90:10).

In the case of sea waters, Oasis HLB cartridges received in the lab were eluted with 5 mL of methanol. The eluates were dried until dryness under N₂ stream and reconstituted with 1 mL H₂O:MeOH (90:10).

3.1.1. Analysis by UHPLC—MS/MS

50 µL of the sample 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. Details about instrumentation and experimental conditions can be found elsewhere (Boix, 2015). Analysis of seawater extracts was made in a similar manner, injecting 50 µL of the SPE extract into the UHPLC-MS/MS.

A total of 29 analytes were investigated: 20 antibiotics (Sulfamethoxazole, Erythromycin, Clarithromycin, Azithromycin, Cloxacillin, Tetracycline, Doxycycline, Ciprofloxacin, Ampicilline, Amoxicillin, Oxacillin, Ceftriaxone, Oxytetracycline, Norfloxacin, Trimethoprim, Clindamycin, Metronidazole, Imipenem, Meropenem, Cefotaxime), 1 antidepressant (Venlafaxine), 3 nonsteroidal anti-inflammatory drugs (NSAID) (Diclofenac, Naproxen, Ketoprofen), 3 angiotensin II receptor antagonists (Losartan, Irbesartan, Valsartan), 1 painkiller (Acetaminophen), and 1 anticonvulsant (Carbamazepine).

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 seawater 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 °C 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 °C for 18-24 h. Trypticase soy broth (Oxoid Ltd.) was then inoculated with the presumptive *E. coli* strains and incubated at 37 °C overnight. Aliquots were stored at 80 °C in 50% (v/v) glycerol in Trypticase soy broth.

3.2.2. Antibiotic susceptibility testing

Antibiotic susceptibility patterns were determined with the disk diffusion method (CLSI, 2012) using different groups of antibiotics: penicillins (ampicillin, AMP), cephalosporins (cephalothin, CEP; cefuroxime, CXM; cefoxitin, FOX; cefotaxime, CTX; ceftazidime, CAZ; cefepime, FEP), carbapenems (ertapenem, ETP; meropenem, MEM; imipenem, IMP), aminoglycosides (streptomycin, STR; kanamycin, KAN; gentamicin, GEN; amikacin, AMK), quinolones (nalidixic acid, NAL; ciprofloxacin, CIP), tetracycline (TET), phenicols (chloramphenicol, CLO), sulfonamide (SUL), and trimethoprim (TMP). *Escherichia coli* ATCC 25922 was used as the control for the susceptibility tests. The results were interpreted according to the CLSI document M100-S24 (CLSI, 2014)

4. Results and discussion

4.1. Bacterial counts

A total of 246 samples were analyzed between 2016 and 2017. Total coliforms and *E. coli* were detected in 76,4 % y 67,5 % of samples, respectively. *E. coli* were detected in 84.8% of the coastal samples and in 47.4% of the open sea samples.

The highest bacterial counts were found in seawater surrounding the sewage outfalls. However, the bacterial counts decreased rapidly with increasing distance from the outfall. *E. coli* were absent in all samples collected further than 150 m from the outfall. Fecal coliforms and *E. coli* were not detected at the control sample.

The counts of total coliforms and *E. coli* in the wastewater samples are in the range 210-120 000 CFU/100mL and 20-50 000 CFU/100mL, respectively.

4.2. Antibiotic susceptibility testing

The strains isolated from seawater showed resistant to 5 of the 20 antibiotics tested (TET, AMP, SUL, TMP, and STR). Twenty-eight strains were susceptible to all the antibiotics tested and 13 showed susceptibility and intermediate susceptibility, mainly to CEP. Twelve strains were resistant to at least one antibiotic, and five strains

were MDR, according to the criteria of Magiorakos (2012). All the MDR strains were resistant to TET.

Among the strains isolated from the WWTPs, 2 strains were resistant to antibiotics from at least three different groups (KAN-TET-SUL and AMP-STR-SUL-TMP), and can therefore be classified as MDR strains. Two strains from the WWTPs showed susceptibility to every compound tested, whereas the other six strains showed intermediate susceptibility to at least one antibiotic, with resistance to STR most common.

4.3. Determination of antibiotics

Our preliminary results show the presence of several pharmaceuticals in wastewater, including some antibiotics. The most frequent compounds were the quinolones ciprofloxacin and norfloxacin (12 and 7 out of 13 samples analyzed, respectively, at average concentrations of 0.89 and 0.75 µg/L) and macrolides (azithromycin and clarithromycin, at average conc. close to 0.4 µg/L, erythromycin, at 0.002 µg/L). Metronidazole and clindamycin were found in just one sample, at conc. near 0.1 µg/L, and trimethoprim was present in two samples, at 0.001 µg/L.

Other pharmaceuticals such as acetaminophen, carbamazepine, venlafaxine, losartan, valsartan, irbesartan, diclofenac and naproxen were also found. Additionally, the presence of two metabolites of dypirone/metamizole (4-AAA, 4-FAA), mefenamic acid and ketoprofen was confirmed by LC-QTOF MS.

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. ILIS added in Antarctic as surrogated also allowed to correct for matrix effects, which was corroborated by the use of QCs prepared in the analytical laboratory.

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

Regarding sea water, only data from 2016 samples are available at the time of writing this paper. Three antibiotics (ampicilline, clarithromycin and azithromycin) were detected in sea water samples collected in the surrounding of wastewater discharges in several of the stations monitored. A more complete set of data from 2017 samples will allow to confirm this fact soon.

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 from conventional treatments applied in most WWTPs, pharmaceuticals- specifically antibiotics- are not completely removed in WWTPs and therefore are released into the seawater nearby the stations. Our preliminary data show that a few antibiotics are also present in the sea water nearby the wastewater discharges at the low ng/L level. Parallel studies made on strains isolated from seawater and wastewater revealed resistance to the same antibiotics found in the samples, a fact that supports the human anthropogenic impact derived from pharmaceutical consumption in the area. Future research is required to confirm this fact and to extend the knowledge on antibiotics occurrence and their potential impact in the Antarctic environment.

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