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Do plastics serve as a possible vector for the spread of antibiotic resistance? First insights from bacteria associated to a polystyrene piece from King George Island (Antarctica)

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ABSTRACT

The retrieval of a polystyrene macro-plastic piece stranded on the shores in King George Island (South Shetlands, Antarctica) gave the opportunity to explore the associated bacterial flora. A total of 27 bacterial isolates were identified by molecular 16s rRNA gene sequencing and 7 strains were selected and screened for their ability to produce biofilm and antibiotic susceptibility profiles. All the bacterial isolates were able to produce biofilm. The Kirby-Bauer disk diffusion susceptibility test to 34 antibiotics showed multiple antibiotic resistances against the molecules cefuroxime and cefazolin (belonging to cephalosporins), cinoxacin (belonging to quinolones) and ampicillin, amoxicillin + clavulanic acid, carbenicillin and mezlocillin (belonging to beta-lactams). The obtained results suggest that plastics can serve as vectors for the spread of multiple resistances to antibiotics across Antarctic marine environments and underline the relevance of future studies on this topic.

1. Introduction

To date, a plethora of studies are documenting the ubiquitous occurrence in diverse environmental matrices of different and relatively new plastic polymers, including low- and high-density polyethylene, polypropylene, polyvinyl chloride, polystyrene and polyethylene terephthalate. This explains why plastics are currently recognized as one of the most widespread contaminants of anthropogenic origin (Plastics Europe, 2017). Most research on plastic pollution has focused on aquatic systems, particularly oceans (Bergmann et al., 2015 and references therein), however most plastic litter originates from beaches and land-based activities (Rillig, 2012; Horton et al., 2017). In Antarctic regions the potential terrestrial sources that contribute to aquatic plastic pollution have rarely been studied, however some reports (Waller et al., 2017) have shown that the anthropic presence related to scientific research stations is the most relevant source of plastic pollution. Both wastewater discharges and inland waters, together with the transport of land litter by wind, represent important routes through which plastic pollution reaches marine environments ((Ryan et al., 2009; Jambek et al., 2015)). Sludge amendment or plastic mulching are relevant sources for plastic contamination in continental systems (Steinmetz et al., 2016). Fishing and more in general maritime activities further contribute to the production of plastic wastes (Cooper and Corcoran, 2010).

Plastics can be divided into three classes: macroplastics (> 20 cm), mesoplastics (0.5 − 20 cm), and microplastics (< 0.5 cm) (NOAA, 2009). Depending on the environmental conditions, type of polymer and weathering, all three size classes undergo both abiotic and biotic transformation processes including photo-oxidation, temperature and mechanical shearing into smaller plastic debris (Andrady, 2017; Dawson et al., 2018; Derraik, 2002; Barnes et al., 2009; Syranidou et al., 2017). Due to their environmental persistence, plastic wastes represent a global concern, in relation to possible detrimental effects on humans and wildlife (Barnes et al., 2009; Science for Environment

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Policy, 2011).

Plastics are spread on a global scale, both in coastal and in offshore areas, in remote and even in previously considered pristine environments, such as in deep seas and polar areas (i.e. Arctic ices) (Browne et al., 2011; Obbard et al., 2014, 2018; Cannon et al., 2016). In remote regions, such as the Antarctic continent, Barnes et al. (2010) first reported the occurrence of plastic objects (fishing buoys and plastic packaging pieces) in the Durmont D'Urville and Davis Seas as well as in the Amundsen Sea; more recently, microplastic debris have been documented in surface waters (Cincinelli et al., 2017) and also in sediments (Van Cauwenberghe et al., 2013; Munari et al., 2017; Reed et al., 2018). Waller et al. (2017) have estimated plastic levels 5 times higher than expected, based on the annual human presence in the Antarctic continent. However, data on plastic debris distribution in Antarctica and on their potential effects in terms of their bioaccumulation, trophic transfer and toxicity on marine biota are still unknown (Reed et al., 2018). Plastic debris discharged as wastes from the Antarctic research stations could remain embedded in shallow and deep sea sediments, but a fraction could be exchanged by hydrodynamic phenomena with the water column and carried through remote Antarctic areas with a dilution effect (Waller et al., 2017).

Plastic debris are known to provide a surface suitable for biological colonization, with Carpenter and Smith (1972) first reporting the occurrence of hydroids and diatoms attaching to plastic surfaces. Since Zettler et al. (2013) and Lobelle and Cunliffe (2011) demonstrated the microbial biofilm produced on a plastic surface, more details on microbial heterotrophs, autotrophs, predators, and symbionts attached to plastic polymers, have been provided. These observations have highlighted that "Plastisphere" communities are structurally different from those of the surrounding environments (Harrison et al., 2011, 2014; Reisser et al., 2013; Caruso, 2015; Oberbeckmann et al., 2014, 2015; Bryant et al., 2016). The formation of a biofilm on submerged surfaces is a preliminary step for the settlement of sessile organisms (Qian et al., 2007). Biofilms colonizing plastic debris, with their regional variability and succession over time, have been reviewed by Mincer et al. (2016). However, except for some studies (Webster and Negri, 2006; Lee et al., 2016), knowledge on microbial colonization of artificial substrates in extreme polar regions is still limited. Plastics can cause mechanical damages due to their physical structure (Kühn et al., 2015). In addition, they are known to sorb highly-toxic hydrophobic organic compounds, including Persistent Organic Pollutants (POPs), PAHs, PCB, phthalates, pesticides, heavy metals, drugs, etc. which can undergo accumulation in fatty tissues and bio-magnification through the marine trophic web (Rios et al., 2007; Teuten et al., 2007; Cole et al., 2011; Li et al., 2016; Avio et al., 2017). Chemical contaminants, in turn, have been reported to be drivers of antibiotic resistance (AR) (Huijbers et al., 2015; Singer et al., 2016), leading to the hypothesis that microplastic debris could support the transmission of antibiotic resistant bacteria (ARB). In natural aquatic environments, migration patterns of AR from their sources to surface waters, coastal and ocean systems are complex (Gao et al., 2018; Grenni et al., 2018). In plastic polluted environments, the main pathway/mechanism of ARB proliferation may switch from active transmission to passive transmission. To date, in Antarctic areas the transmission of AR in bacterial communities attached to plastics has never been investigated. In such regions, human-derived bacteria have been documented in sewage wastes, and the presence of ARB has been reported close to the scientific stations (Hernández and González-Acuña, 2016). In view of the increasing anthropogenic impact related to the ongoing research activities in this continent, specific measures such as the delivery of a Protocol to the Antarctic Treaty on Environmental Protection, have been adopted to reduce possible negative effects on this fragile ecosystem (Champ et al., 1992).

Exposure of bacteria to contaminants (such as drugs/pharmaceuticals) sorbed to plastics is expected to affect the AR profiles of the plastic-attached bacterial communities; consequently, plastics could serve as a possible vector for the spread of ARB. Investigating the antibiotic susceptibility of *Vibrio* spp. attached on microplastics collected from a tributary of the lower Chesapeake Bay, Darr et al. (2016) found that Vibrio isolates (most identified as *Vibrio parahaemolyticus*) were still susceptible to three or more of the six tested antibiotics and all were susceptible to tetracycline and chloramphenicol. In Antarctic regions, the presence of plastics and the ability of bacterial isolates to produce biofilms with their antibiotic susceptibility must be evaluated. In the framework of the PLANET project (PLastics in ANtarctic EnvironmenT) funded by the Italian National Antarctic Research Programme (PNRA15_00090) the presence, abundance, metabolism and antibiotic-resistance profiles of the bacterial flora attached on microplastics were studied. The hypothesis tested in this context was that plastics from macro- to nano- and micro-sizes could serve as a habitat for bacterial species expressing resistances to antibiotics. In particular, this study focuses on biofilm production and on the antibiotic susceptibility of bacteria associated to the surface of some pieces of polystyrene recovered from King George Island in 2016.

2. Materials and methods

2.1. Sample collection and treatment

The sampling activities were conducted in February 2016 at King George Island, belonging to the South Shetlands archipelago (Antarctica) along the coast of Maxwell Bay (62° 11′ 53.5" S, 058° 56′ 29.6″ W), close to the Antarctic stations "Bellingshausen" (Russia) and "Profesor Julio Escudero" (Chile) (Fig. 1). This region is strongly affected by anthropic impact due to the various research stations present and, consequently, to the potential uncontrolled dispersion of waste and plastics in the environment (Waller et al., 2017).

A polystyrene (PS) macro-sized block (34 × 31 × 5 cm) covered by microalgae, mosses (predominantly *Sanionia uncinata*) and lichens (Fig. 2) was collected in the framework of the PLANET project. After collection, 4 sub-samples were isolated from the polystyrene fragment, further indicated as 1B, 2B, 3B and 4B, which were treated separately as replicates. From each sub-sample, sections of the plastic debris of 1 cm^2 were excised using a sterile scalpel, taking care to avoid bacterial contamination, and immediately stored at −20 °C and +4 °C for further microbiological analyses.

2.2. Chemical and physical characterization of the plastic' samples

Fourier transform infrared spectroscopy (FTIR) measurements in attenuated total reflectance (ATR) geometry were performed on the plastic' samples in the 600 ÷ 4000 cm^{-1} wavenumber range. Spectra were recorded at room temperature by a Bomem DA8 FT-IR spectrometer using a thermoelectrically cooled deuterated triglycene sulphate (DTGS) detector in combination with a KBr beamsplitter and a Globar source. Samples without any *a priori* treatment were contained in a Golden Gate diamond ATR system, based on ATR technique (Crupi et al., 2006, Bio-Rad KnowItAll IR spectral library). Each spectrum was recorded in dry atmosphere in order to avoid unnecessary contributions from air humidity, with a resolution of 4 cm^{-1} and an average of 100 repetitive scans to ensure high reproducibility and good signal-to-noise ratio. All spectra were normalized for taking into account the effective number of absorbers, no mathematical corrections were done (e.g. smoothing), while baseline adjustment and normalization were performed using a Spectracalc software package GRAMS (Galactic Industries, Salem, NH, USA). For the identification of microplastics, the BIO-RAD KnowItAll IR Spectral Library was used (Bio-Rad KnowItAll IR spectral library).

2.3. Isolation and selection of bacteria

Using sterile tweezers, each section (~1 gr each) of polystyrene was rinsed 3 times with sterile Phosphate Buffer Solution (PBS 1×) to

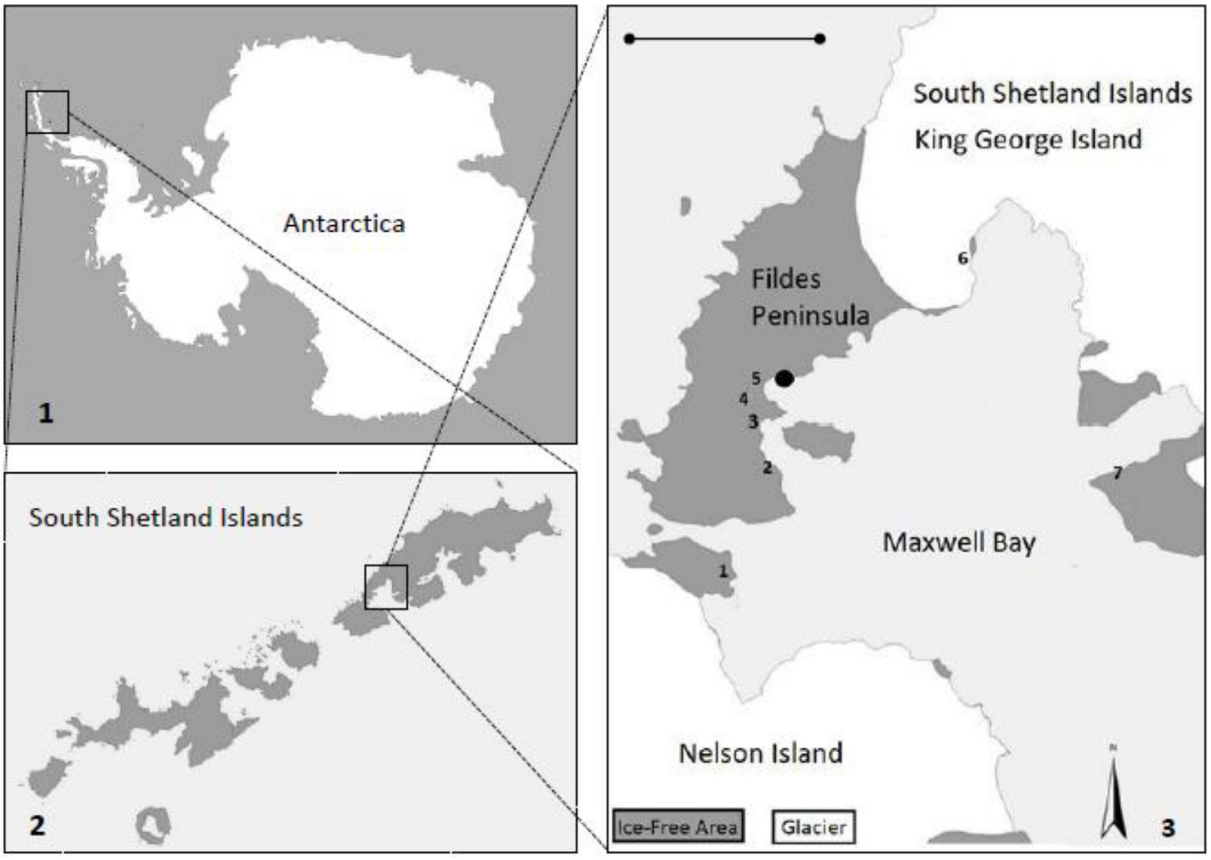

Fig. 1. - Map of the Fildes Peninsula (King George Island in the South Shetlands archipelago). The black point indicates the place where the polystyrene piece was collected. Legend: 1, Eco-Nelson Station (non-governmental Czech facility); 2, Great Wall Station (China); 3, "Profesor Julio Escudero" Station (Chile); 4, Presidente Eduardo Montalva Station (Chile); 5, Bellingshausen Station (Russian); 6, Artigas Antarctic Station (Uruguay); 7, King Sejong Station (Korea).

detach loosely attached bacteria. The sections were immersed into a volume 10 times the weight of the same solution (PBS 1×) and vortexed for 15 min at 2800 × g. The obtained supernatant was treated like a bacterial suspension, namely small volumes (0.1 ml) were spread in duplicate on the surface of Marine Agar 2216 (Difco S. p.a, Milan, Italy) plates which were incubated at 4 ± 1 °C for 15 days. Bacterial strains grown on the plates were randomly isolated and purified by successive streaking on the same culture medium until an axenic culture was obtained. Bacterial isolates were maintained at 4 ± 1 °C on Marine Agar slants for further molecular and biochemical analysis of biofilm production and antibiotic susceptibility assays.

2.4. Molecular identification

2.4.1. DNA extraction and PCR amplification

Analysis of the 16S rRNA gene was performed for the taxonomic characterization of the isolated strains. Total DNA was extracted from the bacterial strains using the CTAB method (Winnepenninckx et al., 1993). The bacterial 16S rRNA loci were amplified using the domain-specific forward primer Bac27_F (5′-AGAGTTTGATCCTGGCTCAG-3′) and the universal reverse primer Uni_1492R (5′-TACGYTACCTTGTTACGACTT-3′). The amplification reaction was performed in a total volume of 50 µl containing 1 × solution Q (Qiagen, Hilden, Germany), 1 × Qiagen reaction buffer, 1 µM of each forward and reverse primer, 10 µM dNTPs (Gibco, Invitrogen Co., Carlsbad, CA), and 2 U of Qiagen

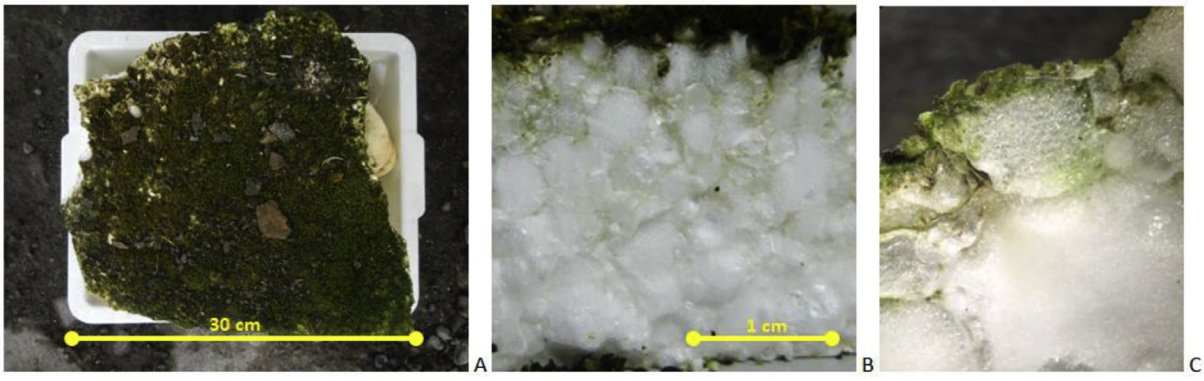

Fig. 2. (A) Polystyrene macro-sized debris collected in King George Island and analysed in this study; (B) and (C) Section of the polystyrene fragment covered by microalgae on the outer layer.

Table 1
- List of the antibiotics tested in this study. Reported are the dose per each disk and the code of the commercial reagent. All were provided by Oxoid, apart those marked by the asterisc, provided by Liofilchem (Italy).

Cell wall antibiotics			
Beta-lactams		Fosfomycin	Glycopeptide antibiotics
Penicillins	Penicillin G (P, 10 Units, CT0043B) Amoxycillin (AML, 10 µg, CT0061B) Carbenicillin (CAR, 100 µg, CT0006B) Methicillin (MET, 5 µg, code 051013053*) Mezlocillin (MEZ, 75 µg, CT0192B) Oxacillin (OX, 1 µg, CT0159B) Piperacillin (PRL, 100 µg, 0199B)	Fosfomycin (FOS, 50 µg, CT0183B)	Teicoplanin (TEC, 30 µg, CT0647B) Vancomycin (VAN, 30 µg, CT0058B)
Cephalosporins	Cefazolin (KZ, 30 µg, CT0011B) Cefotaxime (CTX, 30 µg, CT0166B) Ceftriaxone (CRO, 30 µg, CT0417B) Cefuroxime (CXM, 30 µg, CT0127B)		
Nucleic acid inhibitors			
Quinolones		DNA inhibitors	RNA synthesis inhibitors
Quinolones Fluoroquinolones	Cinoxacin (CIN, 100 µg, code 05113056*) Levofloxacin (LEV, 5 µg, CT1587B) Ciprofloxacin (CIP, 5 µg, CT0425B) Norfloxacin (NOR, 10 µg, CT0434B) Ofloxacin (OFX, 5 µg, CT0446B)	Nitrofurantoin (F, 300 µg, CT0036B)	Rifampicin (RD, 30 µg, CT0104B)
Protein synthesis inhibitors			
Aminoglycoside antibiotics	Glicilglicines	Macrolides	Phenicol derivatives
Gentamycin (CN, 10 µg, CT0024B) Sisomicin (SIS, 30 µg, code 052312077*)	Tigecycline (TGC, 15 µg, CT1841B)	Azithromycin (AZM, 15 µg, CT0906B) Erythromycin (E, 15 µg, CT0020B) Lincomycin (MY, 2 µg, CT0027B)	Chloramphenicol (C, 30 µg, CT0013B)
Tetracyclines	Oxazolidinones		
Doxycycline (DXT, 30 µg, CT0018B) Minocycline (MN, 30 µg, CT0030B) Tetracycline (TE, 30 µg, CT0054B)	Linezolid (LNZ, 10 µg, CT1694B)		
Other molecules			
Beta-lactamase inhibitor and semi-synthetic penicillin association	Carbapenems		
Amoxicillin + clavulanic acid (Augmentin, AUG, 30 µg, CT0223B)	Imipenem (IMI,10 µg, CT0455B)		

Taq polymerase (Qiagen). Amplification for 35 cycles was performed in a GeneAmp 5700 thermocycler (PE Applied Biosystems, Foster City, CA, USA). The temperature profile for PCR was 95 °C for 5 min (1 cycle); 94 °C for 1 min and 72 °C for 2 min (35 cycles); and 72 °C for 10 min after the final cycle (Troussellier et al., 2005).

2.4.2. Restriction fragment length polymorphism (RFLP) analysis

Each PCR amplified product (5 µl) was digested with restriction endonucleases (RsaI/AluI; Boehringer Mannheim), as previously indicated (Yakimov et al., 2006) and according to manufacturer's indications. Amplified DNA was detected by electrophoresis on a 0.8% (w/v) agarose gel in TBE 1 × buffer, while the restriction fragments were separated on a 3% (w/v) agarose gel. Short restriction fragments (~400 bp) were resolved better on a 5% (w/v) agarose gel (Yakimov et al., 2002, 2006). The gels were stained with ethidium bromide and photographed. The lengths of both amplified products and restriction fragments were estimated by comparison against a 100 bp DNA ladder (Gibco-BRL).

2.4.3. 16S rDNA taxonomical analysis

The amplified 16S rRNA fragment was sequenced using Macrogen Service (Korea). The similarity rank from the Ribosomal Database Project (RDP) (Cappello et al., 2012) and FASTA Nucleotide Database Queries (Pearson and Lipman, 1988) were used to estimate the degree of similarity to other 16S rRNA gene sequences. Phylogenetic analysis of the sequences was performed as previously described by Yakimov et al. (2006).

2.4.4. Biochemical characterization

To identify and characterize the bacteria isolates, biochemical tests such as Gram staining and tests for oxidation/fermentation, the production of acid from carbohydrates and the hydrolysis of gelatin and citrate were performed according to Bergey's Manual of Systematic Bacteriology (taxonomy) (Hassanshahian et al., 2012).

2.4.5. Screening for biofilm production

Bacterial isolate biofilm production was assayed by a method described by O'Toole (2011), and previously used by Spanò et al., 2016. The wells of a sterile 96-well microplate were filled (eight replicates per each isolate, for a greater reliability of the data to quantify biofilm formation and to get statistically significant results) with 100 µl of a bacterial suspension (concentration 1.5×10^8 CFU ml^{-1}), using Triptic Soy Broth (TSB, Oxoid) as a negative control (without bacterial inoculum). The plates were incubated for 48 h at 5 ± 1 °C without shaking, and non-adherent bacteria were removed by washing three times with sterile physiological saline solution (0.9% NaCl, w/v). The adherent bacteria (biofilms) were then stained for 45 min with a solution of 1% Crystal Violet (Sigma-Aldrich, w/v). After washing and air drying, the dye bound to the adherent cells was dissolved into ethanol and the biofilm mass, per each well, was measured as the optical density (OD_{550nm}) using a Multiskan GO Microplate Spectrophotometer (Thermo Scientific) (Laganà et al., 2015). The average OD from the control wells was subtracted from the OD of all tested wells. According to the OD values, the bacterial strains were assigned to one of four categories related to their different adherence capabilities (non-adherent, weakly, moderately or strongly adherent), as suggested by Stepanovic et al. (2000). As the interpretation of the results requires the definition of a cut-off value that separates biofilm-producing from non-biofilm-producing bacteria (Stepanovic et al., 2007), a value of OD equal to three standard deviations above the mean OD of the negative control was defined as the cut-off OD (ODc) for the microtiter-plate test For the interpretation of the results, a strain was considered as no biofilm producer when its OD was ≤ ODc, while it was considered as a strong biofilm producer when its OD was > 4xODc. Weak and moderate biofilm producers were those strains having an OD > 2xODc and 2xODc < OD ≤ 4xODc, respectively.

2.4.6. Screening for antibiotic susceptibility

The bacterial isolates were screened for antibiotic susceptibility by the Kirby-Bauer test (Bauer et al., 1966), performed with three replicates per each isolate. The isolates were grown for 48 h on plates of Tryptic Soy Agar (TSA, Oxoid), harvested and then suspended in sterile water adjusted to a 0.5 McFarland turbidity standard (bioMérieux), corresponding to 1.5×10^8 CFU ml^{-1}. The inoculum was streaked onto plates of Mueller-Hinton agar using a cotton swab; the produced diameters of inhibition were measured after 48 h of incubation at 5 ± 1 °C and averaged. Commercially available antibacterial disks (Oxoid) were used to determine the resistance patterns of the isolates against 34 different antibacterials (listed in Table 1), grouped into three specific categories (Cell wall antibiotics, Nucleic acid inhibitors and Protein synthesis inhibitors) according to their different mechanisms of action (Laganà et al., 2018).

The diameter of the zone of inhibition around each disk was measured with a precision calliper (Mitutoyo, Andover, UK). Each bacterial species was classified as resistant (R), intermediately resistant (I) or sensitive (S) according to the breakpoints established by the EUCAST (2017). For cinoxacin and sisomicin molecules, the breakpoints established by Clinical Laboratory Standards Institute (CLSI, 2012) were used.

3. Results

3.1. Chemical-physical characterization of the polystyrene sample

The analysis of the experimental FTIR-ATR spectrum of the macroplastic sample allowed us to recognize two main constituents (score 85.7%): PS, in amount ≅ 70%, and water, in amount ≅ 30%. Fig. 3 (a) and (b) show the experimental FTIR-ATR spectrum of this sample, in the low (600 ÷ 1800 cm^{-1}) and high (2700 ÷ 3800 cm^{-1}) wavenumber regions, respectively.

The low wavenumber region of the spectrum revealed a very intense peak centred at ~ 697 cm^{-1}, followed by a second peak at ~ 751 cm^{-1}. Both of them can be ascribed to the out of plane bending δ(C-H) vibrations. Another peak at ~ 1447 cm^{-1} was ascribed to the bending $\delta(CH_2)$ vibration of PS chain. The peak at ~ 1492 cm^{-1} was due to the stretching ν(C=C) vibrational modes of PS ring. Finally, the band between ~ 1550 cm^{-1} and ~ 1700 cm^{-1} reflected the bending δ_{HOH} mode of water molecules. It appeared convoluted with another peak at ~ 1599 cm^{-1}, due to breathing vibrations of PS ring. Regarding the high wavenumber region of the spectrum, the well-evident peaks at ~ 2847 cm^{-1} and ~ 2920 cm^{-1} were respectively associated to the symmetric and antisymmetric $\nu(CH_2)$ stretching modes of PS. The peaks centred at ~ 3025 cm^{-1}, ~ 3059 cm^{-1} and ~ 3084 cm^{-1} reflected the contributions of aromatic ν(C-H) stretch of PS. Finally, the broad band detected between ~ 3100 cm^{-1} and ~ 3700 cm^{-1} was attributed to the ν(O-H) stretching vibrational mode of H_2O molecules.

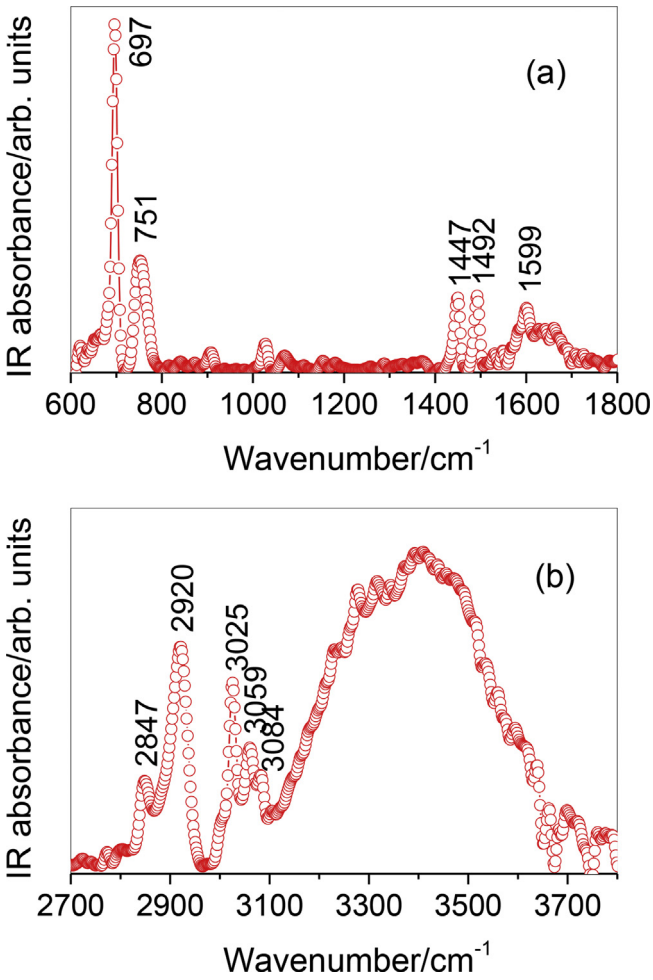

Fig. 3. - Experimental FTIR-ATR spectrum, in the 600 ÷ 1800 cm^{-1} (a) and 2700 ÷ 3800 cm^{-1} (b) wavenumber regions, of the PS sample.

3.2. Bacterial isolation, identification, and screening for biofilm production and susceptibility to antibiotics

A total of twenty-seven bacterial strains were isolated from the PS sub-sample fragments. 16S rRNA inserts were de-replicated using restriction fragment length polymorphism (RFLP) analysis. The obtained data, on the basis of different bands profiling, identified 6 operative taxonomic units (OTUs). From the total of the isolates, seven bacterial strains were selected according to their RFLP profile. These bacterial isolates were identified by classical biochemical tests as well as by molecular methods (Table 2). The amplification and sequencing of their

Table 2
- Table summarizing the main taxonomic and physiological traits of the bacterial isolates. The closest relatives of the 16S rRNA gene sequences of bacteria isolated in this study are reported. The code indicates the origin of each isolate (polystyrene fragments 1B–4B), whereas the number reported within brackets refers to the plate of isolation.

Code	Isolate	Gram	Shape	Oxydase	Catalase	Mobility	%, identity	Accession N°	Reference
ANT-1B (1)	*Alteromonas hispanica* strain CIBAAG11	–	Rod	+	+	+	99	EU529840	Thillai et al. (2008)[a]
ANT-1B (2)	*Pseudomonas balearica* DSM 6083	–	Rod	+	+	+	98	CP007511	Bennasar-Figueras et al. (2016)
ANT-2 B (1)	*Thalassospira lohafexi* strain 139Z-12	–	Rod	+	+	+	99	KP231598	Shivaji et al. (2015)
ANT-2 B (2)	*Shewanella* sp. 204Z-25	–	Rod	+	+	+	99	GU584178	Bozal et al. (2002)
ANT-3 B	*Halomonas* sp. 204Z-23	–	Rod	+	+	+	98	GU584176	Bozal et al. (2002)
ANT-4 B (1)	*Pseudoalteromonas* sp. 204Z-21	–	Rod	+	+	+	99	GU584174	Bozal et al. (2002)
ANT-4 B (2)	*Pseudomonas balearica* DSM 6083	–	Rod	+	+	+	99	CP007511	Bennasar-Figueras et al. (2016)

Institute of Brackishwater Aquaculture, Aquatic Animal Health Division, Central, Chemmai, India. Submitted (27-FEB-2008).
[a] Thillai Sekar, V., Singarave l,R., Joseph Sahaya Rajan, J., Alavandi, S.V., Kalaimani, N. and Santiago, T.C., 2008. Direct Submission to NCBI.

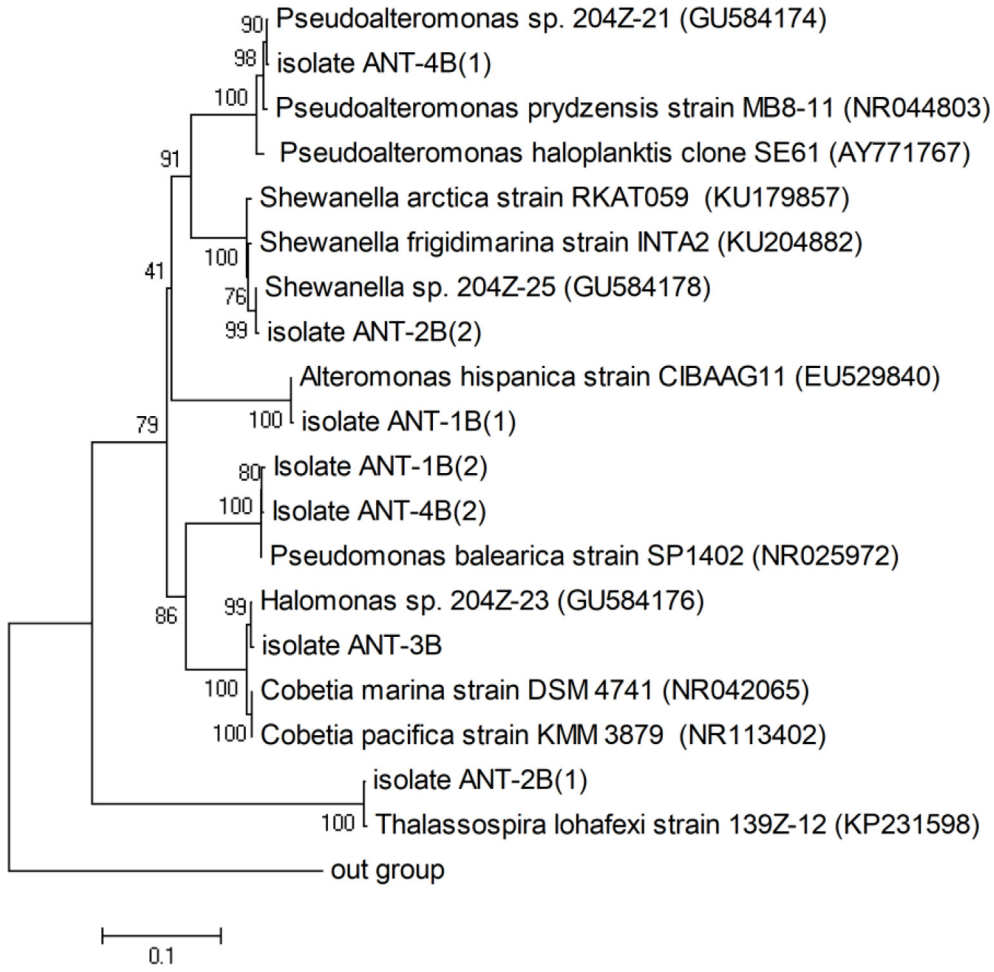

Fig. 4. - Phylogenetic tree based on 16S rRNA gene sequences for bacterial strains [isolates ANT-4B(1), ANT-2B(2), ANT-1B(1), ANT-1B(2), ANT-4B(2), ANT-3B, ANT-2B(1)] isolated from the antartic macroplastic. Percentages of 100 bootstrap resampling that supported the branching orders in each analysis are shown above or near the relevant nodes. The tree was rooted and outgrouped by using the 16S rRNA sequences of *Methanococcus jannaschii* (M59126). Evolutionary distance is indicated by vertical lines; each scale bar length corresponds to 0.05 fixed point mutations per sequence position (Cytophaga– Flavobacteria—Bacterioides).

16S rRNA genes and comparison of the sequences to the database of known 16S rRNA sequences yielded the phylogenetic tree shown in Fig. 4. The sequences of the seven studied bacteria were submitted to the genetic sequence database at the National Center for Biotechnical Information (NCBI).

16S rRNA sequences revealed that among the 7 isolates, strains were identified to be related to *Pseudomonas balearica* DSM 6083 [isolates ANT-1B(2) and ANT-4B(2)]; moreover, other isolates were related to *Alteromonas hispanica* [isolate ANT-1B(1)] and *Pseudoalteromonas* [isolate ANT-4B(1)] and *Shewanella* [ANT-2B(2)] genera. Strains related to *Halomonas* (isolate ANT-3B) and *Thalassospira lohafexi* [ANT-2B(1)] were also isolated.

All the bacterial strains isolated in this study were active in biofilm production (Fig. 5). The values of Optical Density (OD_{550nm}), as a measure of the expression of this ability, fall in a narrow range, oscillating between 0.20 and 0.26 nm. The bacteria isolated from the fragment 2B [strain ANT-2B(1) and strain ANT-2B(2)] showed the lowest O.D. value (~0.20) compared to the biofilm formation measured in the strain ANT-1B(1) (~0.24), although no statistically significant differences were found.

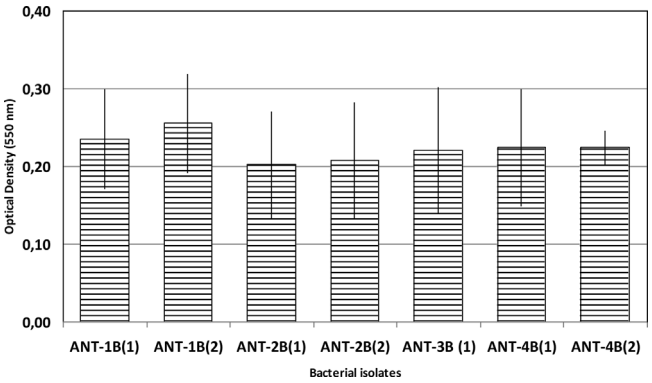

Fig. 5. - Optical density values measured in the biofilm production assay performed on the seven bacterial strains isolated from the polystyrene block collected from Maxwell Bay, King George Island. Each sample of plastic was assayed in replicate. The number reported within brackets refers to the plate of isolation (two replicates for each polystyrene sub-fragment, labeled with the codes 1B–4B).

As shown in Table 3 , the bacteria under study were fully sensitive (100%) to CIP, DXT, CN, LEV, NOR, OFX, PRL, SIS, TGC, but fully resistant (100%) to CXM, E, FOS, MY, LNZ, MET, OX, P, TEC, VAN. A high percentage of strains (67% of the total) were also resistant to AML, AMP, AUG, AZM, CAR, KZ, CIN, C, MEZ, F.

Grouping the antibiotics into major categories beta-lactams, cephalosporins, aminoglycosides, the assayed bacterial strains showed resistance against ampicillin, amoxicillin + clavulanic acid, carbenicillin and mezlocillin belonging to beta-lactams, against both cefuroxime and cefazolin belonging to cephalosporins, and against cinoxacin belonging to quinolones (Fig. 6).

4. Discussion

PS is a thermoplastic homopolymer, available as both a typical solid plastic as well in the form of a rigid foam material. PS plastic is commonly used for a variety of functions including in rigid items such as refrigerator crispers and DVD cases. PS foam, which is particularly useful for commercial packaging and in products such as insulated disposable cups, meat trays and panel insulation, is bulky and non-degradable. Being composed of around 95% air, it is highly mobile and escapes from garbage bins and landfill. It tends to flake, with small pieces of litter travelling long distances and harming wildlife upon ingestion. In addition, the porous structure of PS favours the adsorption of biological and chemical pollutants such as biofouling microorganisms and POPs (Castaldo et al., 2017).

In marine environments, the composition of the microbial communities associated with the plastics shows variations related not only to the nature of the plastic substrate, but also to the geographical area of collection and the season (Oberbeckmann et al., 2014). Antarctic waters host communities of psychrophilic microorganisms which are extremophiles, having molecular and biochemical adaptations which enable them to multiply and to grow at temperature below 0 °C (D'Amico et al., 2002; Deming, 2002).

Since the scientific approach of the present research focused specifically on culturable bacterial strains, no culture independent approaches (like metagenomic sequencing) were applied, being beyond the specific discussed topics. In this study the taxonomical analysis of the PS-attached bacterial isolates revealed the presence of species affiliated to the genera *Pseudoalteromonas* and *Shewanella*. Both genera are hydrocarbonoclastic bacteria commonly found in areas contaminated by oil. In Admiralty Bay, which is an irregular bay located in the southern coast of King George Island, Martins et al. (2004) indicated human wastes and fossil fuel combustion as the main causes for the release of aliphatic and polycyclic aromatic hydrocarbons in surface sediments. This evidence within the King George area could imply a proliferation of obligate hydrocarbon-degrading bacteria, specialized members of the microflora that typically proliferate and become dominant following oil contamination (Cappello et al., 2007; Cappello and Yakimov, 2010).

From the PS sample a total of twenty-seven bacterial strains were isolated, with different colony morphology and characteristics. The taxonomical analysis of 16S rRNA, performed by restriction fragment length polymorphism (RFLP) and 16S rRNA sequencing allowed to identify six OTUs typical of cold marine environments. From the total of the isolates, seven bacterial strains were selected for this study.

Table 3
Antibiotic susceptibility of the bacterial isolates. Reported is the percentage of resistant, intermediate or sensitive bacterial strains compared to the total of the isolates. See the text for the abbreviations of the tested antibiotics.

	AML	AMP	AUG	AZM		MET	MEZ	MN	F
Resistant	67	67	67	67	Resistant	100	67	0	67
Intermediate	0	0	0	0	Intermediate	0	0	33	0
Sensitive	33	33	33	33	Sensitive	0	33	66	33
	CAR	**KZ**	**CTX**	**CRO**		**NOR**	**OFX**	**OX**	**P**
Resistant	67	67	33	33	Resistant	0	0	100	100
Intermediate	0	0	66	0	Intermediate	0	0	0	0
Sensitive	33	33	0	66	Sensitive	100	100	0	0
	CXM	**CIN**	**CIP**	**C**		**PRL**	**RD**	**SIS**	**TEC**
Resistant	100	67	0	67	Resistant	0	0	0	100
Intermediate	0	0	0	0	Intermediate	0	33	0	0
Sensitive	0	33	100	33	Sensitive	100	67	100	0
	DXT	**E**	**FOS**	**CN**		**TE**	**TGC**	**VAN**	
Resistant	0	100	100	0	Resistant	33	0	100	
Intermediate	0	0	0	0	Intermediate	33	0	0	
Sensitive	100	0	0	100	Sensitive	33	100	0	
	IMI	**LEV**	**MY**	**LNZ**					
Resistant	33	0	100	100					
Intermediate	33	0	0	0					
Sensitive	33	100	0	0					

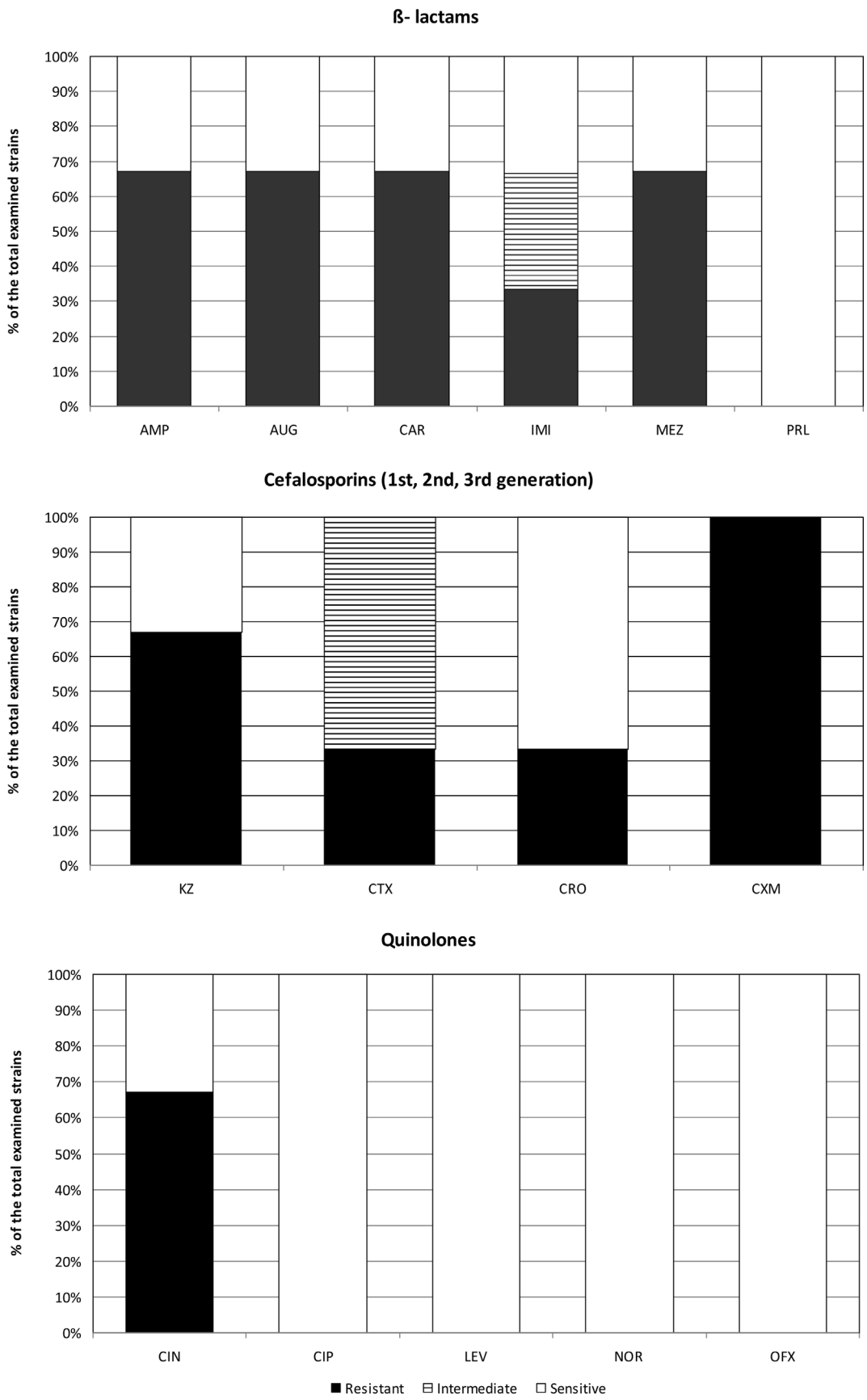

Fig. 6. Profiles of susceptibility of bacterial isolates to selected classes of antibiotics: beta-lactams, cefalosporins and quinolones. Reported is the percentage of resistant, intermediate or sensitive strains on the total of the isolates.

16S rRNA sequence of the ANT-1B(1) strain revealed that this isolate was related to the bacterium *Alteromonas hispanica.* As indicated by Martínez-Checa et al. (2005), this strain which produces exopolysaccharides and contains polyunsaturated fatty acids, was isolated from a hypersaline water sample collected from Fuente de Piedra (southern Spain). It is moderately halophilic, growing better with 7.5–10% w/v of NaCl; it grows at 4 °C, therefore under conditions similar to those of the Antarctic environment.

ANT-1B(2) and ANT-4B(2) isolates appeared to be related to *Pseudomonas balearica* DSM 6083. This strain was originally isolated from the water of a lagooning wastewater treatment plant by enrichment with 2-methylnaphthalene band and classified as a new denitrifying species of *Pseudomonas* (Bennasar-Figueras et al., 2016). Its physiological tolerance to 8.5% NaCl suggested that *P. balearica* represents a true marine *Pseudomonas* species; other strains of P. balearica were previously isolated from marine areas (Rossello et al., 1991) and salt marshes (Mulet et al., 2008).

ANT-2B(1) strain was related to Thalassospira lohafexi, a gram-negative, aerobic, obligatory halophilic bacterium, which was isolated also from the Southern Ocean water samples surrounding Antarctica (Shivaji et al., 2015). Also 16S rRNA sequences of *Shewanella* [ANT-2B (2)], *Halomonas* (ANT-3B) and *Pseudoalteromonas* [ANT-4B(1)] isolates were related to sequences of bacteria isolated from the Southern Ocean surrounding Antarctica. In particular, the genus *Shewanella* included Gram-negative, chemo-organotrophic, oxidase positive bacteria, generally associated with aquatic or marine habitats, and psychrophilic strains isolated from the coastal Antarctic marine environments (Bozal et al., 2002).

According to Webster and Bourne (2007), the majority of Antarctic bacteria belongs to Gammaproteobacteria, Alpha and Beta Proteobacteria, Bacterioidetes, Firmicutes, Actinomicetales, Planctomycetes, Chlorobi. The screening of the bacterial isolates showed that all they were able to produce biofilm. This finding confirmed that bacteria can survive extreme conditions through the colonization of plastic debris. Moreover, the production of a biofilm matrix, made of exopolysaccharides, is recognized to be crucial in the generation/outbreaks of AR phenomena (Balcazar et al., 2015).

The antibiotic susceptibility profiles of the bacterial strains isolated from the analysed PS sample highlighted the occurrence by multiple AR against cefuroxime and cefazolin, two molecules that belong to cephalosporins, against cinoxacin that belongs to quinolones as well as against ampicillin, amoxicillin + clavulanic acid, carbenicillin and mezlocillin that belong to beta-lactams. The spread of antibiotic resistant bacteria across marine and terrestrial ecosystems can be considered as the natural consequence of antibiotic misuse, through the enrichment for pre-existing resistant bacteria as well as through the selection for new antibiotic resistant strains (Grenni et al., 2018). After entering natural environments, antibiotics usually undergo natural attenuation due to physico-chemical processes like hydrolysis, photolysis, oxidation and reduction, and/or microbial biodegradation (Sun et al., 2018). On the other hand, marine microbes are sentinels of environmental impacts due to their genome plasticity making them able to respond to xenobiotics and chemical contaminants (Nogales et al., 2011; Caruso et al., 2016 and references therein).

The presence of antibiotic residues in highly anthropogenically impacted areas, or in areas affected by hospital influence, is known to act as a selective pressure for AR (Davies and Davies, 2010). Remote environments are generally considered as pristine areas; nevertheless in Antarctic environments AR is not an unusual finding. In Admiralty Bay, close to Palmer Station, Miller et al. (2009) reported that bacteria isolated from seawater and penguin faeces exhibited multidrug resistance to five common antibiotics such as tetracycline, kanamycin, ampicillin, nalidixic acid and streptomycin. In addition, the frequency of resistance increased with the increased anthropogenic impact. In another previous study, Santos et al. (2004) suggested that environmental contamination by antibiotics was mainly caused by the research activities and the peculiar circulation of sea currents in the Antarctic Peninsula. Again, close to the Brazilian Antarctic Station Comandante Ferraz, Santos et al. (2005) reported the occurrence of trace metal contamination in the sediments of this region, suggesting as sources for such pollution the disposal of sewage wastes or oil and paint residuals from human settlements. Since the occurrence of co-selection mechanisms between antibiotic and heavy metal resistance genes (Baker-Austin et al., 2006; Wright et al., 2006; Seiler and Berendonk, 2012; Zhang et al., 2018) has been reported in different environmental contexts, the co-occurrence of AR with resistance to heavy metals could be a possible explanation for the multiple AR observed in this study.

In a previous study performed in Antarctic areas (Estrellas lake of King George Island and Crater lake of Deception Island) close to those examined in the present research, Tam et al. (2015) detected the occurrence of multiple AR in most of the bacterial isolates [affiliated to *Aeromicrobium*, *Arthrobacter*, *Bacillus*, *Brevundimonas*, *Cryobacterium*, *Flavobacterium*, *Pedobacter*, *Pseudomonas*, *Rhodococcus*, and *Sphingomonas*], with 43 strains showing resistance to at least three antibiotics, and 26 strains to 10 or more antibiotics. According to this finding, Antarctic bacteria were suggested to be potential reservoirs for AR genes. In aquatic environments, contamination from antibiotics poses an emerging challenge for the control of AR gene transmission (Baquero et al., 2008); although the presence of these contaminants in King George waters was not assessed during our research, antibiotic residues have been detected in Northern Antarctic Peninsula surface waters, a territory close to our study area (González-Alonso et al., 2017). Absorption of antibiotics to plastic surface has recently been documented (Li et al., 2018; Xu et al., 2018). In spite of the lack of data on antibiotic residues in the King George area, we may speculate that harbouring so many AR could be a response to the presence of antibiotics in the waters, even if till now it cannot be stated whether the presence of ARGs could represent a potential advantage for bacterial survival under extreme conditions.

The occurrence of AR in the plastisphere present in Antarctic environments further stresses the importance of studying such phenomenon in areas faced with the effects of a previously unexpected anthropogenic pressure. To date, however, no data exists on the potential effects of mixed contamination by microplastics and antibiotics on AR spread. Future experiments such as plasmid isolation, ARG identification, conjugation, and sequencing are needed to explore the possible mobility of the AR components across the environment, to give insights on the possible transference of the isolated strains and their AR traits. Furthermore, identification of antibiotic residues would help establish whether a selective pressure was placed on the isolated strains to help determine their AR profiles.

5. Conclusions

Multiple AR was detected in our study, reinforcing the idea that plastics act as potential reservoirs of resistance traits, also in extreme environments like Antarctic areas. Plastics are known as potential vectors of chemical pollutants and our findings show that they also can represent a carrier for AR bacteria. . This study investigates an area of research that has thus far remained neglected. Further research on this topic is recommended within current International monitoring plans, in order to assess the role of macro-, micro- and nanoplastics as possible substrates for the colonization by the microbial community and their role as potential vehicles for the transmission of AR.

Declarations of interest

None.

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Abbreviations

FTIR	Fourier transform infrared spectroscopy
ATR	attenuated total reflectance
DTGS	deuterated triglycene sulphate
P	penicillin G
AML	amoxycillin
AMP	ampicillin
CAR	carbenicillin
MET	methicillin
MEZ	mezlocillin
OX	oxacillin
PRL	piperacillin
KZ	cefazolin
CTX	cefotaxime,
CRO	ceftriaxone
CXM	cefuroxime,
FOS	fosfomycin
TEC	teicoplanin
VAN	vancomycin
CIN	cinoxacin
LEV	levofloxacin
OFX	ofloxacin
CIP	ciprofloxacin
NOR	norfloxacin
F	nitrofurantoin
RD	rifampicin
CN	gentamycin
SIS	sisomicin
TGC	tigecycline,
AZM	azithromycin
E	erythromycin
MY	lincomycin
C	chloramphenicol
DXT	doxycycline,
MN	minocycline,
TE	tetracycline,
LNZ	linezolid
AUG	augmentin
IMI	imipenem
R	resistant
I	intermediately resistant
S	sensitive,

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