Marine Biology Research

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/smar20

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MARINE BIOLOGY RESEARCH
2021, VOL. 17, NOS. 7–8, 537–544
https://doi.org/10.1080/17451000.2021.1990960

Presence of microplastics and antimicrobial-resistant bacteria in sea cucumbers under different anthropogenic influences in Gran Canaria (Canary Islands, Spain)

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ABSTRACT
Microplastics and antimicrobial-resistant bacteria are a matter of concern, especially in aquatic environments. In this study, we compared the presence of microplastics and antibiotic-resistant bacteria in the intestine of the sea cucumber *Holothuria sanctori* at sites under different levels of pollution in Gran Canaria Island (Canary Islands, Spain, eastern Atlantic). We sampled animals at two offshore sites (controls) under low organic pollution and at a site under high organic pollution, i.e. directly affected by sewage water. From a total of 79 collected animals, 133 Gram-negative bacteria were isolated; 20 were *Enterobacteriaceae*, and the rest Non-Fermenting Bacilli (NFB). We detected *Enterobacteriaceae* resistant to critically important antimicrobials, such as ceftazidime or fluoroquinolones, particularly at the polluted site. In addition, we observed a significantly higher number (□2 orders of magnitude) of microplastics in the gut of sea cucumbers sampled at the polluted site, relative to the two controls. Our preliminary results point towards integrating microbiological and ecological approaches to analyse the mutual influence of both pollutants in aquatic ecosystems.

ARTICLE HISTORY
Received 28 July 2021
Accepted 4 October 2021
Published online 30 November 2021

KEYWORDS
Microplastics; antimicrobials; bacteria; sea cucumber; Canary Islands; *Holothuria sanctori*

Introduction

Antimicrobial resistance is considered a 'One Health' issue (Robinson et al. 2016). Human, animal and environmental health are interconnected and the presence of bacteria resistant to antibiotics mirrors antimicrobial resistance in human and veterinary medicine (WHO 2019). Antibiotics have been massively used, as therapeutic or preventive drugs for human and animal health, leading to a selective pressure that promotes the selection of antimicrobial resistant (AMR) bacteria in their intestines (Robinson et al. 2016). These AMR bacteria mostly reach aquatic environments via wastewater, for example via sewage effluents in nearshore areas. Aquatic microorganisms are then exposed to antimicrobials or their metabolites, and, under this selective pressure, AMR become more prevalent (Berkner et al. 2014).

Across coastal areas of the world, pollution of aquatic environments by AMR and microplastics is of growing concern (Guo et al. 2020). The widespread discharge of sewage effluents into seawater is considered one of the main routes of antibiotic-resistant bacteria from humans and livestock to aquatic environments (Al-Bahry et al. 2009), while sewage favours bacteria biofilm formation on microplastic fragments (Martínez-Campos et al. 2021).

The impact of microplastics on aquatic environments results from their incorporation, via small animals, into food chains and the adsorption of pollutants on microplastic surfaces (Anbumani and Kakkar 2018). Microplastics can influence antimicrobial resistance, spreading microorganisms that are resistant to antibiotics (Bank and Hansson 2020; Guo et al. 2020; Moore et al. 2020). In addition to this, several antimicrobials can be adsorbed on the surface of microplastics (Li et al. 2018; Alprol et al. 2021; Santana-Viera et al. 2021), contributing to the selection of resistant bacteria.

In this sense, Arias-Andres et al. (2018) demonstrated that there is an increased frequency of plasmid transfer, including antibiotic resistance

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Published online 30 Nov 2021

genes, in bacteria associated with microplastics, compared with free-living bacteria.

Holothurians, popularly known as 'sea cucumbers', are found in almost every marine environment of the world, and play an important role in nutrient recycling, by consuming sediments and retaining organic matter (Uthicke 2001; Zhang et al. 2015). Holothurians are deposit-feeders, and, therefore, it is plausible that sewage discharges into the ocean may induce antibiotic resistance in their intestinal bacteria (Jiang et al. 2014). Sea cucumbers are high-value animals for the aquaculture industry, and bacterial diseases are frequent, promoting the use of antibiotics as therapeutic or preventive solutions (Dang et al. 2006). The presence of AMR bacteria in these animals could, therefore, complicate the treatment of diseases. For example, many *Vibrio parahaemolyticus* isolates from cultured sea cucumbers (*Apostichopus japonicas*) showed large resistance to ampicillin and cefazolin, while less resistance was observed to streptomycin, cefuroxime sodium, tetracycline, sulphamethoxazole/trimethoprim and quinolones (Jiang et al. 2014). At the same time, such a presence could also be a risk for human health, by transferring this resistance either to consumers (De-la-Torre 2020), or other animals.

Sea cucumbers are non-selective feeders (Uthicke 2001; Navarro et al. 2013, 2014), ingesting large quantities of sediment to extract nutrients, including microorganisms and organic debris. It has been demonstrated that holothurians not only ingest microplastics present in sediments, but they selectively ingest microplastics over sediment grains (Graham and Thompson 2009).

Ingestion of microplastics by benthic deposit-feeders is, in turn, a way to introduce microplastics in the food chain (van Cauwenberghe and Janssen 2014; Reguera et al. 2019; Teng et al. 2019).

The aim of this study was to compare the presence of microplastics and antibiotic-resistant bacteria in the intestine of the sea cucumber *Holothuria sanctori* at sites under different levels of pollution in Gran Canaria Island (Canarian Archipelago, eastern Atlantic, Spain).

Specifically, we compared the presence of microplastics, and antimicrobial resistant bacteria, between two offshore sites (controls) under low organic pollution and a site under large organic pollution.

Materials and methods

Study area

The study was carried out, between November 2019 and July 2020, in Gran Canaria, Canary Islands, Spain (28°N, eastern Atlantic Ocean) (Figure 1). Three sites, between 7 and 14 m depth, were selected: Baja de Pasito Blanco (P) (27°44′422″N, 15°37′858″W), Baja de Arguineguín (A) (27°44′48.36″N, 15°41′2.32″W) and Taliarte (T) (27°59′27.95″N, 15°22′05.04″W). The first two sites were outside direct human influences and, therefore, were considered as the control sites for this study. Both sites were located between 2 and 3 km offshore the coast and are mainly dominated by rocky-sandy bottoms. On the contrary, T was directly onshore with bottoms that were mainly rocky to sandy. This site was located near two submarine sewage outlets that directly discharged wastewater into the sea (IDECanarias visor 4.5.1, s. f.). On each sampling site, a team of scuba divers randomly collected between 23 and 28 *Holothuria sanctori*. Overall, 79 sea cucumbers were collected. Each sea cucumber was introduced in a Ziploc bag and, once out of the water, immersed in a plastic container with seawater until reaching the laboratory.

Animal dissection and intestinal sampling

Each holothurian was placed on a plastic tray, where its total length was measured (to the nearest cm) with a ruler. Sea cucumbers were opened with rounded-tip scissors, or a scalpel, exerting some force longitudinally through the ventral area (Díaz-Sol Sol et al. 2019). The intestine was collected with tweezers and placed on a tray covered with a sanitized bag. Then, each intestine was washed off with a syringe filled with sterile saline solution.

With a punch, a small hole was opened in the luminal epithelium of the anterior intestine (Pagán-Jiménez et al. 2019) and a small faecal sample was collected using a sterile swab. Samples were then sent to the microbiology laboratory, always before 24 h after sampling. The rest of the intestine was stored in bottles with alcohol for later analysis of microplastics.

Microbiological procedures

Samples were cultured on McConkey agar (MC), McConkey Agar + cefotaxime 2 mg/L (MC + CTX), and Mannitol Salt Agar (MSA), and then incubated (24–48 h) at 37°C. Selenite Broth was used as an enrichment medium for *Salmonella* and, after 24 h at 37°C, a loop of this medium was streaked onto *Salmonella–Shigella* Agar (SS). All media were obtained from BD Difco, Detroit, MI, USA.

An oxidase test was carried out on bacteria growing on MC and/or MC + CTX. If the oxidase test was

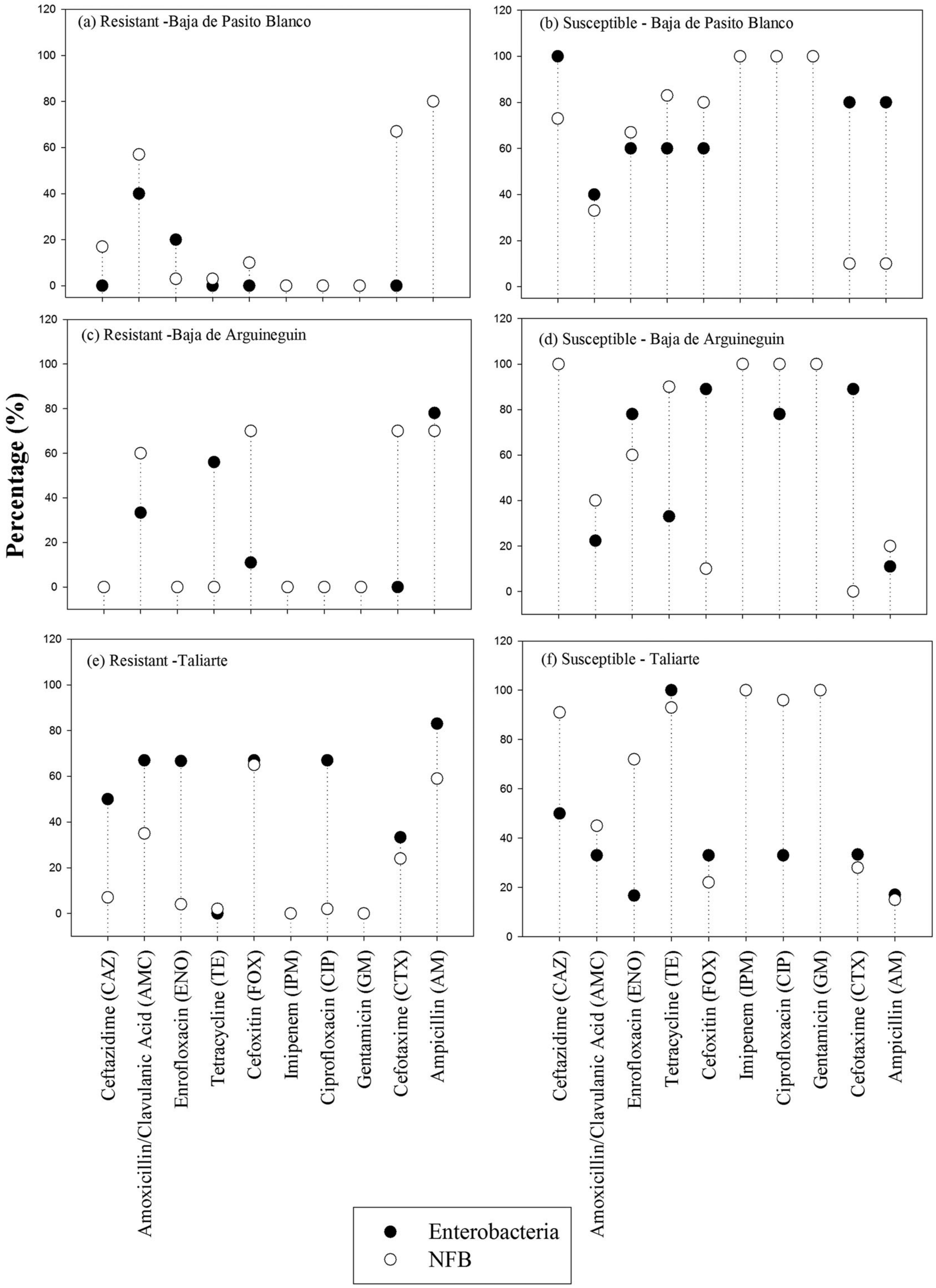

Figure 1. Percentages of resistance and susceptibility to antibiotics in *Enterobacteriaceae* and NFB for each sampling site. (a) Resistant bacteria at Baja de Pasito Blanco. (b) Susceptible bacteria at Baja de Pasito Blanco. *Enterobacteriaceae* and NFB have the same susceptibility percentage (100%) for IPM, CIP and GM. (c) Resistant bacteria at Baja de Arguineguín. (d) Susceptible bacteria at Baja de Arguineguín. *Enterobacteriaceae* and NFB have the same susceptibility percentage (100%) for CAZ, IPM and GM. (e) Resistant bacteria at Taliarte. (f) Susceptible bacteria at Taliarte. *Enterobacteriaceae* and NFB have the same susceptibility percentage (100%) for IPM and GM.

positive, a Kligler iron agar medium (Difco) was used to screen fermentation of glucose and lactose. Bacteria that yielded an oxidase test as positive, and did not ferment glucose and lactose, were identified as Non-Fermenter Bacilli (NFB). Oxidase negative bacteria growing on MC and/or MC + CTX, and bacteria suspected to be *Salmonella* from SS Agar, were identified using an API 20E system (bioMérieux, Marcy L'Etoile, France).

Gram staining, catalase and coagulase tests were implemented on bacteria suspected to be *Staphylococcus aureus* growing on MSA.

Antimicrobial susceptibility of isolates was studied on Müeller–Hinton Agar (Difco), using the disk diffusion method (CLSI 2015). Antibiotics tested were: ampicillin (AM 10 µg), amoxicillin/clavulanic acid (AMC 20 µg + 10 µg), cefoxitin (FOX 30 µg), cefotaxime (CTX 30 µg), ceftazidime (CAZ 30 µg), ciprofloxacin (CIP 5 µg), enrofloxacin (ENO 5 µg), gentamicin (GM 30 µg), imipenem (IPM 10 µg) and tetracycline (TE 30 µg).

Presence of microplastics

Sea cucumbers' intestines, initially stored in bottles with alcohol, were passed onto a plastic tray and opened through the anterior to the end (posterior part) of the cloacae with a pair of scissors. A plastic pipette with water was used to spread the faecal content on the plastic tray. The faecal content was observed with the help of a magnifying glass to tear apart microplastics (1–5 mm) fragments; this is the size fraction that dominates in coastal habitats of the study region (Herrera et al. 2018). Similar proportions of synthetic fibres, resin pellets and fragments from larger plastics were observed, including a wide range of colours and shapes. The number of fragments per sea cucumber was then annotated. All microplastic fragments were removed with tweezers, allocated on a piece of aluminium foil, and weighed. Generalized Linear Models (GLMs), using a 'Poisson' distribution with a 'log' link function, then tested for differences in the number and weight of microplastics between the three sites. The total length of each sea cucumber was included as a covariate. Models were implemented using the R 'Flexplot' library (Fife, 2019) in R studio (RStudio Team, 2020).

Results

Microbiological results

None of the bacteria colonies growing on MSA was identified as *Staphylococcus aureus*. When Gram staining was carried out, all bacteria were Gram positive bacilli. Colonies suspected to be *Salmonella* were not observed on SS Agar. A total of 89 Gram negative isolates were recovered from MC and 44 from MC + CTX. Of these 133 Gram negative bacteria, 20 were identified as *Enterobacteriaceae* (6 *Citrobacter freundii*, 1 *Enterobacter cloacae*, 6 *Leclercia adecarboxylata* and 7 *Escherichia coli*). One oxidase positive isolate was identified as *Vibrio alginolyticus*. The remaining isolates were considered NFB.

Antimicrobial susceptibility tests were carried out on all *Enterobacteriaceae*, one *Vibrio alginolyticus* and 77 NFB isolates (Table I). For *Citrobacter freundii*, the highest percentages of resistance were found to amoxicillin + clavulanic acid, enrofloxacin, cefoxitin, ciprofloxacin and ampicillin (>50%). All of them were, however, susceptible to tetracycline, imipenem and gentamicin (100%). *Enterobacter cloacae* showed resistance to ampicillin but were susceptible to the rest of the antimicrobials tested. For *Leclercia adecarboxylata*, 83% of isolates showed intermediate resistance to enrofloxacin. High percentages of susceptibility to imipenem, ceftazidime and gentamicin (100%) and amoxicillin + clavulanic acid, tetracycline, cefoxitin and ciprofloxacin (>60%) were detected.

For *E. coli*, the highest percentages of resistance were found to ampicillin (100%) and tetracycline (72%). All the isolates were susceptible to ceftazidime, enrofloxacin, imipenem, ciprofloxacin, and gentamicin. High percentages of susceptibilities to cefoxitin and cefotaxime (86%) were also observed.

Among NFB, the higher percentages of resistance were found to cefoxitin (74%) and ampicillin (68%). All of them were susceptible to imipenem and gentamicin (100%). High percentages of susceptibility to ceftazidime, tetracycline and ciprofloxacin (>85%) were also found.

The strain of *Vibrio alginolyticus* isolated was resistant to ampicillin and susceptible to all the rest of the antibiotics tested.

Percentages of AMR isolates for the three sampling sites are presented in Figure 1. In general, for *Enterobacteriaceae*, higher percentages of resistant isolates were found at the polluted site (T) for most of the antibiotics tested. However, the pattern of antimicrobial resistance seems to not differ between the polluted and the control sites for NFB.

Presence of microplastics

Large differences (□ 2 orders of magnitude) in the amount of microplastics between sites were observed. Sea cucumbers from the control sites (P and A) contained a very low amount of microplastics in their intestines, i.e. only three individuals. However, a high number of plastic particles was observed in the intestinal content of individuals from the polluted site (T, i.e. all individuals contained microplastics) (Figures 2 and 3), which

Table I: Percentages of susceptible isolates to different antimicrobials tested.

Antibiotic	Species	Resistant	Intermediate	Susceptible
	Citrobacter freundii	50	–	50
	Enterobacter cloacae	–	–	100
	Leclercia adecarboxylata	–	–	100
Ceftazidime (CAZ)	*E. coli*	–	–	100
	Vibrio	–	–	100
	Non-fermenting bacilli (NFB)	10	5	85
	Citrobacter freundii	67	–	33
	Enterobacter cloacae	100	–	–
Amoxicillin/Clavulanic acid (AMC)	*Leclercia adecarboxylata*	17	16	67
	E. coli	43	57	–
	Vibrio	–	–	100
	Non-fermenting bacilli (NFB)	49	15	36
	Citrobacter freundii	66.67	16.67	16.67
	Enterobacter cloacae	–	100	–
Enrofloxacin (ENO)	*Leclercia adecarboxylata*	17	83	–
	E. coli	–	–	100
	Vibrio	–	–	100
	Non-fermenting bacilli (NFB)	6	25	69
	Citrobacter freundii	–	–	100
	Enterobacter cloacae	–	100	–
	Leclercia adecarboxylata	–	17	83
Tetracycline (TE)	*E. coli*	72	14	14
	Vibrio	–	–	100
	Non-fermenting bacilli (NFB)	4	6	90
	Citrobacter freundii	67	–	33
	Enterobacter cloacae	–	–	100
	Leclercia adecarboxylata	–	33	67
Cefoxitin (FOX)	*E. coli*	14	–	86
	Vibrio	–	–	100
	Non-fermenting bacilli (NFB)	74	12	14
	Citrobacter freundii	–	–	100
	Enterobacter cloacae	–	–	100
	Leclercia adecarboxylata	–	–	100
Imipenem (IPM)	*E. coli*	–	–	100
	Vibrio	–	–	100
	Non-fermenting bacilli (NFB)	–	–	100

resulted in statistically significant differences (Tables II and III).

Discussion

Nowadays, antimicrobial resistance and microplastics are two subjects of major interest and concern. The presence of AMR bacteria and microplastics in marine environments are a risk for human, animal and environmental health (De-la-Torre 2020; Alprol et al. 2021; Ugwu et al. 2021). In turn, antibiotic-resistant bacteria and microplastics can act as indicators of pollution in aquatic environments (Al-Bahry et al. 2009; Pico et al. 2019). In addition to this, biofilms colonizing microplastics' surface can become a reservoir for

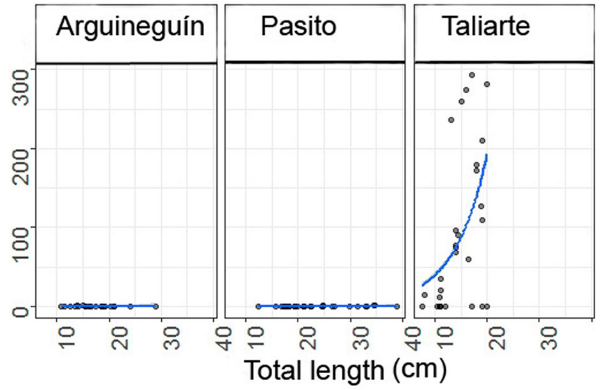

Figure 2. Number of microplastic particles in the intestinal contents of the specimens collected at each site, according to their size (total length).

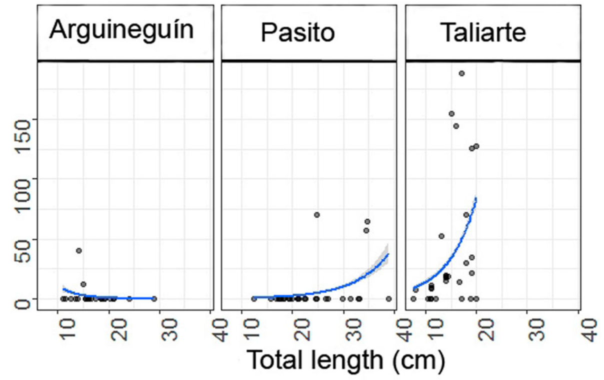

Figure 3. Weight (micrograms) of microplastics found in the intestinal content of the specimens collected at each site, according to their size (total length).

Table II. Results of the GLM testing for differences in the number of microplastic particles between sites. The total length of each individual was included as a covariate. Asterisks denote statistically significant *P* values.

	Estimate	Std. Error	z value	Pr (>|z|)
Intercept	−5.412603	0.718312	−7.535	4.88e^{-14} ***
Total length	0.157503	0.006099	25.823	<2e^{-16} ***
Location: B. Pasito	−1.167126	0.915183	−1.275	0.202
Location: Taliarte	7.522329	0.707840	10.627	<2e^{-16} ***

*** $P < 0.001$.

pathogens or faecal contamination indicator microorganisms, spreading through the aquatic environment (Arias-Andres et al. 2019; De-la-Torre 2020; Yang et al. 2020). In this study, we have determined the presence of AMR bacteria and microplastics in the intestine of the sea cucumber *Holothuria sanctori* collected at three sampling sites, under different pollution levels, in Gran Canaria (Spain).

In addition to the identification of microplastics in farmed holothurians (Mohsen et al. 2019), the presence of microplastics in the guts of wild holothurians has been described previously (Graham and Thompson 2009; Renzi et al. 2018). Initially, our study observed a clear and positive effect of individual size on the amount of microplastics in the intestines of holothurians. This contrast with results observed for the holothurian *Apostichopus japonicus* in China (Mohsen et al. 2019). A larger individual size implies a wider mouth and, therefore, larger sediment ingestion rates (Navarro et al. 2013). The number and total weight of microplastic particles observed in our study were significantly higher (several orders of magnitude) in the guts of animals sampled at the polluted site, reflecting, therefore, the large amount (availability) of microplastics in a nearshore site under high organic pollution. It is particularly noteworthy that, despite animals at the impacted site being mostly small-sized, relative to controls, they consumed large amounts of microplastics. Despite the presence of microplastics being widespread in the coastal regions of the Canary Islands (Herrera et al. 2018; Reinold et al. 2020), this result may concurrently reflect a deficient depuration process of sewage waters released to the ocean. Without a doubt, this outcome points towards an urgent necessity to improve depuration of wastewater released by coastal outlets. These microplastic particles can act as carriers of pollutants (Camacho et al. 2019) and, by bioaccumulation, are moving through the food web (Alprol et al. 2021). Sea cucumbers are part of the human diet, especially in Asia, and, as a result, the presence of microplastics in these animals could represent a risk for human health (De-la-Torre 2020).

Table III. Results of the GLM testing for differences in the weight of microplastic particles between sites. The total length of each individual was included as a covariate. Asterisks denote statistically significant *P* values.

	Estimate	Std. Error	z value	Pr (>|z|)
Intercept	−1.983072	0.198826	−9.974	<2e^{-16} ***
Total length	0.149185	0.006951	21.463	<2e^{-16} ***
Location: B. Pasito	−0.178171	0.172629	−1.032	0.302
Location: Taliarte	3.324204	0.144782	22.960	<2e^{-16} ***

*** $P < 0.001$.

The sea cucumber *Holothuria sanctori* is a species of interest for aquaculture. The presence of AMR bacteria could complicate treatments of infectious diseases and it could also represent a risk for consumers of sea cucumbers. Some authors (Yang et al. 2019; Dong et al. 2021) emphasized the environmental effects and risks derived from interactions between microplastics and antimicrobials, calling for a reduction of both pollutants, especially in aquaculture environments.

Large percentages (>50%) of resistant isolates to several antimicrobials (AM, AMC, CAZ, FOX, CIP, ENO) were found for *Enterobacteriaceae* from holothurians sampled at the polluted site (T). *Enterobacteriaceae*, and more specifically coliforms, are often used as indicators of faecal pollution of water (Al-Bahry et al. 2009). Detection of *Enterobacteriaceae* resistance to antimicrobials, considered of 'critical importance' by the World Health Organization (WHO 2019), in holothurians reinforces the need for better control of pollution of aquatic environments, particularly to avoid possible risks for human and animal health derived from sewage discharges (Al-Bahry et al. 2009).

Significantly higher quantities of microplastic particles were also found in the intestine of animals sampled at the polluted site (T), relative to control sites. The role of microplastics as a reservoir of antimicrobial resistance genes (ARGs) in the marine environment has been studied (Yang et al. 2019) and a significantly greater presence and diversity of ARGs in plastics microbiota than in surrounding seawater microbiota was found. Of particular concern is that multidrug resistance genes were the main class of ARGs detected in microplastics. In conclusion, by combining two sources of data in the gut contents of a holothurian species, this study has pointed towards clear effects of pollution on a nearshore site directly affected by a sewage outlet. This preliminary result stresses that it is important to integrate microbiological and ecological approaches to analyse the combined influence of microplastics and microbiological pollution in aquatic ecosystems.

Acknowledgements

We appreciate the constructive comments and corrections by two anonymous reviewers.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This research received no external funding.

Conflicts of interest

The authors declare no conflict of interest.

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