PRESENCE OF PATHOGENIC BACTERIA, BACTERIA RESISTANT TO ANTIBIOTICS AND MICROPLASTICS IN SEA CUCUMBERS UNDER DIFFERENT LEVELS OF ANTHROPOGENIZATION AT GRAN CANARIA ISLAND (SPAIN)

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ABSTRACT

The aim of this study was to assess the presence of pathogenic bacteria and bacteria resistant to antibiotics from the intestinal tract of the sea cucumber *Holothuria sanctori* (Delle Chiaje, 1823), a conspicuous marine invertebrate in coastal waters of the Canary Islands. A total of 79 specimens were collected from three sites at the island of Gran Canaria, Canary Islands, Spain. One site is directly influenced by continuous discharges of organic sewage through two coastal underwater sewage outlets, while the other two sites where at control sites with no direct sources of organic pollution. Individuals were dissected longitudinally through the ventral area and the intestinal tract removed. With the help of a swab, a small faecal sample was removed from the luminal epithelium of the anterior intestine. The swabs with faecal material were then seeded in different media (McConkey Agar (MC), McConkey Agar with Cefotaxime (MC+CTX), Mannitol Salt Agar (MSA) and Selenite Broth). Gram stain, catalase and coagulase tests were done to suspected *Staphylococcus* growing in MSA. Oxidase tests, Kligler Iron Agar (KIA) and biochemical identification were used for identification of Gram-negative bacteria. Susceptibility to several antimicrobial agents was determined. In addition, we compared the amount (number and weight) of microplastics in the intestinal contents of sea cucumbers in different sites. No presence of *Staphylococcus aureus* and *Salmonella* spp. was registered, but there is presence of others pathogenic bacteria (Enterobacteria and NFB) at all sites. The low concentrations of bacteria could be explained because sea cucumbers could have the capacity to phagocytize bacteria. In terms of resistance, almost all bacteria were resistant to ampicillin (>50%), while all of them were susceptible to imipenem and gentamicin (100%). Also, there was a greater resistance to antibiotics in polluted waters than in clean waters for Enterobacteria but not for NFB; still, our sample size was too low to support robust conclusions. A very small amount of microplastics were found on control sites, whereas sea cucumbers from the polluted site contained large amounts of these. As a result, *Holothuria sanctori* seems an ideal candidate for monitoring pollution impacts on nearshore waters of the Canary Islands.

Keywords: sea cucumber; *Holothuria sanctori*; antimicrobial susceptibility; pathogenic bacteria, microplastics.

ANNEX I (ABBREVIATION GUIDE)

- **A:** Baja de Arguineguín
- **AM:** Ampicillin
- **AMC:** Amoxicillin/Clavulanic Acid.
- **B-:** Gram-negative bacilli
- **B+:** Gram-positive bacilli
- **BHI:** Brain-Heart Infusion
- **CAZ:** Ceftazidime
- **CIP:** Ciprofloxacin
- **Coag -:** coagulase negative
- **Coag +:** coagulase positive
- **C_T-:** Catalase negative
- **C_T+:** Catalase positive
- **CTX:** Cefotaxime
- **ENO:** Enrofloxacin
- **FOX:** Cefoxitin
- **GM:** Gentamicin
- **I:** Intermediate
- **IPM:** Imipenem
- **L-:** Lactose negative
- **L+:** Lactose positive
- **MC:** McConkey Agar
- **MC+CTX:** McConkey Agar with Cefotaxime
- **MH:** Müeller-Hinton Agar
- **MSA:** Mannitol Salt Agar
- **NFB:** Non-fermenting bacilli
- **Ox-:** Oxidase negative
- **Ox+:** Oxidase positive
- **R:** Resistant
- **S+:** Bacteria suspected to be *Staphylococcus aureus*
- **P:** Baja de Pasito Blanco
- **S:** Susceptible
- **SS:** Bacteria suspected to be *Salmonella*
- **SS+:** *Salmonella* positive
- **T:** Taliarte

- **TE:** Tetracycline

1. INTRODUCTION

Antibiotics are frequently used by humans for different purposes in a range of fields, e.g. medicine, veterinary, agriculture, aquaculture, etc., which are more or less metabolized subsequently by humans or domestic animals. The remains of these antibiotics, which are not metabolized, are excreted and then reach waste-water treatment plants. Antibiotics are, however, only partially eliminated in these waste-water treatment plants. If they are not eliminated during the purification process, they pass through the waste-water system and end up in the environment through surface waters, ground waters or sediments, mainly reaching the seawater through underwater outfalls affecting nearshore marine ecosystems (Kümmerer, 2009).

Extensive usage of antimicrobials has resulted in the development of antibacterial resistance to pathogens by marine organisms, which has rendered many known antimicrobials ineffective (Jiang et al., 2014). When antibiotic residues enter the environment, they may suppress susceptible species and strains (Marinho et al., 2014). Bacterial resistance has motivated the scientific world to search for new bioactive compounds with antibacterial activity. The ocean is the main target of this search, as there is a great variety of marine organism bioactive compound holders, which could be used as future suppliers for the world of drugs (Jha & Zi-Rong, 2004).

Echinoderms, in particular Holothurians (sea-cucumbers), have an innate immune mechanism against high concentrations of bacteria, viruses and fungi found in coastal sediments. The main intestinal bacteria in echinoderms are *Vibrio*, *Pseudomonas*, *Flavobacterium* and *Aeromonas*, in addition to *Enterococcus* spp., *Salmonella* spp. and *Escherichia coli (E. coli)*, due to their presence in the environment (Marinho et al., 2014; Marinho et al., 2013).

Enterococcus spp. and *E. coli* can act as a reservoir of antimicrobial resistance that may be transmitted to other pathogenic bacteria. In fact, both species are specialists in obtaining and transmitting resistance genes to phylogenetically distant bacteria (Barros et al., 2011; Marinho et al., 2014). Antibiotic resistance in enterococci and *E. coli* is not only limited to the clinical setting, but also to other environments such as the intestinal tract of healthy animals (Barros et al., 2011; Poeta et al., 2006; Romalde et al., 1996). Faecal samples from echinoderms show a higher concentration of *Enterococcus* spp. bacteria than *E. coli*, because *E. coli* are Gram-negative bacteria, more susceptible to adverse situations than Gram-positive bacteria (Marinho et al., 2014; Marinho et al., 2013). *Salmonella* spp., family *Enterobacteriaceae*, are gram-negative bacilli, which ferment glucose, maltose and mannitol. These bacteria are present in waste-water coming from agriculture, and it is potentially pathogenic to echinoderms (Arifin et al., 2013; López et al., 2016; Omran & Allam, 2013).

Preceding studies analysing antibiotic resistant bacteria, as bio-indicators of pollution, have given clear indications that coastal sewage effluents can affect a range of marine fauna. The analysis of faecal samples in fish, such as those from coast of the Gulf of Oman (Al-Bahry et al., 2009), showed bacteria resistant to ampicillin. In Gilthead seabream (*Sparus aurata*) from the Atlantic Ocean (Barros et al., 2011), the strains showed high percentages of resistance to erythromycin and tetracycline.

With respect to echinoderms, sea cucumbers and sea urchins from aquaculture facilities in China were described to carry abundant antibiotic resistant bacteria, especially marine vibrio (Dang et al., 2006). Many *Vibrio parahaemolyticus*, from cultured sea cucumbers (*Apostichopus japonicas*), showed resistance to ampicillin and cefazolin; less of them were resistant to streptomycin, cefuroxime sodium, tetracycline, sulphamethoxazole/trimethoprim and quinolones (Jiang et al., 2014). In a study with echinoderms (*Echinoidea* and *Holothuroidea*) from the Azores islands (Portugal), antibiotic resistance of *Enterococcus* spp. and *E. coli* was evaluated in a total of 250 faecal samples. High percentages of resistance in enterococci were found for erythromycin, ampicillin, tetracycline and ciprofloxacin and in *E. coli* isolates resistance was detected for streptomycin, amikacin, tetracycline and tobramycin (Marinho et al., 2013).

A study on microbial contamination in a sea cucumber from the Mediterranean Sea, revealed the presence of isolates of five human Gram-negative pathogenic bacteria, *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella* sp. and *Shigella* sp. (Omran & Allam, 2013).

Holothurians, marine organisms popularly known as “sea cucumbers”, are found in almost every marine environment of the world. Sea cucumbers play an important role in the nutrient cycling of coastal ecosystems by consuming sediments and moving sand and retaining organic matter (Zhang et al., 2013). Holothurians are deposit-feeders, and, therefore, it is plausible that sewage discharges into the ocean may induce antibiotic resistant bacteria and the parallel ingestion of multiple plastic particles (Assidqi, 2015; Grossmann, 2014; Miliou et al., 2016; Mohsen et al., 2019; Seary et al., 2013). Microplastic ingestion by sea cucumbers is unavoidable, due to their filtration of marine sediments (Assidqi, 2015; Miliou et al., 2016; Mohsen et al., 2019); this is particularly common nowdays in coastal areas that are full of plastics released by humans. A study conducted with *Holothuria sanctori*, however, suggested that the ingestion of microplastics does not substantially harm the animal (Grossmann, 2014).

Holothurians have a high commercial value, mainly in nutrition, but also have medicinal purposes. The main consumer is Asia (China, Hong Kong, Taiwan, Singapore, Korea and Malaysia) (Kim et al., 2017; Marinho et al., 2014; Navarro, 2012; Navarro et al., 2012). In China, they are used as traditional remedies because of their high

nutritional value, due to their high protein content, low fat content, amino acid profile and presence of trace elements. As medicine, they have several chemical compounds that are used to treat anaemia, prevent some cancers, promote strong immune function and reduce arthritis pain (Jiang et al., 2014; Navarro, 2012).

In this study, I evaluated the presence of pathogenic bacteria (*Salmonella* spp., *Staphylococcus aureus*) and the antimicrobial resistance of bacteria isolated from intestinal contents of *Holothuria sanctori* in Gran Canaria, Canarian Archipelago, Spain. My objective was to determine whether the presence of pathogenic bacteria and bacteria resistant to antibiotics differed between three populations of *H. sanctori*, one population from a site under large organic inflow (polluted site) and two populations from sites (unpolluted, controls) with low organic inflow. In addition, I compared the amount (number and weight) of microplastics in the intestinal contents of collected sea cucumbers at the three sites.

2. MATERIALS AND METHODS

2.1. Target species

Figure 1. Holothuria sanctori.

This study focused on *Holothuria sanctori* (Delle Chiaje, 1823) (Figure 1), a conspicuous marine invertebrate in the coastal waters around the Canary Islands (Navarro, 2012; Navarro et al., 2012). This holothurian is characterized by an elongated body, more or less tubular and centrally depressed forming a ventral area pedicle sole well differential from the back. This species is light-dark brown in colour, with whitish rings around the dorsal papillae that cover the back in a disorderly manner, or light brown with dark brown rings. They are about 15-20 cm in total size (length), but can reach up to 30 cm and are about 6-7 cm wide (Navarro et al., 2013). This species has a tegument quite thick and hard. They have one or two vesicles of Poli and copious Cuvier's tubes (Navarro, 2012). This sea cucumber is abundant on sandy and rocky bottoms from

intertidal pools down to 70 metres depth. They are nocturnal animals, hiding in crevices during the day and feeding at dusk. The species is distributed across the Mediterranean, the eastern Atlantic, Biscayan Gulf, Portugal, Santa Helena, Madeira, Cape Verde, the Azores and the Canary Islands (Entrambasaguas Monsell, 2008; Navarro, 2012). This is the most abundant species of Holothurian in Gran Canaria (Navarro et al., 2012).

2.2. Area of study and sampling

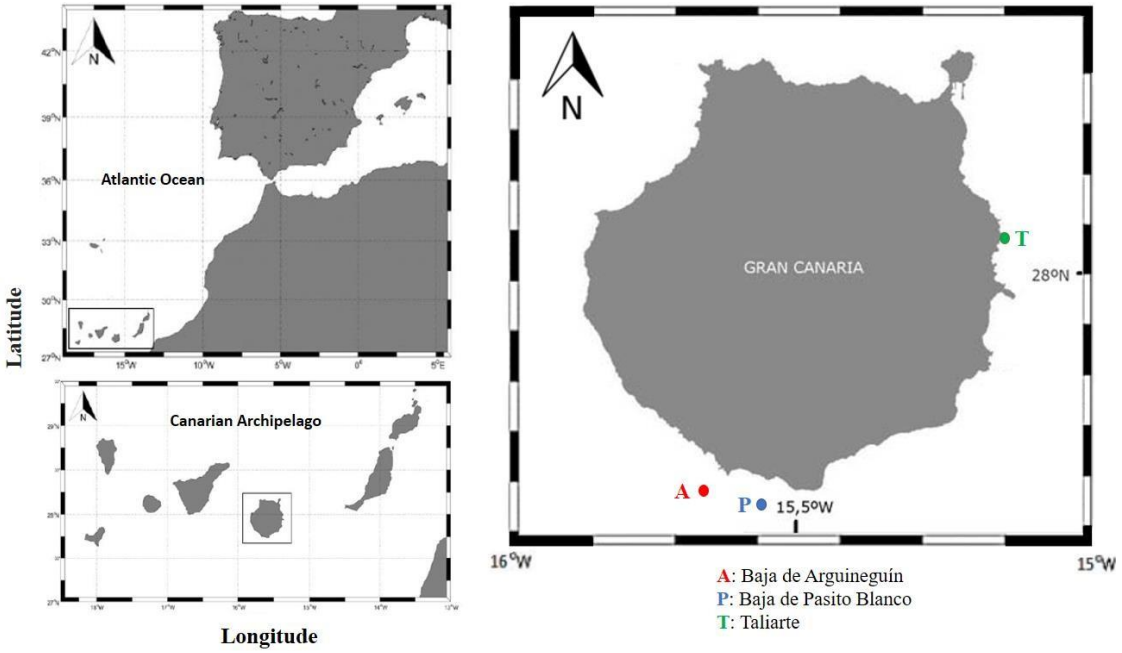

Figure 2. Map of study sites in Gran Canaria island, Spain (QGIS, 2020).

This study was carried out, between November 2019 and July 2020, in Gran Canaria, Canarian Archipelago, Spain (28°N, eastern Atlantic Ocean) (Figure 2). Three sites between 7 and 14 m depth were selected: Baja de Pasito Blanco (P) (27º 44,422'N 15º 37,858'W), Baja de Arguineguín (A) (27º 44'48.36''N 15º 41'2.32"W) and Taliarte (T) (27º 59'27.95"N 15º 22'05.04"W). The first two sites are outside direct human influences and, therefore, were considered as the control sites for this study. Both sites are located between 2 and 3 km offshore the nearby coast and are mainly dominated by rocky-sandy bottoms with high-relief rocky ledges. On the contrary, Taliarte is directly onshore with bottoms that are mainly rocky to sandy. This site is located near two submarine sewage outlets that discharge waste-water into the environment (IDECanarias visor 4.5.1, s. f.).

On each sampling occasion, a team of SCUBA divers moved to the sampling area with the help of a marine boat and about 23 to 28 individuals were randomly collected. Each individual was introduced in a Ziploc bag and, once out of the water, immersed in a plastic container with sea water until reaching the laboratory (Navarro et al., 2012).

2.3. Animals dissection

In the laboratory, all instruments (scissors, forceps, scalpel and punch) were initially sterilized with alcohol before each dissection, allowing the alcohol to evaporate every time in order to not killing bacteria when animals were opened.

I took each animal from the bag previously collected, which was allocated on a plastic tray where its total length was measured in centimetres with a ruler (Figure 3. (a)). Then the animal was processed: opened while still alive (this not considered animal mistreatment because it is an underdeveloped specimen) with a rounded tip scissors, or a scalpel, so I exerted some force longitudinally through the ventral area (Figure 3. (b)) (Díaz-Sol Sol et al., 2019). The intestine of the animal was collected with tweezers and allocated on to a tray covered with a previously placed sanitized bag. The intestine was washed with a syringe filled with sea water or a sterile saline solution. With a punch, a small hole was opened in the luminal epithelium of the anterior intestine, which is the part that connects with the cloacae (Figure 3. (c)) (Pagán-Jiménez et al., 2019). Then, a small faecal sample was collected from this area using a sterile swab (Figure 3. (d)). Samples were then sent to the microbiology laboratory, as quick as possible, and always before 48 hours after sampling. Swabs were labelled with a code that indicates the number of the animal and the area of collection.

The rest of the intestine was stored in bottles with alcohol and the rest of the animal was discarded.

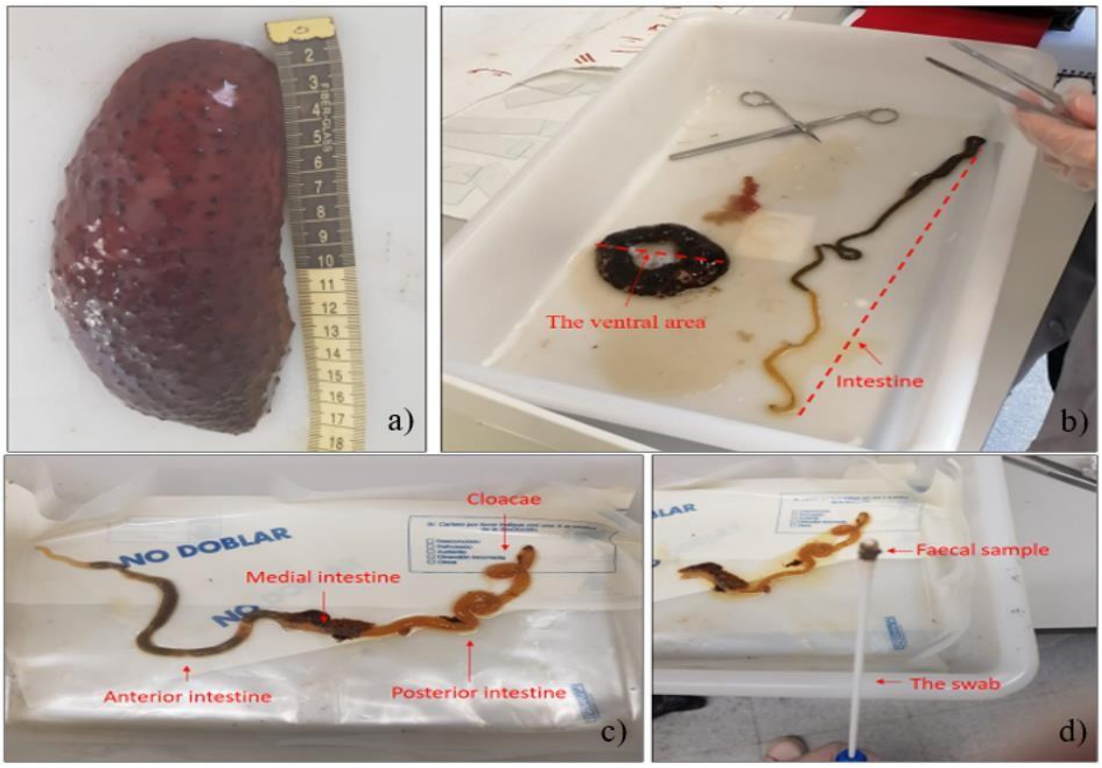

Figure 3. Holothuria sanctori dissection process. (a) Measurement of the specimen (b) Dissection (c) *Transfer and cleaning of the intestine (d) Collection of the faecal sample.*

2.4. Seeding in different culture media

Once in the microbiology laboratory, swabs were seeded by isolation on McConkey Agar (MC), Mannitol Salt Agar (MSA), McConkey Agar with Cefotaxime (MC+CTX) and were inoculated in Selenite Broth, in that order. All media were obtained from DIFCO (MI, USA). Plates were incubated in a stove at 37ºC for 24 hours. Selenite Broth was later seeded by isolation in *Salmonella-Shigella* Agar (SS Agar) and the SS plates were left in the stove at 37ºC for 24 hours. Finally, the reading of the culture medium was done at 24 and 48 hours (Díaz et al., 1994).

2.5. Readings of culture media

MacConkey Agar (MC) is a selective and differential culture medium for Gram negative bacteria. The majority of Enterobacteria and other Gram-negative microorganisms, such as *Pseudomonas*, *Aeromonas*, etc. can grow on MC agar.

It acts as a differential medium because it contains lactose and a pH indicator. Bacteria capable of fermenting this sugar will cause a change in the pH of the medium by the release of acidic products (Díaz et al., 1994). On the basis of the colour of the colonies, this medium allows the differentiation between bacteria that ferment lactose (colour pink or red) than the ones that do not ferment this sugar (other colours) (Figure 4. (a)). Colonies of bacteria that do not ferment lactose (L-) (yellow-orange) and colonies of bacteria that ferment lactose (L+) (red).

McConkey Agar with Cefotaxime (MC+CTX) is used to facilitate the detection of bacteria possessing broad-spectrum beta-lactamases, which allow them to be resistant to numerous beta-lactam antibiotics. As with MC agar, colour of the colonies, allows the differentiation of bacteria that ferment or not Lactose. In the Figure 4. (b), was illustrated colonies of bacteria that ferment Lactose (L+).

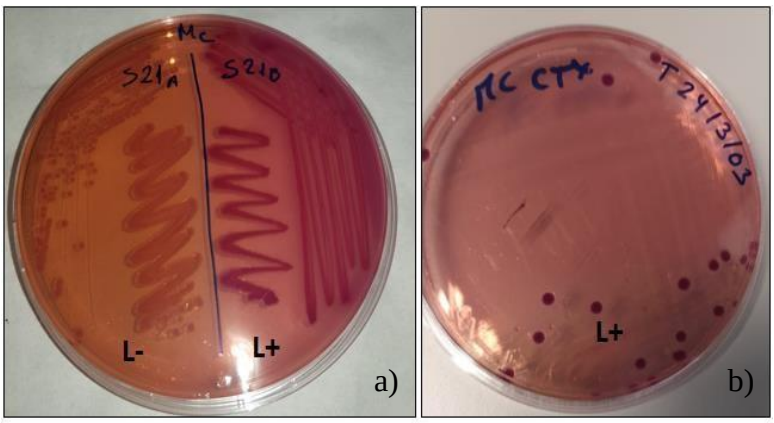

Figure 4. Lactose fermentation in MC and MC+CTX media. (a) Lactose non-fermenting colonies (left) and lactose fermenting colonies (right) in MC agar. (b) Lactose fermenting colonies in MC+CTX agar.

The second seeding (MC) **The first seeding (MC+CTX)**

Mannitol Salt Agar (MSA) allows the growth of bacteria that resist high concentrations of NaCl, while inhibiting the growth of other Gram-positive or Gram-

negative ones. It contains a high concentration of salt (NaCl) causing inhibition of other microorganisms, except of halo-tolerant bacteria. In this medium, we were looking for *Staphylococcus aureus*, that can grow under these conditions. Bacteria suspected to be *Staphylococcus aureus* (S+) grow giving yellow colonies surrounded by a yellow zone, because they ferment mannitol (Figure 5) (Díaz et al., 1994).

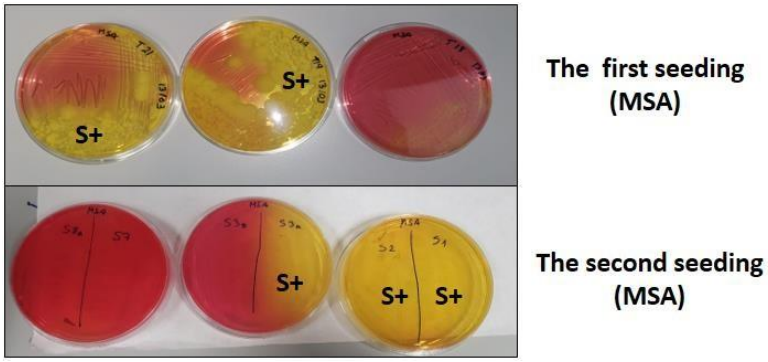

Figure 5. Suspected Staphylococcus aureus in MSA media.

The use of Selenite Broth as enrichment media and subsequent seeding in SS agar is intended to facilitate the detection of bacteria of the genus *Salmonella* spp. In SS agar, bacteria suspected of being *Salmonella* are observed as transparent colonies (because they do not ferment lactose) and with a black precipitate due to the production of SH_2. Presence of doll's eye (black) colonies (Figure 6) indicates possibility of *Salmonella* (SS+), and colonies should be further identified.

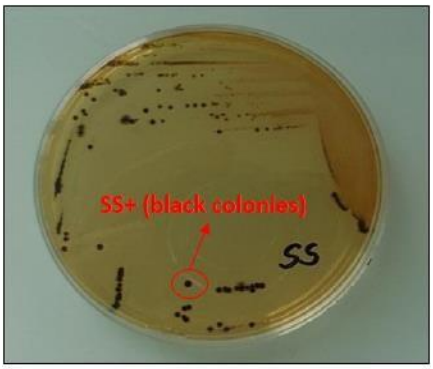

Figure 6. Suspected Salmonella in SS media (Díaz et al., 1994).

2.6. Second seeding in culture media

After the first seed and growth on MC, MSA, MC+CTX and SS plates, one colony per plate was chose and isolated in a new plate, to obtain pure cultures. If two different colonies were observed, the new Petri dishes were divided in half to seed two bacteria (A or B) according to their different appearance. Plates were incubated in a stove at 37ºC for 24 hours.

2.7. Bacteriological analysis

Gram stain is a technique that allows the differentiation of two large groups of Eubacteria: Gram-positive or Gram-negative, according to the cell-wall composition. This provides very useful information to choose the appropriate identification tests and

to guide the antibiotic treatment. When observed under microscope after Gram stain, Gram-positive bacteria appear blue-violet, and Gram-negative appear red-pink (Díaz et al., 1994). Morphology of bacterial cells can also be observed.

2.7.1. Identification of suspected *Staphylococcus aureus*:

Colonies of yellow colour on MSA are suspected of being *Staphylococcus aureus.* To confirm this identification, it is necessary to do Gram staining. If Gram positive cocci grouped in bunches are observed, catalase test should be done and if positive, coagulase test should be done finally. If this later one is also positive, probably the bacterium can be identified as *Staphylococcus aureus*.

Catalase is an enzyme characteristic of aerobic bacteria. It breaks down hydrogen peroxide into water and oxygen. The release of bubbles of oxygen indicates that the test is positive and the bacterium possess this enzyme. Among Gram positive cocci of clinical interest, catalase test makes it easy to distinguish between *Streptococcus* and *Staphylococcus. Staphylococcus* possess catalase and *Streptococcus* and *Enterococcus* do not possess catalase (Figure 7) (Díaz et al., 1994).

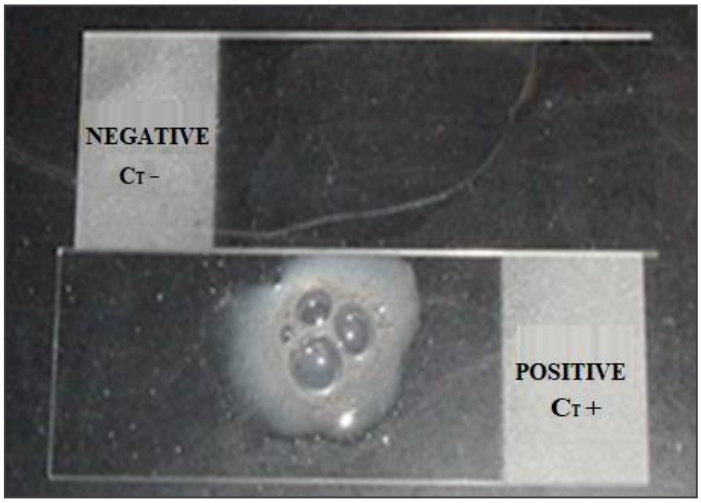

Figure 7. Catalase test.

Coagulase is a protein produced by several microorganisms that allows the conversion of fibrinogen into fibrin. This technique allows us to differentiate *Staphylococcus aureus*, which is possess coagulase (Coag +), from other *Staphylococcus* species, which are coagulase negative (Coag -). This test can be performed using two techniques: the slide technique (Figure 8. (a)), which detects bound coagulase or aggregating factor, and the tube technique (Figure 8. (b)), which detects free coagulase and bound coagulase (Díaz et al., 1994).

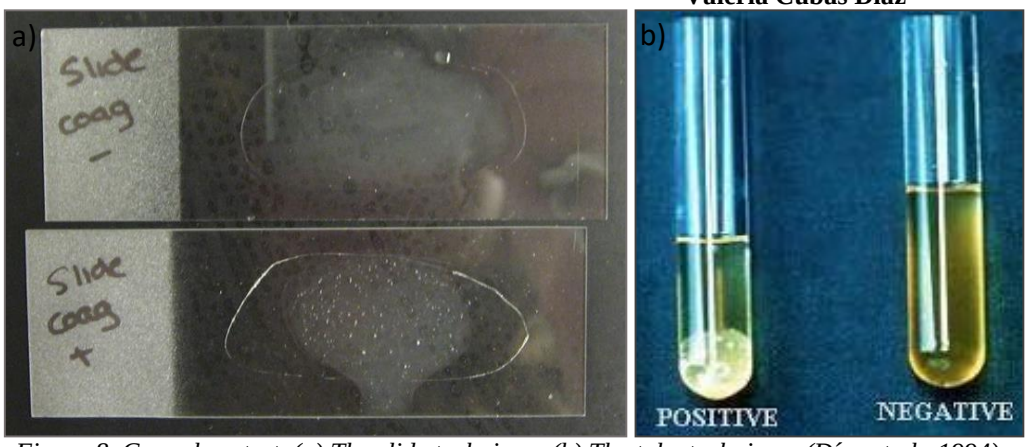

Figure 8. Coagulase test. (a) The slide technique. (b) The tube technique. (Díaz et al., 1994).

2.7.2. Gram-negative bacteria in MC, MC+CTX and SS:

Pseudomonas aeruginosa is a Gram-negative bacterium, can be isolated as clear colonies on MC and MC+CTX agar (as it does not ferment lactose) and will test positive for oxidase test. This test helps to identify cytochrome C oxidase, which oxidizes to NNN'N', tetramethyl,1-4, phenylenediamine (1% (w/v) aqueous solution). The oxidation was detected as blue colour (Figure 9). This test allows to differentiate the group *Enterobacteriaceae* (lacking cytochrome C) from the genus *Pseudomonas* and other Non-fermenting bacilli (possessing cytochrome C) (Díaz et al., 1994).

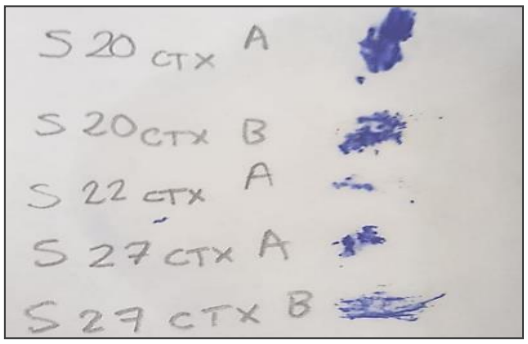

Figure 9. Oxidase test of colonies on MC+CTX.

If oxidase test was positive, a Kligler iron agar medium was used to detect fermentation of glucose. Bacteria that yield an oxidase test positive and did not ferment glucose and lactose were identified as Non-Fermenter Bacilli. If it does not ferment lactose, we need to know if ferment glucose or not. If it does not ferment glucose and is oxidase positive, it is a non-fermenting bacillus (e.g. *Pseudomonas*). Whether it does not ferment glucose but is oxidase negative, it may also be a Non-fermenting bacilli (NFB) (for example, some species of *Acinetobacter* do not have oxidase, but are NFB). But if it is oxidase positive and fermented glucose but not lactose, some other possibilities could be considered (*Vibrio, Aeromonas, Plesiomonas).* In our study, it is *Vibrio alginolyticus.*

Oxidase test was performed to colonies growing in MC and MC+CTX. If they do not possess oxidase and ferment lactose, they were identified using API 20E system

(bioMerieux, France), and all of them belong to the order *Enterobacterales*. This system was also used for identification of suspected *Salmonella* in SS agar. API 20E system allows the identification of bacteria by biochemical characteristics. Each API 20E, strip consists of 20 wells containing dehydrated media. The isolate to be tested was suspended in sterile saline solution and added to each well. The inoculated strip was incubated for 16–24 h at 37ºC and the reactions were noted either positive or negative based on changes of colour (Figure 10) (Omran & Allam, 2013).

Figure 10. Example of the API 20E system (MCA11a. E. coli).

2.8. Antibiotic susceptibility Tests

Antibiotic susceptibility was studied by the disk diffusion technique (antibiogram) using Müeller-Hinton (MH) agar as culture media (Díaz et al., 1994). An inoculum of isolated bacteria in saline solution was prepared and turbidity was adjusted to 0.5 McFarland standard. A sterile cotton swab was dipped on this inoculum and later used to inoculate the surface of MH agar plate by streaking the swab over the entire agar surface. The final step consisted on application of antimicrobial disks.

The antibiotics used were Ceftazidime (CAZ), Amoxicillin/Clavulanic Acid (AMC), Enrofloxacin (ENO), Tetracycline (TE), Cefoxitin (FOX), Imipenem (IPM), Ciprofloxacin (CIP), Gentamicin (GM), Cefotaxime (CTX) and Ampicillin (AM). Distance between disk should be higher than 24 mm from centre to centre. After incubation at 37ºC for 24 hours, diameters of growth inhibition zones were measured using a ruler and were compared with reference tables to determine if the bacteria were susceptible (S), resistant (R) or showed an intermediate susceptibility (I) to each antibiotic (Figure 11) (Díaz et al., 1994).

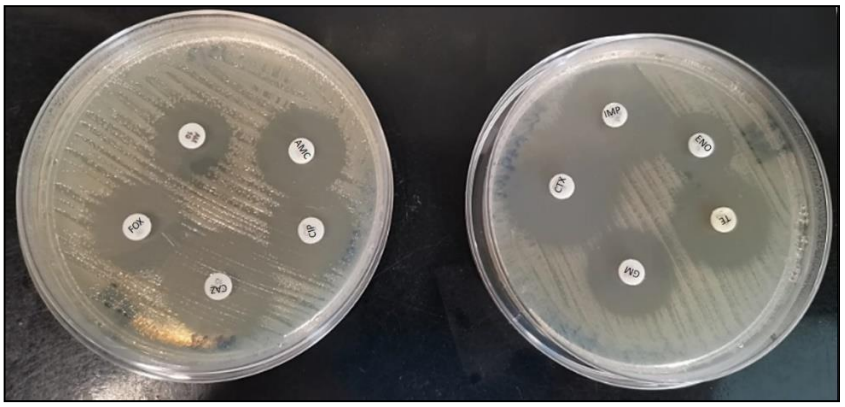

Figure 11. Example of the disk diffusion technique (Antibiogram).

2.9. Kligler Iron Agar (KIA)

Kligler Iron Agar allows to determinate the following tests: glucose fermentation, lactose fermentation, production of H_2S and gas production. If the bacterium does not ferment sugars, can grow using peptones present in the medium, but this growth does not produce acid and no colour change will be observed. Gram negative bacteria isolated from MC or MC+CTX and with a positive oxidase tests were seed in KIA. After incubation at 37ºC for 24 hours, results were interpreted as described in Table 1.

Table 1. Reading of Kligler Iron Agar (KIA).

	Lactose	**Glucose**
All of colour yellow	+	+
Yellow bottom and red slant	-	+
All of colour red or pink	-	-
Black precipitates	Production of SH_2	
Displacement or fracture of the culture media	Gas production	

2.10. Sample preservation

Finally, bacterial strains were preserved. They were inoculated in Brain-Heart Infusion (BHI) agar and were left in the stove at 37ºC for 24 hours. Later, 900 microliters were taken with automatic pipette and placed in an Eppendorf tube. Once all the Eppendorf tubes were filled, 100 microliters of sterile glycerol were added to each one and they were placed in the -20ºC freezer.

2.11. Analysis of microplastics fragments in the intestinal contents

Sea cucumber intestines, initially stored in bottles with alcohol, were passed on to a plastic tray, where they were processed. I opened intestines from the anterior part to the end (posterior part) of the cloacae with a pair of scissors. A plastic pippete (500 ml) with water facilitated 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 mm) fragments. The number of fragments per sea cucumber was then annotated; all fragments were removed with tweezers, allocated on a piece of aluminium foil and their weight obtained.

2.12. Statistical analysis

For the analysis of the data obtained, I employed Rstudio (RStudio Team, 2020), a programming language and free software, in particular for the data of the microplastics. The library used in Rstudio was Flexplot (Fife, 2019). Generalized Linear Models (GLMs), using a Poisson distribution with a log link function, tested for differences in the

number and weight of microplastics between sites. The total length of each sea cucumber was including as a covariate.

SigmaPlot Version 14 (SigmaPlot version 14, 2020) was the package used to graph the microbiological data.

3. RESULTS

3.1. Microbiology data

At all sites, bacteria growing on MSA were not *Staphylococcus aureus*. When Gram stain was applied to yellow colonies from MSA, bacilli morphology was observed in all cases (Gram-positive bacilli) (Figure 12). For 63 MSA isolates I analysed, 28 were positive bacilli (B+), 29 were negative bacilli (B-) and 6 samples had both.

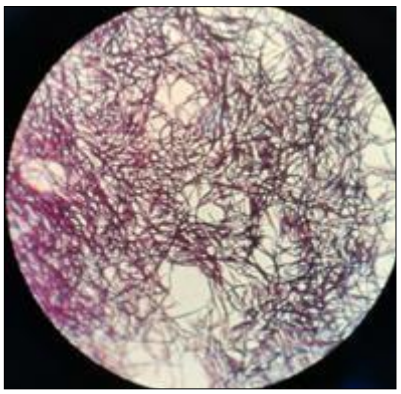

Figure 12. Gram-positive bacilli.

Globally, we identified 20 *Enterobacterales*, all of them belonging to the family *Enterobacteriaceae* and 91 Non-fermenter bacilli (40 *Pseudomonas*, 22 did not have oxidase and did not ferment glucose and 29 unidentified). We isolated also a strain of *Vibrio alginolyticus*.

Among *Enterobacterales*, we identified 6 *Citrobacter freundii*, 1 *Enterobacter cloacae*, 6 *Leclercia adecarboxylata* and 7 *E. coli*.

At the first site, Baja de Pasito Blanco (P), we obtained 28 animals. A total of 29 strains were isolated in MC, where 5 fermented lactose (17.24%) and 24 did not ferment (82.76%). Lactose fermenter were all *Enterobacteriaceae* (4 *Leclercia adecarboxylata* and 1 *Enterobacter cloacae*). From the 24 strains that did not ferment lactose, 75% were NFB (62.5% were *Pseudomonas* and 12.5% did not have oxidase and did not ferment glucose) and 25% were unidentified (they did not grow back). Regarding MC+CTX, 10 strains were isolated that did not ferment lactose, but had oxidase, being 100% Non-fermenting bacilli (NFB) (50% *Pseudomonas* and 50% not grow back).

At the second site, Baja de Arguineguín (A), we obtained 23 animals. A total of 26 strains were isolated in MC, where 7 fermented lactose (26.92%) and 19 did not ferment (73.08%). All the lactose fermenters were *Enterobacteriaceae* (5 *E. coli* and 2 *Leclercia adecarboxylata*) and from the 19 strains that did not ferment lactose, 100% were NFB. Concerning MC+CTX, 12 strains were isolated, being 16.67% *Enterobacteriaceae* (2 *E. coli*) and 83.33% were NFB.

At the third site, Taliarte (T), we obtained 28 animals. A total of 34 strains were isolated in MC, where 5 fermented lactose (14.71%) and 29 did not ferment (85.29%). All the lactose fermenters were *Enterobacteriaceae* (5 *Citrobacter freundii*), while that of the 29 strains that did not ferment lactose, 62.06% were NFB (31.03% are *Pseudomonas* and 31.03% did not have oxidase and did not ferment glucose); 34.48% were unidentified (not grow back) and 3.46% (1 isolate) was a *Vibrio alginolyticus.* Concerning MC+CTX, 22 strains were isolated, being 4.55% *Enterobacteriaceae* (1 *Citrobacter freundii*) and 95.45% did not ferment lactose, 100% were NFB (52.38% *Pseudomonas* and 47.62% did not have oxidase and did not ferment glucose).

Zones 1 and 3 were the only sites for which the analysis were complete. A total of 77 antimicrobial susceptibility tests for NFB, 20 for Enterobacteria and one for *Vibrio alginolyticus* were performed.

Antibiotic susceptibility of isolates from the three sites are shown in Table 2.

Table 2. Percentages of antimicrobial susceptibility in different bacteria for all sites.

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

Imipenem (IPM)	*Citrobacter freundii*	-	-	100
	Enterobacter cloacae	-	-	100
	Leclercia adecarboxylata	-	-	100
	E. coli	-	-	100
	Vibrio	-	-	100
	Non-fermenting bacilli (NFB)	-	-	100
Ciprofloxacin (CIP)	*Citrobacter freundii*	67	-	33
	Enterobacter cloacae	-	-	100
	Leclercia adecarboxylata	-	33	67
	E. coli	-	-	100
	Vibrio	-	-	100
	Non-fermenting bacilli (NFB)	-	1	99
Gentamicin (GM)	*Citrobacter freundii*	-	-	100
	Enterobacter cloacae	-	-	100
	Leclercia adecarboxylata	-	-	100
	E. coli	-	-	100
	Vibrio	-	-	100
	Non-fermenting bacilli (NFB)	-	-	100
Cefotaxime (CTX)	*Citrobacter freundii*	33.33	33.33	33.33
	Enterobacter cloacae	-	-	100
	Leclercia adecarboxylata	-	17	83
	E. coli	-	14	86
	Vibrio	-	-	100
	Non-fermenting bacilli (NFB)	54	35	11
Ampicillin (AM)	*Citrobacter freundii*	83	-	17
	Enterobacter cloacae	-	-	100
	Leclercia adecarboxylata	-	50	50
	E. coli	100	-	-
	Vibrio	100	-	-
	Non-fermenting bacilli (NFB)	68	19	13

*Non-fermenting bacilli (NFB) (n=77), *Vibrio alginolyticus* (n=1), *E. coli* (n=7), *Leclercia adecarboxylata* (n=6), *Enterobacter cloacae (n=1)* and *Citrobacter freundii* (n=6).

For *Citrobacter freundii* higher percentages of resistance were found to amoxicillin-clavulanic acid, enrofloxacin, cefoxitin, ciprofloxacin and ampicillin (> 50%). All of them were susceptible to tetracycline, imipenem and gentamicin (100%).

The strain of *Enterobacter cloacae* isolated showed resistance to amoxicillin-clavulanic acid but was susceptible to all the rest of antimicrobial tested.

About *Leclercia adecarboxylata*, 83% showed intermediate resistance to enrofloxacin. High percentages of susceptibility to imipenem, ceftazidime and gentamicin (100%) and to amoxicillin-clavulanic acid, tetracycline, cefoxitin, cefotaxime and ciprofloxacin (> 60%) were detected.

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

Among Non-fermenting bacilli (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* isolated was resistant to ampicillin and susceptible to all the rest of antibiotics tested.

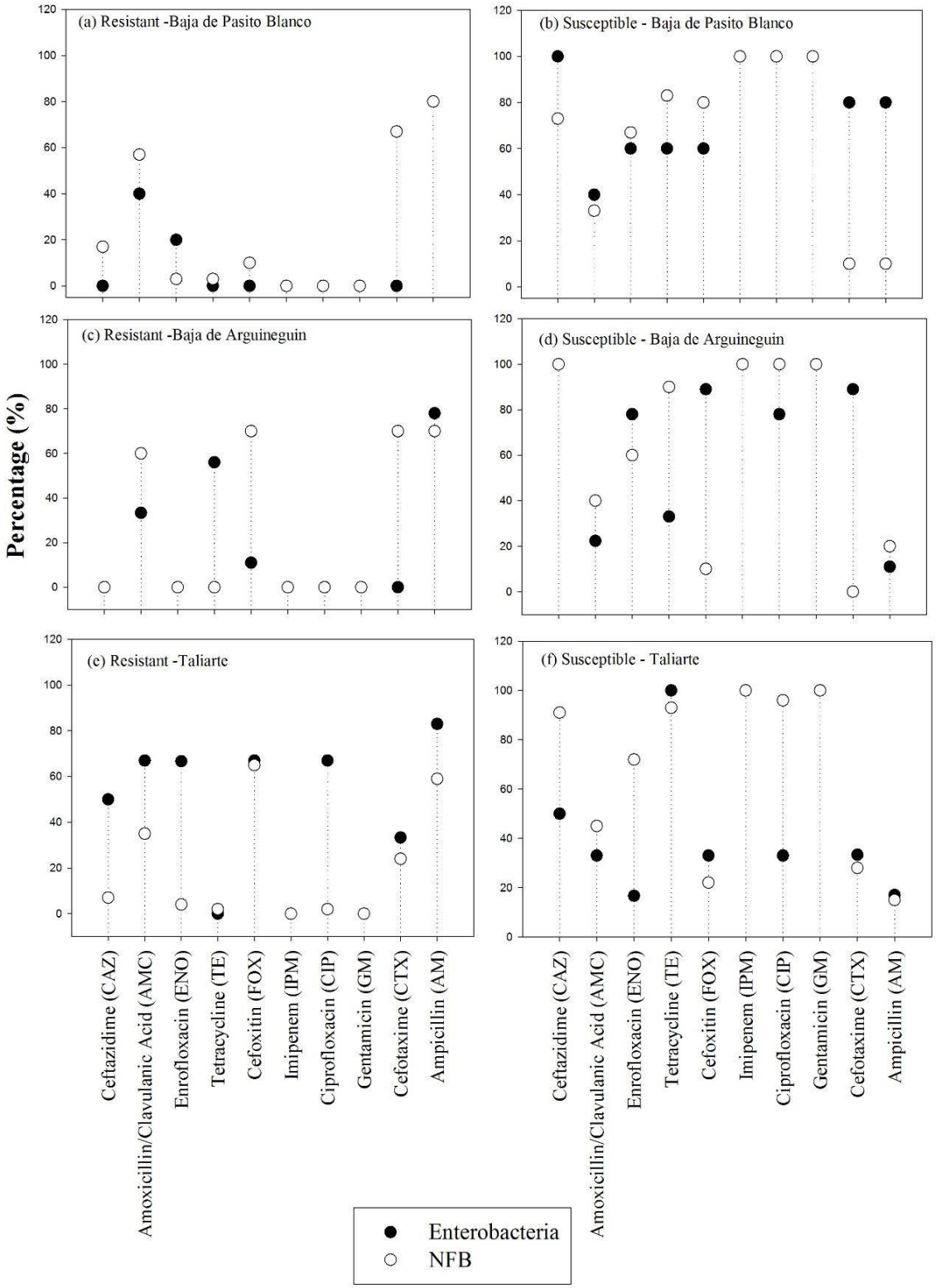

Figure 13. Percentages of resistant and susceptible bacteria to antibiotics in Enterobacteria and NFB for each site. (a) Resistant bacteria at Baja de Pasito Blanco (b) Susceptible bacteria at Baja de Pasito Blanco. Enterobacteria and NFB have the same percentage (100%) for IPM, CIP and GM. (c) Resistant bacteria at Baja de Arguineguín. (d) Susceptible bacteria at Baja de Arguineguín. Enterobacteria and NFB have the same percentage (100%) for CAZ, IPM and GM. (e) Resistant bacteria at Taliarte. (f) Susceptible bacteria at Taliarte. Enterobacteria and NFB have the same percentage (100%) for IPM and GM.

Enterobacteria from Taliarte was majorly resistant, at least > 50%, to 6 out of 8 antibiotics (Figure 13 (a)). However, Enterobacteria from Baja de Arguineguín only showed resistance, i.e. > 50%, to 2 out of 8 antibiotics (Figure 13 (c)) and Enterobacteria from Baja de Pasito Blanco overall displayed very low resistance to antibiotics (Figure 13 (e)). This pattern of larger resistance to antibiotics at the polluted site, however, was not observed for NFB. On the contrary, more than 50% of the NFB from the three sites have a percentage of susceptibility greater than 50% to 6 or 7 antibiotics (Figure 13 (b), (d) and (f)).

Table 3 (Annex II, Appendix A) shows the antimicrobial susceptibility of different bacteria species from the Baja de Pasito Blanco. Among Enterobacteria, the higher percentages of susceptibility were found to ceftazidime, ciprofloxacin, imipenem and gentamicin (100%) (Figure 13 (b)). Among Non-fermenting bacilli (NFB), the higher percentages of resistance were found to ampicillin (80%) and cefotaxime (67%) (Figure 13 (a)). Most of them were susceptible to imipenem, ciprofloxacin and gentamicin (100%) and cefoxitin (80%) (Figure 13 (b)).

Table 4 (Annex II, Appendix B) shows the antimicrobial susceptibility of different bacteria species from Baja de Arguineguín. Among Enterobacteria, the higher percentages of resistance were found to ampicillin (78%) (Figure 13 (c)). Most of the isolates were susceptible to ceftazidime, imipenem and gentamicin (100%) and to cefoxitin and cefotaxime with 89% (Figure 13 (d)). Among Non-fermenting bacilli (NFB), the higher percentages of resistance were found to cefoxitin and ampicillin (70%) (Figure 13 (c)). Most of them were susceptible to ceftazidime, imipenem, ciprofloxacin and gentamicin (100%) (Figure 13 (d)).

Table 5 (Annex II, Appendix C) shows the antimicrobial susceptibility of different bacteria species from Taliarte. Between Enterobacteria, the higher percentages of resistance were found to ampicillin (83%) (Figure 13 (e)). All the isolates were susceptible to tetracycline, imipenem and gentamicin (100%) (Figure 13 (f)). On the contrary, among Non-fermenting bacilli (NFB), the higher percentages of resistance were found to cefoxitin (65%) and ampicillin (59%) (Figure 13 (e)). Most of them were susceptible to imipenem and gentamicin (100%) and ciprofloxacin with 96% (Figure 13 (f)).

3.2. Presence of microplastics in the intestinal content

The analysis of the microplastics in the intestinal contents of sea cucumbers showed large differences between sites. Sea cucumbers from Baja de Pasito Blanco (P) and Baja de Arguineguín (A) contained a very low amount of microplastics in their intestines; they mainly contained sand, fragments of small rocks and some fragments of shells. However, the intestinal contents of individuals from Taliarte (T) had a large amount of plastic particles (Figures 14 and 15, tables 6 and 7).

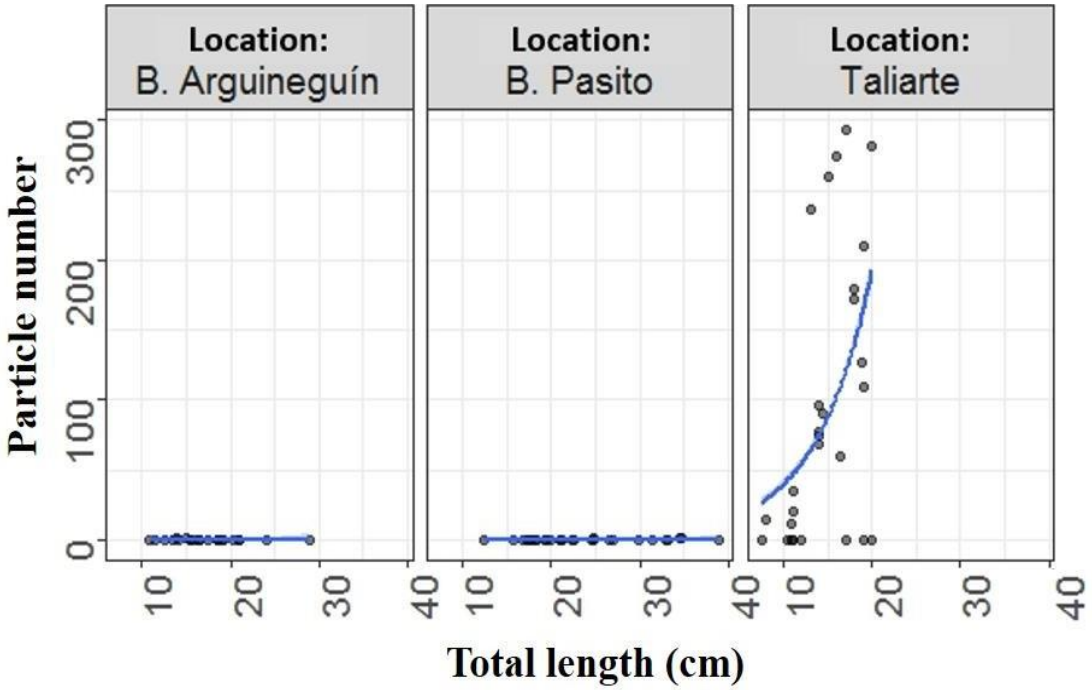

Figure 14. Number of microplastic particles in the intestinal contents of the specimens collected at each site, according to their size (total length).

Table 6. 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 (cm)	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	$< 2e^{-16}$ ***

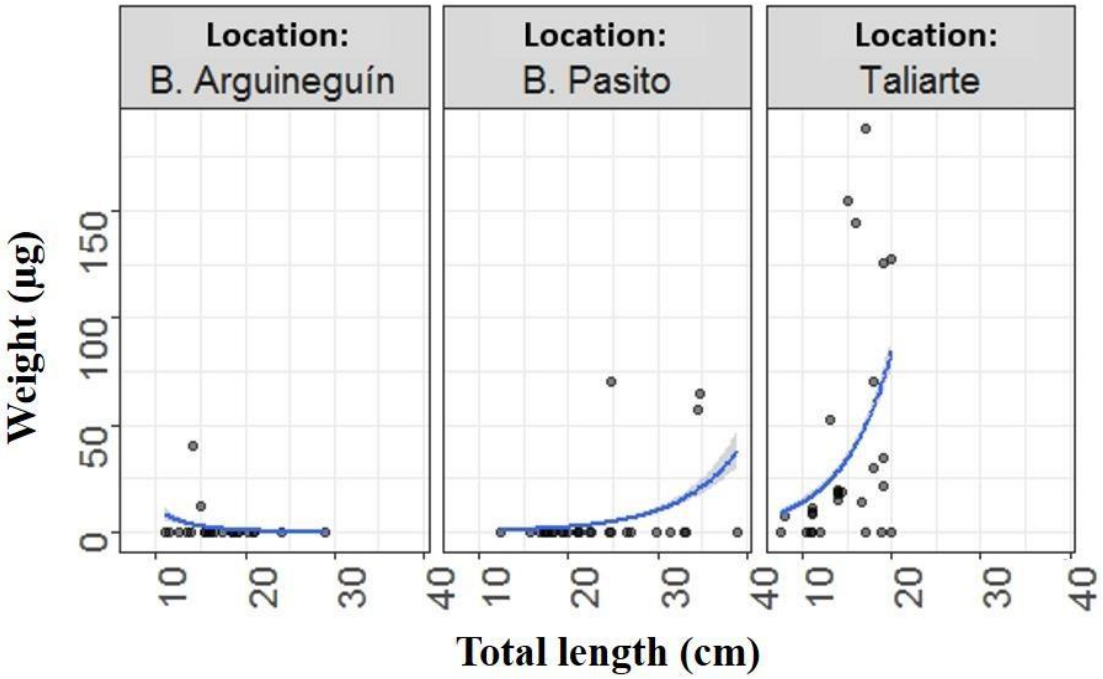

Figure 15. Weight (micrograms) of microplastics found in the intestinal content of the specimens collected at each site, according to their size (total length).

Table 7. 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 (cm)	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	$<2e^{-16}$ ***

In Taliarte, a total of 2695 microplastic particles were found (n= 20 specimens). The average number of microplastic particles was 96.25 particles. The largest particles (1-7 mm) were the most abundant, corresponding to 73.68%, while small particles (<1 mm) only accounted for 26.32% of the total. In general, the larger (total length) the holothurians from Taliarte, the larger the number and weight of particles (Figures 14 and 15, tables 6 and 7).

4. DISCUSSION

4.1. Presence of pathogenic bacteria and antimicrobial resistance of bacteria

Holothurians are filter-feeder animals, which consume sediment, taking advantage of the microbiota, organic matter and nutrients of the environment they inhabit (Navarro, 2012). Therefore, they are exposed to multiple pathogenic bacteria from the environment, or from any source of pollution (e.g. sewage water) near the coast.

With regard to the overall results of antimicrobial susceptibility in different bacteria (Table 2), all isolates were susceptible to imipenem and gentamicin (100%). For *Vibrio* and *E. coli*, high percentages of resistance were found to ampicillin (100%). Also, high percentages of resistance were found to ampicillin in *Citrobacter freundii* (83 %) and NFB (68%). Moreover, high percentages of resistance for *Enterobacter cloacae* to amoxicillin-clavulanic acid were found (100%). For *Leclercia adecarboxylata,* 83% showed intermediate resistance to enrofloxacin and high percentages of susceptibility to imipenem, ceftazidime and gentamicin were found (100%).

Staphylococcus aureus was not present in any of the samples. A total of 63 MSA isolates, suspected to be *Staphylococcus aureus* were not confirmed. In addition, no *Salmonella* spp. was isolated from any samples of the three sites.

Vibrio alginolyticus is a Gram-negative marine bacterium that belongs to the genus *Vibrio,* of the family *Vibrionaceae*; *V. alginolyticus* is a pathogen in fish and bivalves. It is associated with massive mortalities in bivalve larvae and presents antibiotic resistance (Díaz-Sol Sol et al., 2019). In a previous study (Jiang et al., 2014), *Vibrio parahaemolyticus,* from cultured sea cucumbers, showed resistance to ampicillin and cefazolin; less of them were resistant to streptomycin, cefuroxime sodium, tetracycline, sulphamethoxazole/trimethoprim and quinolones. In 20 samples of the sea cucumber *Stichopus horrens* from the Pangkor Island (Malaysia), authors isolated different *Vibrio* spp. (only 5% were *V. alginolyticus*). The outcome of the antimicrobial susceptibility pattern indicated that 100% were resistant to ampicillin (Chanderan et al., 2019). This confirms the findings of this project.

The presence of *Enterobacteriaceae*, was very low, 20 in total from 79 animals. In zone 1, we found one *Enterobacter cloacae* and four *Leclercia adecarboxylata*. In zone 2, we found seven *E. coli* and two *Leclercia adecarboxylata* and in zone 3, six *Citrobacter freundii*. All of them belong to family *Enterobacteriaceae* and are Gram-negative bacteria present (as a local microbiota) in the digestive system of animals and humans.

A study that investigated the frequency of isolation and antimicrobial susceptibility profiles of bacteria isolated from cloaca of migratory Passeriformes (Basilicata, Italy), identified *Enterobacter cloacae* (21 strains), *Leclercia adecarboxylata* (16 strains) and *Citrobacter freundii* (4 strains). Strains showed resistance to amoxicillin as the most frequent, followed by ampicillin, rifampicin and amoxicillin–clavulanic acid. *Enterobacter cloacae* showed resistance to 18 molecules (100%), *Citrobacter* spp. and *Leclercia adecarboxylata* showed resistance to 13 molecules (72.2%) (Foti et al., 2017). A study with the bacteria *Leclercia adecarboxylata* and *Citrobacter freundii* isolated from poultry, in South Western Nigeria (Akinbami et al., 2018) showed all *Leclercia adecarboxylata* and *Citrobacter freundi* strains were sensitive to imipenem, meropenem and amikacin. Besides, all *Leclercia adecarboxylata* strains were resistant to moxifloxacin, ceftazidime, cefepime and fosfomycin. And the only *Citrobacter freundii* strain had additional resistance to ceftazidime and cefepime.

The data from these studies is not consistent with results from this project, despite there is a clear relationship between higher levels of resistance to amoxicillin-clavulanic acid and ampicillin with these *Enterobacteria*. The results may not coincide when comparing the degree of resistance of two very different animals, such as birds and sea cucumbers.

In a study with echinoderms (*Echinoidea* and *Holothuroidea*) from the Azores islands (Portugal), antibiotic resistance of *Enterococcus* spp. and *E. coli* was evaluated in a total of 250 faecal samples. Higher percentages of resistance in *E. coli* isolates were found for streptomycin, amikacin, tetracycline and tobramycin. Also, they found a very low prevalence of *E. coli*, i.e. only 10 isolates were recovered (the number of animals in which *E. coli* is isolated represents a very small % of the total number of animals sampled) (Marinho et al., 2013). This outcome is similar to this study. In a study focusing on antimicrobial substances of potential biomedical importance from holothurian species, different bacteria were obtained, among them *E. coli*, which had a higher resistance to ampicillin and an intermediate resistance to chloramphenicol (Abraham et al., 2002). That confirms the higher percentages resistance of *E. coli* to ampicillin and tetracycline (>50%) in Table 2 and Table 4.

It should also be noted that zone 2 (Baja de Arguineguín) was the only site to show the presence of this bacterium, which makes me suppose that this species of bacteria proliferates in these waters or belongs to the biota of the marine species that inhabit this site (Table 4).

Two studies with bivalves also showed a possible alternative explanation. *Anodonta cygnea* (Da Costa et al., 2013), as a filter-feeder, is exposed to a

constant challenge by various pathogenic bacteria when growing in polluted waters. The persistence of these bacteria within bivalve tissues largely depends on their sensitivity to the bactericidal activity of the hemolymph. The bivalves had numerous *E. coli* bacilli inside granulocytes, suggesting that these freshwater mussels had the capacity to actively phagocyte *E. coli*. Also, the mussel *Mytilus galloprovincialis* (Cavallo et al., 2009) has the ability to filter and concentrate bacteria, which could contribute to the reduction of bacterial concentrations in seawater. This would explain the shortage of *E. coli* and the rest of Enterobacteria in this study; i.e., that is, the small percentage of pathogenic bacteria compared to the total number of animals sampled. Sea cucumbers could have, as filter-feeders, the capacity to phagocytize bacteria, similarly to these bivalves, which then pass *E. coli* to hemolymph and have scarce values of bacteria in their faecal material.

Overall, a lower antibiotic resistance was found in Enterobacteria and NFB from zones 1 and 2 (control zones) relative to zone 3 (contaminated zone). In the first site, Baja de Pasito Blanco, Enterobacteria did not show higher values of antibiotic resistance, while Non-fermenting bacilli (NFB) were found the higher percentages of resistance to ampicillin (80%) and cefotaxime (67%). In the second site, Baja de Arguineguín, the higher percentages of resistance were found to ampicillin (78%) for Enterobacteria, though for Non-fermenting bacilli (NFB), the higher percentages of resistance were found for cefoxitin and ampicillin (70%). On the contrary, at the third site, Taliarte, for Enterobacteria, the higher percentages of resistance were found to ampicillin (83%), while Non-fermenting bacilli (NFB) had the higher percentages of resistance to cefoxitin (65%) and ampicillin (59%). Therefore, the presence of waste-water interfere to some extent with the presence of pathogenic bacteria and antimicrobial resistance. Hence, there is a greater percentage of resistance to antibiotics in dirty (polluted) waters than in clean waters; this is true for Enterobacteria in Taliarte, but not NFB. Still, sample size is modest to confirm any effect of the waste-water at a microbiological level on these animals.

Besides, the percentage of resistance in Enterobacteria was higher in Taliarte, while in the two control sites this resistance is subtler or sparse. In contrast, at least more than 50% of the NFB in the three sites were susceptible to almost all antibiotics and in all three cases, imipenem and gentamicin are the most susceptible (100%). Also, in all sites, both Enterobacteria and NFB have higher percentages of resistance to ampicillin (>50%), except Enterobacteria of Baja de Pasito Blanco, which have the higher percentage of susceptibility to ampicillin (80%).

4.2. Presence of microplastics

This study has showed a clear difference in the amount (number and weight) of microplastics found in the intestines of sea-cucumbers between a site under large pollution (Taliarte) and two unpolluted controls. Sea-cucumbers from those sites in the south of Gran Canaria presented a low amount of microplastic particles due to their distance from the coast and their clean waters, in comparison with Taliarte, which was more strongly affected by anthropogenic activities. Taliarte is affected by direct contamination, both from the two submarine outfalls near the port and from the plastic wastes generated by boats, fishermen (anglers) and sunbathers that often are found in the site.

In a previous study, the presence of microplastics was evaluated in holothurians of 114 beaches of the Greek islands, where an average of 4.68 ingested particles per individual was found. The most contaminated sites were at the Attica peninsula and the island of Ikaria, with an average amount of microplastics of 20.13 ± 16.03 (SD) and 19.79 ± 10.74 (SD) particles per 50 ml of sediment (Seary et al., 2013). On the island of Samos, samples of *Holothuria poly* and *Holothuria tubulosa* were taken at 5 sites in the coastal zone and microplastics were found in all samples, with an average of 1.388 ± 0.041 (SD) particles per individual (Miliou et al., 2016).

Research from eight *Apostichopus japonicus* farms in the waters of China showed that the abundance of microplastics in the dry sediment ranged from 20 to 1040 particles; the ingestion of these particles ranged from 0 to 30 per gut, and the filtering the coelomic fluid varied from 0 to 19 particles (Mohsen et al., 2019).

Comparing all these data with the average number of particles we here obtained in individuals from Taliarte (96.25 particles), suggests that the abundance of particles in the intestines depends on their proximity to the coast, the degree of pollution and the industrial and tourist activity of the sites where the study is carried out. This study has not assessed whether ingestion of microplastics can affect holothurians. However, it is possible that there will be effects at the cellular or molecular level in the long term (Assidqi, 2015; Grossmann, 2014).

5. CONCLUSIONS

There is no presence of *Staphylococcus aureus* and *Salmonella* spp. from any of the three sites. However, bacteria (Enterobacteria and NFB) is present in individuals from the three sites. Still, they are found in very low concentrations compared to the total number of animals sampled. These low concentrations of bacteria could be explained

because sea cucumbers have, as filter-feeder, the capacity to phagocytize bacteria, similar to bivalves, e.g. *Anodonta cygnea* and *Mytilus galloprovincialis*, which passes *E. coli* to hemolymph.

High to resistance to ampicillin (>50%) was found at the three sites. At the three sites, 100% of susceptible bacteria to imipenem and gentamicin were found.

Also, there is an overall greater resistance to antibiotics in the polluted site than in the controls, in particular for Enterobacteria. This confirms the objective of this study, i.e. whether bacteria were more resistant in contaminated than in clean waters. This result deserves caution, because our sample sizes were small and only 1 polluted site was investigated.

A very small amount of microplastics were found on control sites, while sea cucumbers from the polluted site contained large amounts of these.

In brief, *Holothuria sanctori* is a very good bio-indicator of the presence of pollution.

ANNEX II

Appendix A: Antimicrobial susceptibility in bacteria from Baja de Pasito Blanco

Table 3. Percentages of antimicrobial susceptibility in different bacteria from Baja de Pasito Blanco.

ZONE 1: Baja de Pasito Blanco				
Antibiotic	**Species**	**Resistant**	**Intermediate**	**Susceptible**
Ceftazidime (CAZ)	Enterobacteria	-	-	100
	Non-fermenting bacilli (NFB)	17	10	73
Amoxicillin/Clavulanic Acid (AMC)	Enterobacteria	40	20	40
	Non-fermenting bacilli (NFB)	57	10	33
Enrofloxacin (ENO)	Enterobacteria	20	20	60
	Non-fermenting bacilli (NFB)	3	30	67
Tetracycline (TE)	Enterobacteria	-	40	60
	Non-fermenting bacilli (NFB)	3	14	83
Cefoxitin (FOX)	Enterobacteria	-	40	60
	Non-fermenting bacilli (NFB)	10	10	80
Imipenem (IPM)	Enterobacteria	-	-	100
	Non-fermenting bacilli (NFB)	-	-	100
Ciprofloxacin (CIP)	Enterobacteria	-	-	100
	Non-fermenting bacilli (NFB)	-	-	100
Gentamicin (GM)	Enterobacteria	-	-	100
	Non-fermenting bacilli (NFB)	-	-	100
Cefotaxime (CTX)	Enterobacteria	-	20	80
	Non-fermenting bacilli (NFB)	67	23	10
Ampicillin (AM)	Enterobacteria	-	20	80
	Non-fermenting bacilli (NFB)	80	10	10

*5 Enterobacteria (1 *Enterobacter cloacae* and 4 *Leclercia adecarboxylata*) and 28 Non-fermenting bacilli (NFB) (20 *Pseudomonas*).

Appendix B: Antimicrobial susceptibility in bacteria from Baja de Arguineguín

Table 4. Percentages of antimicrobial susceptibility in different bacteria from Baja de Arguineguín.

ZONE 2: Baja de Arguineguín				
Antibiotic	**Species**	**Resistant**	**Intermediate**	**Susceptible**
Ceftazidime (CAZ)	Enterobacteria	-	-	100
	Non-fermenting bacilli (NFB)	-	-	100
Amoxicillin/Clavulanic Acid (AMC)	Enterobacteria	33.33	44.33	22.33
	Non-fermenting bacilli (NFB)	60	-	40
Enrofloxacin (ENO)	Enterobacteria	-	22	78
	Non-fermenting bacilli (NFB)	-	40	60
Tetracycline (TE)	Enterobacteria	56	11	33
	Non-fermenting bacilli (NFB)	-	10	90
Cefoxitin (FOX)	Enterobacteria	11	-	89
	Non-fermenting bacilli (NFB)	70	20	10
Imipenem (IPM)	Enterobacteria	-	-	100
	Non-fermenting bacilli (NFB)	-	-	100
Ciprofloxacin (CIP)	Enterobacteria	-	22	78
	Non-fermenting bacilli (NFB)	-	-	100
Gentamicin (GM)	Enterobacteria	-	-	100
	Non-fermenting bacilli (NFB)	-	-	100
Cefotaxime (CTX)	Enterobacteria	-	11	89
	Non-fermenting bacilli (NFB)	70	30	-
Ampicillin (AM)	Enterobacteria	78	11	11
	Non-fermenting bacilli (NFB)	70	10	20

*9 Enterobacteria (7 *E. coli* and 2 *Leclercia adecarboxylata*) and 10 Non-fermenting bacilli (NFB) with antibiogram.

Appendix C: Antimicrobial susceptibility in bacteria from Taliarte

Table 5. Percentages of antimicrobial susceptibility in different bacteria from Taliarte.

ZONE 3: Taliarte				
Antibiotic	**Species**	**Resistant**	**Intermediate**	**Susceptible**
Ceftazidime (CAZ)	Enterobacteria	50	-	50
	Non-fermenting bacilli (NFB)	7	2	91
Amoxicillin/Clavulanic Acid (AMC)	Enterobacteria	67	-	33
	Non-fermenting bacilli (NFB)	35	20	45
Enrofloxacin (ENO)	Enterobacteria	66.67	16.67	16.67
	Non-fermenting bacilli (NFB)	4	24	72
Tetracycline (TE)	Enterobacteria	-	-	100
	Non-fermenting bacilli (NFB)	2	4	93
Cefoxitin (FOX)	Enterobacteria	67	0	33
	Non-fermenting bacilli (NFB)	65	13	22
Imipenem (IPM)	Enterobacteria	-	-	100
	Non-fermenting bacilli (NFB)	-	-	100
Ciprofloxacin (CIP)	Enterobacteria	67	0	33
	Non-fermenting bacilli (NFB)	2	2	96
Gentamicin (GM)	Enterobacteria	-	-	100
	Non-fermenting bacilli (NFB)	-	-	100
Cefotaxime (CTX)	Enterobacteria	33.33	33.33	33.33
	Non-fermenting bacilli (NFB)	24	48	28
Ampicillin (AM)	Enterobacteria	83	-	17
	Non-fermenting bacilli (NFB)	59	26	15

*6 Enterobacteria (6 *Citrobacter freundii*) and 39 Non-fermenting bacilli (NFB) (20 *Pseudomonas*).

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VALORACIÓN PERSONAL

1. **Descripción detallada de las actividades desarrolladas durante la realización del TFT.**
    - Búsqueda y recopilación de artículos científicos relacionados con el proyecto.
    - Lectura y desarrollo de la bibliografía de la memoria.
    - Lectura, recolección y organización de datos de laboratorio.
    - Realización del mapa de situación (área de estudio) con QGIS y Visor Grafcan (IDECanarias).
    - Tratamiento de datos de laboratorio con Microsoft Excel para la obtención de porcentajes de los datos de susceptibilidad antimicrobiana para las diferentes bacterias (Tablas).
    - Graficación de los datos de susceptibilidad antimicrobiana para las diferentes bacterias (Rstudio).
    - Graficación y desarrollo estadístico de los datos del contenido en microplásticos (Rstudio y Sigmaplot).
    - Redacción del manuscrito en inglés.
    - Realización de la presentación oral.

2. **Formación recibida (cursos, programas informáticos, etc.).**

A lo largo de toda la realización del TFG he utilizado múltiples de páginas webs y programas informáticos que me han facilitado la redacción de la memoria escrita.

- Visor Grafcan (IDECanarias) y QGIS para la realización de los mapas.
- Microsoft Excel para el tratamiento y recogida de los datos de laboratorio.
- Rstudio para la graficación de los datos de susceptibilidad antimicrobiana y de los datos de los microplásticos del contenido fecal de las holoturias.
- Sigmaplot para el desarrollo estadístico de los datos de los microplásticos del contenido fecal de las holoturias.
- Mendeley y Zotero para el desarrollo de la bibliografía de la memoria.

3. **Nivel de integración e implicación dentro del departamento y relaciones con el personal.**

El nivel de integración e implicación desde el primer momento ha sido muy bueno y correcto. Desde el primer día me han hecho sentir integrada y tengo muy buena relación tanto con mis tutores como con todas las personas que colaboran en el laboratorio de la FCCS. Me han explicado siempre todo aquello en lo que he tenido dudas, recomendándome lecturas científicas para llevar a cabo el TFG, explicándome toda la

metodología en detalle y disponiendo de un contacto telefónico que me ha facilitado una comunicación más rápida con ambos tutores.

4. **Aspectos positivos y negativos más significativos relacionados con el desarrollo del TFT.**

Aspectos positivos:

- Tener conocimientos previos sobre la temática.
- Facilidad a la hora de pedir tutorías.
- Disponer de un contacto telefónico con ambos tutores que me ha llevado a tener una comunicación más rápida con ellos.
- Recomendaciones de lecturas científicas.
- Ayuda por parte de los tutores a la hora de graficar y del desarrollo estadístico de los datos con los programas Rstudio y Sigmaplot.
- Ayuda por parte de los tutores con la redacción en ingles de la memoria escrita.
- Recomendación de uso del programa Mendeley para la bibliografía.

Aspectos negativos:

- Pérdida de tiempo en el muestro, análisis de las muestras y su lectura causada por el parón producido por la epidemia del COVID-19.
- No haber terminado con el análisis de las muestras y no haber realizado el muestro de una cuarta zona de estudio (sitio contaminado) para poder tener un TFG más completo.

5. **Valoración personal del aprendizaje conseguido a lo largo del TFT.**

La valoración de este TFG en general ha sido buena debido a que tengo una relación correcta con ambos tutores, que me han ayudado a lo largo de todo el proceso. También he aprendido a tener más paciencia debido a que la cosas nunca suceden como uno planea y tienes que buscar otras alternativas para poder avanzar y terminar. Pero el parón causado por esta emergencia nacional me ha llevado a perder mucho tiempo de análisis en el laboratorio, lo que me ha perjudicado de manera negativa en el desarrollo de los resultados al no haber podido terminar el análisis de la cuarta zona del proyecto; lo que ha repercutido en tener que presentar mi TFG en la convocatoria extraordinaria y no en la convocatoria ordinaria como deseaba.