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Research article

Distribution of antibiotic-resistant bacteria in the Abyssal sea and the strengthening of resistance traits by exposure to microplastics

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Abstract

Although residues from the antibiotics found in marine products were originally examined as a pressing problem facing marine life, marine bacteria's resistance to antibiotics has recently come to the fore as a central environmental issue. This study was conducted to examine antibiotic resistance levels among different marine samples and to show the connection between marine bacteria's resistance to antibiotics and their exposure to microplastics. Marine bacteria were collected from coastal seawater, abyssal seawater, and coastal sand, and intestinal bacteria were collected from deep-sea fish, coastal fish, shellfish, and rotten fish; each of these samples of bacteria was tested for resistance to four types of antibiotics (meropenem, streptomycin, penicillin, and vancomycin). Among the types of bacteria collected from the coastal sand, 87.1% to 100% showed resistance to one or more of the selected antibiotics, and among the types of bacteria collected from the coastal waters, 84.6% to 100% showed antibiotic resistance. In addition, when the distribution of resistant bacteria was observed in intestinal bacteria collected from coastal and abyssal fish, the intestinal bacteria of abyssal fish had lower rates of resistance to antibiotics than that of coastal fish; moreover, 100% of versatile resistant bacteria (which showed resistance to more than three types of antibiotics) found in the intestines of coastal fish were resistant to antibiotics while, among abyssal fish, only 92% showed resistance. Among the intestinal bacteria found in shellfish and decomposing fish except for one type of sensitive bacteria, all types of bacteria were resistant to vancomycin and meropenem. Finally, as a result of culturing antibiotic-resistant bacteria with microplastics, 11 out of 12 types of bacteria developed antibiotic resistance, showing that exposure to microplastics can cause antibiotic resistance. Based on these results, it can be concluded that microplastics contribute to the development of antibiotic resistance among marine bacteria.

Keywords: Antibiotic resistance, marine bacteria, microplastics

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Introduction

Dr. Anne Leonard (2018) of Exeter University in England collected and analyzed 273 stool samples of English surfers and reported that they had three times more cefotaxime-resistant E.coli than among samples of people who did not surf, possibly due to surfers drinking so much more seawater than non-surfers. Leonard et al. (2018) reported the growing amount of antibiotic residues found in marine products due to the abuse or misuse of antibiotics on marine farms arose as a problem (Cabello, 2006) and now, 13 years later, the number of antibiotic-resistant bacteria appearing among marine bacteria has become a more significant problem.

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Bacteria found in coastal waters often achieve resistance to antibiotics due to the overuse of antibiotics on marine farms; on the other hand, significant distributions of antibiotic-resistant bacteria in the oceanic zone, particularly in deep-sea waters, have not been discovered. When bacteria in the oceanic zone show antibiotic resistance, two hypotheses can be suggested regarding their cause: First, the bacteria can develop antibiotic resistance due to gene splicing with antibiotic-resistant bacteria in coastal waters and the propagation of resistant traits. A research team from Uppsala University, Sweden, reported in *PLOS Pathogens* that "even antibiotics with very low concentration can increase the resistant bacteria (Koffmar, 2018) This research shows that antibiotics released by humans and animals can be the cause of the appearance of antibiotic-resistant bacteria: Bacteria that spread from marine farms increase the pollution of the sea nearby, and this can cause marine bacteria to gain antibiotic resistance; these antibiotic-resistant bacteria then transfer the resistance gene to other bacteria, propagating the trait to the bacteria living offshore.

Second, the antibiotic-resistant bacteria can develop resistance because of the chemical substances produced by microplastics, which are generated by the plastics found in polluted waters. Marine pollution is defined as the water quality change or impact on the physical, chemical, and biological environment by humans injecting substances or energy into the sea (Lebreton et al. 2018). The size of the garbage patch on the ocean was about half of South Korea in 2011, but now it has increased to 1,550,000km^2, which is seven times larger than North and South Korea combined (Lebreton et al. 2018) Among the trash found in the ocean, micro plastics have been raised as the most serious problem. It is estimated that about 150 million metric tons of plastic are floating on the ocean, and the plastic does not decompose but breaks into smaller pieces (Derraik (2002). In 2014, a Chinese research team reported that 1-butyl-3-methylimidazolium hexafluorophosphate, a non-water-soluble ionic liquid, strengthens the transfer of antibiotic resistance genes between bacteria. Likewise, some types of chemical substances, including certain detergents and pesticides, can strengthen the propagation of antibiotic resistance genes (Liu et al. 2014). In recent years, organosynthetic chemical substances contained in microplastics and different chemical substances from marine pollution have been detected in the seawater (Connor, 2009; Koelmans et al. 2016). If antibiotic resistance genes can be strengthened by those substances, it will have an impact on marine bacteria in the abyssal waters.

The purpose of this study is to address this issue through the following: First, the antibiotic resistance of bacteria collected from coastal fish, coastal and abyssal waters, and coastal sand was tested and will be discussed. Second, the antibiotic resistance of bacteria collected from deep-sea fish was tested and will be compared with that of coastal fish. Third, the antibiotic resistance of marine bacteria exposed to microplastics was studied and will be examined as a way to prove that the development of antibiotic-resistant bacteria can be caused by marine pollution.

Materials and Methods

1. Preparing Samples

Samples of bacteria from seawater, fish, and shellfish of different regions were prepared. More than 2L of seawater from the coastal ocean and deep sea near Incheon, Gangleung, and Samcheok were collected, and fish and shellfish were obtained from fishing boats and fish markets near these areas. Different types of fish from coastal and abyssal ocean waters were prepared, and they were transported in an icebox to the lab with no dissecting procedure. Decomposition fish were also transported in an icebox to the lab.

Table 1: Types of Fish and Shellfish from Each Location

Samples	Region	Location		
		Ulsan	Samcheok	Incheon
Fish	Coastal	flounder, yellowtail	flounder, sandfish, codfish etc.	croaker, gizzard
	Deep sea	-	moray	croaker
	Decomposed	-	flounder, codfish	-
Shellfish	Aerobic	clam	conch, abalone, scallop, clam, lanceolaria grayana	abalone

Fig. 1: Classifying different fish and shellfish from different areas 1.

Fig. 2: Classifying different fish and shellfish from different areas 2.

2. Producing Bacteria Media

Marine agar (MA) media with a similar concentration and composition to seawater were produced. After 55.1g of MA media was mixed with 1L of distilled water, the mixture was sterilized in a sterilizer at 121℃, 1.2atm for 15 minutes. The mixture was moved to a clean bench, and after it cooled down to 50℃, it was poured into petri dishes and solidified.

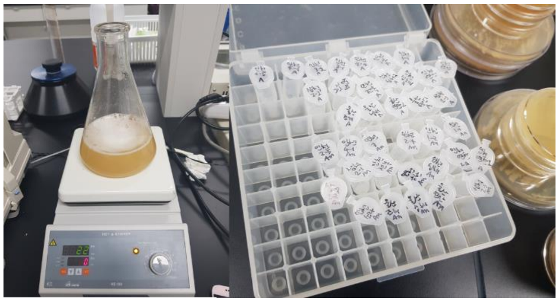

Fig. 3: Producing MA media and culturing bacteria.

3. Culturing Bacteria

Bacteria from seawater, sand, and the internal organs of fish were cultured in MA media. When the bacteria colonies were made, the colonies were separated and cultured. After dissecting the fish with sterilized tools and separating the internal organs, the contents in the organs were smeared on MA media by a streaking method and were cultured at room temperature for 1 week. The contents in the organs of the shellfish were also cultured by the same method, and seawater was cultured by dipping a loop into the water and smearing the sample on MA media. In the case of the sand, it was mixed with distilled water in a ratio of 1:4, and the supernatant was smeared on MA media. After 1 week, the colonies grown in all media were classified, and each type was moved into new media and cultured separately.

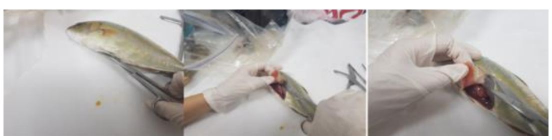

Fig. 4. Dissecting fish and shellfish in order to separate intestinal bacteria.

4. Antibiotic Resistance Test – Agar Diffusion Test

Four types of antibiotics, including meropenem and vancomycin, which are known to be effective on super bacteria, and streptomycin and penicillin, which are traditional antibiotics, were used to observe the antibiotic resistance of each type of bacteria. A bacteria solution of 20uL, which went through a separation culture, was dropped and smeared onto MA media. After the holes were made on four different locations of the media with the top part of the white tip, 10uL of each type of antibiotic was put into each hole. The samples were cultured at room temperature for 1 week, and afterward, the size of the zones surrounding each hole was observed and recorded.

5. Obtaining Antibiotic Resistance from Microplastics

Following the tests described above, the intestinal bacteria that proved to be sensitive to the select antibiotics were cultured with microplastics to observe their antibiotic resistance. First, 5g of facial cleanser was mixed with 20mL of distilled water, and 1mL of this solution was put into each micro tube. Second, centrifugation was conducted on the solution at 13000rpm for 5 minutes. The supernatant was discarded and 1mL of distilled water was added to each micro tube. This procedure was repeated five times so that the pure micro plastic can be separated. Third, 500uL of the micro biz solution and 500uL of the bacterial solution were mixed, and the mixture was cultured at room temperature for 1 week. Finally, the antibiotic resistance test was conducted using the four types of antibiotics.

Results

1. Separation of Bacteria from Seawater and Soil in Each Location and Distribution of Antibiotic-Resistant Bacteria

To test the significance of the antibiotic-resistant bacteria in the marine ecosystem, coastal sand and seawater were collected in Samcheok and Ulsan, which are in the East Sea area, and in Incheon, which is in the West Sea area; in Samcheok, seawater along the shoreline and from deep-sea areas was also collected.

A. Separating Marine Bacteria

As a result of separating the bacteria from the sand samples using MA media, 16 types of aerobic bacteria and 15 types of anaerobic bacteria were found. Fifteen types of bacteria were separated from the sand from Incheon, which is twice as much as the number of types separated from each of the other areas. Through the same separation method, six types of aerobic and seven types of anaerobic bacteria were separated from the seawater samples, and among them, one type of aerobic and two types of anaerobic bacteria were separated from deep-sea water near Samcheok. Each type was classified by the shapes of its colonies and named using numerals and letters.

B. Checking Antibiotic Resistance

An agar diffusion test was conducted to check the resistance of each type of bacteria separated from the procedure described in section A using four types of antibiotics: meropenem, streptomycin, penicillin, and vancomycin.

Table 2: Number of Species of Marine Bacteria Separated from Sand and Seawater from Different Areas

Samples	Culture Condition	Location		
		Ulsan	Samcheok	Incheon
Coastal Sand	Aerobic	2	5	9
	Anaerobic	6	3	6
Seawater	Aerobic	2	2(1)*	2
	Anaerobic	2	4(2)*	1

*Among bacteria in Samcheok, one type of aerobic and two types of anaerobic bacteria were separated from within the deep sea water

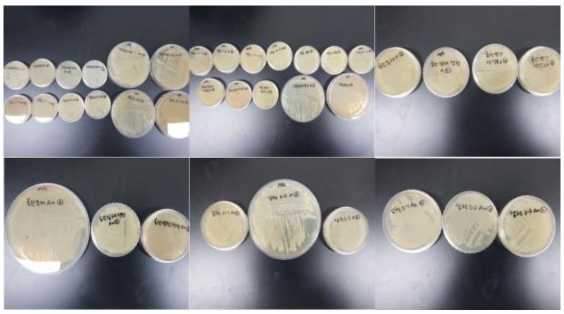

Fig. 5. Separated and cultured marine bacteria.

Fig. 6. Bacteria that dissolve agar were separated but could not be used in the experiment.

As a result, among the 16 types of aerobic bacteria separated from the sand, all types showed resistance to meropenem and streptomycin. Twelve types showed resistance to penicillin, and 11 types showed resistance to vancomycin. Among 15 types of anaerobic bacteria found in the samples of sand, 11 types showed resistance to meropenem, and all showed resistance to streptomycin, penicillin, and vancomycin. All six types of aerobic bacteria found in the seawater showed resistance to meropenem and penicillin, and five types showed resistance to streptomycin and vancomycin. All seven types of anaerobic bacteria found in the seawater showed resistance to streptomycin and penicillin, and six types showed resistance to meropenem and vancomycin.

Among aerobic bacteria, all types of bacteria separated from the samples of sand were resistant to meropenem and streptomycin, and all types of bacteria in the seawater samples were resistant to meropenem and penicillin. Among the bacteria found in the samples of sand, 75% were resistant to penicillin and 68.8% to vancomycin. Among the seawater samples, 83.3% of the bacteria collected were resistant to streptomycin and vancomycin.

Table 3: Number of Antibiotic-Resistant Bacteria Separated from Coastal Sand and Seawater

Bacterial Origin	Culture Condition	Species	Antibiotics			
			Meropenem	Streptomycin	Penicillin	Vancomycin
Coastal Sand	Aerobic	16	16	16	12	11
	Anaerobic	15	11	15	15	15
Seawater	Aerobic	6	6	5	6	5
	Anaerobic	7	6	7	7	6

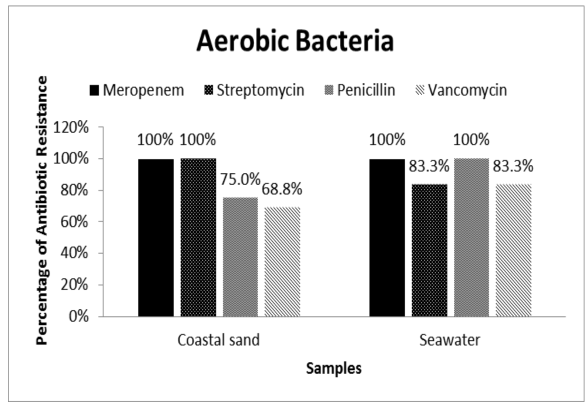

Fig. 8. Percentage of antibiotic resistance among aerobic bacteria separated from coastal sand and seawater.

Among anaerobic bacteria, all types of bacteria found in the samples of sand were resistant to streptomycin, penicillin and vancomycin, and all types of bacteria found in the seawater were resistant to streptomycin and penicillin. Among the bacteria separated from the sand, 73.3% were resistant to penicillin, and among the bacteria separated from the seawater, 85.7% were resistant to meropenem and vancomycin.

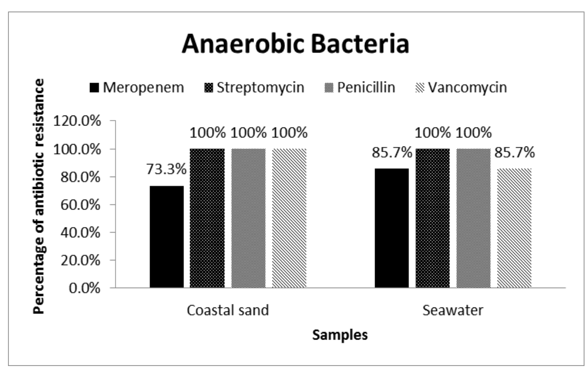

Fig. 9: Percentage of antibiotic resistance among anaerobic bacteria separated from coastal sand and seawater.

All bacteria observed were multi-drug resistant bacteria, which include bacteria that have resistance to more than two types of antibiotics. Specifically, 23% of the aerobic bacteria and 27% of the anaerobic bacteria tested had resistance to three types of antibiotics, and 63% of the aerobic bacteria and 73% of the anaerobic bacteria tested had resistance to all four types of antibiotics used in the experiment.

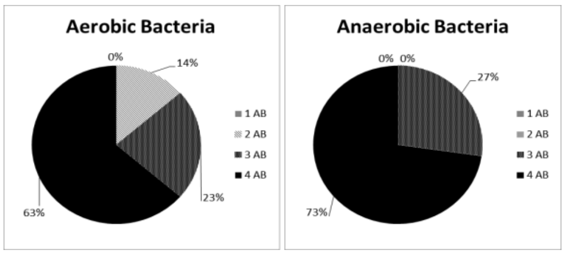

Fig. 10: Percentage of aerobic and anaerobic bacteria resistant to multiple antibiotics (from one antibiotic [1AB] to four antibiotics [4AB]).

2. Separation of the Intestinal Bacteria of Fish and Shellfish and Distribution of Antibiotic-Resistant Bacteria Among Intestinal Bacteria According to the Habitats

Experiment 1 showed that the distribution of marine bacteria containing antibiotic resistance and the percentage of multi-drug resistant bacteria among the samples collected were both considerably high. To check whether this problem is confined to the coastal area or is pervasive in the ecosystem, samples of intestinal bacteria from fish and shellfish from different habitats were collected and prepared, and the distribution of antibiotic resistance among the samples was observed.

Table 4: Distribution of Intestinal Antibiotic-Resistant Bacteria Separated from Fish and Shellfish

Bacterial Origin	Culture Condition	Species	Antibiotics			
			Meropenem	Streptomycin	Penicillin	Vancomycin
Coastal Fish	Aerobic	9	9	9	9	7
	Anaerobic	6	5	6	6	5
Deep-Sea Fish	Aerobic	7	4	7	7	5
	Anaerobic	7	7	7	7	3
Rotten Fish	Aerobic	1	1	1	1	1
	Anaerobic	1	0	1	1	1
Shellfish	Aerobic	3	3	3	3	3
	Anaerobic	4	4	4	4	3

A. Distribution of Antibiotic Resistance Among Intestinal Bacteria Collected from Coastal Fish

Nine types of aerobic bacteria and six types of anaerobic bacteria were separated from the intestines of eight types of coastal fish, including flounder and yellowtail, and the resistance of their intestinal bacteria to four types of antibiotics was tested. Except for one type of sensitive bacteria (anaerobic), which responded to meropenem, and three types of sensitive bacteria (two aerobic and one anaerobic), all other types of bacteria tested were resistant to the antibiotics used. All types of bacteria tested were resistant to streptomycin and penicillin, which have been used for a long period of time, and a few types were sensitive to meropenem and vancomycin, types of antibiotics developed relatively recently.

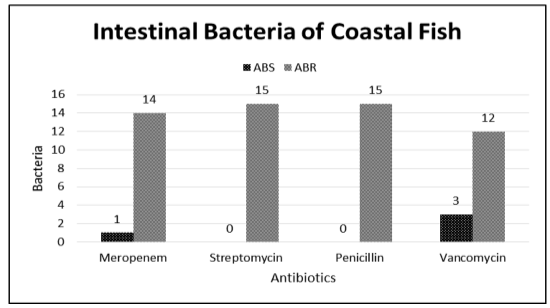

Fig. 11: Types of antibiotic-resistant intestinal bacteria collected from coastal fish.

B. Distribution of Antibiotic Resistance Among Intestinal Bacteria Collected from Abyssal Fish

Most of the intestinal bacteria collected from the coastal fish were antibiotic-resistant bacteria, and the percentage of those bacteria that were multi-drug resistant bacteria was high. To check whether the results with abyssal fish would be the same, seven types of aerobic and anaerobic bacteria were collected from the intestines of moray and croaker, and the bacteria's resistance to four types of antibiotics was checked.

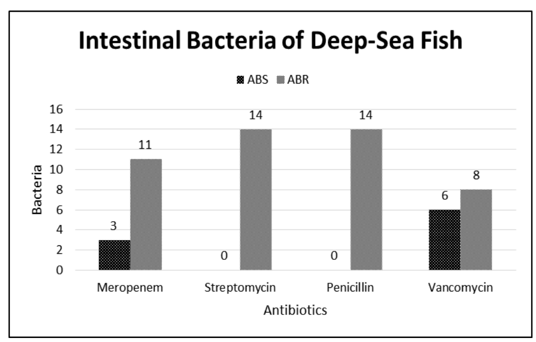

Fig. 12: Types of antibiotic-resistant intestinal bacteria collected from abyssal fish.

While all types of bacteria were resistant to streptomycin and penicillin, which have been used for a long period time, three types of bacteria (aerobic) were sensitive to meropenem, and six types (two aerobic and four anaerobic) were sensitive to vancomycin. Compared to the intestinal bacteria of coastal fish, the intestinal bacteria of abyssal fish had a higher ratio of sensitive bacteria.

However, the existence of antibiotic-resistant bacteria in the internal organs of abyssal fish shows that the antibiotic pollution of the ocean is so serious that it is affecting the deep sea as well as the seashore.

C. Distribution of Antibiotic Resistance Among Intestinal Bacteria Collected from Shellfish and Rotten Fish

The antibiotic resistance of intestinal bacteria contained in shellfish was observed. In addition, to check whether antibiotic resistance is caused by the environment of the fish market, the antibiotic resistance of internal bacteria contained in decomposed fish was also observed.

Only one of seven types of bacteria collected from shellfish showed sensitivity to vancomycin, and the rest of them were resistant to all antibiotics, including meropenem. Three types of aerobic and anaerobic bacteria were separated from the rotten fish, and only one had sensitivity to meropenem; the rest showed resistance to all types of antibiotics, including vancomycin.

Shellfish are thought to reveal the state of antibiotic pollution as they live in an environment that can be easily exposed to pollutants. Moreover, it was also checked whether the fish market, where a number of marine organisms are sold, could also be a medium for antibiotic pollution.

D. Distribution of Multi-Drug Resistant Bacteria

A number of resistant bacteria existed in the internal organs of coastal fish and abyssal fish, and all of them were multi-drug resistant bacteria, which are resistant to more than two types of antibiotics. In the case of the intestinal bacteria of coastal fish, the percentage of bacteria resistant to three types of antibiotics was 33% and 67% were resistant to four types; however, in the case of abyssal fish, the percentages were 54% and 38%, respectively.

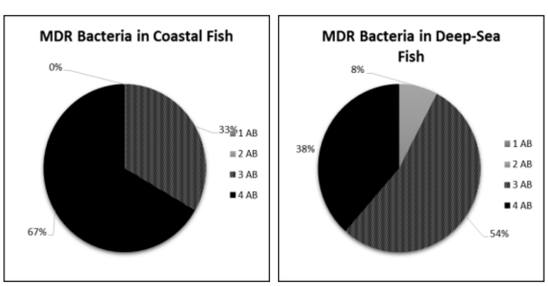

Fig. 13. Percentage of multi-drug resistant bacteria found in coastal fish and deep-sea fish (from one antibiotic [1AB] to four antibiotics [4AB]).

Most of the intestinal bacteria found in shellfish and rotten fish were resistant bacteria, and all of them had resistance to more than three types of antibiotics. In the case of the intestinal bacteria found in shellfish, the percentage of bacteria resistant to three types of antibiotics was 14%, and 86% were resistant to all four types. In the case of the intestinal bacteria found in rotten fish, 33% (1 type) of bacteria collected were resistant to three types of antibiotics, and 67% (2 types) were resistant to all four types of antibiotics used in the study.

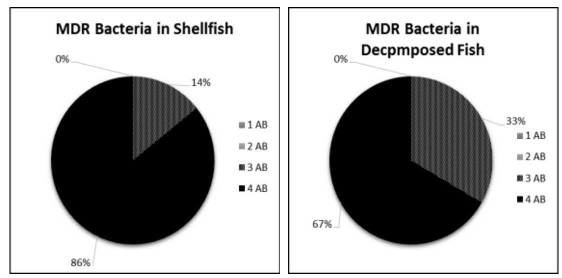

Fig. 14. Percentage of multi-drug resistant bacteria collected from shellfish and rotten fish (from one antibiotic [1AB] to four antibiotics [4AB]).

As shown in the result of the experiment, it is highly possible that the propagation of antibiotic-resistant bacteria is caused by the spread of antibiotics and related substances among marine habitats. However, as multi-drug resistant bacteria exist in abyssal fish and deep-sea waters at high rates, the study further explored whether the cause of this resistance is related to marine pollution—specifically, to microplastics.

3. Impact of Microplastics on Marine Bacteria's Development of Antibiotic Resistance

1-butyl-3-methylimidazolium hexafluorophosphate, the substance contained in many detergents and pesticides, is known to strengthen the transfer of antibiotic resistance genes among bacteria; microplastics also contain different chemical substances. As such, whether exposure to microplastics can cause sensitive bacteria to gain antibiotic resistance was tested.

A. Developing Resistance to Meropenem

Meropenem, a carbapenem-type antimicrobial, impedes the production of bacteria's cell walls and causes the bacteria to perish. It was recently developed: The patent was applied in 1983, and the first medical use was in 1996.

After the intestinal bacteria that showed sensitivity to meropenem were cultured with microplastics, a test of their antibiotic resistance test was conducted. The sensitive bacteria that originally showed a clear zone of 0.7 to 1.8cm around the meropenem injected in the agar diffusion test was not able to produce clear zone after their exposure to microplastics; in other words, the bacteria developed antibiotic resistance.

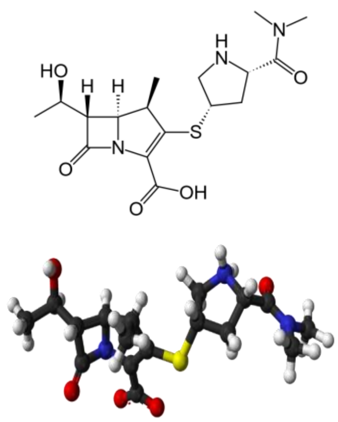

Fig. 15. Chemical structure of meropenem.

B. Developing Resistance to Vancomycin

While Vancomycin was discovered in 1956, it was not widely used for 20 years due to problems in the manufacturing process. Its effectiveness was reviewed after the manufacturing process was improved, and vancomycin proved to be effective on MRSA, a type of super bacteria; however, vancomycin-resistant enterococci (VRE) was reported in 1986.

Table 5: Change of Sensitivity to Meropenem Among Antibiotic-Sensitive Bacteria After Exposure to Microplastics (Size of Clear Zone in Centimeters)

Bacteria \ Microplastics Exposure	Sensitivity to Meropenem	
	Pre-Exposure to Microplastics	Post-Exposure to Microplastics
A7 (Deep-Sea Fish)	Antibiotic sensitive (1.8 cm)	Antibiotic resistant (0 cm)
A8 (Deep-Sea Fish)	Antibiotic sensitive (1.3 cm)	Antibiotic resistant (0 cm)
A9 (Deep-Sea Fish)	Antibiotic sensitive (0.9 cm)	Antibiotic resistant (0 cm)
An2 (Coastal Fish)	Antibiotic sensitive (1.8 cm)	Antibiotic resistant (0 cm)
An6 (Rotten Fish)	Antibiotic sensitive (0.7 cm)	Antibiotic resistant (0 cm)

Among the intestinal bacteria found in coastal fish, deep-sea fish, and shellfish, seven types of bacteria that were sensitive to vancomycin were detected, and among those seven types, four were from intestinal bacteria found in deep-sea fish. During the agar diffusion test using bacteria cultured with microplastics, six of these seven types of bacteria were not able to produce a clear zone around the vancomycin, showing that they had developed resistance to the antibiotic. Therefore, it was confirmed that microplastics can cause antibiotic resistance.

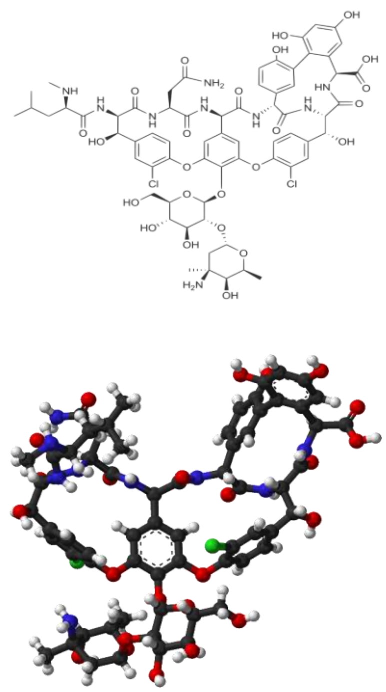

Fig. 16: Chemical structure of vancomycin.

Table 6: Change of Sensitivity to Vancomycin Among Antibiotic-Sensitive Bacteria After Exposure to Microplastics (Size of Clear Zone in Centimeters)

Bacteria \ Microplastics Exposure	Sensitivity to Meropenem	
	Pre-Exposure to Microplastics	Post-Exposure to Microplastics
A20 (Coastal Fish)	Antibiotic sensitive (0.6 cm)	Antibiotic sensitive (1.4 cm)
A9 (Deep-Sea Fish)	Antibiotic sensitive (0.7 cm)	Antibiotic resistant (0 cm)
A16 (Deep-Sea Fish)	Antibiotic sensitive (0.6 cm)	Antibiotic resistant (0 cm)
An1 (Coastal Fish)	Antibiotic sensitive (0.7 cm)	Antibiotic resistant (0 cm)
An3 (Deep-Sea Fish)	Antibiotic sensitive (1.2 cm)	Antibiotic resistant (0 cm)
An15 (Deep-Sea Fish)	Antibiotic sensitive (1.1 cm)	Antibiotic resistant (0 cm)
An22 (Shellfish)	Antibiotic sensitive (1.1 cm)	Antibiotic resistant (0 cm)

Discussion

First, the bacteria found in coastal sand were found to be resistant to all four types of antibiotics used in this experiment at the following rates: 87.1% were resistant to meropenem, 100% were resistant to streptomycin, 87.1% were resistant to penicillin, and 88.9% were resistant to vancomycin. Bacteria collected from coastal seawater were also resistant to all four types of antibiotics: 92.3% were resistant to meropenem, 92.3% were resistant to streptomycin, 100% were resistant to penicillin, and 84.6% were resistant to vancomycin. All of the bacteria tested in these samples were multi-drug resistant, and 86% of aerobic and 100% of anaerobic bacteria had resistance to more than three types of antibiotics.

Second, as a result of collecting samples of the intestinal bacteria of fish and shellfish in both coastal and abyssal waters and testing their resistance to the four selected antibiotics, the bacteria from coastal fish and shellfish revealed a higher rate of resistance to antibiotics than that of abyssal fish. In the case of the coastal fish, one type of bacteria showed sensitivity to meropenem, and three types of bacteria showed sensitivity to vancomycin. In case of the abyssal fish, three types of intestinal bacteria showed sensitivity to meropenem and six types showed sensitivity to vancomycin; despite this, the percentage of antibiotic-resistant bacteria found among the intestinal bacteria of abyssal fish was also high. Many of the bacteria tested from both coastal abyssal fish were multi-drug resistant bacteria: Among the intestinal bacteria tested from the coastal fish and abyssal fish, 100% and 92% were resistant to more than three types of antibiotics, respectively. Among intestinal bacteria collected from shellfish, one type was sensitive to vancomycin; furthermore, one type of intestinal bacteria from the rotten fish was sensitive to meropenem. The rest of the bacteria tested from shellfish and rotten fish showed resistance to antibiotics, confirming that environmental pollution is one cause of the bacteria's resistance to antibiotics.

Finally, all five types of bacteria that were sensitive to meropenem and six of the seven types of bacteria that were sensitive to vancomycin developed a resistance to antibiotics after they were cultured with microplastics. This confirms that microplastics are one cause of the development of antibiotic resistance among bacteria.

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