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Characterization of Heavy Metal and Antibiotic Resistant Bacteria Isolated from Aliaga Ship Dismantling Zone, Eastern Aegean Sea, Turkey

Kacar, A.[1*] and Kocyigit, A.[2]

[1] DEU, Institute of Marine Sciences and Technology, Baku Bul. No:100, 35340, Inciralti, Izmir, Turkey

[2] Ege University, Faculty of Science, Biology Department, Basic and Industrial Microbiology Section, 35040, Bornova, Izmir, Turkey

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ABSTRACT: In this study, it was aimed to determine the effects of ship dismantling zones on distribution of heavy metal resistance and level of antibiotic resistance of bacterial isolates from Eastern Aegean Sea coast. Thirteen isolates were identified by phylogenetic analysis using 16S rDNA sequences which indicated that the isolates belonged to genus *Bacillus*. These strains were investigated in respect of the minimum inhibitory concentrations (MICs) of heavy metals (Hg, Cu, Cd, Cr, Zn, Co, Ni, Pb and As) and susceptibility of some antibiotics (ampicillin, tetracycline, chloramphenicol, vancomycin, gentamicin and tobramycin). The MICs of heavy metals were different for each strain but the general order of resistance to the metals was found to be as Pb>As>Ni>Co>Cu>Zn>Cr>Cd>Hg and the toxic effects of these metals increased with increasing concentration. It can be concluded that all isolates were sensitive to Hg but were highly resistant to Pb, As and Ni. Additionally, it was found that the strains were resistant to gentamicin followed by tobramicin. The studies suggest that sediment bacteria in a ship dismantling area can be biological indicators of heavy metal contamination.

Key words: Hazardous wastes, Heavy metal resistance, Sediments, Ship dismantling zone

INTRODUCTION

Ship dismantling activities lead to many problems such as the discharge of detrimental and persistent pollutants affecting the coastal zone where dismantling is conducted, deep sea water and sediment. Such activities mostly cause pollutants such as heavy metals and petroleum hydrocarbons to spread into environment, and the severity of the problem depends on the size and function of the ship (Hossain and Islam, 2006; Reddy *et al.*, 2005). Heavy metals are found in many parts of ships such as in batteries (Pb, Cd, Ni) coatings and paint (Cu, Zn, Cl, TBT) and electrical system (Cu, Pb, Hg). Heavy metals and their compounds are major pollutants in the coastal environment (Ghaderi *et al.*, 2012; Serbaji *et al.*, 2012, Ajibola. and Ladipo, 2011). Presence of these heavy metals in the marine environment may pose a serious threat to the environment because of their ability to persist for several decades (Kamala-Kannan and Lee, 2008; Matyar, 2012). The main threats to human health from heavy metals are associated with exposure to lead, cadmium, chromium, mercury and arsenic (Nithya *et al.*, 2011). Arsenic and chrome are classified as priority pollutants by the United States Environmental Protection Agency (US EPA) with a carcinogenicity classification A (human carcinogen), while cadmium and lead are classified in the same list with a carcinogenicity classification B (probable human carcinogen) (US EPA, 1999; Pazi, 2011). Persistent toxic metals that settle down on the sediment are a threat to the survival of all organisms and to biodiversity (Nasrabadi *et al.*, 2010; Haruna *et al.*, 2011). Through bioaccumulation such metals enter in the food chain and bio-magnified. Concentration of some metals exceeding the tolerance limit is a threat to the fisheries, found in a more recent investigation in the fishes from ship breaking area (Hossain and Islam, 2006).

Although microorganisms are sensitive to various concentrations of heavy metals, they have mechanisms which enable them to proliferate in the environment by rapidly adapting to that environment and to convert heavy metals into harmless forms through biosorption or enzymatic transformation. Heavy metal resistant microorganisms may be useful as indicators of potential toxicity to other forms of life and are important in studies of genetic transfer in heavy metal resistance mechanism (Jansen *et al.*, 1994;

*Corresponding author E-mail:asli.kacar@deu.edu.tr

De Rore *et al.*, 1994). Thus, individual bacterial strains will develop their capacity to survive under toxicological stress and will be of importance in future bioremediation strategies (Kamala-Kannan and Lee, 2008; Valls and De Lorenzo, 2002; Gadd, 2000).

In this study, we examined isolation and identification of sediment bacteria from the ship dismantling area in Aliaga (Aegean Coastline of Turkey). These strains were investigated in respect of the minimum inhibitory concentrations (MICs) of heavy metals (Hg, Cu, Cd, Cr, Zn, Co, Ni, Pb and As) and susceptibility of some antibiotics (ampicillin, tetracycline, chloramphenicol, vancomycin, gentamicin and tobramycin).

MATERIALS & METHODS

Aliaga is the main ship dismantling site of Turkey around 50 km north of Izmir at the Aegean coast. Ship breaking in Aliaga began in mid-1970s and officially in 1984 (Hossain and Islam, 2006). Ship dismantling facilities are located on a peninsula, together with a petrochemical complex. In another area, some 15 km south of the peninsula, a number of steel works are located that use scrap iron and steel mainly from ship recyclers as their main raw material. Apart from steel, other scrap metals and alloys such as copper, bronze, brass and aluminum are also obtained, as well as certain outfit and machinery from ships that are re-used by the maritime industry (Esen *et al.*, 2010; Neser *et al.*, 2008). The study area and the locations are shown in the Fig. 1.

Sediment samples were collected during the October 2010 cruise of R.V *K. Piri Reis* using Van Veen Grab from surface sediments in Aliaga ship dismantling zone at eight locations which are showed in Fig. 1. Samples for microbiological analyses were aseptically collected and maintained at 4° C until processing in the laboratory. Diluted samples (10^{-1}-10^{-7}) were inoculated on ZoBell 2216e Medium by the spread plate method and R2A Agar Medium. The agar plates were incubated at 26° C. Bacterial colonies showing different morphological characteristics were selected and purified on ZoBell 2216 Agar plates (Nithya *et al.*, 2011; Nithya and Pandian, 2009; Kacar *et al.*, 2009). The bacterial isolates were characterized by biochemical tests (oxidase and catalase reactions, motility, utilization of sugars, growth at pH 4.5 and 9.0, tolerance to 5%, 10% and 15% NaCl, growth at 4 and 45° C).

Genomic DNA was extracted from log-phase cells of all isolates using a Bacterial Genomic DNA Purification Kit (Zymo Research, USA) after incubation in ZoBell 2216 Broth for 1 day at 26° C. The universal bacterial primers 27F and 1522R were used for polymerase chain reaction (PCR) amplification of 16S rDNA. FastStart Taq DNA Polymerase, the dNTPack Kit (Roche, Germany), 0.2 M of primers, and 10 ng of template DNA were used for PCR. PCR was performed for 30 cycles (5 min denaturing step at 95° C in the first cycle; 55 s denaturing at 95° C, 40 s annealing at 52°C, and 1.5 min polymerization at 72° C, with a final extension step at 72° C for 7 min (Kacar *et al.*, 2009). The DNA sequence analyses of purified PCR products were performed using BigDye terminator technology and an automatic sequence analyzer system (ABI Prism 3100) (REFGEN Biyoteknoloji, Turkey). The single-stranded 16S rRNA gene sequences of the 13 isolates were matched with those in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) by BLAST searching. The most similar reference sequences were downloaded and aligned with the isolate sequences using Clustal W (Version 1.81, available from (http://www.ebi.ac.ukclustalw) program. A phylogenetic tree was constructed by the neighbor-joining method, and was evaluated by bootstrap sampling (1,000 replicates) using the MEGA 4 program (Altschul *et al.*, 1997; Thompson *et al.*, 1994).

Determination of minimum inhibitory concentrations (MICs) of heavy metals by the microdilution method The Minimum Inhibitory Concentration (MIC) for each bacterial isolate for nine heavy metals was determined using Mueller–Hinton Agar (Difco, USA) containing $HgCl_2$, $CdSO_4.8/3H_2O$, $K_2Cr_2O_7$, $CuCl_2$, $ZnCl_2$, $NiCl_2.6H_2O$, $CoCl_2$, $Pb(NO_3)_2$ and $NaAsO_2$. Metal stock solutions were sterilized at 0.2 µm membrane filter. These solutions, in various concentrations according to the tested metal, were kept at 4°C for no longer than a month. Concentrations for the metals in mmol L^{-1} were ranged from 0.01-12.0. Plates were incubated at 26° C for 24-48 h. Before inoculation, the bacterial strains were adjusted to the turbidity of the 0.5 McFarland standards. Experiments were carried out in duplicate (Matyar *et al.*, 2008).

Susceptibility testing was performed by an agar diffusion test, using Mueller–Hinton Agar (Difco, USA) (Matyar *et al.*, 2008) and 6 different of antibiotics: ampicillin (AM, 10 µg), chloramphenicol (C, 30 µg), tetracycline (TE, 30 µg), gentamicin (CN, 10 µg), tobramycin (TM, 10 µg), and vancomycin (VA, 30 µg) (Bioanalyse, Turkey).

RESULTS & DISCUSSION

Thirteen bacterial isolates were obtained from Aliaga ship dismantling zone sediments. Some biochemical tests are shown in Table 1. Phylogenetic analysis using 16S rDNA indicated that the 13 strains belong to genus *Bacillus* (*Bacillus* sp. Asd1, *Bacillus* sp. Asd2, *Bacillus* sp. Asd3, *B. fusiformis* strain Asd5,

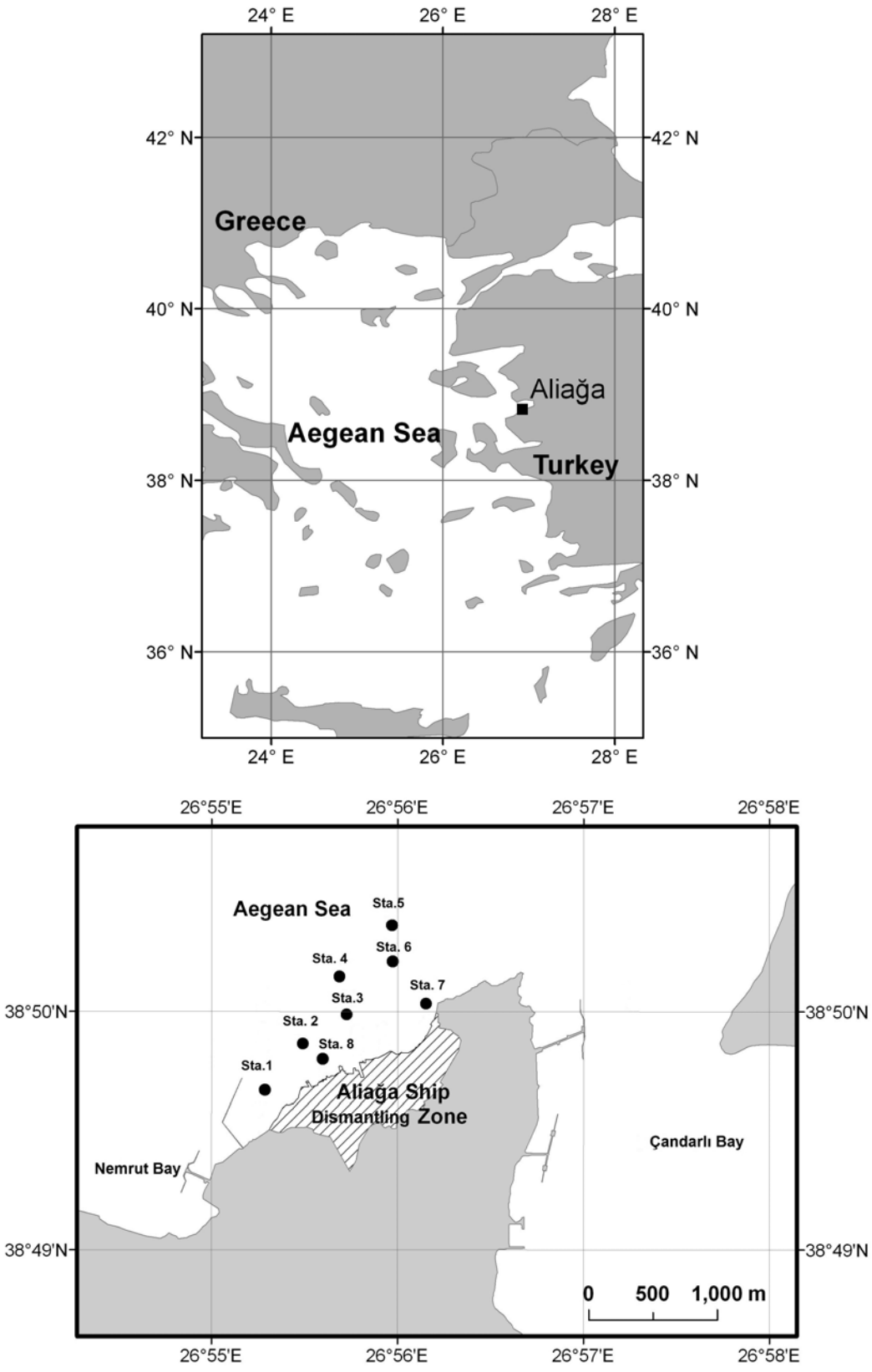

Fig. 1. Location of sampling stations in Aliaga ship dismantling zone

Table 1. Biochemical characteristics of sediment isolates

Tests	Isolates												
	Asd1	Asd2	Asd3	Asd5	Asd7	Asd8	Asd9	Asd10	Asd11	Asd12	Asd13	Asd14	Asd15
Catalase	+	+	+	-	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	+	-	-	-	-	-	+	-	+	+
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	-	-	w	-	+	+	-	+
Raff.	+	+	+	-	+	+	+	w	+	+	+	+	+
Melibi.	+	+	+	-	+	+	+	+	+	w	+	+	+
Ribose	+	+	+	+	+	+	+	w	+	w	+	+	+
Mann.	+	+	+	-	+	+	+	w	+	+	-	w	w
Galac.	+	+	+	w*	+	+	+	-	+	+	-	+	+
pH: 4.5	+	+	-	-	-	-	-	-	-	-	-	-	-
pH: 9.0	+	+	+	+	+	+	+	+	+	+	+	+	+
5%NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+
10%NaCl	+	+	+	+	+	+	+	+	-	+	-	+	+
15%NaCl	-	-	-	+	-	-	+	+	-	-	-	+	+
4°C	-	-	-	-	-	-	-	-	-	-	+	-	-
45°C	+	+	+	+	+	-	-	w	-	+	w	+	+

*w: weakly positive growth

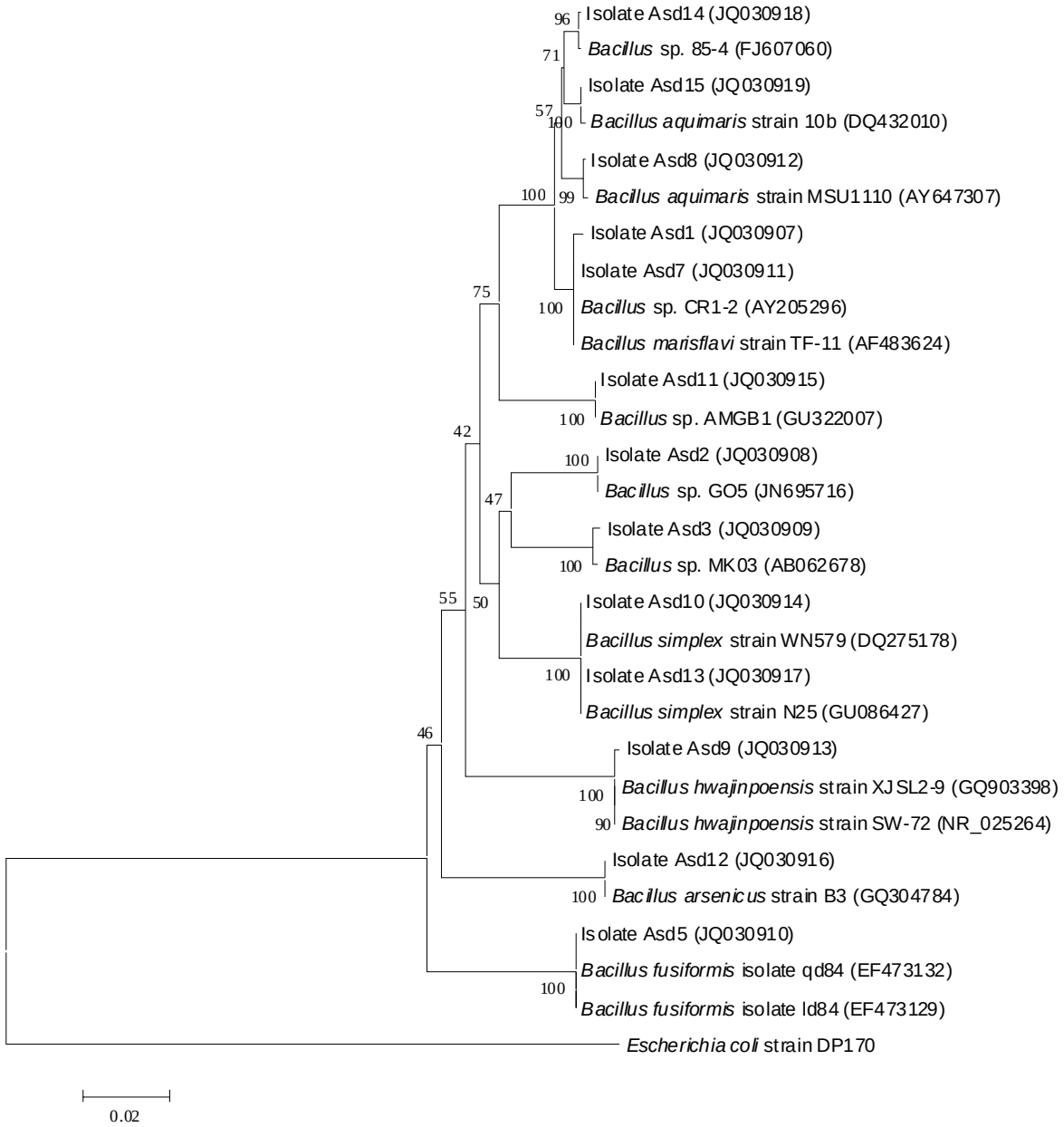

Fig. 2. The phylogenetic tree of 13 isolates based on 16S rRNA genes. *E. coli* strain DP-100 was given as an outgroup. Note: numbers in the parentheses represent the sequences accession number in GenBank. Numbers at the nodes indicate the bootstrap values on neighborhood-joining analysis of 1,000 resampled datasets

B. marisflavi strain Asd7, *B. aquimaris* strain Asd8, *B. hwajinpoensis* strain Asd9, *B. simplex* strain Asd10, *Bacillus* sp. Asd11, *B. arsenicus* strain Asd12, *B. simplex* strain Asd13, *Bacillus* sp. Asd14, *B. aquimaris* strain Asd15 reported in this article were submitted to GenBank and assigned the accession numbers JQ030907, JQ030908, JQ030909, JQ030910, JQ030911, JQ030912, JQ030913, JQ030914, JQ030915, JQ030916, JQ030917, JQ030918 and JQ030919, respectively) (Fig. 2). In previous studies, the presence of *Bacillus* species were reported in metal contaminated environments (Nithya et al. 2011; Kamala-Kaanan and Lee 2008; Gul-Seker and Mater 2009).

Minimum inhibitory concentrations of heavy metals are shown in Table 2. *Bacillus* strains showed higher tolerance to Pb, As, and Ni. All the isolates were highly resistant to lead and they showed 100% no growth in 10mM concentration. Among them, three *Bacillus* strains (*B. aquimaris* strain Asd15, *B. hwajinpoensis* strain Asd9 and *B. marisflavi* strain

Asd7) exhibited high tolerance to arsenic. The isolates *B. aquimaris* strain Asd15, *B. hwajinpoensis* strain Asd9 and *B. marisflavi* strain Asd7 showed no growth in 12mM, 11mM, and 10mM of arsenic concentration, respectively. In the case of nickel, *Bacillus* sp. Asd1, *Bacillus* sp. Asd2, and *B. hwajinpoensis* strain Asd9 strains exhibited high resistance to nickel compared to other isolates. Solely, isolate *Bacillus* sp. Asd2 did not grow in 10mM of cobalt concentration.

On the other hand, mercury was the highest toxic metal against to the all isolates. Zolgharnein *et al.*, (2007) showed that some bacteria which were isolated from samples belonging to the marine environment and enclosed industrial areas were capable of taking up heavy metals at high concentrations when exposed to 0.5 mM and 1.0 mM Zn, Cu, Pb and Cd. Many researchers have also reported the association between heavy metal and antibiotic resistance (Kamala-Kaanan and Lee, 2008; Matyar *et al.*, 2008; Gul-Seker and Mater, 2009). Similarly, in the present study, we found a relationship between heavy metals and antibiotic resistance. In antibiotic resistant assay, the isolates exhibited a maximum resistance to gentamicin and tobramicin. Especially, the isolates *B. fusiformis* strain Asd5 and *Bacillus* sp. Asd11 showed high resistance compared to the other isolates (Table 3). In addition, this is the first study which detects the heavy metal resistance from *B. hwajinpoensis*.

In various studies, it has been reported that the sediment bacteria present in heavy metals contaminate environments. Nithya *et al.*, (2011) found that sediment bacteria which were isolated from Palk Bay (India) and which exhibited high resistance against arsenic, mercury, cobalt, cadmium, lead and selenium were subjected to microbiological, biochemical and 16S rRNA analyses and identified as *B. arsenicus, B. pumilus, B. indicus, B. clausii* and they suggest that sediment bacteria could be biological indicators of

Table 2. MIC concentrations of sediment isolates against metals

	MIC concentrations of sediment isolates against metals (mM)								
Isolates	**Hg**	**Cd**	**Cr**	**Co**	**Cu**	**Zn**	**Ni**	**Pb**	**As**
Asd1	0.08	0.2	2.0	4.0	2.0	1.0	10.0	10.0	6.0
Asd2	0.01	0.1	2.0	10.0	2.0	2.0	10.0	10.0	2.0
Asd3	0.01	0.06	0.8	4.0	1.0	2.0	4.0	10.0	4.0
Asd5	0.08	0.6	0.8	6.0	2.0	2.0	8.0	10.0	4.0
Asd7	0.02	0.08	2.0	4.0	2.0	1.0	6.0	10.0	10.0
Asd8	0.08	0.4	0.8	4.0	1.0	1.0	8.0	10.0	6.0
Asd 9	0.02	0.2	2.0	6.0	2.0	4.0	10.0	10.0	11.0
Asd 10	0.01	0.2	0.8	4.0	2.0	2.0	6.0	10.0	8.0
Asd 11	0.02	1.0	0.8	2.0	2.0	1.0	8.0	10.0	6.0
Asd 12	0.08	0.1	0.8	4.0	2.0	1.0	4.0	10.0	6.0
Asd 13	0.06	0.08	0.8	4.0	2.0	1.0	6.0	10.0	6.0
Asd 14	0.02	0.2	0.8	4.0	2.0	1.0	4.0	10.0	6.0
Asd 15	0.08	0.6	2.0	4.0	2.0	1.0	4.0	10.0	12.0

Table 3. Antibiotic resistance of sediment isolates

	Antibiotic Resistance (mm)					
Isolates	**Ampicillin**	**Tetracycline**	**Chloramphenicol**	**Vancomycin**	**Gentamicin**	**Tobramycin**
Asd 1	34	22	34	25	14	15
Asd 2	22	20	30	25	18	15
Asd 3	16	30	38	25	13	18
Asd 5	18	20	25	6	10	8
Asd 7	30	15	30	22	8	12
Asd 8	-	25	30	28	18	16
Asd 9	30	25	30	20	13	15
Asd 10	17	25	35	23	20	25
Asd 11	8	10	22	18	13	15
Asd 12	20	25	35	20	10	15
Asd 13	14	23	25	20	18	22
Asd 14	35	25	40	25	15	13
Asd 15	32	25	35	22	14	18

heavy metal contamination. Kamala-Kaanan and Lee (2008) found that *Bacillus* species which isolated from the Sunchan Bay sediments exhibited high tolerance to manganese and a maximum resistance to ampicillin, tetracycline, kanamycin and streptomycin. Gul-Seker and Mater (2009) isolated the marine bacteria from Izmit Bay, the Black Sea and the Marmara Sea entrance of the Bosphorus (Turkey) and determined the heavy metal and antibiotic resistance of these bacteria. They found that most of the isolates belonged to the genera *Acinetobacter* and *Bacillus* which can be referred to metal toxicity indicators with their significant resistance to Cu, Cd and Cr, and majority of the metal-resistant isolates were also resistant to at least 2 antibiotics, and the incidence of metal-antibiotic double resistance against the metals Cd and Cu and the antibiotics chloramphenicol and ampicillin was 100%. In another study, Matyar *et al.*, (2008) studied the level of antibiotic resistance patterns and distribution of heavy metal resistance of bacterial isolates from seawater, sediment and shrimps in the industrially polluted Iskenderun Bay (Turkey), on the south coast of Turkey. In their study, it was observed that the metals given in the following order of toxicity Cd>Cu>Cr>Pb>Mn were toxic to the species collected and these species had a high incidence of resistance to ampicillin, streptomycin, cefazolin, but a low incidence of resistance to imipenem, meropenem and cefepime.

In our study area, several studies have focused on heavy metal (Hg, As, Pb, Ni, Cu, Mn, Cr and Zn) concentrations measured in sediment samples. For instance; Pazi (2011) found that As, Hg, Pb, and Zn concentrations in the surface sediments collected in Çandarli Bay were higher than the elemental background concentrations. In another similar result, Esen *et al.*, (2010) found that the Hg, As, Pb, and Zn concentrations measured in sediment samples were above the background levels in the Nemrut Bay. In a recent study conducted in a ship breaking are within the scope of DIVEST project, it was found that Hg, Cd, Pb and Cu levels measured were high and this situation revealed that sediment samples from the zone close to Aliaga facilities were polluted with heavy metals (DIVEST, 2011). These studies showed that heavy metal contamination resulting from the establishment of industrial enterprises and a ship dismantling area is becoming environmental problems, and if the industrialization and economic development in the region increases with the present rate, this will cause even more severe effects on the marine ecosystem (Pazi, 2011).

Although bacteria are sensitive to low concentrations of heavy metals, in specific environmental conditions, they rapidly adapt to the conditions so that they can reproduce in the presence of heavy metals. Therefore, they can be used as the indicator of heavy metal pollution. When viewed from a positive aspect, it can be proposed that these bacteria can be used to clean the areas contaminated with metals. In studies focused on this issue, first the resistance of the bacteria isolated from seas to heavy metals and antibiotics is tested (Nithya *et al.*, 2011; Nithya and Pandian, 2009), and then their plasmid genes which enable them to resist antibiotics and heavy metals and the mechanisms of these genes are investigated (Matyar *et al.*, 2008).

CONCLUSION

It is concluded that the MICs of heavy metals were different for each strain but the general order of resistance to the metals was found to be as Pb>As>Ni > Co > Cu > Zn > Cr > Cd > Hg. The toxic effects of the metals increased with their high concentrations and the strains were resistant to gentamicin followed by tobramycin. All these results suggest that the isolates could survive in heavy metal contaminated sediments. Therefore, the isolates may be useful as indicators of potential toxicity of heavy metals in coastal area such as ship dismantling zones to other forms of life and they could be designed as bioremediation tools by advanced studies.

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