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RESEARCH ARTICLE ARAŞTIRMA MAKALESİ

A mutagenicity investigation of sediment from İzmir Inner Bay using Ames genotoxicity assay

İzmir İç Körfezi sedimentinin Ames genotoksisite testi kullanılarak mutajenitesinin incelenmesi

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Abstract: İzmir Bay is one of the most important ecosystems of Aegean Region. Impacts of environmental pollution in the aquatic environments, especially impacts of pollution with mutagenic and carcinogenic substances on human health is an important area of research. Thus, it is required to incorporate short-term biological research methods to the molecular chemical analysis methods. By means of Ames's assay, it is possible to determine mutagenic potential of several chemicals, environmental pollutants, sediments, and waste waters. After the treatment facility was established in İzmir Bay in 2000, no mutagenicity studies were carried out in the sediment. Ames's mutagenicity assay without S9 fraction using TA98 and TA100 strains of *Salmonella typhimurium* was done at four different concentrations (125 µg, 250 µg, 375 µg, and 500 µg) in the sediment samples from six stations on İzmir Inner Bay in order to detect presence of chemicals that may cause mutagenic effects. Based on the results of Ames's assay, it was found that especially Turan Area (Station 3, on which shipyard is located) among 6 stations on İzmir Bay was under mutagenic and toxic effect and Bostanlı Area (Station 6) was under intense toxic effect. Turan Area was under the influence of environmental pollutants that may cause frameshift mutations. According to the present study, İzmir Inner Bay was contaminated by mutagenic and toxic substances.

Keywords: *Salmonella typhimurium*, TA98-TA100, sediment, pollution, Ames test, İzmir Bay

Öz İzmir Körfezi, Ege Bölgesi'nin en önemli ekosistemlerinden biridir. Su ortamlarında çevre kirliliğinin etkileri, özellikle mutajenik ve kanserojen maddelerle oluşan kirliliğin insan sağlığı üzerindeki etkileri önemli bir araştırma konusudur. Bu nedenle kısa süreli biyolojik araştırma yöntemlerinin moleküler kimyasal analiz yöntemleriyle birleştirilmesi gerekmektedir. Ames testi ile çeşitli kimyasalların, çevresel kirleticilerin, sediment ve atık suların mutajenik potansiyelini belirlemek mümkündür. İzmir Körfezi'nde 2000 yılında arıtma tesisi kurulduktan sonra sedimentte mutajenite çalışması yapılmamıştır. Bu amaçla İç Körfez'deki altı istasyondan alınan sediment örneklerinde, mutajenik etki potansiyeline sahip kimyasal maddelerin olup olmadığını tespit etmek için dört farklı konsantrasyonda (125 µg, 250 µg, 375 µg ve 500 µg) *Salmonella typhimurium* TA98 ve TA100 suşları kullanılarak S9 fraksiyonu olmadan Ames'in mutajenite tarama testi yapıldı. Ames testinin sonuçlarına göre, İzmir Körfezi'ndeki altı istasyondan özellikle Turan Bölgesi'nin (tersanenin bulunduğu İstasyon 3) mutajenik ve toksik etki altında olduğu, Bostanlı Bölgesi'nin (İstasyon 6) ise yoğun toksik etki altında olduğu belirlendi. Turan Bölgesi, çerçeve kayması mutasyonlarına sebep olabilen çevresel kirleticilerin etkisi altındadır. Bu çalışmaya göre, İzmir İç Körfezi mutajenik ve toksik etkili maddelerle kirlenmiştir.

Anahtar kelimeler: *Salmonella typhimurium*, TA98-TA100, sediment, kirlilik, Ames testi, İzmir Körfezi

INTRODUCTION

Aquatic ecosystems have been exposed to several pollutants and pollution has increased rapidly in recent years as a consequence of rapidly increasing production and use of artificial chemicals in progressively increasing urban population, and intense industrial activities.

The sediment layer underlying the seas is an appropriate substance to understand history of the pollution in the aquatic environments and to interpret on its future situation (Atgin et al., 2000). Many hazardous toxic substances are known to be accumulated in the sediment by several mechanisms of transportation (i.e., direct solid/fluid discharge, drainage from the land, atmospheric precipitates, shipping activities). Currently, the chemicals have become indispensable part of daily life. Hazardous chemicals are those leading to acute or chronic damage to the environment. The registry of Chemical Abstracts Service (CAS) includes more than 140 million chemicals. Annually, about twenty-five thousand new chemicals are added to the list. Number of the chemicals used in the market has been estimated to be around 350.000, but it has been reported that only about one per cent of them has been evaluated in terms of safety on human health and environment.

As can be seen readily, procedures for assessing the chemical substances are not up-dated and sufficient for steadily increasing number of chemicals currently available in the market (Anonymous, 2018). This is the case chemicals contaminants, including organochlorine compounds, herbicides, domestic and municipal wastes, petroleum products and heavy metals are now recognized to have adverse ffects on ocean and sea environment, even when released at low levels (Haynes and Johnson, 2000; Pinto et al., 2003).

Determining the chemicals with impacts on human health

and on the environment is an important research object in environmental and medical science. Impacts of environmental pollution, especially the pollution from the mutagenic and carcinogenic chemicals on human health is an important research object. The most important source of mutagenic and carcinogenic substances is industrial and agricultural activities. Xenobiotics originating from these sources sooner or later come into contact with aquatic ecosystems. Environmentally relevant foreign chemicals, named xenobiotics, are members of very different categories, including polyaromatic hydrocarbons (PAHs), polychlorinated biphenlys (PCBs), dioxins, dib enzofurans, organometals, arylamines, nitroaromatics, organophosphates, phatalate esters, organochlorines, etc. (Sheehan, 2007; Livingstone, 1993).

Although considered as an important part of the basic mechanisms of evolution, mutations more often have a detrimental effect for individuals and their offspring. Furthermore, increased mutation rates due to environmental pollution may adversely impact populations. There is consensus on close proximity of DNA damage, mutations and the induction of various types of cancer. It is the dominant paradigm in genetic toxicology that the ability of a chemical to cause mutation presages its ability to cause cancer (Zeiger, 2001). Even though carcinogenesis is a complex, multi-step process that is still not fully unravelled, growing evidence shows that it involves multiple mutations eventually leading to uncontrolled cell proliferation (OSPAR, 2002).

Detecting the chemicals exactly in the aquatic environments, however, is challenging and practically impossible if not literally impossible because of complex molecular structure of the organic substances. On the other hand, using the pollutants detected so far as a base and searching them in certain environments is a practical way to predict pollution and toxicity in those environments. Thus, incorporating the short-term biological research methods to molecular chemical analysis methods makes a practical and validated method to explore toxic substances in the environmental samples (Shuetzle and Lewtas, 1986). Among them, Ames's assay is one of the most important assays continuing to be relevant. Several chemicals can be investigated using Ames's assay (Grifoll et al., 1990). Aquatic sediments act as repositories for a variety of industrial and domestic pollutants. Our primary objective was to enhance the utility of the Ames assay for screening these complex chemical mixtures for mutagenicity (Papoulias et al., 1996). In most cases, mutagenic potential of pure chemicals has been studied. Ames's assay, however, has also been used to analyze complex mixtures extracted from a variety of sources, such as effluents from pulp mills (Douglas et al.,1980; Kamra, et al., 1983), petroleum refineries (Metcalfe,1985), and wastes from the wood-preserving industry (Donnelly, 1987). Several studies have been carried out in order to detect mutagenicity of sediment samples using the *Salmonella* Mutagenicity Assay (Ames's Assay) (Hollert et al.,1999; Vargas et al., 2001; Boyacıoğlu et al., 2008; Çakmak and Demir, 2018).

The first study on mutagenic characteristics of sediment from İzmir Bay was conducted by Boyacıoğlu as a doctoral dissertation along with BAP project in 1999 followed by a few genotoxicity studies (Boyacıoğlu, 2004; Arslan et al., 2010; Boyacıoğlu et al., 2011). Pollution status of the sediment which was a complex mixture hasn't been explored mutagenically in İzmir Inner Bay since the treatment facility (Big Canal Project) was launched in 2000. Thus, a mutagenicity study was aimed to be performed in order to re-explore potential mutagenicity status of sediment from İzmir Inner Bay 20 years after the Treatment Facility was launched.

MATERIALS AND METHODS

İzmir Inner Bay being polluted rapidly from 1960s was one of the areas with heaviest pollution in Mediterranean Sea before the treatment facility run as a part of Big Canal Project. Organic matters, hydrocarbons, metals, and pathogenic organisms created big threats in terms of aesthetic and health. Fifty percent of these basic pollutants affecting the water quality of İzmir Bay came from industrial wastes, 15% from raindrop, 10% from the rivers, 10% from the agricultural sources, and 15% from other sources (İZSU, 2012; İZSU, 2016). Until the establishment of the Treatment Facility, there were approximately 10 creek and discharge points in the Inner Bay (Boyacıoğlu,1999).

Study area

İzmir Bay is located on the coast of Aegean Sea on 38º25´–38º42´ North Altitudes and 29º 25´–27º 10´ East Longitudes. Its total length is 64 km with surface area of 500 km^2. The part of İzmir Bay between Karaburun Peninsula and Gediz Delta is called "Outer Bay" whereas the part of it from the end of Outer Bay to Yenikale Lighthouse is called "Middle Bay" and rest of it from Yenikale Lighthouse is called Inner Bay.

In the present study, sediment samples collected using a grab sampler at 6 stations in the Inner Bay were used. (St.1=Bayraklı, St.2=Alsancak, St.3=Turan, St.4=Pasaport, St.5=Konak, St.6=Bostanlı) (Table 1) (Figure 1).

Table 1. Data on the stations from which the sediment samples were taken and their coordinates

Sampling Stations	Latitude	Longitude
Bayraklı (St. 1)	38˚ 27' 34.93'' N	27˚ 9' 0.05'' E
Alsancak (St. 2)	38˚ 26' 59.19'' N	27˚ 9' 35.85'' E
Turan (St. 3)	38˚ 27' 18.1'' N	27˚ 8' 24.23'' E
Pasaport (St. 4)	38˚ 26' 23.14'' N	27˚ 7' 47.88'' E
Konak (St. 5)	38˚ 25' 11.88'' N	27˚ 7' 12.07'' E
Bostanlı (St. 6)	38˚ 26' 41.56'' N	27˚ 6' 36.26'' E

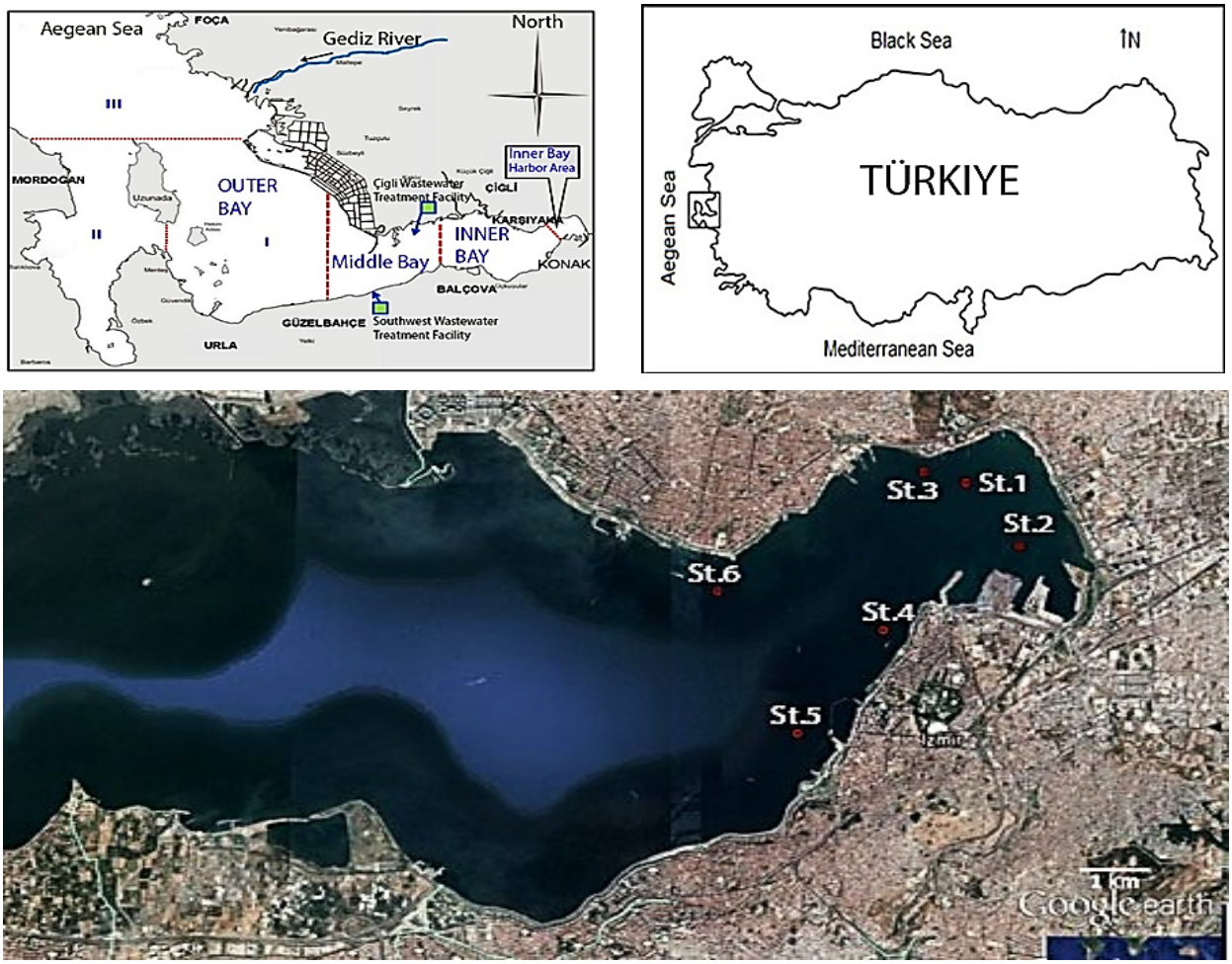

Figure 1. Six stations selected from İzmir Inner Bay

Extraction of the samples

Sediment samples were collected by a grab sampler from 6 stations in İzmir Bay (Figure 1). They were kept cool in ice-box until returning to the laboratory. Sediment samples were kept cool in ice-box at 4°C until returning to the laboratory. Air-dried samples for 24-48 hours in a fume-hood prior to extraction were crash to powder using net with pore size of 63 µm and then placed in portions of 2 g into teflon tubes and mixed with hexane/chloroform/acetone (1:1:1: v: v: v) (Maccubin, 1991). Subsequently, supernatants were taken after centrifugation for 10 Min at 4°C at 5600 g in *Sigma K3* cooled centrifuge. This procedure was repeated for 3 times and supernatant was collected. Organic solvents of the sediment samples were evaporated and dissolved with 2 mL Dimethylsulfoxide (DMSO) and added to give a final residue concentration of 2 g sediment mL (Kotelevtsev and Stepanova, 1995).

The mutagenicity assay

Salmonella mutagenicity assay was performed using the standard plate incorporation method (Maron and Ames, 1983) with the TA98 and TA100 strains of *Salmonella typhimurium,* and without S9-derived metabolic activation. Organic extracts (sediments) were diluted to 125 µg 250 µg, 375 µg, and 500 µg. of residue for each sample and tested for mutagenicity using the standard plate incorporation protocol (Maron and Ames, 1983). For testing mutagenicity, 100 µl of organic extract of sediment was mixed with 100 µl of an overnight culture of bacteria and 2 ml of melted agar containing 0.5 mM histidine and biotin. The molten top agar was then poured onto a minimal glucose agar base plate and incubated at 37°C for 2 days. Daunomicyne (0.6 µg/plate) and Mitomycin-C (0.5 µg/plate) were used as positive controls. Each dilution of extracts and controls was assayed in triplicate. Following incubation, the number of revertant colonies (His^- revertants) was counted (Maron, and Ames,1983).

Statistical methods

In the present study, the mean and standard deviation of 3 parallel results for each concentration were given in Table 2.3. The results were not subtracted from the results of negative controls. Analysis of variance (ANOVA) was used to test the significance of the difference among the variables. Calculations

with TA100 strain showed a statistically significant difference compared to negative controls at each concentration of all stations ($p < 0.005$). In mutagenicity assay with TA98 strain, statistically significant difference was found for 3 concentrations on Station 3 (Turan Area) and for concentration of 500 µg on Station 6 ($p < 0.005$).

RESULTS

According to the results of the present study, mutagenicity was observed on the Station 3 (Turan area), meaning that the number of revertant colonies of TA98 strain of *S. typhimurium* exceeded 10 folds of the control negative ($p<0.005$). Highest level of toxicity was observed on Turan and Bostanlı (Station 3, Station 6) at concentration of 500 µg (based on Ames's mutagenicity criteria) (Dugan et al., 1990) (Table 2, Figure 2).

Based on the results for TA100 strain, numbers of the revertant colonies were found to be below the negative controls at all concentrations from all 6 stations ($p< 0.005$), and the numbers fallen below half of the negative controls at concentration of 500 µg on Turan and Bostanlı Areas ($p<0.005$) (based on Ames's mutagenicity criteria) (Dugan et al., 1990), (St.6 and St. 3) (Table 3, Figure 2).

Table 2. Number of the revertant colonies in the mutagenicity analysis of sediment samples using *S. typhimurium* assay with TA98 strain in the absence of metabolic activation.

Stations	Concentration of the sediment extracts per plate					
	NC[a]	125 µg	250 µg	375 µg	500 µg	A-Cr**
Bayraklı St. 1	23.6 ± 3.21	35 ± 2	19.66±4.04	17.66 ± 4.1	21 ± 0	NM
Alsancak St. 2	23.6 ± 3.21,	33.3 ± 6.5	34.3±5.5	27 ± 5.5	20 ± 5	NM
Turan St. 3	23.6 ± 3.21	639.33 ±120*	402.6± 20.5*	391.66±9.5*	0 ± 0*	M
Pasaport St. 4	23.6 ± 3.21	26 ± 3	28.33±3.78	26 ± 3	23 ± 3	NM
Konak St. 5	23.6 ± 3.21	20 ± 3	26 ± 1	29.33 ± 3.51	24 ± 2	NM
Bostanlı St. 6	23.6 ± 3.21	22 ± 6.08	19.33 ± 3.51	18 ± 1	7.6±5.6*	TOX

[a] NC (Negative Control): DMSO (not subtracted from the values in the table)
Number of Spontaneously revertant colonies: 32-38 (not subtracted from the values in the table)
Number of the revertant colonies of positive control with Mitomycin-C (0.5µg/plate): No growth
*Analysis of mutagenic activity of sediments from Inner Bay as the number of His^+ revertant colonies in Ames test without metabolic activation system; significant data are shown in (*) ($p<0.005$)
A-Cr**: Ames Criteria, SM: strongly mutagenic; M: moderately mutagenic; WM: weakly mutagenic; NM: non-mutagenic; TOX: toxic (Dugan et al.,1990).

Table 3. Number of the revertant colonies in the mutagenicity analysis of sediment samples using *S. typhimurium* assay with TA100 strain in the absence of metabolic activation.

Stations	Concentration of the sediment extracts per plate					
	NC[a]	125 µg	250 µg	375 µg	500 µg	A-Cr**
Bayraklı St. 1	180.6±1.5	109±17.3*	117±1.5*	104±14*	100.6±6.6*	NM
Alsancak St. 2	180.6±1.5	117±10*	135±5*	112±11*	121±2.8*	NM
Turan St. 3	180.6±1.5	54±9.2*	47±3.05*	67±15.3*	79±7.2*	NM
Pasaport St. 4	180.6±1.5	137±11.26*	124±3.6*	110.33±9.2*	110.33±14.4*	NM
Konak St. 5	180.6±1.5	171±16.04	114±11.5*	109±5.1*	118±7.8*	NM
Bostanlı St. 6	180.6±1.5	107.3±11.5*	83.33±0.5*	97.33±5.8*	81.66±8.5*	NM

[a]NC (Negative Control): DMSO (not subtracted from the values in the table)
Number of the spontaneously revertant colonies: 165-218 (not subtracted from the values in the table)
Number of the revertant colonies of pozitive control with Daunomycine (6.0 µg/plate) :47-68
*Analysis of mutagenic activity of sediments from Inner Bay as the number of His^+ revertants in Ames test without metabolic activation system; significant data are shown in (*) ($p<0.005$).
**A-Cr (Ames criteria): SM: strongly mutagenic; M: moderately mutagenic; WM: weakly mutagenic; NM: non-mutagenic; TOX: toxic (Dugan et al.,1990).

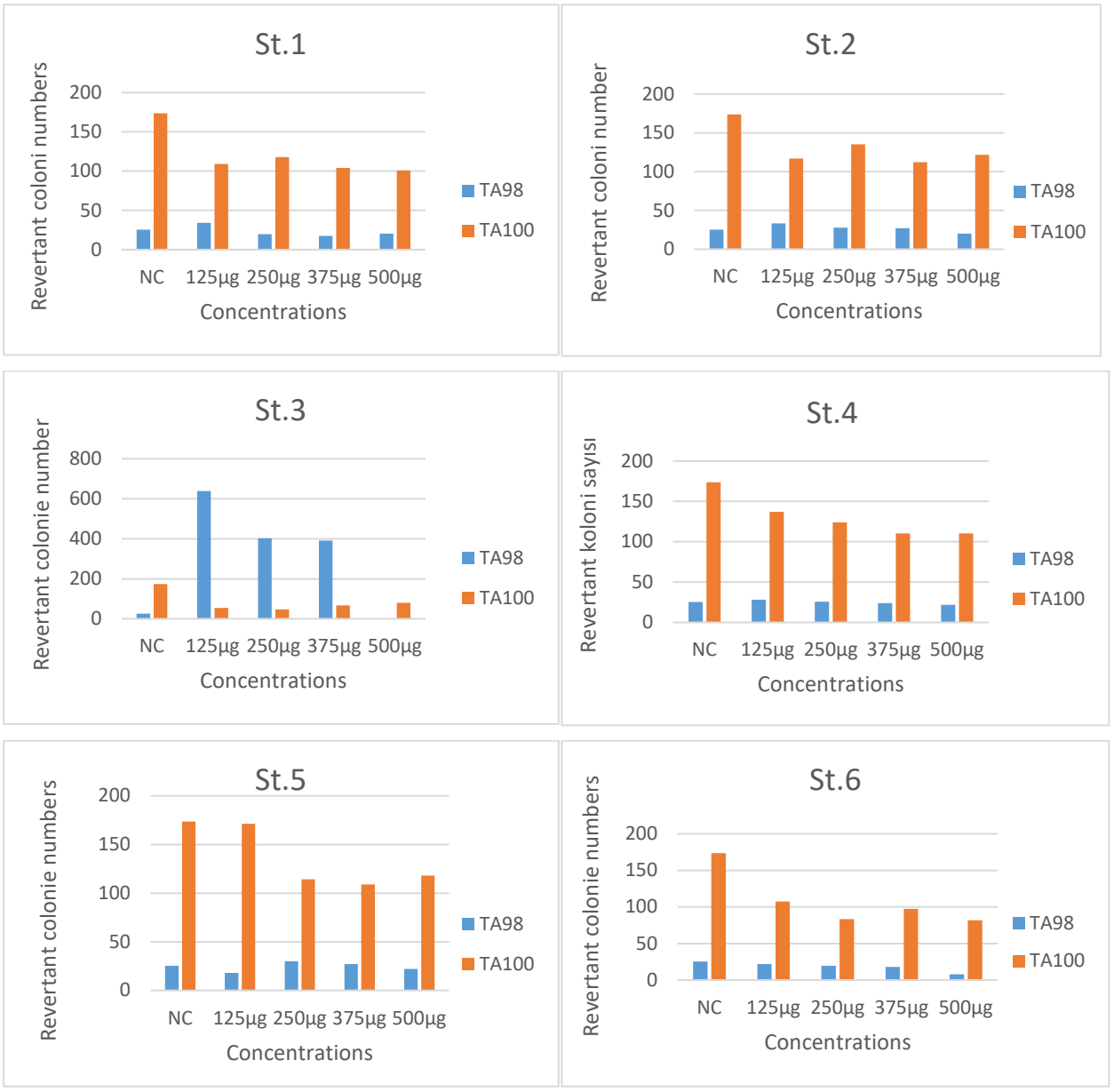

Figure 2. Number of the revertant colonies in the mutagenicity analysis of sediment samples using *S. typhimurium* assay with TA98 and TA100 strain in the absence of metabolic activation [(NC (Negative Control): DMSO (not subtracted from the values in the graphic); Concentrations: Concentration of the sediment extracts per plate (125 µg, 250 µg, 375 µg, 500 µg)]

DISCUSSION

İzmir Bay is known to be contaminated by organic materials, hydrocarbons, heavy metals, nutrients and pathogenic organisms (Balcı and Türkoğlu, 1993; Balkas and Juhasz, 1993; Kaymakci et al., 2001).

Aközcan and Görgün (2013) determined trace metals in surface sediments collected from two important areas (İzmir Bay and Didim Area) from the eastern Aegean coast and measured trace metals (Cd, Cr, Cu, Fe, Mn, Ni, Pb and Zn) in the sediment samples. The trace metal results showed that the İzmir Bay was exposed to trace metal pollution.

Küçüksezgin et al. (2006) reported that the heavy metal concentrations in various fish in İzmir Bay ranged between 4.5 and 520 µg/kg for Hg, 0.10 and 10 µg/kg for Cd, and 0.10 and 491 µg/kg for Pb µg/kg; in the same study, metal concentrations in the sediments were found to be as follows: Hg, 0.05-1.3; Cd, 0.005-0.82; Pb, 14-113, and Cr, 29-316 µg/g.

Cellular biomarkers and heavy metal concentration were investigated in mussels (*Mytilus galloprovincialis* L.) collected from eight different regions of İzmir Bay, and as a result, different levels of heavy metal pollution in İzmir Bay are an environmental problem (Katalay et al., 2022). Especially the

coastal areas of the gulf have been heavily affected by anthropogenic effects due to the increasing population.

Oral et al. (2012) found the highest concentrations of heavy metals as well as total PAH from the same stations on İzmir Bay (anthracene [A], fluoranthene [Fluo], benzo(ghi)perylene [BPer], benzo(b) fluoranthene [BbF], benzo(k)fluoranthene [BkF], benzo(a)pyrene [BaP] and indeno(1,2,3-cd) pyrene [IP]). Total PAH concentration was reported as 885,5+-244,5 ng/g dw.

There is a scarcity of ecotoxicological studies sediment of İzmir Bay sediment (Boyacıoğlu, 1999; Boyacıoğlu, 2004; Kutlu et al., 2008, Arslan et.al., 2010). The mutagenicity study of Ames conducted by Boyacıoğlu on İzmir Bay in 1999, is the first study conducted before the establishment of the Treatment Facility (2000) in the bay. This study was carried out approximately 18 years after the establishment of the Treatment Facility.

Many studies have been reported about mutagenic characters of sea, river and lake waters. It has also been reported that this mutagenic character was primary originated from PAHs (Kutlu et al., 2008)

Kutlu et al. (2008) performed *Salmonella* mutagenicity analysis on water samples in İzmir Çamaltı saltern without S9 fraction. They concluded that low PAH toxicity of central İzmir Bay might be considered as a reason of negative results on the mutagenicity investigation of Çamaltı Saltern.

As well known, TA100 strain of *S. typhimurium* is used to detect the mutagens causing alterations in base-pairs of the DNA chain while TA98 strain is used to detect the mutagens causing frameshift mutations (Maron and Ames, 1983). TA98 strain is rather sensitive to the PAH derivatives (i. e. benz(a)anthracene, 7-ethylbenz (a)anthracene) (Ames et al., 1972). Technically TA 98 strain of *S. typhimurium* indicate and sensitive to the occurrence of PAH in the media (Chen and White 2004). TA100 strain, on the other hand, is rather sensitive to the compounds causing alterations in the base-pairs such as acridine dyes, nitrous acid, hydroxylamine, alkylating agents, aldehydes, hydroperoxidases, aromatic amines, benzydine, toluidine, and dianisidine (Prival et al., 1979) (Prival and Mitchell,1982). The present study showed that Turan area (Station 3) was polluted by the mutagens against which TA98 strain of *S. typhimurium* were sensitive (causing frameshift mutations). This was considered to be due to presence of a military shipyard on Turan area. Repair and maintenance activities commonly carried out at shipyards include hull cleaning, repair and painting, electrical and machine work, carpentry, steel fabrication, pipe-fitting, and sand blasting of parts. Paint stripping and painting activities are significant sources of pollution from shipyards, and their waterfront locations increase the potential for pollutants to reach bodies of water. Many of the coatings used on hulls contain anti-fouling. heavy metals, such as copper and zinc. The metals are toxins added to marine coatings to prevent marine organisms from building up on ship hulls, which reduces speed and fuel efficiency (Turner, 2010).

It was found that amount of the mutagens responding to the TA98 strain of *Salmonella typhimurium* increased remarkably since 1999 and toxic effect was found on Station 6 (Bostanlı Area). It is possible to explain the reason for toxic effect on Bostanlı Area (Station 6) as follows: Cyclonic gyre exists on Inner İzmir Bay (Beşiktepe et al., 2011) and pollution burden from Turan Area (Station 3) as a result of this cyclonic gyre possibly affects this area as well.

When results of the present study were compared to those from the mutagenicity study conducted by Boyacioglu (1999) using TA98 strain of *S. typhimurium*, the mutagens responding to TA98 strain of *S. typhimurium* were observed to again mutagen in sediments from Station 3 (Table 2, Figure 2).

In the study with TA100 strain, numbers of the revertant colonies at all concentrations from six stations were found to be lower than the negative control and numbers of revertant colonies were observed to decrease almost to half values at the highest concentration of 500 µg in the samples from Turan Area (St.3) and Bostanlı Area (St.6). Although the values were non-mutagenic, toxic effect was observed according mutagenicity criteria of the Ames' Assay ($P < 0.005$) (Dugan et al., 1990) (Table 3; Figure 2).

When results of the present study were compared to those from the mutagenicity study conducted by Boyacıoğlu (1999) using TA100 strain of *S. typhimurium*, the mutagens responding to TA100 strain of *S. typhimurium* were observed to change from mutagenic to toxic characteristics (Dugan et al., 1990) in sediments from all 6 stations (Table 3, Figure 2).

CONCLUSION

In conclusion, based on the results of Ames's assay, it was found that especially Turan Area (Station 3, on which shipyard is located) and Bostanlı Area (Station 6) among 6 stations on İzmir Bay was under frameshift mutagenic and toxic effect. Although the pollution burden from the Inner Bay decreased after İzmir Treatment Facility was operated in 2000, according to the results of this study, the pollution burden from the shipyard continues to pollute the Inner Bay with mutagenic and toxic influences. The military shipyard needs to take some precautions about environmental pollution as soon as possible. Additional experiments should be performed using rat liver homogenate (S9) in order to determine the genotoxic potentials in mammals. We believe that this study will shed light on future ecotoxic studies.

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AUTHORSHIP CONTRIBUTIONS

Meltem Boyacıoğlu: Conceptualization, funding acquisition, methodology, resources, investigation, writing-

reviewing and editing. Yiğit Egüz: Conceptualization, investigation, methodology, formal analysis, writing-reviewing.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest or competing interests.

ETHICS APPROVAL

No specific ethical approval was necessary for this study.

DATA AVAILABILITY

All relevant data is inside the article.

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