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Pla*Bacter:* Determination of microbial diversity on plastic polymer in Topçu Pond

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Abstract

One of the measures taken to solve the climate change crisis is to reduce the consumption of oil and oil derivatives, which are considered among fossil resources. However, with the increasing population, oil companies have turned to the plastics and packaging sector in order to meet the demand for plastic consumption. This situation inevitably increases the environmental damage of plastic waste. In particular, the inability to use water resources correctly and the pollution of the wastes left in the environment negatively affect the lives of living things on earth. Within the scope of the study, it was aimed to determine the microbial diversity living on polyethylene terephthalate (PET), which is known as the raw material of tools such as plastic bottles, food containers, and fishing line, which are left as waste in the environment, especially on river and lake shores. In this context, Topçu Pond, which is an important water source for agricultural irrigation in Yozgat province, was selected as the study area. Genomic DNA (gDNA) was isolated from biofilm layers formed on PET samples, water, and sediment collected from the study area. Bacterial communities living in the samples and 16S rDNAs of gDNAs were analysed by next-generation sequencing. The results show that the dominant phylum in all three samples is Pseudomonadota. Nonetheless, variations in relative abundance at the species level were detected using metagenomic analysis. Additionally, several species were identified in the PET sample that were absent from the water and sediment samples. In conclusion, we performed the first microbial diversity investigation in Topçu Pond and characterized the bacterial composition on PET samples within the pond.

Keywords: Polyethylene terephthalate, 16S rDNA, Next Generation Sequencing, Microbial diversity, Lentic system

1. INTRODUCTION

Climate change is defined as the phenomenon of increasing the average temperature of the Earth and changing the climate on Earth as a result of the accumulation of gases such as carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O), hydrofluoride carbons (HFCs), sulphurhexa fluoride (SF_6) etc. in the atmosphere [1]. As a result of climate changes, increases in some meteorological events such as floods, droughts, storms, desertification, typhoons, uneven rainfall and tornadoes occur [2]. The Paris Agreement, which is an indicator of the war against climate change in the world, has been signed by developed countries and has revealed what countries should pay attention to for our world. According to the Paris Agreement, countries should reduce the consumption of meat products and the use of fossil fuels to reduce CO_2 consumption.

The automotive sector is shown as the largest market share of fossil resources. After the Paris Agreement, oil producers have searched for a different market due to the automotive companies' orientation towards alternative energy sources [3]. Plastic and packaging sector is shown as the new market of oil producers. Plastic materials are petroleum derivatives and today they appear as low-cost, durable polymers that can be used and applied in all kinds of areas [4]. Due to the increasing population density, there is a considerable increase in the amount of production of plastic materials and an increase in the amount of consumption at the same rate. It is known that plastic consumption, which has reached 368 million tonnes per year today, has increased 187 times in the last seventy years [5]. The fact that it is cost-effective and applicable to every field has enabled plastic polymers to find a place in different areas of use (Table 1). With the unconscious increase in the use of plastics, plastic wastes on Earth are also increasing in direct proportion. Plastic materials, which have a long disintegration and destruction process, are not only harmful to the environment, but also cause disruptions in the balance of aquatic and terrestrial ecosystems by mixing into the food chain.

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Table 1. Plastic polymers and application areas [5]

Resin Identification Codes	Types of Plastic	Material	Usage areas
	PET / PETE	Polyethylene Terephthalate	Food containers, plastic bottles, textiles, films, fishing rods, microwave trays
	HDPE	High Density Polyethylene	Shampoo and detergent bottles, cables, bags
	V/PVC	Vinyl / Polyvinyl Chloride	Medical products, packaging, window frames
	LDPE	Low Density Polyethylene	Bags, cling film, toys, lids of milk bottles
	PP	Polypropylene	Pipettes, medicine boxes, bottle lids, electrical Devices
	PS	Polystyrene	Plastic plates, forks, spoons, knives and cups, egg boxes, CD containers
	0	Others	Recyclable plastic water bottles, packaging, cooking bags

In addition to being used in many fields, plastic wastes, which can remain in nature for a long time without dissolving, are found in oceans, lakes, atmosphere and sediments in microplastic and macroplastic forms with effects such as wind, flood, erosion [7]. It is estimated that plastic wastes will be 15 million metric tonnes per year in 2050 and it is inevitable that these wastes will enter aquatic environments [8]. Plastic materials mixed into aquatic environments cause environmental pollution, as well as interacting with living organisms in the environment and may restrict their movements or be ingested by them. As a result, this environmental pollution also negatively affects the organisms living in the aquatic ecosystem [9].

Lake systems play an active role in many stages of the ecosystem, such as the supply of drinking water, energy production, aquaculture, drought control and irrigation of agricultural lands. Increasing temperature due to global climate change causes an increase in the temperature of surface waters, thus causing a significant negative change in the water levels and surface areas of lakes [10]. Recently, the decrease in water resources in the world has emerged because of climate change. Wastewater discharges from industries and residential areas, insecticides/pesticides used in agriculture and the use of water for recreational purposes are defined as the main pollutants that degrade the quality of lake waters [11]. In lake systems called lentic systems, many pathogenic and non-pathogenic aquatic microorganisms survive due to decreased water quality [12].

The reproduction of microorganisms by adhering to a surface and the community they form on the surface as a result of this reproduction is called biofilm. Biofilms formed by microorganisms have negative effects such as product contamination, energy losses, especially infectious diseases. Plastics are also an ideal habitat for microorganisms to form biofilms [13]. With the formation of stress tolerance on plastic materials, biofilm structure, which forms the basis of microbial diversity, is formed [14].

Plastisphere is the name given to biofilm layers formed on plastic materials [15]. The habitat formed on plastic materials is not only for biofilm formation but also hosts environmental pollutants such as antibiotic resistance genes and metal deposits [16]. Recent studies have revealed that different types of plastics have variable properties such as different surface areas, different electrostatic charges and different hydrophobic properties, which are the most important factors for biofilm layer formation by microorganisms. For these reasons, the diversity of bacteria that will adhere to the surfaces of various plastic materials and form a biofilm layer will also vary significantly. Although the existence of this effect has been recognised, there is currently very limited literature information on the characteristics and mechanisms of bacterial adhesion on plastics [17].

The growth of microorganisms on different plastic substrates is an indication that different microbial diversity may occur in aquatic environments [17]. In recent scientific studies in aquatic ecosystems using different plastic substrates, it has been revealed that there are significant differences in the microbial diversity of plastic material kept in the aquatic environment, water environment and sediment samples taken from the same environment [18]. Current studies have shown that pathogenic Vibrio, Pseudomonas, Escherichia, Acinetobacter and human pathogenic *Morganella morganii* bacteria species grow on plastics. The presence of pathogenic microorganisms in aquaculture and drinking water use poses significant risks to human health [19]. Likewise, the differentiation of microbial diversity due to environmental pollution may lead to the emergence of new genes, especially antibiotic resistance genes, which may have a major negative impact on human beings. For all these reasons, it is quite clear that the negative effects of plastic should not be ignored.

Topçu Pond, located within Yozgat province borders, is geographically located at 39° 44' 00" N, 34° 48' 54" E (Figure 1). The pond has the largest surface area in the region and is fed by Karacaoğlan Stream and Zaptiye Stream. The pond, which helps aquaculture and irrigation of agricultural land, is actively used for irrigation of the lands of Topçu, Divanlı and Gökçekışla villages in its immediate vicinity. Despite this strategic importance, only one study on the algal flora of Topçu Pond has found a place in the literature [20]. However, revealing the microbial presence of Topçu Pond and comparing the microbial diversity of plastic materials in the pond exposed to plastic pollution will provide important information in terms of biotechnological and public health as well as agricultural aspects for more efficient use of lake waters.

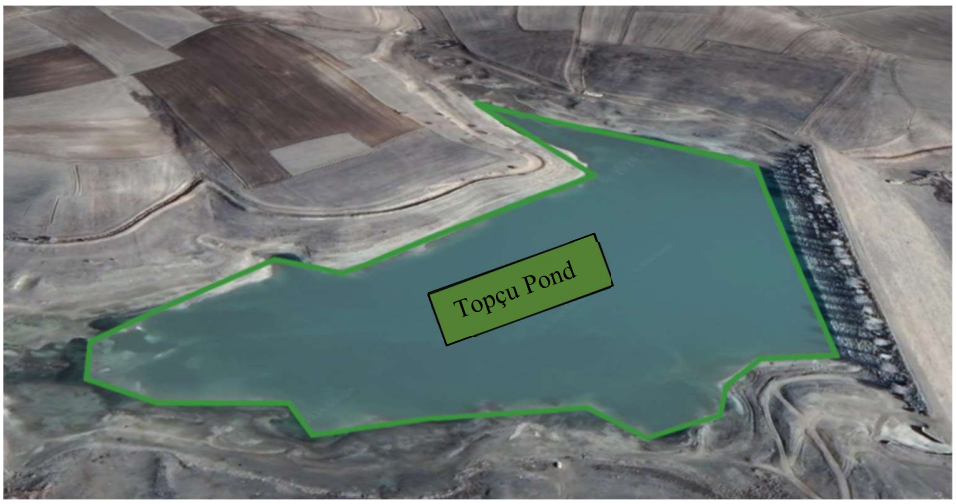


Figure 1. An image of the Topçu Pond

Today, it is essential to protect and carefully use lake systems, which are an important source of surface waters and have their own habitat, due to increasing environmental pollution. Within the scope of this study, it is aimed to compare the microbial diversity of microorganisms found on lentic systems exposed to plastic pollution by human hands with the microbial diversity of polyethylene terephthalate (PET) plastic material, which is the most polluting plastic material in the environment, with the microorganism diversity living in the lake system by using the next generation sequencing method and to reveal the differences.

2. MATERIAL AND METHODS

2.1 Collection of samples

Polyethylene terephthalate (PET) samples, which are one of the environmental pollutants, were cut into 27mm×4mm and sterilised with 70% ethanol. The samples were placed in Topçu Pond approximately 10cm below the surface area with the help of a device that can float on the lake. The experiment was organised in triplicate. The samples were left for 30 days for the biofilm layer to form on the samples at 39° 43' 55" N, 34° 48' 56" E. At the end of the period, the samples were carefully removed from the environment and taken into sterile falcon containers. In addition, samples of lake water were collected from the depth of PET samples into sterile bottles. The sediment sample of the lake was taken from 5 metres away from the shore into a 50 ml sterile falcon tube. All samples were brought to Yozgat Bozok University Molecular Biology Laboratory by cold chain.

2.2 Extraction of Genomic DNA (gDNA)

Each of the water, sediment and PET samples collected from the lake and brought to the laboratory by cold chain were cultured using Luria- Bertani (LB) medium. Cultivation was carried out at 270 rpm at 10°C for 16 hours. After incubation, growth was observed in the samples. Cultured bacterial gDNA was isolated from water, sediment and PET samples using the AMBRD Microbial DNA Isolation Kit (Istanbul, Türkiye) protocol.

2.3 Qualitative and Quantitative Determination of Isolated gDNA

Qualitative analysis of the isolated DNA samples was performed by agarose gel electrophoresis. Agarose gel electrophoresis was performed with 0.7% agarose gel at 120V for 20 min. Quantitative analysis of the samples was performed with Avans Bio brand UVS-99 UVISDrop nanodrop device.

2.4 Next Generation Sequencing (NGS)

Ligation sequencing kit (SQK-LSK109; Oxford Nanopore Technologies) protocol was used for library preparation of gDNAs. Firstly, dA tails were added to the samples, and nick parts were repaired in a 60 µl reaction. The samples were then purified using MobiomX MagBeads, and adapters were attached to the repaired ends. The samples were then purified again and quantified spectrofluorimetrically. The total library was loaded into a Spot-On flow cell (FLO-MIN106D) after priming and sequencing were initiated on an Mk1C™ instrument (Oxford Nanopore Technologies) using the latest MinKNOW™ software. Sequencing was stopped when sufficient data were obtained or when the maximum run time of 72 hours was completed.

2.5 Bioinformatics for the Determination of Microbial Diversity

Following sequencing, the findings in fast5 format were converted to fastq format utilizing the newest version of guppy software for base-calling and de-multiplexing. The barcode and adapter sequences were cleaned using guppy software, and universal primers and labels were also deleted, 15 bases from each end of the sequences. After cleaning the arrays, reads between 1000-2000 bp in length were filtered with Trimmomatic, and the remaining reads were excluded from the analysis. The cleaned reads were analysed with a customised workflow using the Python programming language. During the filtering process, each sequence was matched with the BLAST algorithm. In the results, a biome file was created by taking the taxonomic data of the sequences with more than 40% coverage and 60% similarity in sequence matching. To perform phylogenetic analyses with the biome file created, krona plots, abundance analysis and alpha diversity analyses were performed with the tools provided by the qiime2 platform. The graphs and tables in the analyses were made with the libraries of the Python programming language.

3. RESULTS AND DISCUSSION

3.1 Collection of Plastic Samples in the Topçu Pond

The samples were placed in the pond after being arranged and set up in May 2023 (Figure 2). The samples were collected from the pond in June 2023.

Figure 2. PET samples placed in the Topçu pond

The pH, temperature, salinity, conductivity, and dissolved oxygen (DO) were determined by the HI-98194 Multiparameter Waterproof Meter (Hanna Instruments) (Table 2).

Table 2. Physiological properties of Topçu pond during the placement of PET samples

Physiological Properties	Values
pH	7.8
Temperature (°C)	10.18
Salinity (‰)	0.22
Conductivity (µS/cm)	323
Dissolved oxygen (DO%)	34.5

3.2 Qualitative and Quantitative Determination of Isolated gDNA

The results of agarose gel electrophoresis from culture-dependent genomic DNA isolation are presented in Figure 3.

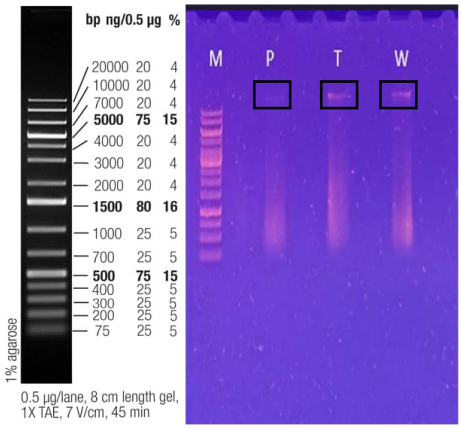


Figure 3. Agarose gel electrophoresis results of gDNAs isolated from culture-dependent samples. M: DNA marker, P: PET samples, T: sediment from the lake, W: Lake water

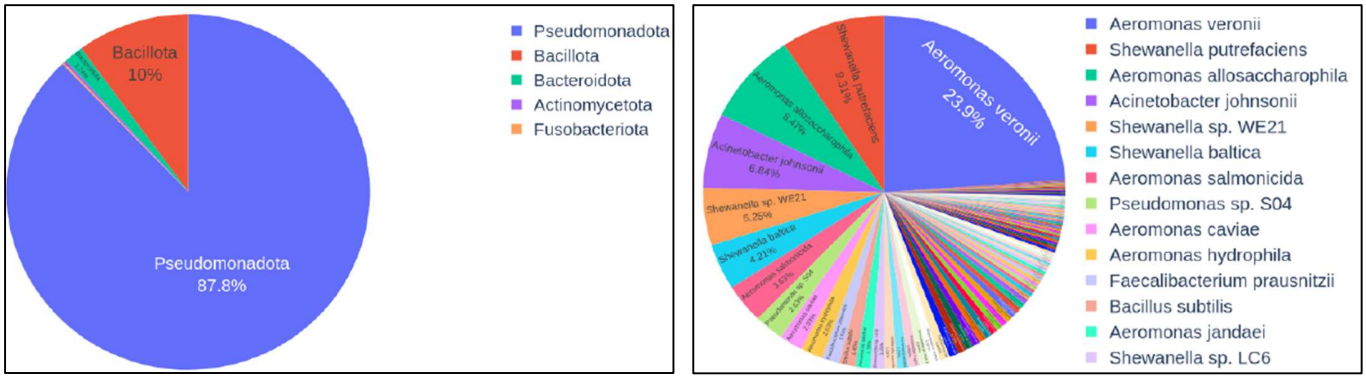
The concentration of isolated gDNAs were determined spectrophotometrically using nanodrop as shown in Table 2.

Table 2. Quantitative data of the samples measured by nanodrop. P: PET samples, T: sediment from the lake, W: Lake water

Sample	260/280	260/230	Concentration (ng/µl)
PET	2.20	2.32	201.0
T	2.21	2.12	241.8
W	2.20	2.22	216.1

3.3 Results of NGS by Nanopore

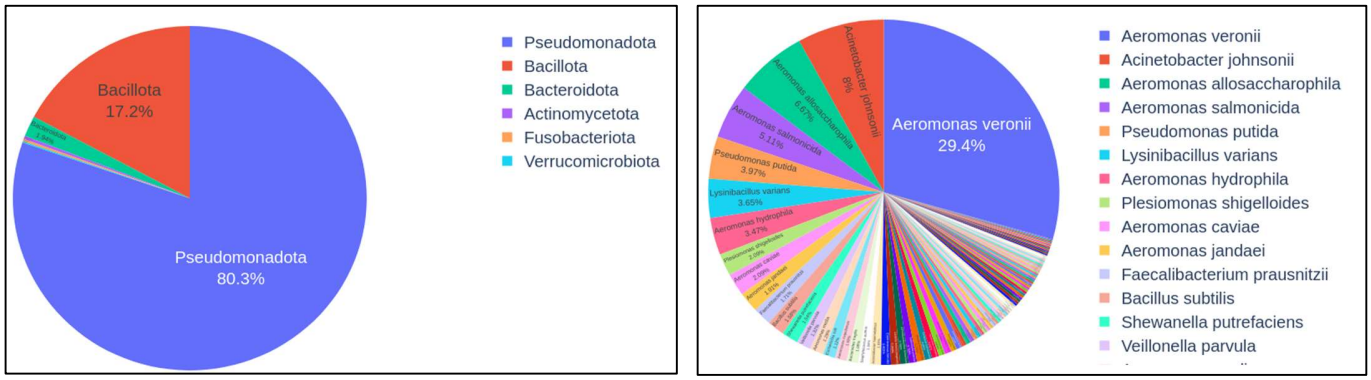
The NGS analysis of 16S rDNA on PET samples revealed the presence of 5 phlyum, 7 classes, 13 orders, 14 families, 14 genera, and 14 species (Figure 4).



(a) **(b)**

Figure 4. Next generation sequencing results of PET sample. (a) Phylum level of PET sample, (b) Species level of PET sample

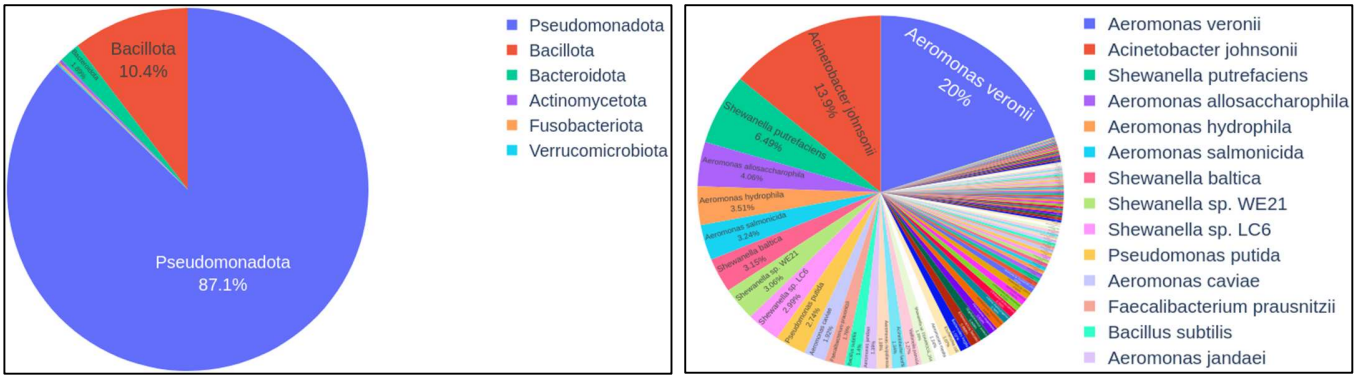
As soon as the 16S rDNA data from the sediment samples were analysed, it was discovered that the cultured sediment samples contained 6 phylum, 8 classes, 13 orders, 14 families, 14 genera, and 14 species (Figure 5).



(a) **(b)**

Figure 5. Next generation sequencing results of sediment sample. (a) Phylum level of T sample, (b) Species level of T sample

As a result of the 16S rDNA analysis of water samples collected from Topçu Pond and cultivation, it was determined that there were 6 phylum, 8 classes, 14 orders, 14 families, 14 genera, and 14 species (Figure 6).



(a) **(b)**

Figure 6. Next generation sequencing results of water sample. (a) Pie chart in Phylum level of W sample, (b) Pie chart in Species level of W sample

Based on the obtained data, we observed the Verrucomicrobiota phylum in the soil and water samples, but not in the PET samples. The sediment, water, and PET samples of Topçu Pond predominantly contained Gammaproteobacteria class bacteria. In contrast to the other samples, the PET sample revealed the presence of Salmonella and Limosilactobacillus genera, along with *Pseudomonas* sp. S04 species. In addition, although Plesiomonas and Citrobacter genera were observed in water and sediment samples. In contrast, they were not observed in PET sample.

The bacterial genera that were found in each of the three samples are comparable to one another. Only differences in their relative abundance were observed. For example, *Aeromonas veronii*, the most dominant species, was found to be 20 % in the PET sample, 29.4 % in the soil sample, and 23.9 % in the water sample. Aeromonas genus is one of the most important pathogens in recent years. It causes many diseases in human and fish [21]. Especially, these species cause diseases such as soft tissue necrosis, hemorrhagic septicaemia, and fin necrosis in fish [22]. Aeromonas species with zoonotic properties include *Aeromonas hydrophila*, *Aeromonas caviae*, and *Aeromonas veronii* [23]. In humans, it is known to cause diarrhea, sepsis, and soft tissue infections [24].

Acinetobacter spp. is a non-fermentative, aerobic, gram-negative bacterium. It can be found widely in various environments, including soil, water, mud, and human skin [25]. *Acinetobacter johnsonii*, which was found 13.9 % in a PET sample, is associated with meningitis cases in humans [26]. *Acinetobacter johnsonii* is relatively preferred to colonise PET samples compared to soil and water samples. This situation demonstrates that plastic material pollution threatens human health as well as affecting the world in an ecological way.

The genus Pseudomonas belongs to the gram-negative bacteria and is well studied in the literature. *Pseudomonas* species are commonly found in our universe, and they could be pathogens as well as commensal species. [27]. *Pseudomonas* sp. S04 strain is firstly defined in a study conducted in Switzerland associated with *Solanum tuberosum* [28]. According to the NGS results obtained within the scope of our study, it was determined that *Pseudomonas* sp. S04 strain was found on PET samples placed in Topçu pond at a rate of 2.63 %. In order to understand why *Pseudomonas* sp. S04 was found in our PET samples, the whole genome of the *Pseudomonas* sp. S04 strain was analysed. [29]. Among the genes encoded by the bacterium, it was observed that there are biofilm formation genes such as murein hydrolase regulator LrgA, CidA/LrgA family protein, and lytic murein transglycosylase [30,31]. Therefore, it is thought that *Pseudomonas* sp. S04 strain could form biofilm. So, only the PET samples create the environment for biofilm formation. Because of that, it was ought that *Pseudomonas* sp. S04 strain was observed on the PET samples.

Within the scope of the study, the microbial diversity of the pond was determined with the findings obtained from sediment, water, and PET samples taken from Topçu Pond in Yozgat province. The findings indicate that the pond is predominantly characterized by pathogenic bacteria. The reason for this situation is thought to be the physical and chemical pollution of the pond by humans.

4. CONCLUSION

This study assessed the microbial diversity of Topçu Pond in Yozgat province, analysing PET samples placed in the pond as well as water and sediment samples obtained from the lake. The differences in microbial diversity derived from soil, water, and PET samples,

recognized as a significant pollutant in Topçu Pond were clarified. The percentage of abundance for bacterial species on the PET sample was comparable to that of other samples. According to the NGS analysis, Topçu Pond includes some of the pathogenic bacteria. So that this situation causes infectious diseases. Additional research should be undertaken on bacterial species living on the PET sample that are absent in water sediment samples. Future research aims to clarify the mechanisms by which bacteria develop biofilms on PET samples.

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AUTHOR'S CONTRIBUTIONS

ETSC: Designing and performing the study, supervision, writing and reviewing the manuscript. BY: Designing and performing the study, writing and editing the manuscript, and funding. ŞG: Sampling, performing the study. ED: Sampling, performing the study. SA: Sampling.

CONFLICTS OF INTEREST

All authors declared that there is no conflict of interest.

RESEARCH AND PUBLICATION ETHICS

All authors declare that this study complies with Research and Publication Ethics.

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