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Evaluation of genotoxic potential induced by marine cage culture

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ABSTRACT

BACKGROUND AND OBJECTIVES: The eutrophication process is increased by anthropogenic or aquaculture facilities in marine ecosystems. DNA damage biomarkers for fish species detect genotoxic parameters for ecological risk assessment. The aim of the present study was to determine genotoxic potential induced by marine cage culture in Iskenderun Bay on gilthead sea bream (*Sparus aurata*) using Comet assay.

METHODS: This study was conducted at cage and reference stations of Iskenderun Bay, Northeastern Mediterranean in January 2017. The wild and cultured samples of *S. aurata* and water samples were collected from wild and fish farm.

FINDINGS: The DNA damages at gill and liver cells of gilthead sea bream in the present study were observed with a higher level of DNA damage in gill cells compared to liver cells, and were determined at the low and minimal scale at the cage and reference stations, respectively. The present study demonstrated that the TP values were recorded at 0.020 and 0.016 mg/L in the cage and reference stations which are at border and below 0.020 mg/L. The DIN values were recorded at 0.097 and 0.075 mg/L in the cage and reference stations, which are at below 0.1 mg/L. The water bodies in the cage and reference stations exhibit Moderate/Mesotrophic water quality The correlations between physical-chemical parameters and DNA damage were shown that DIN, NH_4-N, NO_3-N and NO_2-N in water revealed significant positive correlations with DNA damage levels in gill cells.

CONCLUSION: The present study provides the first data set on genotoxic damage induced by marine cage culture in Iskenderun Bay on gilthead sea bream. The result of this research is an early warning for the marine system and further detailed research is needed to establish the source of the pollution and monitor environmental pollution.

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INTRODUCTION

There is increasing demand in culturing aquatic livings in coastal and inland waters. Aquaculture, likely the fastest growing food-producing sector, presently represents almost 50 percent of the world's food fish. Aquaculture production in world reached 80.1 million tons and valued at USD 237.5 billion by 2017. It is clearly accepted that world aquaculture production will remain to increase, especially in the developing countries of Asia and Africa, through the expansion of semi-intensive, small-scale pond aquaculture (FAO, 2018). Gilthead Sea bream, a species of great economic importance in Mediterranean aquaculture, thrives naturally in the coastal waters of the Mediterranean and Eastern Atlantic, often in marine lagoons, and is a fish commonly cultivated in marine cages and recirculating aquaculture systems (Basurco *et al.,* 2011). Gilthead sea bream is benthopelagic inhabiting shallow waters as well as various kinds of bottoms of coastal areas up to 100-150 m depth. Being eurythermic (2/5-32°C) and euryhaline (3‰- to full strength sea water) species. This fish is also a carnivorous species, but with a lower trophic level 3.3–3.5 (FishBase), that feeds preferentially on shellfish (mussels and oysters), crustaceous, fish and sometimes algae. Aquaculture as any aquaculture production activity, if not well managed, it can lead to ecological distraction (Gorlach-Lira *et al.,* 2013; FAO, 2014). Conventional aquaculture systems command aquaculture production in many areas, yet these are currently gradually being supplanted by intensive production approaches. Fast scale development of intensive farming systems usually causes adverse effects on surrounding environments. Intensive aquaculture has a non-stop or pulse release of nutrients that contribute to eutrophication. The main source of potentially polluting waste was discharged farm waste, uneaten feed and fish faeces (Cripps and Bergheim, 2000). Nutrients load and suspended solids in aquaculture effluent can cause eutrophication (Cho *et al.,* 1991; Ozbay *et al.,* 2014), oxygen depletion and algae blooms problems in the surrounding aquatic environments. Moreover, releasing aquaculture effluent of poor water quality may have an important effect on the marine organisms in ecosystems (Stephens and Farris, 2004). The amount of these pollutants in the effluent depends on a wide range of factors. In recent years, both worldwide and in our country, the assessment of the possible unfavourable environmental effects of aquaculture has been a salient issue. Feed-derived wastes are either dissolved, such as phosphorus (P) and nitrogen (N) based nutrients, or suspended as solids (Cripps and Bergheim, 2000). Their environmental impact can be decreased either by improved farm management, or by physical and/or biological treatment of the effluent (Moustafa *et al.,* 2020). The assessments in ecological risks, relied on molecular or biochemical markers, has been highlighted in eco-toxicological and genotoxic studies (Connon *et al.,* 2012; Baudou *et al.,* 2019) handling the reviews of marine ecologies impacted as a result of publicity to at least one or more pollution along with anthropogenic or agriculture/aquaculture services (Chapman, 2007; Kroon *et al.*, 2017). The detection procedures of DNA damage on the degree of a character eukaryotic cellular have been formerly utilized to a diffusion of research regions together with plant sciences and mammal toxicology research (Nehls and Segner, 2005; Olive and Banáth, 2006). Currently, comet assay is a standard and flexible approach, followed for eco-toxicological studies that can be applied to truly any animal and plant tissue that may be disaggregated into single cells, measuring the breaks in the DNA chain prompted via natural or inorganic pollution (De Lapuente *et al.,* 2015). The single-cell gel electrophoresis counts the DNA breaks, alkali labile sites, DNA crosslinks, damage in base or base pairs, and apoptosis in the cells of living organisms. The Comet assay was started by Ostling and Johanson (1984) and then modified via Singh *et al.* (1988). Standard dose-reaction tracking method for determining of molecular-level damages in marine animals has now commonly been used (Li *et al*., 2013; Turan and Ergenler 2019). Hallare *et al.* (2016) used the comet analysis and micronucleus test for the genotoxic effects induced by means of intensive cage aquaculture in Taal Lake (Philippines) on Nile tilapia (*Oreochromis niloticus*) that comet assay was reported as high-quality biomarkers for investigating the hazardous effects of cage-culture on freshwater quality. Arslan *et al.* (2016) stated that rainbow trout which grown in cage culture may be more pronounced with genetic damage, depending on the cage stress and concluded that these changes may be associated with nutritional conditions. Likewise, Demir *et al.,* (2015) reported that there is a correlation between water pollution that was caused by over-feeding in fish breeding farms and in vivo

genotoxicity in rainbow trout (*O. mykiss*). Different anthropogenic activities (such as fish breeding farms and fertilizers) increased the genotoxicity in rainbow trout lymphocytes according to pollution rate. Despite the number of the studies, there is lack of information about evaluation of genotoxic damage in cultured and wild marine fishes, especially. The main aim of this study was to evaluate the genotoxic damage in cultured and wild gilthead sea bream (*Sparus aurata*) from Iskenderun Bay by Comet assay. This study has been carried out in Hatay, Turkey, in 2017.

MATERIAL AND METHODS

Sampling area

This study was conducted at Cage and Reference station in January 2017. As Cage station, cultured gilthead sea bream (*Sparus aurata*) and cage water samples were collected from a fish farm located in Iskenderun Bay (36°29'57.4"N 35°57'42.8"E). Wild specimens and sea water samples as Reference station were collected from the coastal zone (36°26'48.6"N 35°53'27.3"E) of Iskenderun Bay, Northeastern Mediterranean using commercial trawler (Fig. 1). Reference Station was assigned an unaffected location of the upstream area about 1 km in distance from the cage station. The Iskenderun bay receiving anthropogenic inputs and surface runoff in winter season, and indicates development of mesotrophic/ eutrophic conditions locally in these semi-enclosed water bodies, due to NOx rich river in flows with modified N/P/Si ratios and direct discharges of urban wastewaters (Tugrul *et al.* 2019).

Sampling procedure and Water quality assessment

Water and fish samples were collected during the winter season in January 2017. The wild and cultured samples of *S. aurata* (10 individuals from each sampling sites) were collected from wild and fish farm, and immediately transported to the laboratory. Measurements such as total body length (cm) and wet weight (g) of sampling sea bream (±SD) were recorded at wild *S. aurata* (410.64±7.05 g, 27.50±0.70 cm) and cultured *S. aurata* (320.45±9.25 g, 24.56±0.50 cm). The sea bream samples were euthanized for gill and liver removal and immediately after dissection they were carefully washed with phosphate buffer. Water samples of sampling sites were collected at 15–30 cm below the surface, following the descriptions of DEA (2012) with Nansen hydrographical bottle. Water samples were collected in triplicate from each of the selected sites in winter season (January) 2017. The water samples were taken in 1000-mL polyethylene bottles after rinsing few times with water from the collection point and later transferred to the laboratory in cooler, containing ice to reducing the degradation of samples before analysis. The samples were filtered as soon as possible through 0.45 µm membrane filter. Immediate analysis is recommended but if it wasn't possible, samples was directly deep frozen till carrying out chemical analysis. The recommended standard methods (APHA, 2005) was used for

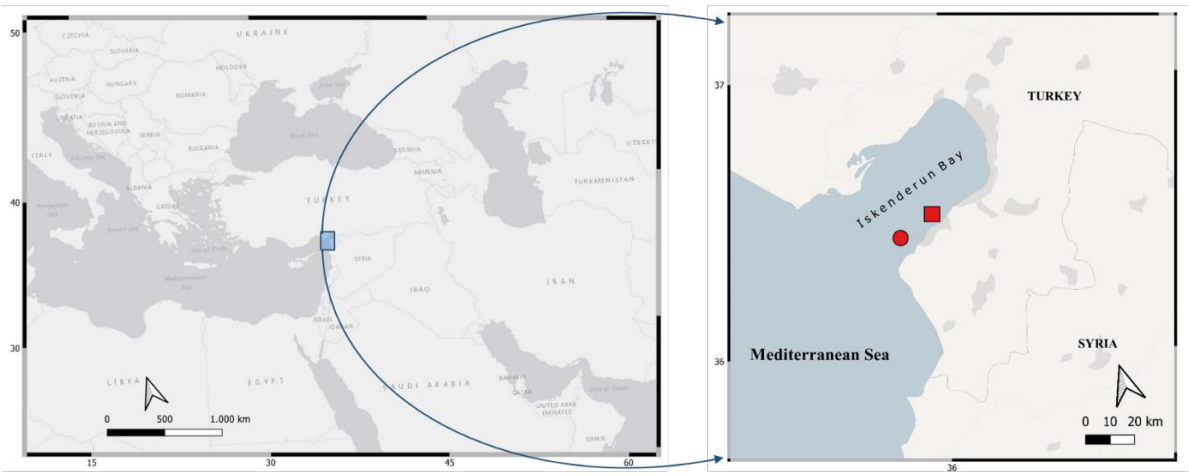

Fig. 1: Geographic location of the study area along with the sampling stations in the Iskenderun Bay, Turkey (reference station; cage station)

physicochemical analyses and preservation of water samples. These water-quality parameters tested include; temperature (ºC), pH, Alkalinity (mg/L), HCO_3 (mg/L), Total phosphate (TP) (mg/L), Dissolved Inorganic Nitrogen (DIN) (mg/L), Ammonium nitrogen (NH_4-N) (mg/L), Nitrate nitrogen (NO_3-N) (mg/L), Nitrite nitrogen (NO_2-N) (mg/L), Ammonia (NH_3-N) (mg/L), Sulphate (SO_4-S) (mg/L) were conducted in triplicates. In the sampling locations, seawater quality parameters such as temperature and pH were measured using YSI model multi probe system during the sampling.

Water quality criteria

National water quality criteria (Turkish Environmental Guideline) (TEG, 2005, 2006) were determined as proposed by Ministry of Agriculture and Forestry as the main authority responsible for regulating marine finfish aquaculture in Turkey. The TEG was determined by measuring some of the analyzed physicochemical parameters (temperature, pH, Nitrate nitrogen, Nitrite nitrogen, Ammonia etc.). In addition, National Mediterranean Coastal Waters Criteria tools were determined by Ministry of Forestry and Water Management (TEG, 2012). The Eutrophication indices obtained were classified as follows: DIN values:<0.020 mg/L; TP values:<0.010 mg/L (Good/Oligotrophic water quality); DIN values:0.020-0.1mg/L; TP values:0.010-0.020mg/L (Moderate/Mesotrophic properties); DIN values:0.1-0.2 mg/L; TP values:>0.02-0.03 mg/L (Poor/eutrophication); DIN values:>0.2 mg/L; TP values:>0.03 mg/L (Bad/ Dystrophic).

Comet assay

Cellular dissociation method modified from Cavalcante *et al.* (2008) was used in Comet assay. Gill and liver tissues of *S. aurata* were homogenized in order to get single-cell suspension and centrifuged at 3000 rpm at 4 °C for 5 min for the cell suspension, and then the cell pellet was retained. Cell viability was evaluated by the Trypan blue exclusion test (Anderson *et al.*, 1994). Singh *et al.* (1988) was followed for performing the single cell gel electrophoresis. The slides were neutralized with ice cold 0.4 M Tris buffer (pH 7.5) and stained with 80 ml ethidium bromide (20 mg/mL) and counted with an image analysis system. Images of 100 cells from each sample were scored with Comet Analysis Software, *V* 3.0. Tail density (%T-DNA), tail moment (µm) and tail migration (TM_i) were taken as the parameter of the nuclear DNA damage.

Statistical analysis

One-way analysis of variance (ANOVA) was used for statistical evaluations of data. Principal component analysis (PCA) was used to get a comprehensive view of the results and define the most important parameters involved in DNA damage (Zheng *et al.*, 2016). All data were executed using IBM SPSS Statistics 21 and R-Studio.

RESULTS AND DISCUSSION

The physical-chemical parameters of the water samples collected from the cage and reference station were given in Table 1. The data related to temperature, pH, Alkalinity, HCO_3, SO_4-S and TP were not significantly different between the cage and reference stations (P>0.05). Dissolved Inorganic Nitrogen (DIN) in cage and sea water samples was 0.097±0.004 mg/L and 0.075±0.005 mg/L, respectively, and the data obtained for DIN was statistically different between the cage and reference stations (P<0.05). NH_4-N, NO_3-N, NO_2-N and NH_3-N parameters were also significantly different between the cage and reference stations (P<0.01). The present study demonstrated that the nutrient concentrations in the cage station were between the applicable high-quality levels of water traits according to the national marine aquaculture limits. The detected pH, temperature, NO_3-N, NO_2-N and NH_3-N parameters did not exceed the national water quality criteria (TEG 2005, 2006).

Furthermore, our findings in terms of nutrients were similar to those of previously performed research in offshore cage systems in the Aegean Sea, Turkey (Gurses *et al.*, 2019). Moreover, the TP values as Eutrophication Criteria (E.C.) tools were recorded at 0.020 mg/L and 0.016 mg/L in the cage and reference stations which are at border and below the TEG (2012) limit, 0.020 mg/L. The DIN values as Eutrophication Criteria (E.C.) tools were recorded at 0.097 mg/L and 0.075 mg/L in the cage and reference stations, respectively in this study, which are at below the TEG (2012) limit, 0.1 mg/L. The water bodies in the cage and reference stations in the Iskenderun Bay exhibit moderate/mesotrophic water quality according to the TEG (2012). Similarly, Tugrul *et al.*

Table 1: Physical-chemical parameters of the water samples collected from the cage and reference stations

Parameters (mg/L)	Reference station	Cage Station	Water quality criteria
pH	8.367±0.153	8.507±0.125	6.5–8.5
Temperature (ºC)	16.0±0.5	16.5±0.5	15–25
Alkalinity	34.333±1.155	33.333±2.887	-
HCO_3	44.000±1.732	43.333±2.887	-
Total phosphate (TP)	0.016±0.003	0.020±0.008	-
Dissolved Inorganic Nitrogen(DIN)*	0.075±0.005	0.097±0.004	-
Ammonium nitrogen (NH_4-N)**	0.039±0.001	0.047±0.001	-
Nitrate nitrogen (NO_3-N)**	0.026±0.001	0.032±0.001	< 40
Nitrite nitrogen (NO_2- N)**	0.009±0.001	0.017±0.001	< 0.5
Ammonia (NH_3- N)*	0.011±0.002	0.017±0.002	0.05–1.5
Sulphate (SO_4-S)	73.333±2.887	70.000±5.000	-

The data are shown as arithmetic mean ± standard deviation. Indicate significance level between the water samples collected from the cage and reference station (*, P<0.05; **, P<0.01). Water quality criteria: Proposed licensing requirements for marine aquaculture in Turkey (TEG, 2005, 2006); Ministry of Agriculture and Forestry as the main authority responsible for regulating marine finfish aquaculture in Turkey.

Table 2: DNA damage in the gill and liver cells of wild and cultured gilthead sea bream from the cage and reference station analyzed by Comet Assay

Gill		
Head Length (µm)**	24.845±0.419	26.668±0.401
Tail Length (µm)***	19.160±0.429	23.930±0.508
H-DNA (%)*	85.871±0.902	82.236±1.125
T-DNA(%)*	14.128±0.902	17.763±1.125
Tail.Moment (µm)**	1.846±0.157	2.958±0.244
Tail Migration (TMi)**	7.157±0.509	11.047±0.606
Liver		
Head Length (µm)**	24.168±0.263	28.423±0.835
Tail Length (µm)**	15.397±0.287	17.560±0.382
H-DNA (%)	91.749±0.530	91.004±1.118
T-DNA(%)	8.250±0.530	8.995±1.118
Tail Moment (µm)	0.793±0.075	0.885±0.005
Tail Migration (TMi)	3.592±0.327	3.349±0.264

The data are shown as arithmetic mean ± standard deviation. Indicate significance level between wild and cultured gilthead sea bream from the cage and reference station (*, P<0.05; **, P<0.01).

(2019) also reported that the Eastern Mediterranean and its offshore waters are in oligotrophic, and the inner sites of the Mersin and Iskenderun Bays are in mesotrophic conditions. DNA damage in the gill and liver cells of wild and cultured gilthead sea bream from the cage and reference stations analyzed by Comet assay are given in Table 2.

A higher level of DNA damage in gill cells

compared to liver cells was observed in sea bream samples (Table 2). The highest level of DNA damage as %T-DNA, TM and TMi in gill cells were 17.763±1.125%, 2.958±0.244µm, 11.047±0.606 TMi at the cage station, respectively (Table 2). Likewise, the highest level of DNA damage as %T-DNA, tail moment and tail migration in liver cells were 8.995±1.118%, 0.885±0.005µm, 3.349±0.264TMi at the cage station, respectively. Significant differences (P<0.01) in DNA damage especially gill cells between the cage and reference stations from Iskenderun Bay (Table 2). The increased concerns on the genotoxicity of organic/ inorganic pollutants lead to the usage of sensitive bioassays as an important instrument to monitor the genotoxicity of polluted water columns. The present study provides the first data set on genotoxic damage induced by marine cage culture in Iskenderun Bay on gilthead sea bream using Comet Assay. The DNA damages at gill and liver cells of gilthead sea bream in the present study were observed with a higher level of DNA damage in gill cells compared to liver cells in both the cage and reference stations. Gills may be more susceptible to pollutants than other tissues owing to a high respiratory blood flow and permanent contact with the water. Gill tissue are commonly used for monitoring water pollution due to their direct contact with the water. Several studies reported that gill was sensitive and target tissue for water pollution monitoring (Lenhardt *et al.,* 2015; Butrimavičienė *et al.,* 2018). On the other hand, the liver also has a high accumulation potential, and therefore, used as an important pollution indicator (Ploetz *et al.,* 2007). The DNA damages at gill and liver cells of gilthead sea bream in the present study were determined at

Table 3: Eigenvalue, proportion and cumulative contribution of physico-chemical variables to DNA damage of sea bream on first two Principal Components

Component	Eigenvalues	Variance (%)	Cumulative (%)
1	13.559	61.632	61.632
2	4.790	21.775	83.407
3	1.758	7.993	91.400
4	1.060	4.816	96.216
5	0.832	3.784	100.000

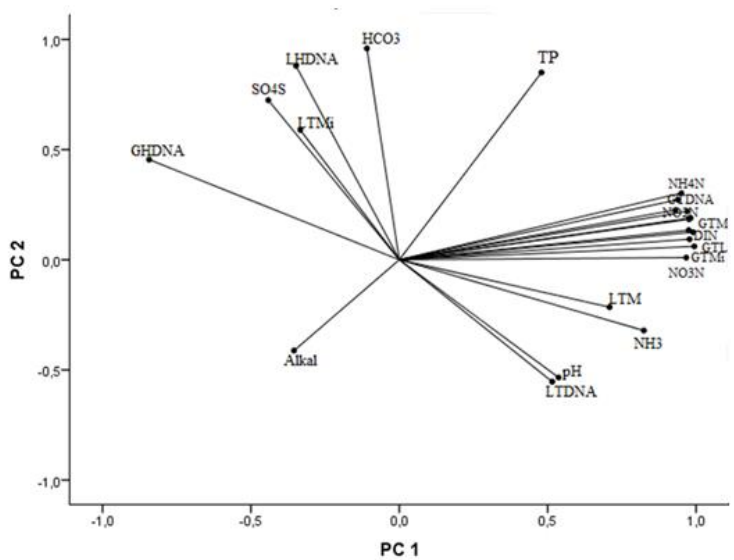

Fig. 2: Contribution and relation of analysed parameters on first two Principal Components (GTDNA: Tail density in gill cells; GTM: tail moment (TM) in gill cells; GTMi: tail migration (TM_i) in gill cells; LTDNA: Tail density in liver cells; LTM: tail moment (TM) in liver cells; LTMi: tail migration (TM_i) in liver cells).

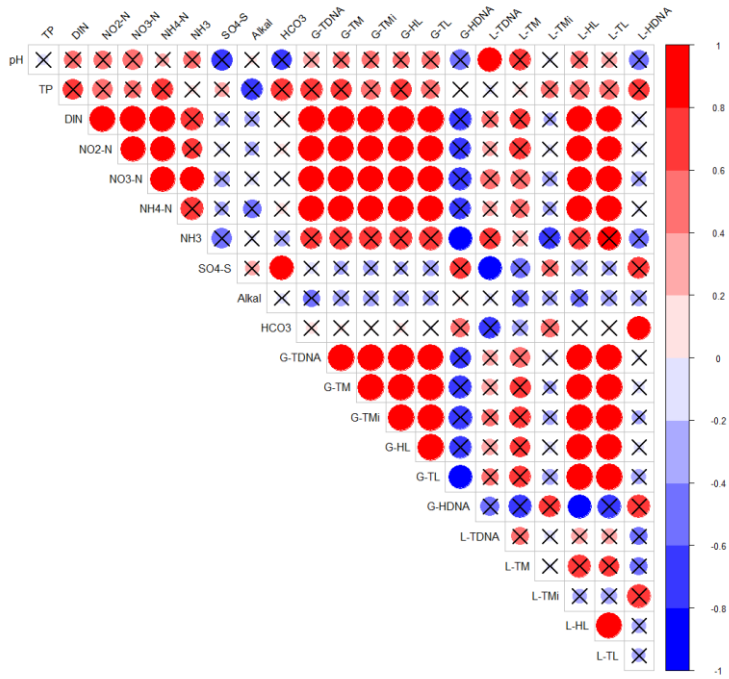

Fig. 3: Heatmap of correlations between parameters. The scale color bar indicate correlation between -1 and +1. Cross (x) indicate the insignificant correlations according to the specified significance level (P>0.05) and non-crossed circle indicate significant correlations according to the specified significance level (P<0.05)

the low and minimal scale at the cage and reference stations, respectively, based on Mitchelmore *et al.* (1998) who reported %T-DNA damage scale as <%10 T-DNA minimal damage, %10-25 T-DNA low damage, %25-50 T-DNA medium damage, %50-70 T-DNA high damage and >%70 T-DNA extreme damage. The DNA damage in fish tissue are commonly applied for detecting the genotoxic pollution of water columns (Colin *et al.,* 2016) being able to adjust exposure to low concentrations of pollutant in candidate sentinel species. Similarly, Gutiérrez *et al.* (2019) reported that mussels from mollusc farm in the Guanabara Bay revealed small amount of genomic destruction, on the other hand, mussels from aquaculture located at Rasa Beach and Forno Bay exhibited values near to zero. Furthermore, Demir *et al.* (2015) reported that the degree of DNA damage was low scale in the blood cells of rainbow trout collected from different sites from Esen stream with nutrient pollution generated from overfed fish farms. On the other hand, first two principal components revealed 61.632 % and 21.775 % of total variations, respectively in Principal component analysis (PCA) (Table 3). When the pattern of strong contribution of the DIN, NH_4-N, NO_3-N and NO_2-N (in the water column) parameters and the DNA damage in gill cells of gilthead sea bream were examined here with the PCA, the DNA damage parameters seems to be correlated with NH_3-N, DIN, NH_4-N, NO_3-N and NO_2-N which are relatively important parameters involved in DNA damage (Fig. 2).

Correlations between physical-chemical parameters of the water samples and DNA damage parameters were shown with Heatmap that DIN, NH_4-N, NO_3-N and NO_2-N in water revealed significant positive correlations (P<0.05) with DNA damage levels in gill cells (Fig. 3).

There were no significant correlations between the other physical-chemical parameters (pH, TP, NH_3-N, SO_4-S, Alkalinity and HCO_3) and DNA damage parameters both in gill and liver cells of *S. aurata*. However, a positive correlation (r^2=0.9) was observed between the gill T-DNA and other DNA damage parameters in all the examined samples (Fig. 3). The similar correlations were also reported with the previous study that Demir *et al.* (2015) showed that

there is a correlation between water pollution and genotoxicity in rainbow trout (*O. mykiss*) in the Esen Stream that was caused by over-feeding in fish farms.

CONCLUSION

In conclusion, this is the first study on genotoxic damage induced by marine cage culture in Iskenderun Bay, Northeastern Mediterranean. The physical-chemical parameters and nutrient concentrations in cage station were between acceptable ranges of water quality characteristics and within the limits suitable for marine aquaculture activities. The water bodies in the cage and reference stations in coastal waters of the Iskenderun Bay, Northeastern Mediterranean exhibit moderate/mesotrophic water quality. The DNA damages at gill and liver cells of gilthead sea bream were determined at the low and minimal scale at the cage and reference stations, respectively. Furthermore, the correlations between physical-chemical parameters and DNA damage were shown that DIN, NH_4-N, NO_3-N and NO_2-N in water revealed significant positive correlations with DNA damage levels in gill cells. Correspondingly, the present study revealed the effectiveness of genotoxic markers for monitoring aqua cultural and environmental pollution by using damage DNA of the gilthead sea bream, *Sparus aurata*. Consequently, the assessment of genotoxicity by Comet Assay from cultured and wild gilthead sea bream denotes as a convenient marker to evaluate the potential pollution effect of aqua cultural activities. Aquaculture is getting an important factor in the global food supply in the future. In intensive cultural events, the main wastes are solid, chemicals and several therapeutics. Potential pollutant properties of aquaculture activities to marine habitats are progressively acknowledged, while they are a lesser quantity to land-based pollutants. Therefore, it is increasingly important to monitor genotoxic effects of any aquaculture activities in related environments. Accurately planned usage of aquaculture waste lightens marine pollution events and not only protects valuable marine assets but also profits the nutrients comprised efflux. Thus, it is greatly challenging to advance sustainable aquaculture that deliberate stocking density and pollution loadings below environmental capacity. From the management point of view, further researches are encouraged in terms of continuous monitoring of cage farm places in order to control water quality and potential culture effects for the maintainable aquaculture in the Mediterranean.

AUTHOR CONTRIBUTIONS

F. Turan performed the conception and design of the study, laboratory studies, water quality analysis acquisition of data, drafting the manuscript, reviewing and editing. M. Turgut performed laboratory studies, sampling procedure and water quality analysis.

CONFLICT OF INTEREST

The authors declare no potential conflict of interest regarding the publication of this work. In addition, the ethical issues including plagiarism, informed consent, misconduct, data fabrication and, or falsification, double publication and, or submission, and redundancy have been completely witnessed by the authors.

ABBREVIATIONS

%H.	DNA Head Density
%T	DNA Tail Density
ANOVA	One-way Analysis of Variance
APHA	American Public Health Association
COMET	The Single-cell Gel Electrophoresis
DEA	Department of Environmental Affairs
DIN	Dissolved Inorganic Nitrogen
FAO	The Food and Agriculture Organization
N	Nitrogen
NH_3- *N*	Ammonia
NH_4-*N*	Ammonium nitrogen
NO_2-*N*	Nitrite nitrogen
NO_3-*N*	Nitrate nitrogen
P	Phosphorus
PCA	Principal Component Analysis
SO_4-*S*	Sulphate
TEG	Turkish Environmental Guideline
TM	Tail Moment
TM_i	Tail Migration
TP	Total Phosphate
GTDNA	Tail density in gill cells
GTM	Tail moment (TM) in gill cells
GTMi	Tail migration (TMi) in gill cells

LTDNA	Tail density in liver cells
LTM	Tail moment (TM) in liver cells
LTMi	Tail migration (TMi) in liver cells

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