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Original Article

Effect of Different Plant Extracts Added to Ice on Sensory Preference of Sliced Salmon

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ABSTRACT

Salmon is usually sold in slices on ice in retail markets. Since ice is in direct contact with fish, it affects the sensory characteristics as well as the physicochemical properties. Improving the properties of ice can make positive contributions to fish flavor and consumer preference. In this study, sliced salmons were treated with ice containing one of either basil (B), rosemary (R), laurel (L), oregano (O) or fennel (F) extracts. Iced salmon without any plant extract was the control (C) group. Displaying sliced fish for sale on ice and placing back to cold room at the end of the day is a common practice. Samples were covered with ice, stored at 18±1°C during daytime and taken to the cold room (2±1°C) at night to simulate marketing conditions. Adding plant extracts to ice resulted in a remarkable change in fish flavor, and R, F, and L were the most popular treatments among all groups. In particular, rosemary-added ice significantly ($P<0.05$) increased the preference of consumption. Panelists emphasized that F samples can be consumed as appetizers. Dominant and pleasant aroma was also stated for L samples. The mesophilic aerobic bacteria count remained below 5 log CFU/g in all samples during 4 days. The total volatile basic nitrogen (TVB-N), thiobarbituric acid reactive substances (TBARS) and pH values of all groups remained within the limits of acceptability. Using plant extract-added-ice provided a suitable quality sale of salmon for 4 days and offered an option to consumers by giving the product different flavors.

Keywords: Plant extract, Icing, Fish, Salmon, Sensory preference

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INTRODUCTION

Chilling of harvested fish is the first thing to do in order to maintain the postmortem quality of flesh. Temperature is the most important factor to extend shelf life by retaining the freshness of fish. Since fish is highly perishable, it is necessary to store it at the minimum temperature just above the freezing point of water. Icing delays microbial growth, slows down enzymatic activity and prevents dehydration. Therefore, it has been the most common handling practice during the transportation, storage and marketing of fish (Huss, 1995).

Atlantic salmon is one of the major species produced in world aquaculture (4.5%). In 2018, the total number of Atlantic salmon production was 2435.9 thousand tonnes, and it is expected to continue growing. Salmon is a high value species and widely marketable, and the importation of it is spread all over the world (FAO, 2020). In most countries, fresh salmon is marketed to consumers in slices on ice in the market. This facilitates the sale and helps the fish to maintain its freshness. The ice melts in time, washes the surface of the fish and prevents the loss of moisture. Based on being in direct contact with the fish, the properties of the ice are important, and it can be claimed that improving the properties of ice can make positive contributions to fish flavor and acceptance.

There are studies that have added plant extracts to ice in order to examine the chemical

quality of fish (Quitral et al., 2009; Yerlikaya, Ucak, Gumus & Gokoglu, 2015) or to create a biofilm (Bensid, Bendeddouche, Toy, Sigirci & Özogul, 2014a). However, it is also important to examine the effect of extract on the taste and appearance of fish in terms of consumer acceptance. Otherwise, it will not be possible to sell the product even if the treatment provides advantages in terms of fish quality and shelf life. Therefore, evaluating the sensory appreciation of treatment is of great importance. There are several methods to evaluate the sensory quality of fish such as QIM, EC or Torry Schemes (Barbosa, Bremner & Vaz-Pires, 2002). However, these methods are not adapted to measure the acceptance–preference when an additional treatment is applied on fish. The nine-point hedonic scale provides data about preference of consumers by evaluating the likes and dislikes of the product. The scale is simple to describe and easy to use for all types of foods (Stone & Sidel, 2004). On the other hand, when there are more than three types of food samples, there is a need for a rank-rating scale in order to determine the comparative perception and preference of consumers. The aim of the ranking method is to evaluate and compare the products with each other and place them in increasing order in terms of consumers' perceived intensities rather than mean scores along a predetermined scale (Cleaver, 2018).

Salmon is usually sold in slices on ice in retail markets. Since ice is in direct contact with the fish, it directly affects the quality and attributes of the fish. In this study, the potential of increasing consumer acceptance by adding various plant extracts to ice was investigated. Thus, the possibility of offering salmon, which has already been sold in slices on ice in retail markets, with extended variations of flavors was examined. It is known that when the stock rotation is not considered during the sale, the sliced fish can be placed back in the cold room at the end of the day and offered for sale on ice the next day. Therefore, while the effects of plant extracts added to ice on sensory properties of salmon were studied, and the changes in quality were also examined by simulating the sales conditions.

MATERIAL AND METHODS

Materials and preparation of ice

Whole gutted Atlantic salmon (*Salmo salar* Linnaeus, 1758) was purchased from Metro Gross market, İstanbul, Turkey. A total of 22.5 kg salmon were used for this study. Metro Gross market imported salmon from Norway in polystyrene boxes with ice within three days after the harvest, without breaking the cold chain. Salmons were wrapped with ice gel packs during purchasing and transferred to the laboratory within 30 minutes, then sliced in accordance with the sales conditions. The average size of a salmon slice was 126±6.55 grams.

Basil (*Ocimum basilicum*), rosemary (*Salvia rosmarinus*), laurel (*Laurus nobilis*), oregano (*Origanum vulgare*) or fennel (*Foeniculum vulgare*) liquid extracts were purchased from Alfasol (Kimbiotek) Chemical Materials Ind. & Trade. Co. Ltd., Turkey. These extracts were added to tap water during ice making, separately. Extracts were dissolved in water at the ratio of 1:100 (v/v). Water with each extract was divided into 1 kg batches and frozen. Each ice block was crushed before use. Salmon slices were divided into 6 groups, each group was layered into a styrofoam box and covered with one of the prepared ice varieties. Salmon treated with basil (B), rosemary (R), laurel (L), oregano (O) or fennel (F) extracts added to ice were grouped by the name of the extracts. Control (C) group was determined as the salmon exposed to ice without any extracts. Fish to ice ratio were always maintained as 1:2 (w/w) and the ice was replenished during storage in case of need.

Experimental conditions and sampling

During day time, salmon slices were laid on the counter and surrounded by ice to simulate the retail sales conditions in a fish market. The ambient temperature was 18±1°C and humidity was 42.9±0.2%. Iced salmon was displayed for approximately eight hours on the counter. In the evening, salmon slices were placed in separate styrofoam boxes and covered with related ice varieties to imitate the conditions in the market. The boxes that allow draining were stored in an isothermal cold room at 2±1°C. Humidity of the cold room was 83±0.2%. The boxes were stored in the cold room for approximately sixteen hours until the morning. In the morning, fish were displayed on ice at 18°C again under retail sales conditions. This cycle continued, and the sampling was carried out every day for five days (including the day zero) at the noon time.

Sensory Tests

Two different tests were carried out for four days. Each day, panelists were asked to evaluate every group of iced salmon according to the hedonic preference test and then to rank for the rank-rating test. The hedonic preference test was performed by 12 experienced panelists using 9 to 1 scale, ranging from extreme dislike (score 1), neither like nor dislike (score 5) to extreme liking (score 9) (Stone & Sidel, 2004) (Figure 1a). The samples taking the sensory score of ≤4 were considered as spoiled. Both raw and cooked salmon were served for the evaluation. Samples were coded with three digit numbers to avoid any prejudgment. All descriptions were also given below the form. For cooked fish assessment, salmon was put in a glass jar which was placed into a water filled pot and heated up in a bain-marie. Thus, the possibility of irrelevant smell contamination that may occur during cooking was eliminated. After 20 minutes of simmering in the bain-marie, salmon was done, and the internal temperature was ~80-85°C. The cooked samples were served to the panelists immediately.

The panelists were also asked to rank the fish samples in ascending order of preference from the most liked (#1) to the disliked (#6) for the ranking preference test (Figure 1b) (Cleaver, 2018). Twelve panelists performed the ranking test. Thus, the panelists have determined the most favorite treatments.

Microbial analysis

Total aerobic bacterial counts were analyzed in triplicate in agar plates with duplicate measurements as described in the Bacteriological Analytical Manual (Maturin & Peeler, 2001). Ten grams of each fish sample were put into a sterile filter bag and stomached in 90 mL peptone water (0.1%). After homogenization, serial dilutions were prepared. For the estimation of aerobic bacteria, 1 mL aliquots from the appropriate dilutions were inoculated onto Plate Count Agar (PCA) (Merck No. 1.05463) plates. Pour plate technique was used. The plates were incubated at 37°C for 48 h. The results were reported as Colony Forming Unit per gram (CFU/g) of sample.

Name: Date:

HEDONIC PREFERENCE TEST

RAW SALMON	Sample Code 720	337	548	219	405	681
Appearance						
Odor						
Texture						

COOKED SALMON	Sample Code 720	337	548	219	405	681
Appearance						
Odor						
Taste						
Texture						

Comments:

Score	Hedonic Scale
9	Like extremely
8	Like very much
7	Like moderately
6	Like slightly
5	Neither like nor dislike
4	Dislike slightly
3	Dislike moderately
2	Dislike very much
1	Dislike extremely

(a)

RANKING PREFERENCE TEST

Please rank the samples from the most liked (#1) to the least liked (#6).

	1	2	3	4	5	6
Sample Code						

(b)

Figure 1. Sensory analysis forms of iced salmon for **(a)** Hedonic preference test and **(b)** Ranking preference test in which the codes present the samples as follows: 720-Basil; 337-Rosemary; 548-Laurel; 219-Control; 405-Oregano and 681-Fennel.

Physicochemical analyzes

Total volatile basic nitrogen (TVB-N), thiobarbituric acid reactive substances (TBARS) and pH measurements were carried out. For TVB-N determination, 10 g fish sample was boiled with magnesium oxide, and 0.1 N hydrochloric acid (HCl) was used to hold the vapor components by the steam distillation. HCl was titrated with 0.1 N sodium hydroxide, and the amount of TVB-N was calculated as mg/100g (Schormüller, 1968). In order to determine the lipid oxidation, 5 g sample was homogenized with 100 µL of butylated hydroxytoluene and 50 mL of distilled water. After the addition of 2.5 mL of 4 N HCl and further 97.5 mL of distilled water, the sample was heated, and steam was distilled. The condensed liquid was mixed with 2-thiobarbituric acid reactive and placed into a hot water bath (80°C) for 30 mins. Optical density was measured at 532 nm followed by cooling. Results were expressed as mg malondialdehyde/kg (mg MA/kg) (Vyncke, 1970). The pH analysis was carried out with the homogenized and 1:10 diluted sample. The pH value was measured using a pH-meter (Jenco, 6173pH, China).

Statistical analyzes

Statistical analyzes were performed using SPSS 21 (IBM SPSS Version 21, IL, USA). The results were analyzed by means of analysis of variance (ANOVA). The significant difference level among batches was chosen as 0.05. Tukey tests were used, and standard errors were presented in the tables. Friedman two-way analysis of variance was used to evaluate the statistics of rank-rating sensory tests. Analyzes were carried out in triplicate, and this study was replicated twice.

RESULTS AND DISCUSSION

Sensory evaluation

The initial quality of salmon was defined as excellent (day 0) since the sensory scores were 8.64 and 8.33 for the raw and cooked samples, respectively. Sales conditions were imitated for 4 days, and panelists evaluated each group of iced salmon according to hedonic preference test and ranked for ranking preference test. After one day on ice, with or without plant extracts, the scores of all groups were similar ($P \geq 0.05$), and the overall acceptability was high. As a result of hedonic preference test, none of the samples were regarded as spoiled during 4-day storage. During this period, salmon slices were displayed on ice –with or without plant extracts– during daytime and taken to the cold store in iced boxes in the evening.

Sensory quality of control samples was found to be similar to the other groups in terms of freshness for 4 days (Table 1). However, the ranking preference test showed that control samples were not preferred by any panelists on the first day, yet they were liked by only 8% of the panelists on the 2nd and 3rd days of the study (Table 2). Likewise, basil-added ice (B) resulted in acceptable quality during sales conditions (Table 1), but it was not among the panelists' favorite options (Table 2). Display and storage on oregano-added ice (O) gained the appreciation of 25% of panelists on the 3rd day. However, none of the panelists preferred this treatment on days 1 and 2, and only 8% preferred it on day 4, according to the ranking preference test. Bensid, Ucar, Bendeddouche & Özogul (2014b) kept anchovies in a cold store with ice containing thyme (0.04% w/v) and oregano (0.03% w/v). These amounts were lower than that of our study, and the samples were highly preferred by the panelists due to their desirable odor. In another cold storage study, 1% thyme or laurel essential oil was added into homogenized bluefish fillets and put in plastic bags which were stored in ice. It was determined that the sensory scores of treated groups were higher than the untreated group (Erkan et al. 2011).

The addition of plant extracts to the ice led to an evident change in fish flavor which was defined as herbal or spicy by the panelists after being cooked. The flavors obtained by adding oregano or basil to the ice were not preferred compared to other treatments. However, the flavors released in other three groups (R, F, and L) were among the most popular ones in the ranking test (Table 2). In particular, addition of rosemary to ice (R) significantly improved the aroma and increased the preference. These samples (R) usually received the highest sensory scores in hedonic preference test, and the panelists expressed a pleasant flavor and aroma. According to the ranking preference test, rosemary

Table 1. Sensory scores of control and basil, rosemary, laurel, oregano or fennel extract added iced raw and cooked salmon samples.

Raw Salmon				
	Day 1	**Day 2**	**Day 3**	**Day 4**
Control	$7.64\pm0.78^{a, X}$	$6.86\pm0.61^{a, X}$	$5.78\pm0.83^{ac, Y}$	$5.78\pm0.80^{a, Y}$
Basil	$7.35\pm0.67^{a, X}$	$6.88\pm0.46^{a, X}$	$5.57\pm1.06^{a, Y}$	$5.69\pm0.78^{a, Y}$
Rosemary	$7.47\pm0.66^{a, X}$	$7.49\pm0.65^{a, X}$	$6.93\pm0.60^{b, X}$	$5.78\pm0.46^{a, Y}$
Laurel	$7.46\pm0.87^{a, X}$	$7.39\pm0.60^{a, X}$	$6.99\pm0.71^{b, X}$	$5.75\pm0.90^{a, Y}$
Oregano	$7.35\pm0.81^{a, X}$	$6.76\pm0.88^{a, X}$	$6.58\pm0.67^{bc, X}$	$5.19\pm0.63^{a, Y}$
Fennel	$7.63\pm0.66^{a, X}$	$7.38\pm0.77^{a, X}$	$6.50\pm0.71^{ab, Y}$	$5.57\pm0.87^{a, Z}$
Cooked Salmon				
	Day 1	**Day 2**	**Day 3**	**Day 4**
Control	$7.14\pm0.85^{a, X}$	$6.78\pm0.59^{a, X}$	$5.76\pm0.67^{a, Y}$	$5.67\pm0.51^{a, Y}$
Basil	$7.52\pm0.73^{a, X}$	$6.94\pm0.43^{ab, X}$	$5.65\pm0.98^{a, Y}$	$5.57\pm0.97^{a, Y}$
Rosemary	$7.99\pm0.67^{a, X}$	$7.63\pm0.75^{b, XY}$	$6.91\pm0.91^{b, Y}$	$5.82\pm0.88^{a, Z}$
Laurel	$7.55\pm0.82^{a, X}$	$7.41\pm0.84^{ab, X}$	$6.87\pm0.63^{b, X}$	$5.57\pm0.80^{a, Y}$
Oregano	$7.48\pm0.67^{a, X}$	$6.66\pm0.66^{a, Y}$	$6.48\pm0.81^{ab, Y}$	$5.25\pm0.81^{a, Z}$
Fennel	$7.89\pm0.80^{a, X}$	$7.40\pm0.80^{ab, XY}$	$6.73\pm0.70^{b, Y}$	$5.37\pm1.01^{a, Z}$

$^{a, b, c}$: Mean values followed by different low-case letters show statistical difference ($P<0.05$) between the groups; $^{X, Y, Z}$: Mean values followed by different capital letters indicate significant ($P<0.05$) differences among the days for the same group.

Table 2. Percentages of the scores of ranking tests for the salmon treated with rosemary, fennel, laurel, oregano, basil extracts-containing and traditional ice on days 1, 2, 3, and 4.

	Percentage of ranking scores (%)			
	Day 1	**Day 2**	**Day 3**	**Day 4**
Rosemary	41	34	25	25
Fennel	25	25	17	17
Laurel	17	33	8	25
Oregano	0	0	25	8
Basil	17	0	17	0
Control	0	8	8	25

treatment was chosen by 41% of the panelists as the most liked treatment on day 1. Similarly, R was the most preferred treatment by 34%, 25%, and 25% of panelists on the 2nd, 3rd and 4th days of storage, respectively. In general, this treatment was regarded as the favorite treatment by the panelists, and samples were acceptable during 4 days of storage simulating selling conditions. In another study, sardines were covered with rosemary extract-added ice and stored in a refrigerated room (Özyurt et al., 2012). Unlike our study, it was not aimed to imitate the sales conditions, and samples were kept in the cold storage continuously. No significant differences were reported between sensory qualities of sardines stored with ice containing 0.05% and 0.1% rosemary extract. Samples were regarded acceptable in terms of sensory quality during 15 days of storage. Ozogul et al. (2011) studied the effect of rosemary extract on the quality of frozen sardines. They treated sardines with 1% and 2% extract, stored at -18°C and reported no significant difference between the control and processed samples in terms of color, odor and tightness during 4 months of storage. In addition, they noted a bitter taste in the samples treated with 2% rosemary extract, considering sensory test results.

As for the samples exposed to fennel-added-ice (F), the panelists emphasized that these salmon slices could be consumed as appetizers because of their nice and distinct fennel taste. Although a significant change in aroma was reported due to the addition of fennel into the ice, this change was mostly regarded as pleasant. On the first two days of storage, F samples were regarded as the most liked treatment by 25% of panelists; while it was liked by 17% on the 3rd and 4th days.

A dominant and pleasant aroma was also stated for the samples treated with laurel-added ice (L), and this aroma was defined as herbal. According to the ranking test of group L, the preference percentages of the panelists were 17%, 33%, 8% and 25% on the 1st, 2nd, 3rd and 4th days of storage, respectively. The panelists stated that the herbal or spicy flavors developed in R, F, and L may be preferred especially by the consumer who does not like the taste and smell of fish. It was also asserted that it could be an advantage for the consumer who prefers products with different flavors.

Quality assessment

The quality of freshly sold salmon depends on its microbiological and physicochemical conditions which can be affected by environmental factors. Even though the sensory characteristics of the fish marketed to the consumer seem very pleasant, they may not be suitable for consumption if there are significant losses in these quality parameters. So, it is necessary to evaluate the microbiological and physicochemical parameters in the sales conditions of the fish treated with plant extract-added ice.

Total viable microbial load of the fresh salmon was 3.32±0.08 log CFU/g on day zero. The microbial counts slightly increased during storage. Significant differences (P>0.05) were not determined between the groups during storage. All groups were slightly above 4 log on the fourth day (Table 3). Likewise, Rey, Garcia-Soto, Fuertes-Gamundi, Aubourg & Barros-Velazquez (2012) determined that the total aerobic count of hake iced with/without 0.4% or 0.8% citric, lactic and ascorbic acid commercial mixtures, was between 3 and 4.5 log CFU/g during 5 days of chilled storage. In another study, the aerobic counts of chilled mackerel (iced containing 0.02% of citric, lactic or ascorbic acids) were lower than the traditionally iced group on the 3rd and 6th days. However, the microbial load of traditionally iced fish was 4.21 log CFU/g on the 6th day (Sanjuas-Rey, Gallardo, Barros-Velazquez & Aubourg, 2012). Similarly, the mesophilic load of group C was higher than that of groups B and R on the 3rd day, but all groups were about 4 log CFU/g at the 4th day of storage in our study. Bensid et al. (2014b) reported that iced anchovy with/without oregano extract on the 6th day had the mesophilic bacteria counts above 7 log CFU/g. Mesophilic bacteria load of sardine with rosemary extract-added ice was also reported above 7 log CFU/g after 15 days (Ozyurt et al., 2012). In our study, mesophilic aerobic bacteria count remained below 5 log CFU/g, and all samples were acceptable for 4 days, when the sales conditions were imitated.

The pH value of the fresh salmon was 6.28±0.02 on day zero. Similar pH values were presented by Duun & Rustad (2008) for super chilled salmon. The acceptance limit for pH value is generally 6.8, and the fish is considered to be spoiled when the value increases to 7.0 (Ludorff & Meyer, 1973; Belitz, Grosch & Schieberle, 2009). In this study, even though there were statistical differences between the groups (P≤0.05), pH values of all samples remained below 6.8 during storage (Table 4). Bensid et al. (2014b) stated that pH was not significantly affected by the presence of thyme, oregano or clove plant extracts in ice during the chilled storage of anchovy unlike our study. Post-mortem pH increases due to the microbial activity which results in the rise of alkaline compounds such as TVB-N and can be between 6.0 to 7.1 (Özden, Inugur & Erkan, 2007). In this study, the microbial activity increased by about 1 log during five days in all groups, and it clearly seemed to affect neither pH nor TVB-N and not exceeded the limit values.

The initial TVB-N value of samples was 13.51±2.04 mg/100 g. Generally 30 mg/100 g TVB-N is considered as the limit of acceptance for fish (Sikorski, Kolakowska & Burt, 1990; European Economic Commission, 1995). It is also stated that the TVB-N parameter is an indicator of the suitability of fish for human consumption (Ozogul & Ozogul, 2000). As it was presented in Figure 2, none of the samples reached 30 mg/100 g. In a previous study, rosemary and oregano extract-icing were used to store Chilean jack mackerel for a 23-day chilling period (Quitral et al., 2009). The TVB-N content of all samples remained 5 mg/100 g during the first 4 days of storage. Especially after 10 days of storage, the TVB-N content of traditionally iced samples increased significantly and was higher than extract-iced groups. However, none of the samples contained TVB-N values above 25 mg/100 g. Likewise, TVB-N content of all samples were well below the acceptability limit in our study. Bensid et al. (2014b) examined the effect of ice containing thyme and oregano extracts on anchovy quality. They reported a small difference between the TVB-N values of control and the plant extract added groups, during the first days of storage. Likewise, no significant difference was reported between the TVB-N values of sardines, chilled with traditional or rosemary extract added ice, during storage (Ozyurt et al., 2012).

In this study, TBARS value was 0.48±0.07 mg MA/kg on Day zero. Fish, having TBARS value above 5–8 mg MA/kg (Nunes et al., 1992) or 8 mg MA/kg (Schormüller, 1968) is defined to be unacceptable. The salmon samples were in good condition in terms of lipid oxidation and did not even get close to the limit (Figure 3). Mixing plant extracts to water during ice making and letting this ice contact with the salmon decelerated lipid oxidation in comparison to control on the 4th day. Sanjuas-Rey, Barros-Velazquez & Aubourg, (2011) also stated that horse mackerel iced with 0.02% of citric, lactic and ascorbic acids had lower TBARS value than the control group during cold storage on the 4th day, similarly. Quitral et al. (2009) exposed Chilean jack mackerel to oregano and rosemary extracts-added ice and compared their quality to traditional ice. The TBARS values of the samples stored with plain ice increased after the 5th day of storage and reached 3 mg MA/kg, but extract-added ice samples' TBARS values were between 0.5-1 mg MA/kg during 23 days storage. In our study, TBARS values remained below 2 mg MA/kg in all groups during 4 days of storage, well below the limit of accept-

Table 3. Total viable aerobic plate counts of control and basil, rosemary, laurel, oregano or fennel extract added iced groups.

	Total viable plate counts (log CFU/g)			
	Day 1	**Day 2**	**Day 3**	**Day 4**
Control	3.46±0.02$^{a, XY}$	3.45±0.10$^{a, X}$	3.54±0.07$^{a, XY}$	4.24±0.23$^{a, Y}$
Basil	3.31±0.06$^{a, X}$	3.18±0.09$^{a, X}$	3.26±0.04$^{b, X}$	4.37±0.13$^{a, Y}$
Rosemary	3.46±0.05$^{a, X}$	3.22±0.03$^{a, Y}$	3.27±0.05$^{b, Y}$	4.27±0.15$^{a, Z}$
Laurel	3.46±0.03$^{a, X}$	3.12±0.03$^{a, Y}$	3.18±0.16$^{ab, XY}$	4.45±0.05$^{a, Z}$
Oregano	3.44±0.20$^{a, X}$	3.34±0.13$^{a, X}$	3.39±0.07^{ab,}	4.39±0.02$^{a, Y}$
Fennel	3.45±0.07$^{a, XZ}$	3.16±0.03$^{a, YW}$	3.20±0.16$^{ab, XY}$	4.46±0.34$^{a, ZW}$

$^{a, b}$ Different letters in the same column indicate statistical difference (P<0.05) between the groups; $^{W, X, Y, Z}$ Different letters in the same raw show statistical difference (P<0.05) among the days for the same group.

Table 4. The pH values of control and basil, rosemary, laurel, oregano or fennel extract-added iced salmon groups.

	Day 1	Day 2	Day 3	Day 4
Control	6.28±0.06$^{abc, X}$	6.30±0.02$^{ac, X}$	6.32±0.03$^{ab, X}$	6.29±0.02$^{a, X}$
Basil	6.31±0.04$^{ac, X}$	6.31±0.01$^{a, X}$	6.30±0.04$^{ab, X}$	6.35±0.06$^{a, X}$
Rosemary	6.30±0.03$^{ac, X}$	6.25±0.01$^{b, Y}$	6.28±0.02$^{a, X}$	6.30±0.03$^{a, X}$
Laurel	6.26±0.02$^{c, X}$	6.33±0.01$^{c, Y}$	6.34±0.03$^{b, Y}$	6.35±0.05$^{a, Y}$
Oregano	6.32±0.01$^{a, X}$	6.29±0.07$^{abc, X}$	6.28±0.07$^{ab, X}$	6.29±0.02$^{a, X}$
Fennel	6.22±0.01$^{b, X}$	6.23±0.02$^{b, X}$	6.31±0.03$^{ab, Y}$	6.30±0.03$^{a, Y}$

$^{a, b, c}$ Mean values followed by lowercase letters show statistical difference (P<0.05) between the groups; $^{X, Y, Z}$ Mean values followed by capital letters indicate statistical difference (P<0.05) for the same group among the days.

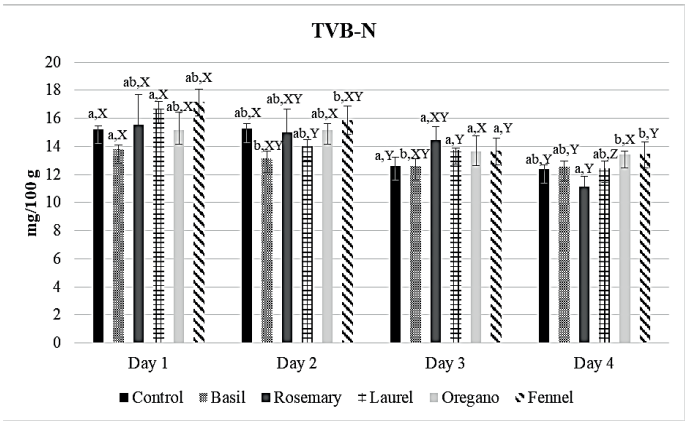

Figure 2. Total volatile basic nitrogen amounts of control and basil, rosemary, laurel, oregano or fennel extract-added iced groups.

$^{a, b, c}$Different lowercase letters indicate statistical difference (P<0.05) between the groups; $^{X, Y, Z}$ Different capital letters indicate statistical difference (P<0.05) among days.

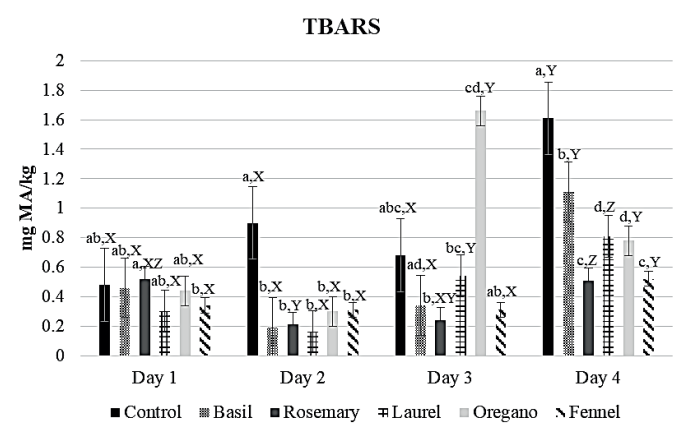

Figure 3. Thiobarbituric acid reactive substances analysis' results of control and basil, rosemary, laurel, oregano or fennel extract-added iced groups.

$^{a, b, c, d}$ Different lowercase letters show statistical difference (P<0.05) between the groups; $^{X, Y, Z}$ Different capital letters show statistical difference (P<0.05) among the days.

ability. Özyurt et al. (2012) stored sardines with traditional ice and ice with 0.05% and 0.1% rosemary extract. After 15 days of cold storage, they reported that TBARS values were above 5 mg MA/kg for traditional and 0.05% extract-added samples, and above 4 mg MA/kg for 0.1% rosemary extract-added samples. However, in our study the sale conditions were imitated, and none of the samples reached TBARS value of 2 mg MA/kg. Ozogul et al. (2011) treated sardines with rosemary extract (1% and 2%) and reported that the antioxidant effect could be achieved at the extract level of 2%. However, they also expressed a bitter taste in the samples treated with this amount of extract. In another study, addition of citrus peel extracts into ice during cold storage of common carp suppressed secondary lipid oxidation products in comparison to the control (Yerlikaya et al., 2015). It was also reported that the rancidity development remained low in pink salmon stored in iced or chilled sea water. No difference (P>0.05) between TBARS values were observed among the samples for ten days of storage (Himelbloom, Crapo, Brown, Babbitt & Reppond, 1994).

CONCLUSION

The effects of plant extract-added-ice on the sensory properties of sliced salmon were studied and quality changes were examined under the imitated sales conditions. Addition of plant extracts to ice was determined to be beneficial both for retaining the quality of fish and creating new flavors. Plant extract-added ice treatment of fillets resulted in a remarkable change in fish flavor, and rosemary, fennel and laurel treatments were the most popular. In particular, rosemary added ice significantly increased sensory preference. All groups were acceptable after 4 days of study, imitating the retail conditions. The use of plant extract-added-ice provided a suitable quality sale of sliced salmon and offered a different option to the consumer by giving the fillets unique flavors for the perfect sensory experience. Good quality and alternative flavored seafood can create a positive perception for consumers and the market.

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Original Article

Comparative Study of Lipid and Fatty Acid Profile in Liver Tissues of Male and Female *Silurus triostegus* During the Catching Seasons

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ABSTRACT

In this study, seasonal variations of total fatty acid (FA), phospholipid (PL) and triacylglycerol (TAG) compositions in liver tissues of catfish (*Silurus triostegus*) were investigated. Samples of *S. triostegus* were obtained from Atatürk Dam Lake, Turkey, in two month periods during one year as from May. The major components were palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1n-9), palmitoleic acid (16:1n-7), docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (AA, 20:4n-6) in total lipid, 16:0, 18:0, 18:1n-9, eicosapentaenoic acid (EPA, 20:5n-3), DHA and AA in PL, 16:0, 16:1n-7, 18:1n-9, linoleic acid (LA, 18:2n-6), AA, EPA, DHA and docosapentaenoic acid (22:5n-3) in TAG extracted from the liver of *S. triostegus* in all seasons. N-3/n-6 ratio was found 2.00-2.61 in females, 1.15-2.75 in males in total lipid. The highest lipid content was found in May (2.39%) in the females. In the males, the highest level was found in September (2.91%). In TAG fraction, the component with the lowest ratio in both sexes' TAG is PUFA. In PL fraction, SFA, MUFA, and PUFA percentages were found at similar rates in both sexes in all months.

Keywords: *Silurus triostegus*, fatty acid, phospholipid, triacylglycerol, comparison, male, female, season, total lipid

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INTRODUCTION

Mesopotamia catfish (*S. triostegus*) is a catfish species from the Siluridae family which is found in the Mesopotamia region namely Syria, Iraq, Iran, and Turkey. In *S. triostegus* living in the Atatürk Dam Lake, it was determined that egg laying started in May and continued until the end of June by utilizing parameters such as gonadosomatic index values and direct observation of gonads. Consequently, it can be said that the reproduction period is May, June, and July (Oymak *et al.*, 2001).

Catfish meat is delicious, and has high protein content. It is a carnivore and aggressive species. Their nutrients usually are water insects and their larvae, worms, frogs, and tadpoles (Geldiay & Balık 1996).

Today, there is a great interest in fish and fish oil due to the polyunsaturated fatty acids (PUFA) they contain. Analyzes usually constitute the fish muscle that forms the food. On the other hand, the fish liver, i.e., the main organ for long-chain PUFA (LC-PUFA), has not been analyzed much (Ackman *et al.*, 2002). Fish liver is the source of the essential oils for the prevention of problems related to vision and growth (Njinkoué *et al.*, 2002). Moreover, livers of some fish species are used for muscle pain and rheumatism in Pakistan (Saify *et al.*, 2003).

The liver is an important organ in terms of lipid metabolism. This organ also has an important role in the uptake, oxidation, and transformation of fatty acids (FA) and the supply of long-chain highly unsaturated fatty acids to other tissues (Rincon-Sanchez *et al.*, 1992).

Phospholipids (PL) and triacylglycerides (TAG) have different roles in fish metabolism. Phospholipids are the main components of cell membrane and structure, and serve as precursors of eicosanoids with the C20 PUFA they contain. Meanwhile, TAG function as energy reserves (Sargent *et al.*, 1995; Kiessling *et al.*, 2001).

Fish oil and fatty acid composition are the most changed biochemical compounds according to the ecological factors and the physiological state of the fish. The total fat content and fatty acid composition in fish vary depending on species, sex, season, nutritional environment, nutrient difference, water temperature, water pollution, and whether the species are in the culture or natural form. Fat and fatty acids differ structurally in different fish species. If the same species of fish live in different geographical regions, they may differ in terms of fatty acid diversity (Ackman *et al*, 2002; Akpınar *et al*., 2009; Kayhan *et al*., 2014). This difference is also seen in different organs of the fish (Crowford *et al.*, 1986; Suzuki *et al*., 1986).

Total, TAG, and PL fatty acids of *S. triostegus* liver have not studied before. Therefore, in this study, it was aimed to compare the seasonal changes of the total, TAG, and PL fatty acid contents of liver tissues of female and male individuals of *S. triostegus* living in Atatürk Dam Lake.

MATERIALS AND METHODS

Samples of *S. triostegus* fish species were gathered from the Atatürk Dam Lake in one year period by using a fishing net (between May, 2008 and March, 2009). On the same day, the caught fish samples were placed in heat-insulated protective containers filled with ice, and brought to the laboratory. The measurements of height and weight of the samples were made. Weight measurements were noted in grams, and height measurements were taken in cm using the fork length of the fish (Kaçar *et al.*, 2016). In this study, three male and three female fish were used. The sexes of fish samples were detected. After determining the wet weights of the liver samples taken out, they were put in tubes and stored in the chloroform-methanol mixture at -80 °C until analysis.

Lipid extraction and conversion of fatty acids to methyl esters

Liver samples homogenized in the chloroform-methanol mixture (Folch *et al.*, 1957). The thin layer chromatography technique was used to fractionate the total lipids in the samples. For this purpose, the mixture of 30 g of silica gel and 50 ml of pure water was applied as a thin layer to the plates of 20X20 size, and they were dried in an oven at 100 °C for one hour. Total lipid extracts of the samples were spotted onto the plates in a single row. The total lipids were run in a mixture of petroleum ether-diethyl ether-acetic acid (80:20:1). After drying the plates in the air, 2´7´ dichlorofluorescein was sprayed to make the lipid fractions visible under the UV lamp. The bands of phospholipids and triacylglycerol fractions that were determined by means of standards were scraped and transferred to the reaction tubes. To each fraction, 3 ml of methanol and 3-5 drops of sulfuric acid were added dropwise, and they were all heated at 85 °C under reflux for 2 hours (Stanley-Samuelson and Dadd,1983). The solution was extracted with methyl esters using hexane. Gas chromatography instrument with a FID detector was used for the analysis of fatty acid methyl esters.

Gas chromatography conditions

Fatty acid analyses of the oil samples converted to methyl esters were performed by using flame ionization detector and DB-23 capillary column in HP6890 model Gas Chromatography device. The features used were as follows. Detector temperature: 280 °C, injector temperature: 270 °C, and injection: split–model 1/20. Gas flow rates were; carrier gas: 2.8 ml/min (constant flow model), hydrogen: 30 ml/min, and air: 300 ml/min. Column (oven) temperature: at 130 °C, stand-by time 1 minute; to 170 °C with 6.5 °C/min, to 215 °C with 2.75 °C/min, standby time 12 minutes; to 230 °C with 40 °C/min, standby time 3 minutes; total analysis time: 38.8 minutes. For example, 1 microliter was injected into the device. A mixture of methyl esters of fatty acids was used as the standard in the detection of fatty acids. Chromatograms of fatty acids methyl esters and total fatty acids were obtained from the computer by the software HP 3365 Chem Station.

Evaluation of data

SPSS 16 computer program was used to compare fatty acid percent rates. All data obtained in our study were obtained from the average of three replicates. In the gas chromatographic analysis of fatty acid methyl esters, three samples of each period were injected separately, and the three values of the same fatty acid were averaged. Comparison of fatty acid percentages was made by one-way analysis of variance. Differences were determined by Tukey HSD test. As a result of the statistics, it was accepted that the differences were significant when the data were $p<0.05$.

RESULT AND DISCUSSION

Lipid content

The quantity of total lipids ranging from 1.29 to 2.39% (breeding period) in *S. triostegus* females decreased in July, September, and January. In male fish, this ratio was found between 0.50-2.91% (post-breeding period). In males, the amount of lipid decreased significantly after September, in November and January. It increased in March (pre-breeding period) and in May (breeding period) (Table 1).

In freshwater fish, the lipid content of the liver varies depending on the season, feeding cycle, and reproductive status (Ackman et al., 2002).

Table 1. Total lipid of liver tissue of female and male *S. triostegus*.

	Total lipid (%)	
	Female	**Male**
May	2.39±0.65a	2.14±0.45a
July	1.78±0.38b	1.04±0.12b
September	1.61±0.40b	2.91±0.13a
November	2.03±0.35a	0.50±0.07c
January	1.29±0.21b	0.60±0.14c
March	1.74±0.41b	2.09±0.52a

Superscript letters (a,b,c) denote significant differences ($p < 0.05$) in lipid content among months.

In the current study, it was found that liver lipid content decreased in the reproductive period of July. The decrease in total lipid and total fatty acid levels in the liver and muscles of the fish in the reproductive period shows that they obtain the energy they need from these lipids. During the reproductive period, the lipids in the liver and muscle are mobilized to the gonad for gonad development (Castell et al., 1972).

In previous studies, the liver lipid content of *Cyprinus carpio* increased in the spring (Kminkova et al., 2001; Akpınar, 1986a). Kandemir & Polat (2007) found the maximum total lipid amount in the liver of *Oncorhynchus mykiss* in the autumn season. In the present study, the highest lipid content of female fish was found in May in accordance with the previous studies.

The amount of lipid in the *S. triostegus* males decreased significantly in November and January, and increased in March and May. Jangaard et al., (1967) reported that seasonal changes in lipid level in liver and other organs are caused by irregular seasonal changes in fish feeding and water temperature.

In our study, the amount of lipid increased in both sexes in the autumn months, which was the post-reproductive period. Stored lipids vary during the breeding and feeding period. Especially when fish find enough food, they can control their reproduction and lipid storage period. The lipid storage cycle is directly related to the abundance of food. Lipid variation is high throughout the year if nutrients are abundant, whereas lipid variation is low if nutrients are scarce (Kluytmans & Zandee, 1973, Ackman & Eaton, 1976, Kinsella et al., 1977, Mute et al., 1989).

The FA composition of total lipid

The total fatty acid content of female and male *S. triostegus* liver tissue is given in Table 2. The highest saturated fatty acids (SFA) in females were found in the summer whereasin males, they were found in spring which is the reproductive period. The lowest SFA was found in January in both sexes. The SFA was found to have 16:0 and 18:0 most commonly. The 16:0 ratio with saturated fatty acids decreased in males and females in January and in the pre-breeding period in March In males, both components were found to be the highest in May, which is in the breeding period. The 18:0 were highest in females during November, yet no statistically significant difference was found in other seasons. This fatty acid in males showed fluctuations throughout the year. In males and females, monounsaturated fatty acids (MUFA) in liver tissue increased in January.

The MUFA and 18:1n-9 ratio was high in January and March, and decreased in September in both males and females. In September, the amount of 16:1n-7 in females reached the lowest value. This fatty acid did not show a significant change except June and September. The highest value for males was found in January. Also, 18:1n-9 did not differ in females in all seasons. In males, it is low in May and September and close to each other. The PUFA in both sexes was found to be high in September. It fell in females in May and in July in males. The 20:5n-3, 22:5n-3, and 22:6n-3 are major n-3PUFA while 18:2n-6 and 20:4n-6 are dominant n-6PUFA. Moreover, the 20: 5n-3 was found to decrease in September after the breeding period, and has the highest value in winter in female *S. triostegus l*iver tissue. It was also observed that it decreased in July and increased in May in males. In May, when the arachidonic acid was the lowest in females, it was the highest in males. Docosahexaenoic acid (DHA) increased in both sexes in July and September. In females, in May, July, and November the highest SFA, in January the highest PUFA and the lowest MUFA was found while in males, in September the highest PUFA and in July, November, January, and March the highest MUFA was determined. The dominant fatty acids throughout the year are 16:0 from SFA, 18:1n-9 from MUFA, and DHA from PUFA. N-3/n-6 ratio was found 2.00-2.61 in females and 1.15-2.75 in males. In both sexes, the highest value was determined in the same month, and it was close to each other.

Fish are not only important protein sources but also contain nutritionally valuable lipids. Analyzes usually focus on the fish muscle that forms the food. But the fish liver is the main organ of long-chain PUFA and has not been analyzed much (Ackman et al., 2002).

In fish liver, lipids major fatty acids are similar to the ones in fish muscle. Most of the fish that have been studied have 16:0 most among the saturated fatty acids, 18:1n-9 among the monounsaturated, and 22:6n-3 among the polyunsaturated (Kminkova et al., 2001; Uysal et al., 2006; Akpınar et al., 2009; Njinkoué et al., 2002; Aras et al., 2003b). Similar results were found in *S. triostegus* liver. However, quantitative fatty acid content in the liver varies. In accordance with the present study, in the previous studies, 14:0 and 18:0 found least among the saturated fatty acids, 16:1n-7 among the monounsaturated fatty acids, 18:2n-6 and 18:3n-3 among the highly unsaturated fatty acids, 20:3n-6 and 20:4n-6 acids which are the precursors of eicosanoids (Tufan et al., 2013; Misir et al., 2016; Kaçar & Başhan, 2017).

Sander lucioperca's n-3 fatty acids increased most in November when the temperature fell (Uysal et al., 2006). In both sexes of *S. triostegus,* PUFA increased in the autumn and winter when the temperature fell (Kaçar *et al*., 2016).

In the previous studies, in line with the current research, it was observed that long-chain unsaturated fatty acids have changed more than saturated fatty acids. It was concluded that gonad development and breeding periods had directly an effect on these changes (Akpınar, 1986b).

The data indicate that the ratio of n-3/n-6 in total lipids of the liver may be different in both sexes. This depends on the ratios of 18:3n-3, 20:5n-3, 22:5n-3, and 22:6n-3, forming n-3PUFA and 18:2n-6, 20:3n- 6, and 20:4n-6, forming n-6PUFA.

In the current investigation, it was determined that the fatty acid content of fish liver lipids is affected by the sex and the season, thus, the reproductive period.

The FA composition of TAG fraction

Table 3 shows the TAG fatty acid content of male and female *S. triostegus* liver. Palmitic acid and therefore the SFA ratio in males increased in September, which is the post-breeding period, and decreased in March, just before the breeding season. There was no significant difference in females for 16:0 throughout the year.

Table 2. Seasonal variations of fatty acid composition of total lipid of liver tissue from female and male *S. triostegus* (% of total FA)*.

	May		July		September		November		January		March	
Fatty Acids	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**
10:00	-	-	-	-	-	0.03±0.01	-	-	-	-	-	-
12:00	0.05±0.02a	0.06±0.02a	-	-	0.01±0.01b	-	-	0.01±0.01b	0.12±0.05c	0.12±0.04c	0.07±0.04d	0.11±0.01c
13:00	0.32±0.03a	0.13±0.05b	0.21±0.02c	0.15±0.03b	-	0.07±0.04d	-	-	-	-	-	-
14:00	1.59±0.16a	2.47±0.21b	1.75±0.11c	1.19±0.11d	1.47±0.17a	2.07±0.25ab	1.10±0.10d	1.45±0.17a	1.14±0.16d	1.26±0.16d	1.98±0.67ab	1.96±0.17ab
15:00	0.72±0.07a	0.54±0.04b	0.26±0.02c	0.17±0.03d	0.47±0.03e	0.54±0.04b	0.01±0.01f	0.11±0.03d	0.35±0.02c	0.25±0.02c	0.47±0.05e	0.60±0.05b
16:00	25.53±1.26a	23.46±1.26a	26.85±1.20a	20.48±1.28b	24.60±1.22a	20.89±1.09b	22.49±1.25b	21.00±1.30b	19.49±1.09b	17.28±1.17c	19.91±1.14b	18.43±1.08b
17:00	0.42±0.03a	0.42±0.04a	0.18±0.01b	0.23±0.01b	0.28±0.02b	0.51±0.05c	0.31±0.03d	0.26±0.01b	0.36±0.02d	0.27±0.03b	0.56±0.05e	0.64±0.05e
18:00	9.95±0.99a	9.94±0.99a	10.47±1.10a	11.55±1.01a	10.66±1.01a	8.58±0.87a	14.15±1.14b	7.78±0.77c	8.95±0.88a	4.84±0.45d	9.21±0.91a	5.97±0.57d
∑SFA***	38.58±1.37a	36.96±1.33a	39.72±1.40a	33.77±1.44b	37.49±1.33a	32.69±1.29b	38.06±1.36a	30.61±1.38b	30.41±1.30b	24.02±1.28c	32.20±1.44b	27.71±1.29c
16:1n-7	7.97±0.77a	5.77±0.55b	4.11±0.41b	4.25±0.43b	3.26±0.30c	5.68±0.45b	7.41±0.66a	5.55±0.56b	7.40±0.59a	8.60±0.83a	7.72±0.69a	6.64±0.63b
18:1n-9	22.24±1.28a	21.80±1.26a	20.93±1.20a	30.91±1.30b	20.57±1.19a	21.59±1.27[a]	20.13±1.10a	30.01±1.38b	21.36±1.17a	30.79±1.36b	23.52±1.22a	29.10±1.28b
20:1n-9	0.71±0.07a	0.86±0.05a	2.17±0.29b	3.16±0.32b	1.64±0.11c	1.31±0.10c	2.07±0.24b	0.99±0.08a	3.21±0.33b	4.78±0.43d	1.93±0.17c	2.70±0.29bc
∑MUFA	30.92±1.33a	28.43±1.27a	27.21±1.28a	38.32±1.43b	25.47±1.26c	28.58±1.26a	29.61±1.30a	36.55±1.33b	31.97±1.38a	44.17±1.54d	33.17±1.42e	38.44±1.45b
18:2n-6	2.52±0.24a	3.51±0.32b	1.40±0.11c	0.88±0.07d	1.69±0.16c	2.96±0.27a	2.55±0.22a	2.79±0.22a	1.94±0.18c	1.55±0.22c	2.14±0.25a	3.80±0.36b
18:3n-3	1.22±0.17a	1.22±0.13a	0.57±0.05b	0.49±0.05b	0.93±0.08a	1.93±0.16c	1.20±0.12a	3.29±0.37c	1.34±0.16a	0.79±0.07ab	1.14±0.18a	2.32±0.25c
20:2n-6	0.45±0.04a	0.49±0.03a	0.61±0.06b	0.34±0.02c	0.76±0.07b	0.80±0.07b	0.90±0.09b	0.38±0.04c	0.54±0.05a	0.70±0.05b	0.72±0.07b	1.26±0.17d
20:3n-6	0.48±0.04a	0.54±0.04a	0.46±0.03a	0.51±0.03a	0.58±0.05a	0.70±0.07b	1.25±0.13c	0.33±0.02d	0.57±0.05a	0.68±0.06b	0.46±0.04a	0.72±0.07b
20:4n-6	4.37±0.39a	11.42±1.01b	7.78±0.76c	5.75±0.56a	9.29±0.91c	8.93±0.88c	4.64±0.44a	6.26±0.65ac	7.09±0.78c	5.28±0.58a	7.05±0.71c	4.96±0.44a
20:5n-3	3.97±0.36a	5.38±0.59b	2.08±0.23c	1.97±0.17c	1.74±0.14c	4.68±0.42a	4.56±0.48a	2.84±0.21c	5.10±0.56b	3.86±0.36a	4.68±0.41a	5.42±0.55b
22:5n-3	1.05±0.10a	3.84±0.37b	2.28±0.22c	2.21±0.20c	3.03±0.38c	4.47±0.45b	3.13±0.33c	2.05±0.36c	5.80±0.59b	3.30±0.31c	4.62±0.48b	3.51±0.33b
22:6n-3	15.42±1.05a	8.05±0.86b	17.80±1.17a	15.67±1.15a	18.96±1.15a	14.21±1.07c	14.02±1.04c	13.91±1.19c	14.27±1.14c	14.69±1.04c	13.80±1.13c	10.86±0.98b
∑PUFA	30.48±1.30a	34.45±1.39a	32.98±1.22a	27.82±1.22b	36.98±1.33c	38.68±1.44c	32.25±1.38a	31.85±1.30c	36.65±1.34c	30.85±1.27a	34.61±1.42aa	32.85±1.43a
n-3	21.66±1.27a	18.49±1.08b	22.73±1.20a	20.34±1.29a	24.66±1.30a	25.29±1.33a	22.91±1.25a	22.09±1.32a	26.51±1.29c	22.64±1.20a	24.24±1.09a	22.11±1.38a
n-6	8.82±0.89a	15.96±1.05b	10.25±0.99c	7.48±0.77a	12.32±1.02c	13.39±1.04c	9.34±0.87a	9.76±0.98a	10.14±1.10c	8.21±0.87a	10.37±1.00c	10.74±1.10c
n-3/n-6	2.45	1.15	2.21	2.71	2.00	1.88	2.45	2.26	2.61	2.75	2.33	2.05

* Means are the averages of 3 replicates ** Values reported are means ±standard deviation; means followed by different letters in same line are significantly different ($p<0.05$) by Tukey's test *** SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids

Table 3. Seasonal variations of fatty acid composition in triacylglycerol fraction of liver tissue from female and male *S. triostegus* (% of total FA)*.

	May		July		September		November		January		March	
Fatty Acids	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**
10:00	0.18±0.01a	0.30±0.03b	0.48±0.03b	0.11±0.01c	-	-	-	-	-	-	-	-
12:00	0.49±0.03a	0.14±0.02b	0.19±0.01c	0.16±0.05b	-	-	-	-	-	-	-	-
13:00	0.94±0.08a	0.34±0.05b	0.50±0.04c	0.33±0.06b	0.66±0.05c	0.77±0.06c	0.92±0.08a	-	-	-	-	-
14:00	4.00±0.40a	2.61±0.22b	2.51±0.25b	2.69±0.26b	2.84±0.24b	4.56±0.44a	2.70±0.22b	1.95±0.19c	2.01±0.20c	2.35±0.26b	3.73±0.33a	2.96±0.30a
15:00	0.92±0.07a	0.52±0.04b	0.47±0.04b	0.35±0.03b	0.88±0.07a	1.16±0.16c	0.40±0.03b	0.31±0.03b	0.74±0.07a	0.40±0.02b	1.09±0.10a	0.70±0.06a
16:00	30.30±1.33a	21.88±1.20b	30.78±1.30a	21.30±1.19b	28.02±1.27a	28.52±1.27a	30.27±1.29a	27.42±1.30a	27.16±1.28a	26.48±1.27a	29.07±1.28a	20.22±1.27b
17:00	0.94±0.09a	0.40±0.04b	0.44±0.04b	0.27±0.01c	0.30±0.03c	0.21±0.08d	1.03±0.10a	0.16±0.01e	0.55±0.04b	0.25±0.01c	0.59±0.05b	0.61±0.05b
18:00	4.09±0.43a	6.69±0.65b	3.62±0.34c	5.23±0.52ab	5.41±0.54ab	7.24±0.77b	4.72±0.44a	6.55±0.56b	4.82±0.40ab	3.75±0.31c	5.19±0.51ab	4.06±0.40a
∑SFA***	41.86±1.40a	32.88±1.33b	38.99±1.33a	30.44±1.30b	38.11±1.38a	42.46±1.40a	40.04±1.44a	36.39±1.44d	35.28±1.33d	33.23±1.36b	39.67±1.42a	28.55±1.29b
16:1n-7	9.01±0.07a	7.32±0.67b	10.54±1.08a	16.74±1.06c	11.62±1.10a	13.99±1.03d	15.17±1.05c	14.00±1.14d	14.05±1.14d	15.99±1.05c	12.59±1.02d	11.09±0.99a
18:1n-9	27.56±1.28a	28.74±1.27a	26.91±1.22a	33.69±1.33b	26.81±1.25a	26.63±1.25a	26.58±1.39a	32.80±1.29b	29.60±1.34a	32.76±1.39b	29.44±1.30a	30.78±1.30ab
20:1n-9	0.92±0.08a	1.23±0.10a	1.88±0.16b	1.07±0.09a	1.47±0.11c	0.83±0.07a	1.35±0.19a	0.42±0.04d	1.74±0.20b	2.09±0.02b	1.07±0.17a	1.69±0.11b
∑MUFA	37.49±1.38a	37.29±1.33a	39.33±1.33a	51.50±1.59b	39.90±1.40a	41.45±1.44c	43.10±1.41c	47.22±1.52d	45.39±1.52d	50.84±1.56b	43.10±1.55c	43.56±1.43c
18:2n-6	3.05±0.34a	4.31±0.46b	2.83±0.22a	3.89±0.32a	2.54±0.35a	3.88±0.33a	2.57±0.29a	2.50±0.22a	2.40±0.28a	1.93±0.19b	2.86±0.27a	5.02±0.56b
18:3n-3	1.77±0.11a	2.14±0.21b	1.07±0.16c	2.50±0.28b	1.45±0.19a	3.38±0.33d	1.07±0.20c	2.27±0.28b	1.39±0.16a	3.95±0.30d	1.27±0.18ac	2.62±0.20b
20:2n-6	0.25±0.02a	0.33±0.03a	0.50±0.04b	0.36±0.02a	1.07±0.19c	0.40±0.03b	0.42±0.04b	0.15±0.01d	0.67±0.05b	0.33±0.02a	0.83±0.07bc	1.81±0.11e
20:3n-6	0.30±0.02a	0.63±0.05b	0.46±0.03a	0.43±0.04a	0.29±0.05a	0.14±0.01c	0.24±0.03a	0.18±0.01d	0.27±0.02a	0.50±0.04b	0.61±0.05b	0.86±0.07e
20:4n-6	4.53±0.44a	5.97±0.55a	5.38±0.50a	2.45±0.22b	3.68±0.36c	2.47±0.28b	2.50±0.20b	2.31±0.30b	3.71±0.32c	2.80±0.31b	3.03±0.33b	3.92±0.38c
20:5n-3	2.54±0.29a	5.73±0.55b	2.64±0.23a	1.28±0.11c	3.49±0.44d	1.75±0.10e	1.86±0.16e	1.31±0.17c	2.35±0.22a	1.48±0.15c	1.74±0.19e	2.59±0.27a
22:5n-3	2.23±0.20a	2.81±0.29a	2.32±0.22a	1.12±0.10b	1.85±0.16c	1.01±0.13b	1.30±0.15c	0.26±0.01d	2.38±0.27a	0.94±0.07b	2.08±0.31a	4.76±0.04e
22:6n-3	5.89±0.55a	7.83±0.71b	6.38±0.34a	5.93±0.56a	7.52±0.77b	2.98±0.23c	6.83±0.66a	6.50±0.68a	6.07±0.60a	3.90±0.33d	4.76±0.45a	6.21±0.56a
∑PUFA	20.56±1.22a	29.75±1.28b	21.58±1.39a	17.96±1.07c	21.89±1.29a	16.01±1.16c	16.79±1.06c	15.48±1.05c	19.24±1.09a	15.83±1.03c	17.18±1.17c	27.79±1.22d
n-3	12.43±1.02a	18.51±1.08b	12.41±1.12a	10.83±1.10a	14.31±1.04c	9.12±0.99a	11.06±1.10a	10.34±1.00a	12.19±1.03a	10.27±1.07a	9.85±0.99a	16.18±1.10b
n-6	8.13±0.78a	11.24±1.12b	9.17±0.88a	7.13±0.88a	7.58±0.70a	6.89±0.65c	5.73±0.51c	5.14±0.51c	7.05±0.69a	5.56±0.45c	7.33±0.77a	11.61±1.03b
n-3/n-6	1.52	1.64	1.35	1.51	1.88	1.32	1.93	2.01	1.72	1.84	1.34	1.39

* Means are the averages of 3 replicates ** Values reported are means ±standard deviation; means followed by different letters in same line are significantly different ($p<0.05$) by Tukey's test *** SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids

Table 4. Seasonal variations of fatty acid composition in phospholipid fraction of liver tissue from female and male *S. triostegus* (% of total FA)*.

	May		July		September		November		January		March	
Fatty Acids	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**
14:00	0.45±0.03a	1.79±0.10b	1.44±0.12b	1.15±0.11c	1.61±0.16b	1.89±0.13b	1.00±0.10c	1.47±0.16b	1.01±0.34c	1.18±0.18c	1.25±0.33c	1.41±0.19b
15:00	0.40±0.03a	0.49±0.04a	0.24±0.02b	0.26±0.02b	0.58±0.05a	0.57±0.03a	0.25±0.02b	0.11±0.01c	0.44±0.03a	0.36±0.03ab	0.43±0.02a	0.74±0.06d
16:00	22.75±1.20a	20.98±1.20a	20.34±1.19a	23.47±1.19a	24.79±1.22a	23.99±1.29a	23.79±1.28a	22.66±1.22a	20.63±1.18a	26.56±1.32b	20.31±1.10a	23.51±1.22a
17:00	0.18±0.01a	0.48±0.04b	0.15±0.02a	0.17±0.01a	0.28±0.03c	0.34±0.03b	0.40±0.04b	0.27±0.02c	0.62±0.05b	0.40±0.03b	0.85±0.07d	0.99±0.01d
18:00	11.71±1.09a	12.56±1.02a	14.74±1.14b	11.38±1.01a	11.86±1.01a	12.78±1.13a	14.43±1.09b	10.05±0.98a	13.81±1.12b	9.00±1.01a	14.68±1.17b	9.13±0.97a
∑SFA***	35.49±1.36a	36.30±1.33a	36.91±1.39a	36.43±1.35a	39.12±1.40a	39.57±1.40a	39.87±1.33a	34.56±1.29a	36.50±1.45a	37.50±1.30a	37.52±1.37a	35.78±1.34a
16:1n-7	2.40±0.29a	3.36±0.34a	3.21±0.31a	3.07±0.32a	3.01±0.33a	3.76±0.31a	3.26±0.27a	5.58±0.54b	2.02±0.20a	4.04±0.40a	3.88±0.41a	3.32±0.32a
18:1n-9	16.01±1.17a	15.74±1.05a	17.24±1.15a	19.83±1.09b	17.05±1.08a	15.66±1.15a	14.48±1.17a	16.72±1.06a	19.16±1.09b	17.00±1.07a	15.66±1.63a	15.09±1.15a
20:1n-9	0.62±0.05a	0.90±0.09b	2.56±0.21c	1.08±0.10b	0.98±0.07b	1.31±0.11b	1.40±0.10b	0.52±0.04a	2.05±0.23c	1.03±0.15b	1.13±0.18b	1.68±0.13d
∑MUFA	19.03±1.09a	20.00±1.20a	23.01±1.22a	23.98±1.26a	21.04±1.20a	20.73±1.19a	19.14±1.09a	22.82±1.29a	23.23±1.29a	22.07±1.21a	20.67±1.30a	20.09±1.25a
18:2n-6	1.58±0.16a	1.24±0.11a	0.79±0.07b	1.72±0.16a	1.42±0.11a	2.18±0.20c	2.58±0.28c	1.24±0.13a	1.66±0.27a	1.22±0.16a	1.39±0.16a	2.05±0.20c
18:3n-3	0.47±0.05a	0.53±0.04a	0.44±0.03a	0.47±0.03a	0.75±0.06b	1.34±0.12c	0.68±0.05b	0.22±0.02d	0.72±0.07b	0.59±0.04b	0.51±0.03b	0.78±0.06b
20:2n-6	0.42±0.04a	0.69±0.05b	0.88±0.07c	0.25±0.02d	0.73±0.05c	0.84±0.07c	0.49±0.04a	0.26±0.02d	0.53±0.04a	0.50±0.03a	0.47±0.03a	0.73±0.07c
20:3n-6	0.76±0.07a	0.83±0.05a	0.59±0.03b	0.55±0.04b	0.57±0.04b	0.71±0.06a	1.25±0.12c	0.31±0.02d	0.46±0.03b	0.75±0.06a	0.28±0.01d	0.50±0.04b
20:4n-6	12.82±1.02a	12.54±1.02a	9.03±0.99b	9.33±0.98b	9.34±0.97b	10.02±1.00b	10.53±1.13b	12.46±1.03a	7.46±0.77c	8.25±0.83bc	10.80±1.01b	8.83±0.78c
20:5n-3	5.23±0.55a	5.35±0.54a	2.08±0.22b	4.69±0.43a	4.02±0.40a	4.46±0.34a	4.55±0.34a	4.86±0.44a	5.11±0.51a	5.31±0.54a	5.28±0.50a	8.89±0.87c
22:5n-3	4.23±0.33a	4.84±0.47a	4.70±0.45a	3.00±0.31a	3.35±0.31a	4.54±0.42a	3.04±0.39a	3.39±0.33a	3.59±0.54a	2.00±0.21b	3.54±0.33a	3.64±0.35a
22:6n-3	19.89±1.09a	17.60±1.07b	21.40±1.20a	19.49±1.09a	19.60±1.20a	15.53±1.15b	17.79±1.07b	19.79±1.20a	20.69±1.27a	21.78±1.21c	19.45±1.08a	18.62±1.08a
∑PUFA	45.40±1.46a	43.62±1.43a	39.91±1.38b	39.50±1.40b	39.78±1.35b	39.62±1.38b	40.91±1.44b	42.53±1.44a	40.22±1.39b	40.40±1.40b	41.72±1.50b	44.04±1.41a
n-3	29.82±1.22a	28.32±1.22a	28.62±1.34a	27.65±1.26a	27.72±1.28a	25.87±1.34a	26.06±1.27a	28.26±1.21a	30.11±1.31a	29.68±1.30a	28.78±1.32a	31.93±1.34b
n-6	15.58±1.05a	15.30±1.54a	11.29±1.01b	11.85±1.01b	12.06±1.12b	13.75±1.03a	14.85±1.04a	14.27±1.14a	10.11±1.10b	10.72±1.01b	12.94±1.02b	12.11±1.12b
n-3/n-6	1.91	1.85	2.53	2.33	2.29	1.88	1.75	1.98	2.97	2.76	2.22	2.63

* Means are the averages of 3 replicates ** Values reported are means ±standard deviation; means followed by different letters in same line are significantly different ($p<0.05$) by Tukey's test *** SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids

In males, it was low in May, July, and March, and it was close to each other,while in September, November, and January it was high and close to each other. Palmitoleic acid and MUFA ratio were found to be highest in females in November and January and in males in July and January. These components decreased in both sexes in May, which is the reproductive period. The 18:1n-9 and 16:1n-7 are the most frequently detected MUFA in both sexes. In addition, 20:1n-9 were detected in both sexes, albeit in small amounts.

In the female TAG fraction, SFA was found most in May, and MUFA was found in November, January, and March. The ratio of these components in the other two months is similar. In males, MUFA was found in all months most commonly in males except for September. The component with the lowest ratio in both sexes' TAG is PUFA. It is noteworthy that MUFA ratios are higher in this fraction. In females, the PUFA ranged 16.79 to 21.89% and in males ranged from 15.48 to 29.75% through the year. The 20:4n-6 and DHA were found as major PUFA. The ratio of n-3/n-6 in the liver TAG fraction of *S. triostegus* was 1.34-1.93 in females and 1.32-2.01 in males. The highest n-3/n-6 ratio was determined in November in both sexes.

Fat-free fish store 50-80% of their fat in their livers in the form of TAG, which are good sources for fat-soluble vitamins, especially A and D vitamins (Jacquot, 1961).

In fish caught in nature, in TAG mostly monoenes, then saturated followed by PUFA are found (Henderson & Tocher, 1987). Based on this, it has been found that fish species accumulate mainly SFA and MUFA as stored lipids (Pinela et al., 2009). The reason for the high rate of monoenes in triacylglycerol is that among the components in this group, the ratio of 18:1-9 is higher. In addition, n-3HUFAs were found to be low in this fraction since the amount of n-3 components such as 20:5n-3 and 22:6n-3 was low (Cejas et al., 2003).

Fatty acids such as palmitic acid, 16:1n-7, and 18:1n-9 are found to be excessively present in storage lipids (Ackman 1967). In the present research, MUFA were found in the liver TAG of both sexes most, then SFA followed by PUFA were found. Similar results were found by Kozlova & Khotimchenko (2000) as well. In May (reproduction period) the percentage of MUFA decreased. Their amounts may be reduced because these components are mobilized to the gonad for reproduction.

In *Comephorus baicalensis*, MUFA were found to be the most, SFA next, and then PUFA the least. Among the monounsaturated fatty acids 18:1-9 were found most. The 16:0 was determined most within the SFA group. Other major fatty acids are 16:1n-7, 18:1n-7, and 20:5n-3(Kozlova & Khotimchenko, 2000). Ackman et al., (2002) identified the 16:0, 18:1n-9, 16:1n-7, 18:2n-2, 18:3n-3, 20:4n-6, 20:5n-3, and 22: 6n-3 as the major fatty acids. This finding is consistent with the findings of the current study as well.

The FA composition of PL fraction

In the liver PL fraction of *S. triostegus,* the seasonal rates of SFA was 35.49-39.87% in females and 34.56-39.57% in males, MUFA was 19.03-23.23% in females and 20.0-23.98% in males, and PUFA was 39.78-45.40% in females and 39.50-44.04% in males. As can be seen from the data, SFA, MUFA, and PUFA percentages were found at similar rates in both sexes in all months. Similarly, the percentages of major components such as 16:0, 18:1n-9, 20:4n-6, 20:5n-3, and 22:6n-3 did not differ within months. In both sexes of *S. triostegus,* PUFA found most, SFA next, and MUFA least, in all seasons. In this fish, the ratio of n-3/n-6 was 1.75-2.97 in females and 1.85-2.76 in males. In both sexes, the n-3/n-6 rate increased in January (Table 4).

The SFA rate is reduced by exposure to low temperature, yet the proportion of unsaturated fatty acids increases (Jobling and Bendiksen 2003). The increased unsaturated fatty acids are either monoene or polyene (Wallaert and Babin, 1994, Fodor *et al.,* 1995, Logue *et al*., 2000).

The increase of the ambient temperature reduces the accumulation of n-3 in PL (Delgado *et al*., 1994). The change in the salinity and temperature of water affects the length and the degree of unsaturation of the fatty acids in the membrane PL in poikilotherms. (Cordier *et al.,* 2002).

In this study, it was observed that PUFA decreased in July and September when the temperature was high.

The 16:0 is dominant in SFA and its ratio in fish tissues is not affected by nutrition (Ackman *et al*., 1975) Ackman *et al*., (2002) detected that 16:0, 18:0, 18:1n-9, 16:1n-7, 20:4n-6, 20:5n-3, and 22:6n-3 were dominant in the PL fraction. The results are consistent with the results of the current study. Buzzi *et al*., (1997) stated that in the liver of *Exos lucius*, linolenic acid was converted into EPA and DHA. This transformation is very important in fish physiology (Arts *et al*., 2001).

AA was found as major n-6 PUFA in both sexes in all seasons. AA is the precursor molecule for the synthesis of thromboxane and prostaglandins (Bell *et al*., 1994).

In the case of migration and gonad development, the amount of EPA and DHA is mostly maintained compared to MUFA. The fact that the percentages of these two components do not change much is necessary to maintain the cell membrane structure and function (Sargent *et al*., 1995, Cejas *et al*., 2004).

CONCLUSION

In the present study, it was found that the amount of DHA and EPA in both sexes did not differ much by season either. PUFA was detected most in the PL fraction in both sexes. According to these results, it can be said that the lipid metabolism of the fish changes depending on the spawning and season.

Conflict of interests: The authors declare that they have no conflict of interest.

Ethics committee approval:-

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Original Article

Is *Gambusia holbrooki* Known as an Invasive Fish Species in Küçük Menderes River Basin Lakes (Turkey)?

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ABSTRACT

The aim of this study was to investigate the level of awareness of the species *Gambusia holbrooki* Girard, 1859, in the Küçük Menderes River Basin lakes, a species which is considered to be a threat, particularly to the persistence of endemic species. Interviews regarding the species in the basin were conducted with a total of 104 people between 2016 and 2017. Data were collected from fishermen (n=52) and local people (n=52) who had an association with the Belevi, Barutçu, Gebekirse, Kocagöz and Kazan lakes in the Selçuk district, and the method used was that of face-to-face questionnaires. The questionnaire included questions about the recognition of the species and the harm that this species might cause. Furthermore, some demographic characteristics (e.g. age, marital status and education level) of participants were included in the present study.

Keywords: Mosquitofish, alien species, fish awareness, Selçuk

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INTRODUCTION

Turkey forms a natural corridor in terms of distribution of biological species (Tarkan, Marr & Ekmekçi, 2015) and with its unique geomorphological feature, it is the only country in the world that can boast hosting 3 of 34 hot spots (Caucasus, Iran-Anatolia and Mediterranean) (Şekercioğlu et al., 2011). However, invasiveness is an increasing threat for the sustainability of biodiversity (Copp et al., 2005), and this threat also applies to freshwater fish species distributed throughout Anatolia (Freyhof et al., 2014; Tarkan, Marr & Ekmekçi, 2015).

The Mosquitofish *Gambusia holbrooki* & *G. affinis* were introduced worldwide for the biological control of malaria (Krumholz 1948). They were introduced into Turkey for the biological control of malaria between the years 1920-1929 (Walton, Henke & Why, 2012). It is claimed that French people brought *G. affinis* from European countries and introduced this species to Lake Amik (Hatay, the southernmost city of Turkey) (Geldiay & Balık, 1996) but the exact year of the introduction is not clear. Mosquitofish was the first "*exotic species*" that was introduced into the Turkey's freshwaters (İnnal & Erk'akan, 2006). The first official introduction of the species into Turkey was in the Çukurova Region (in the southern part of Turkey) in 1960 (Bahadıroğlu & Büyükçapar, 1997).

Although the potential ecological impact of mosquitofish on other species was kept secret last century, today this is a well-known phenomenon. *Gambusia* is one of about 29 aquarium species listed as harmful to native fauna and aquatic ecosystem collectively (Arthington & Marshal, 1999). They cause harm to the native fish fauna, especially in terms of endemic species persistence (Milton & Arthington, 1983; Rupp 1996; Ling 2004; Pyke 2008; Buttermore et al., 2011).

Turkey's geography is divided into 25 major river basins. Küçük Menderes River Basin is one of these river basins and is located within the bor-

ders of the Aegean Region. This basin is adjacent to the Büyük Menderes River Basin in the south and the Gediz River Basin in the north. Küçük Menderes River Basin borders are in 3 cities; İzmir (95%), Aydın (4%) and Manisa (1%); more than half of the lands in the basin are forest and semi-natural areas, 39% are agricultural areas and 1% are water surfaces (OSİB, 2016).

Selçuk district is a very rich place in terms of natural lakes. There are 5 lakes in the district, Belevi, Barutçu (Çatal), Gebekirse, Kazan, and Kocagöz lakes. Belevi Lake is an important natural lake located at the entrance of the Selçuk district, next to Belevi and Halkapınar villages. Barutçu and Gebekirse lakes are located in Zeytinköy village, and they are composed of alluvium brought by the Küçük Menderes River, as are the other main lakes in the same location . Kazan Lake is a famous touristic lake which is located in the same village. Finally, Kocagöz is the other lake in the district which is closest to the Aegean Sea. In some of the dried marshes, the land can be cultivated in all seasons and used for agricultural purposes by the people in the region (OSİB, 2016).

These lakes, especially Belevi and Barutçu lakes, are important water bodies in terms of regional fishing activities. Gebekirse Lake and its surroundings was established as a Wildlife Protection Area (1000 ha) on 31 December, 1984 and was declared to be a Wildlife Development Area (545.3 ha) with the decision of the Council of Ministers (TOB, 2019). Fishing is the mainstay of some of the local people. In addition, a significant number of local people are occupied in doing different kinds of fishing activities in these lakes.

The present study aimed to determine awareness of the invasive fish species in two groups of people who have contact with the lakes in the Küçük Menderes River Basin. The aim was to investigate whether or not fishermen and local people knew the harm caused by the *G. holbrooki*. Public survey studies are substantial for determining the status of invaders and such surveys contribute to fisheries management.

MATERIAL AND METHODS

In this research , two study groups were chosen due to the fact that they live closest to the five lakes (Belevi, Barutçu, Gebekirse, Kocagöz, Kazan lakes) which are located in the Selçuk district (Figure 1 and Figure 2). The first group consisted of local fishermen living close to Belevi, Halkapınar and Zeytinköy villages. The second group consisted of local people living in the same villages. All fishermen and local people who were interviewed had had previous contact in at least some with a lake.

In reviews with local headmen of the villages, it was found that a maximum of 60 fisherman were living there. The sample volume to be selected was calculated with the formula given below (95% confidence level, $\alpha = 0.05$; $p = 0.5$; $d = 0.05$) (Sümbüloğlu & Sümbüloğlu, 2005). As a result of this formula, n = 52 fishermen of sample size were found to be sufficient for the research . Interviews were conducted with almost a full count of the Fishermen in the years 2016 and 2017. In addition, for the second group (consisting of local people) the same sample number (n = 52) was targeted as similar to the number of fishermen who were interviewed.

Formula:

$$n = Np(1-p)t^2 / (d^2(N-1)) + (t^2p(1-p))$$

N: Number of individuals in the target.

n: Number of individuals to be sampled.

p: Frequency of occurrence of the examined event.

t: t-table value.

d: The frequency of occurrence of the event.

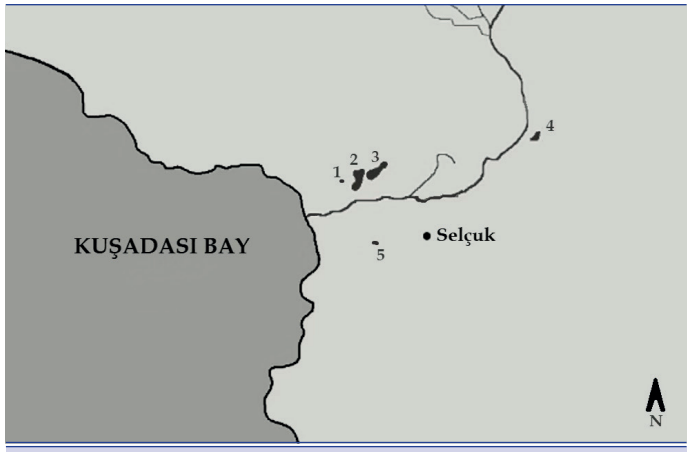

Figure 1. The map of the locations (1: Kazan Lake, 2: Gebekirse Lake, 3: Barutçu Lake, 4: Belevi Lake, 5: Kocagöz Lake).

Figure 2. The photos of Belevi Lake (upper) and Kazan Lake (lower) (Selçuk, İzmir).

The purpose of the questions was to specify the recognition level of the species in the study area. Face-to-face questionnaires gathered information about whether the fish had been seen before, whether the name of the fish was known or not, and whether the fish was known for the harm that it caused. The 6 questions used for the survey are given below (Table 1). Photos of both male and female *G. holbrooki* were shown to all the people who were interviewed.

Table 1. The questions about awareness of *G. holbrooki* and their harm.

No	Questions
1	Have you seen the fish in the photo before?
2	Do you know the name of the fish in the photo?
3	Are mosquitofish useful?
4	Are mosquitofish harmful?
5	Do you know how the fish were introduced into the water resource?
6	Have you ever caught mosquitofish?

To test the aforementioned awareness of the species using fishermen and local people knowledge level differences, Kolmogorov-Smirnov test (95%) was performed to analyse the differences in the two groups *(Chi-square test could not be applied because of the frequency in some parts <5)*. The answers of fishermen and local people were grouped as sufficient and insufficient according to the level of knowledge. Statistical analyses were performed with the SPSS 20.0. The mean ages of fishermen and people from the public were given with their standard deviation (± SD).

RESULTS AND DISCUSSION

All fishermen who attended the face-to-face questionnaire were males and during the study females were not interviewed in either group. The mean age of the fishermen was 51.11 ± 2.95 years, and 45.09% of fishermen were aged between 50 and 59. Almost all fishermen were married, whilst a few were single. Regarding the education level of the fishermen, 92.15% had at least completed primary school, whilst a few fishermen had a high school degree.

The mean age of people from the public was 49.03 ± 3.48 years, and 34.07% of them were aged between 50 and 59. Many of the people who were interviewed were married. The replies of fishermen and people from the public, and total reply numbers, are given below according to questions (Table 2).

In reply to the first question regarding having seen the *G. holbrooki* before in any water resources the fishermen mostly replied in the affirmative. The results showed that 83% of fishermen declared that they had seen the fish before in any water resources, and that 86% of people from the public had not seen the fish before (Figure 2). In both groups the results seem almost similar for the second question, with 83% of fishermen and 98% of people from public declaring that they had never heard the name of "mosquitofish" before.

Table 2. The replies of fishermen and people from the public.

Reply numbers (n_{total} = 104)									
	Fishermen			Local People			Total		
No	Y	N	?	Y	N	?	Y	N	?
1	43	7	2	4	45	3	47	52	5
2	9	43	0	1	51	0	10	94	0
3	14	3	35	4	0	48	18	3	83
4	2	13	37	0	4	48	2	17	85
5	0	52	0	0	52	0	0	104	0
6	19	33	0	0	52	0	19	85	0

Y: Yes, N: No, ?: I do not know.

Almost all people from the public had no idea about the threat of the species. Furthermore, all participants interviewed had no idea about how the fish had been introduced into the waters . In addition, 36.53% of fishermen had caught the species before.

Kolmogorov-Smirnov test (95%) was performed to analyse the differences between the replies of the fishermen and local people according to the six questions (Table 3).

There were significant differences between the replies of the fishermen and those of local people regarding having seen the invasive *G. holbrooki* before or knowing the name of the species (for the 1st and 2nd questions). Moreover, there was no significant difference between the fisherman and local people in terms of their awareness of how harmful the species is (for the 3rd question). There were significant differences between the fishermen and the local people regarding having caught the species before (for the 6th question).

In the present study, it was determined that the fishermen were aware of the *G. holbrooki*. In addition, it was observed that the local people in the study area were mostly not aware of the mosquitofish; also they had no idea about their negative effects on native fish fauna.

Throughout this research, the most dramatic result might be the replies for the 4th question about the harmful effects of the *G. holbrooki*. Almost all the people interviewed did not known about the *G. holbrooki*'s harm to the ecosystem. In particular, many fishermen did not even know the name of the fish.

G. holbrooki has a very large distribution in the inland waters of Turkey, and is well known in Turkey's western freshwater resources. They were recorded in Yuvarlakçay (Muğla) (Balık et al., 2005) and Afyonkarahisar water resources (Yeğen et al., 2007) as well as in Marmara Lake (Manisa) (İlhan & Sarı, 2015). The first record of this species from the study area was done more than 30 years earlier and was conducted in Barutçu Lake (Balık & Ustaoğlu, 1988). In addition, the first study of the species was recorded in the region in question (namely, Belevi, Gebekirse, Kocagöz and Kazan lakes) in 2016 and 2017 years for the first time (Kurtul & Sarı, 2019). Despite its wide distribution in Turkey and in the Küçük Menderes River Basin, it is not a harmful species and the

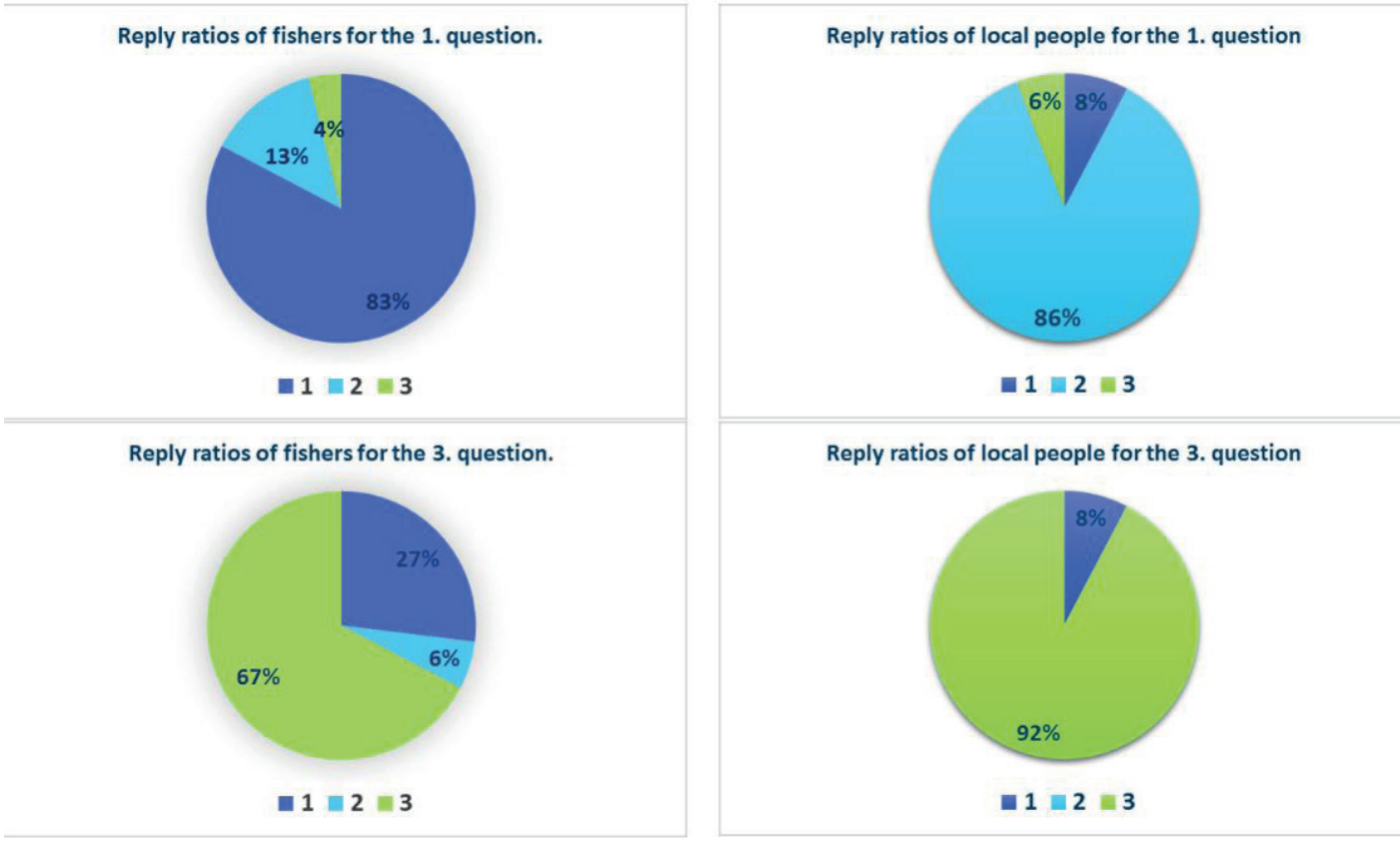

Figure 2. Reply ratios of fishermen and local people for the 1st and 3rd questions..

Table 3. Comparison of the level of knowledge of fishermen and local people.

1 Question	Knowledge Level		4 Question	Knowledge Level	
Group	**Sufficient**	**Insufficient**	**Group**	**Sufficient**	**Insufficient**
Fishermen	43	9	Fishermen	2	50
Local People	4	48	Local People	0	52
p<0.05			*p<0.05*		
2 Question	**Knowledge Level**		**5 Question**	**Knowledge Level**	
Group	**Sufficient**	**Insufficient**	**Group**	**Sufficient**	**Insufficient**
Fishermen	9	43	Fishermen	0	52
Local People	1	51	Local People	0	52
p<0.05			*p>0.05*		
3 Question	**Knowledge Level**		**6 Question**	**Knowledge Level**	
Group	**Sufficient**	**Insufficient**	**Group**	**Sufficient**	**Insufficient**
Fishermen	14	38	Fishermen	19	33
Local People	4	48	Local People	0	52
p>0.05			*p<0.05*		

negative effects of the species are not known by fishermen and public. Despite an increase in the population of the *G. holbrooki* in the river basin over the years, awareness of the invader was found to be very low.

The law prohibits the movement of *Gambusia* by humans in Turkey. After *G. holbrooki* is collected from any kind of water resource it must be disposed of promptly, never introduced or transported to any other water resources. Although the Republic of Turkey's Ministry of Agriculture and Forestry have declared the

negative effects of the species, most of the fishermen and local people do not care about laws.

There are some literature regarding awareness studies of some other invasive species in Turkey and in the world, but there is no similar study for *G. holbrooki*. For example, angling is known as one way to raise public awareness about invasive fish species and also it is used to eliminate invaders from water resources. For example, in some countries where the *Carassius gibelio*, Prussian Carp is spread, awareness of the fish is created in the public through fishing competitions. There were some competitions for Prussian Carp in Asia, Africa, Australia and Europe (Næsje, Hay & Kapirika, 2001; Allendorf & Lundquist, 2003; Vrdoljack, 2014). Today, the Prussian Carp is a very famous invasive species and this species is left out of every kind of fishing limit. These competitions about *C. gibelio* have enabled species to be recognized. There should be such kinds of public studies for *Gambusia*.

In Turkey, there were two Prussian Carp fishing competitions in 2014 and 2015 held by Konya Amateur Fishery Association (KOABDER) in Konya. In addition, a third one was held in Denizli (Topkara, Bayhan & Sayğı, 2020).

CONCLUSION

Invasive species demonstrate both ecological and economic impacts in both marine and freshwater ecosystems (Oreska & Aldridge, 2011; Birkan & Öndes, 2020). As a result of deliberate attempts to improve fisheries, this threat is further exacerbated by uncontrolled studies for aquaculture, or for biological control (Gaygusuz et al., 2015). They cause some changes in the food web, exhibit negative effects on native species, damage fishing gear and some of them even cause health problems in case of their consumption by humans (Öndes et al., 2018). Hence, it is important to monitor these alien species and determine their distribution and impacts in their new localities.

The most important way to prevent the spread of the invasive mosquitofish species is to raise awareness of the public and of fishermen. Furthermore, it should be noted that if the fishermen do not recognize the species, it is impossible to prevent them from spreading. Scientific projects about invaders should carry the responsibility of raising the awareness of the public (Topkara et al., 2020).

The main conservation ideas for *G. affinis* and *G. holbrooki* were declared as awareness and education. Involving the community in struggle activities ensures that local people have a sense of ownership of the potential harm of certain fish and an improved understanding of the difficulties of managing invasive fish species. Scientific literature, posters and prospectuses might be used to inform the public about the harm of invasive fish, in particular those known to be noxious under state and commonwealth statute.

For example, in Australia, the *Gambusia* species are being monitored as part of official stream and river health monitoring programs (Kennard et al., 2005). Monitoring programs similar to these might be accomplished for Turkey's water resources, and this kind of practice would contribute to awareness and control of the invasive species.

By way of a suggestion, some part of the budget which is used for the management of fisheries in the freshwaters of Turkey, might be used for the training of fishermen. It would be extremely important for the sustainable management of freshwater fisheries. The important point here is that the goals of training programs should be for very good planning and access to previous experiences.

Although this option is economically challenging at the beginning, further economic loss can only be prevented in the long-term. It is well known that, if an invasive species is introduced into a freshwater environment suddenly, it is next to impossible to remove it again.

Conflicts of interest: The authors have no conflicts of interest to declare.

Ethics committee approval: This study was conducted in accordance with ethics committee procedures.

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Original Article

Impacts of a Garbage Disposal Facility on the Water Quality of Çavuşlu Stream in Giresun, Turkey: A Health Risk Assessment Study by a Validated ICP-MS Assay

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ABSTRACT

Environmental concerns have been raised because of a garbage disposal facility (GDF) constructed near Çavuşlu Stream in Giresun, Turkey. This article proposes a fully validated ICP-MS technique to investigate the impacts of the GDF on the water quality of the stream and provide a human health risk estimation. Water sampling was carried out at four different stations and in tap water since the stream is the primary source of drinking water for the inhabitants of the town. Quantification of selected toxic metals (As, Pb, Cd, Hg, Sb, Al, and Ni) in conjunction with the essential and other elements (Se, Cu, Fe, Mg, Mn, Zn, and Co) was performed by the use of the previously validated ICP-MS method. Once water quality index (WQI), heavy metal pollution index (HPI), and heavy metal evaluation index (HEI) were computed, the health risk assessment was studied according to the US EPA's method. Although the stations (2, 3, 4, and tap water) showed excellent water quality, station-1, which is the closest one to the facility, was classified as poor water quality. Lifetime cancer risk (LCR) was only significant both for adults and children in station-1. In addition, low risk regarding non-carcinogenic health hazards was found for children. The results indicate that the facility decreases the water quality of this stream and is possibly responsible for LCR. In conclusion, the ecological environment and human health should be protected by further monitoring the effect of the GDF on the ecological system.

Keywords: ICP-MS, water quality, health risk assessment, toxic metals, industrial pollution

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INTRODUCTION

Ecological pollution is an increasing difficulty all over the world as a result of industrialization, which influences each lifeform (Mutlu et al., 2016; Aydın et al., 2021). In particular, water pollution is one of the most significant issues, since the accessibility of high-quality drinking water is necessary for environmental and human health (Taş & Şişman, 2020). Nowadays, many water supplies are at risk because of uncontrolled industrialization and urbanization (Küçükosmanoglu & Filazi, 2020; Egbueri & Mgbenu, 2020). Hence, pollution is a global complication that affects surface water like rivers and streams (Taş & Kolören, 2017; Hadi et al., 2019). Continuing the release of heavy metals into surface waters can cause various chemical, physical, and biological problems (Ustaoğlu et al., 2020a). Contamination may originate from anthropogenic and geogenic sources (Ustaoğlu et al., 2017; Ustaoğlu & Tepe, 2018; Yuksel & Arica, 2018). Pollution in drinking water sources is associated with organic and inorganic impurities involving heavy metals and chemical ions (Egbueri & Mgbenu, 2020). In water ecosystems, excess amounts of metals with high persistence in nature are responsible for toxicity, as they can accumulate in aquatic organisms (Mutlu & Kurnaz, 2018; Ali et al., 2019).

Exposure to arsenic generally occurs through drinking water supplies polluted by natural, geological, and anthropogenic sources of inorganic arsenic. Today, the limit recommended by the World Health Organization (WHO) is 10 µg/L (WHO, 2011). Yet, it is likely that millions of individuals are using drinking water with an arsenic concentration above this safety standard. The relationship between the consumption of arsenic-contaminated drinking water and health disorders has been studied all around the world. Hence, arsenic exposure has been linked with certain cancer types such as liver, kidney, bladder, lung, and skin cancers, as well as other medical disorders like adverse effects in pregnancy, neurological disorders, and cardiovascular complaints (Ustaoğlu & Aydın, 2020; Thakur et al., 2020; Zhang et al., 2018; Yüksel et al., 2018). New technics are used to reduce arsenic concentration to a safe limit in the water treatment process (Zhu et al., 2018). Even so, previous studies proposed the possible toxicity of even low-level arsenic exposure because of its life-long accumulation capability in organisms (Roh et al., 2017; Tsuji et al., 2015).

Lead is a toxic heavy metal, and studies regarding lead exposure in drinking water have been well documented over the past few decades as the number of lead pollution cases has risen (Ding-Quan et al., 2020). It may distress nearly every body system, but chiefly, the hematologic, gastrointestinal, and nervous systems are affected. Furthermore, children are extra vulnerable to medical disorders due to lead exposure (Bozalan et al., 2019, Yüksel et al., 2016), as it damages children's behavioral and mental health (Redmon et al., 2020; Dórea, 2019).

Investigating the mercury levels in surface water such as rivers and lakes, as well as tap and bottled water, is important in water quality assessment because it is a toxic element having no biological or physiological function in humans. However, it is responsible for different sorts of health problems, such as neuropathological degradation, kidney deficiency, renal system failure, and leukemia (Marinho et al., 2020, Yüksel et al., 2017a).

According to the US EPA, cadmium is another toxic element that has been classified as Group B1 (probable human carcinogen). Cadmium pollution in drinking water occurs because of industrial debris and agricultural fertilizers. Specific examples of health disorders that have possibly been linked with cadmium exposure are renal failure, liver injury, muscle cramps, diarrhea, nausea, and vomiting (ATSDR, 2012; Cai et al., 2019). The permissible limit for cadmium in drinking water is 5.0 µg/L, according to the US EPA, EU, and TSE. However, the WHO has established this limit as 3.0 µg/L (WHO, 2011) since cadmium levels in uncontaminated drinking water are usually below 1.0 µg/L.

Certain metals are fundamental for aquatic life and other living organisms. The essential metals may be classified in two groups: micronutrients (Cu, Cr, Co, Fe, Se, Mn, Mo, and Zn) and macronutrients (Mg, Ca, Na, S, and P). However, elevated concentrations of these metals may exert toxicity by distressing reproduction, biotransformation, and growth in living organisms, including human beings (Gheorghe et al., 2017; Stankovic et al., 2014).

Origin characterization of metals dissolved in river water, as well as computing the proportional participation, is required to ensure environmental safety of aquatic ecosystems (Tepe & Aydın 2017; Tokatlı et al., 2019; Tokatlı et al., 2020). It is therefore beneficial to employ principal component analysis (PCA), hierarchical cluster analysis (HCA), and Pearson's correlation coefficient (PCC) analysis to assess the source and spread of metals in river waters (Köse et al., 2014; Çiçek et al., 2019; Ustaoğlu, 2020). There are critical activations to monitor the water quality of rivers. For instance, source identification of pollution, determining water quality status, and controlling water pollution are employed for effective water management (Taş et al., 2019; Varol, 2020). Since high-quality freshwater sources have rapidly deteriorated, water quality assessment in Turkey has become a significant issue in recent years. Water quality index (WQI), heavy metal pollution index (HPI), heavy metal evaluation index (HEI), hazard quotient (HQ), hazard index (HI), and carcinogenic risk (CR) are techniques that are widely used in water quality assessment (Ustaoğlu & Tepe, 2019). Therefore, they play an essential role in water resources management.

Çavuşlu Stream, which flows from northeastern Turkey into the Black Sea, is one of the principal watercourses in the region, as the water needs of the town of Çavuşlu are met by caisson wells in the basin. Çavuşlu Stream, also known as Yalakoda Stream, may be exposed to contamination through industrial, domestic, and medical waste from the GDF located near the stream. No environmental studies of water quality and health risk from the Çavuşlu Stream's water have been performed. Since the GDF is suspected to be the origin of water pollution in the town of Çavuşlu, environmental concerns have been rising day by day. For this reason, the primary objective of this research is to investigate the impact of the GDF on the water quality of Çavuşlu Stream and the human health risk. The complementary tools used in this study are to validate an ICP-MS assay to determine metal levels in stream water in the scope of ISO/IEC 17025 certification, and to address the probable origins of metal pollution through statistical tests such as PCC, PCA, and HCA.

MATERIAL AND METHODS

Research items and sample preparation

Sample collection was performed in early May 2020. Sample raw volume was 300 mL. Triple sampling was conducted at four different stations, starting from the closest point to the GDF near Çavuşlu Stream in Görele, Giresun, Turkey (Figure 1). In addition, tap water samples from three different houses in the town were utilized in this study. After 10-mL amounts of the samples were filtered through the Acrodisc® Minispike PTFE membrane with a pore size 0.45 µm (Merck, Germany), they were mixed with the same amounts of 8% (v/v) nitric acid. Calibration standards, at the concentrations of 1.0, 5.0, 10.0, 25.0, 50.0, and 100.0 µg/L, were produced by diluting a multi-element calibration mother solution (VHG LABS, Manchester, NH, USA) with an appropriate amount of 4% (v/v) nitric acid. To avoid any sort of cross-contamination, all glassware (Analitik Kimya, Istanbul, Turkey) was stored in 10.0% (v/v) nitric acid for 24 hours before the analysis.

Instrumentation

The metal levels in the water samples were quantified utilizing an ICP-MS (7700x ICP-MS, Agilent Corporation, USA). The generation of ultrapure water with a resistivity of 18MΩ cm was provided

Figure 1. Map of Çavuşlu Town, located in Görele, Giresun, Turkey (The garbage disposal facility and sampling stations are labeled).

for the sample preparation step employing Direct-Q8 (Merck-Millipore, Germany). The operating parameters of the instrument were set as follows: The water specimens were injected (60 s, 0.3 rps) by means of a Meinhard® nebulizer as well as a chilled spray chamber. The autosampler was promptly established to rest in the sampling stand for the specified time, and no flow injection valve was employed. As for the argon gas plasma conditions, reflected power and forward power were 7 W and 1300 W, respectively. The gas flow rates of plasma, auxiliary, and nebulizerwere set at 16.0, 1.0, and 1.0 L/min, respectively. Next, nickel interface cones were utilized. The peak jumping mode was employed when running the instrument. The autosampler pump was cleaned between injections in three steps, as follows: i) washing with ultrapure water for 30 seconds, ii) rinsing with 2% (v/v) nitric acid for 50 seconds, and iii) concluding the cleaning by using ultrapure water for 50 seconds.

Standard solutions and reagents

To plot the calibration graphs, VHG LABS (Manchester, NH, USA) multi-element standards solutions at the concentration of 10 mg/L of each element were employed. The internal standard multi-element stock solution to control the quantification stability of the instrument was obtained from Agilent® (USA). Nitric acid (HNO_3, 65% v/v) was purchased from Merck (Darmstadt, Germany) to prepare calibration standards and sample solutions. The certified reference material (CRM), ERM®-CA713 (Sigma-Aldrich, Germany), was utilized to test the validation of the assay. Finally, argon gas with an analytical purity (99.999%) was obtained from a local supplier in Turkey.

Assay optimization

As was well documented in our previous paper (Yüksel & Arica, 2018), quantification of trace element levels in natural water subjects is challenging with the matrix elements (Na, Mg, Ca, K, and Cl) in water. Thus, the water specimens were diluted by the amount of 10 mL 8% (v:v) nitric acid to weaken the matrix effects. The most favorable signal intensity throughout multi-element quantification at very low concentrations was achieved, employing the three elements ^{7}Li at low mass, ^{89}Y at medium mass, and ^{205}Tl at high mass.

Validation

Having taken into account the validation guide of the ISO/IEC 17025 standard (Gisbert Albaga et al., 2017), CRM ERM-CA713 Waste Water (Sigma-Aldrich, Taufkirchen, Germany) was analyzed 11 times to validate the assay based on accuracy, precision, recovery, and limit of detection. To improve the validation study, an in-house secondary reference standard solution at the concentration of 100 µg/L was produced by diluting the multi-element calibration mother solution with an appropriate amount of 4% (v:v) nitric acid. As described in previous papers, precision was calculated in terms of the coefficient of variation, while accuracy was expressed by relative error (Yüksel et al. 2020; Arica et al. 2018; Horwitz, 1982). The results of the validation study are given in Table 1, demonstrating that the assay is accurate and precise.

Statistical analysis

The use of different statistical approaches evaluated elemental quantifications in water samples. The PCC analysis was employed to assess the association between metals and their probable origin. Next, HCA was utilized to interpret the correlation among metals. Finally, PCA was employed to decrease data sets and uncover novel factors. SPSS® software version 22.0 was used throughout the statistical analysis.

RESULTS AND DISCUSSION

Quantification of metal levels

Metal levels in aquatic specimens can be measured by various techniques. However, ICP-MS is one of the most widely used assays because of its multi-element analysis capability (Yüksel & Arica, 2018). Most environmental research does not provide sufficient information regarding the accuracy of the method utilized. The ICP-MS method was validated with respect to accuracy and precision before the determination of metal levels in water samples, which improved the significance of this study. In this research, certified reference material and in-house secondary reference standard solutions were employed to perform the validation procedure. As a result, relative error, the variation of coefficient, and recovery were calculated between 0.5-2.7%, 0.9-4.0%, and 97.3-102.4%, confirming that the ICP-MS assay was accurate and precise. Quantified metal concentrations versus WHO, EU, US EPA, and TSE standards are shown in Table 2. Aluminum, iron, and manganese in station-1 exceeded all limits.

Aluminum levels in natural waters may alter dramatically contingent upon numerous mineralogical and physicochemical circumstances. For instance, aluminum levels typically vary between 1.0 to 50 µg/L in water with a neutral pH value, while they may be as high as 500–1000 u/L in increased acidity or organic content

Table 1. Validation Study for the ICP-MS method (Values are given in µg/L).

Metals	Reference Material	Certified Value	Measured Value	RE %	CV %	R %	LOD
Al	In-House Reference	100.0±0.3	98.4±2.1	1.6	2.1	98.4	1.58
Sb	In-House Reference	100.0±0.1	99.5±1.6	0.5	1.6	99.5	0.01
As	ERM-CA713	10.8±0.3	11.0±0.2	1.9	1.8	101.9	0.02
Cu	ERM-CA713	101±7	100.2±0.9	0.8	0.9	99.2	0.02
Hg	ERM-CA713	1.84±0.1	1.79±0.05	2.7	2.8	97.3	0.01
Zn	In-House Reference	100.0±0.4	101.7±2.2	1.7	2.2	101.7	0.38
Fe	ERM-CA713	445±27	451.4±6.7	1.4	1.5	101.4	5.50
Cd	ERM-CA713	5.09±0.2	4.97±0.2	2.4	4.0	97.6	0.02
Pb	ERM-CA713	49.7±1.7	50.2±1.1	1.0	2.2	101.0	0.02
Mn	ERM-CA713	95±4	96.3±2.2	1.4	2.3	101.4	0.20
Ni	ERM-CA713	50.3±1.4	49.4±1.9	1.8	3.9	98.2	0.03
Se	ERM-CA713	4.9±1.1	5.0±0.2	2.0	4.0	102.0	0.10
Ca	In-House Reference	100.0±0.5	100.5±1.9	0.5	1.9	100.5	12.89
Mg	In-House Reference	100.0±0.2	97.9±3.1	2.1	3.2	97.9	0.05
Co	In-House Reference	100.0±0.5	102.4±2.2	2.4	2.2	102.4	0.01

RE, CV, R, and LOD refer to relative error, variation of coefficient, recovery, and limit of detection, respectively.

Table 2. Measured metal concentration versus WHO, EU, US EPA, and TSE standards. Values are given in µg/L.

Metals	Station-1	Station-2	Station-3	Station-4	Tap Water	WHO, 2011	EU	USEPA	TSE
Aluminum	**1168.33±15.22**	51.34±1.20	**215.33±2.5**	4.32±0.3	16.80± 0.45	200	200	200	200
Antimony	0.70±0.01	0.02±0.00	0.01±0.00	<LOD	0.01±0.00	20	5	6	5
Arsenic	2.86±0.02	0.79±0.01	0.14±0.00	0.49±0.01	0.23±0.01	10	10	10	10
Copper	11.78±0.13	9.69±0.13	1.88±0.01	9.05±0.01	2.12±0.02	2000	2000	1300	2000
Mercury	0.01±0.00	0.02±0.00	<LOD	<LOD	0.01±0.00	6	1	2	1
Zinc	148.7±1.25	22.39±0.33	64.66±0.16	122.44±1.82	9.76±0.18	3000	…	5000	…
Iron	**1273.40±1.25**	85.42±3.45	88.4±3.1	176.52±6.14	2.98±0.13	200	200	300	200
Cadmium	1.53±0.04	1.57±0.03	0.03±0.00	1.36±0.07	0.63±0.04	3	5	5	5
Lead	1.78±0.05	0.28±0.01	1.36±0.13	0.92±0.04	1.28±0.05	10	10	15	10
Manganese	**150.72±1.10**	**60.27±0.45**	3.68±0.06	2.51±0.03	1.64±0.02	50	50	50	50
Nickel	10.27±0.26	5.31±0.07	1.88±0.01	7.19±0.12	2.68±0.04	20	20	…	20
Selenium	1.57±0.05	3.25±0.17	2.04±0.14	<LOD	0.62±0.04	10	10	50	10
Calcium	21958.93±229.6	18305±141	4048.41±82.65	26336±256	12252.68±5.5	75000	…	…	…
Magnesium	6826.06±62.20	5060.10±15.90	1728±34	13694±39.88	5362.75±38	50000	…	…	…
Cobalt	5.12±0.01	6.27±0.00	3.3±0.14	5.97±0.01	3.46±0.01	50	…	…	…

(Yavuz et al., 2013). Therefore, elevated aluminum concentration in station-1 may be linked with rich organic content that has originated from the GDF.

Iron is an essential element for humans in terms of cellular biochemical processes, as long as its amount is at trace levels. Nevertheless, iron can also become toxic when its concentration is elevated (Yüksel et al., 2017b). Iron concentrations in natural waters are varied between 500 to 50000 µg/L, while its levels in drinking water are generally below 300 µg/L (WHO, 2011). Raised iron levels in station-1 compared to other stations and tap water may be related to high metallic content in the GDF.

Manganese is a naturally rich essential element necessary for many integral biological processes in humans. Manganese concentration in drinking water is usually lower than 100 µg/L while it may be more than 1000 µg/L in freshwater. Drinking water with manganese levels of more than 100 µg/L results in an unpleasant flavor. Although manganese toxicity seldom happens, its level in drinking water should be quantified to avoid toxic exposures (Evans & Masullo, 2020; WHO, 2011).

Other measured essential metals and toxic metals were not found to be above the maximum contaminant limits. However, toxic metals like arsenic, lead, mercury, and cadmium can have toxic effects even at very low concentrations, as was described in the introduction section.

Water quality index (WQI)

WQI is one of the best classifying methods computed by taking into account the collective effect of different water quality factors on overall water quality. Therefore, it provides an inclusive and actual perception of the water quality.Initially, WQI was established by Horton (1965) in the USA, and today this approach is broadly benefitted through water quality examiners (Kükrer & Mutlu, 2019; Ustaoğlu et al., 2020b; Tokatli & Ustaoğlu 2020). Hence, WQI, in this study, was computed with the formula below (1).

$$WQI = \sum \left[W_i \times \left(\frac{C_i}{S_i} \right) \times 100 \right] \quad 1$$

$W_i = w_i / \Sigma w_i$ refers to relative weight. Taking into consideration the comparatively critical impacts of heavy metals on public health, the W_i values are designated by minimum and maximum magnitudes of 1 and 5, respectively. C_i represents the microelement level quantified in the water where Si expresses the reference values reported by WHO (2011) in respect of drinking water. Concerning WQI, water quality is assessed in five categories: WQI ≥ 300, undrinkable; 200 ≤ WQI <300, very poor; 100 ≤ WQI<200, poor; 50 ≤ WQI <100, good; WQI <50, excellent (Xiao et al., 2019). Assigned weight (AS) and weight relative (WR) are presented in Table 3. Hence, Station-1, which is the closest one to the GDF, showed poor water quality (Table 4).

Table 3. Relative weight of each heavy metal.

Metals	Assigned Weight (AW)	Weight Relative (RW)
Al	4	0.073
Sb	4	0.073
As	5	0.091
Cu	2	0.036
Hg	5	0.091
Zn	3	0.055
Fe	4	0.073
Cd	5	0.091
Pb	5	0.091
Mn	5	0.091
Ni	5	0.091
Se	2	0.036
Ca	2	0.036
Mg	2	0.036
Co	2	0.036
	55	1.0

Table 4. List of calculated WQI, HPI, and HEI in all stations along with tap water.

	Station-1	Station-2	Station-3	Station-4	Tap Water
WQI	**131.42**	28.43	15.45	18.68	7.02
HPI	38.71	29.07	5.66	20.16	10.60
HEI	**16.91**	3.40	2.14	2.08	0.76

Heavy metal pollution index (HPI)

Since HPI is a valuation approach considering the united impact of every single heavy metal on total water quality, it has been employed by most scientists to expansively evaluate the overall water quality. HPI, in this study, was calculated with formulas (2 and 3), proposed as follows (Mohan et al., 1996):

$$HPI = \frac{\sum_{i=1}^{n}(Q_i W_i)}{\sum_{i=1}^{n} W_i} \qquad 2$$

$$Q_i = \sum_{i=1}^{n} \frac{M_i}{S_i} \times 100 \qquad 3$$

In formulas 2 and 3, Wi expresses the unit weight of the ith factor, Qi refers to the sub-index of the trace-toxic metal, Si states the reference values of the factor, Mi represents the screened values of toxic metals, and n stands for the number of factors taken into account. When HPI<100, it is, therefore, a low level of heavy metal pollution that is possibly not responsible for severe health effects (Saleh et al., 2018). Apparently, in this study, all stations, along with the tap water, showed HPI<100, which is a low level of heavy metal contamination (Table 4).

Heavy metal evaluation index (HEI)

Similar to HPI, HEI defines the general trend in the examination of water quality with reference to heavy metal pollution in water. Thereby, it may simply be employed to interpret the pollution degree in water (Edet & Offiong, 2002). In this study, HEI was computed based on the formula below (4).

$$HEI = \sum_{i=1}^{n} \frac{H_C}{H_{MAC}} \qquad 4$$

In this formula, Hc refers to the value detected for every single factor, where Hmac expresses the magnitude of maximum admissible concentration (MAC) for all variables (WHO, 2011). With reference to MAC, the elevated levels of the metal lead to further unpleasant water quality (Goher et al. 2014). As a general rule, when the level of individual metal exceeds the MAC value (HEI > 10), the water is not advisable for consumption. The water quality diminishes because of other impacts when metal levels are not exceeding but in the vicinity of the MAC values. Therefore, HEI is checked out by three classifications as follows: 20 < HEI means high contamination, 10 < HEI < 20 means medium contamination, and HEI < 10 means low contamination (Saleh et al., 2018). In our study, only station-1 showed medium contamination since it is suspected that the leaking from the GDF contaminates Çavuşlu Stream, whereas other stations and tap water showed low contamination (Table 4). The reason why other stations and tap water have low contamination is possibly the dilution of toxic metals with fresh water in the stream.

Health risk assessment: Hazard quotient, Hazard index, and cancer risk

Although current health risk assessment approaches, along with the mathematical patterns, may vary in different countries and organizations, the principle employed for this assessment remains the same. Throughout this research, the health risk assessment procedure proposed by the US EPA (2004) was applied. With regard to the trace elements in water, the health risk assessment process was performed by taking into consideration the amount of ingestion along with dermal absorption. To calculate the average daily dose (ADD) as a result of dermal absorption (ADD dermal) and direct digestion (ADD ingestion), the following formulas (5 and 6) offered by the US EPA (2004) were enforced:

$$ADD_{ingestion} = \frac{C_{Water} \times IR \times ABS_g \times EF \times ED}{BW \times AT} \qquad 5$$

$$ADD_{dermal} = \frac{C_{Water} \times SA \times K_p \times ET \times EF \times ED \times CF}{BW \times AT} \qquad 6$$

In these formulas, $ADD_{ingestion}$ means average daily dose by ingestion and ADD_{dermal} expresses average daily dose by ingestion in the unit of µg/kg/day. In addition, C_{water} refers to the concentration of the metals in surface water in the unit of µg/L. IR is ingestion rate (L/day), which, in our paper, is 2.0 for adult and 0.64 for children. As a parameter, ABSg refers to the unitless gastrointestinal absorption factor. EF is exposure frequency, which is set in our paper at 365 days/year. Next, ED represents exposure length in years, which is set at 70 for adults and 6 for children. BW displays average body weight in the unit of kg, and it was 70 for adults and 20 for children. AT is another parameter expressing the averaging time in days, and it is set in our paper at 25550 and 2190 for adults and children, respectively. SA is a parameter in the formula that stands for exposed skin area in the unit of cm^2, and it is 18,000 and 6600 for adults and children, respectively. Where Kp is the dermal permeability coefficient in water in the unit of cm/h, ET represents the exposure time throughout shower and bathing, which is 0.6 h/day in this paper. Finally, CF is the unit conversion factor, which is 1 L/1000 cm^3 (Xiao et al., 2019). Values of metals along with toxicological parameters employed for health risk assessment are illustrated in Table 5.

With regard to heavy metals, the non-carcinogenic risk by means of ingestion and dermal absorption was computed, and assessment was performed both for children and adults. As well described in a previous paper (Das et al., 2018), the risk hazard quotient formula (HQ) divides the average daily dose (ADD) by reference dose (RfD), while hazard index (HI) refers to the overall quantity of HQs as well as probable non-carcinogenic effects originatingfrom whole heavy metals.

Table 5. Hazard quotient and cancer risk for each element of the Çavuşlu Stream.

	HQ_{ing}		HQ_{derm}		HI		LCR(Ingestion+Dermal)	
Station-1	**Adult**	**Child**	**Adult**	**Child**	**Adult**	**Child**	**Adult**	**Child**
Al	3.04E-02	3.41E-02	8.35E-04	1.85E-03	3.12E-02	3.59E-02		
Sb	4.79E-02	5.37E-02	1.25E-02	2.77E-02	6.05E-02	8.14E-02		
As	2.48E-01	2.78E-01	1.44E-03	3.18E-03	2.50E-01	2.81E-01	**1.13E-04**	**1.28E-04**
Cu	4.60E-03	5.15E-03	2.11E-04	4.66E-04	4.81E-03	5.62E-03		
Hg	6.39E-05	7.16E-05	6.81E-05	1.51E-04	1.32E-04	2.22E-04		
Zn	2.72E-03	3.04E-03	2.13E-04	4.71E-04	2.93E-03	3.51E-03		
Fe	6.98E-04	7.81E-04	1.30E-03	2.88E-03	2.00E-03	3.66E-03		
Cd	4.19E-03	4.69E-03	8.75E-03	1.94E-02	1.29E-02	2.41E-02		
Pb	6.41E-04	7.18E-04	9.53E-06	2.11E-05	6.51E-04	7.39E-04		
Mn	1.03E-02	1.16E-02	2.25E-02	4.97E-02	3.28E-02	6.12E-02		
Ni	5.63E-04	6.30E-04	3.67E-04	8.12E-04	9.30E-04	1.44E-03		
Co	4.68E-01	5.24E-01	4.88E-03	1.08E-02	4.72E-01	5.34E-01		
				HI_{total}	8.71E-01	**1.03E+00**		
Station-2								
Al	1.34E-03	1.50E-03	3.67E-05	8.12E-05	1.37E-03	1.58E-03		
Sb	1.37E-03	1.53E-03	3.58E-04	7.91E-04	1.73E-03	2.33E-03		
As	6.85E-02	7.68E-02	3.96E-04	8.77E-04	6.89E-02	7.76E-02	3.13E-05	3.55E-05
Cu	3.78E-03	4.24E-03	1.73E-04	3.83E-04	3.96E-03	4.62E-03		
Hg	1.28E-04	1.43E-04	1.36E-04	3.01E-04	2.64E-04	4.45E-04		
Zn	4.09E-04	4.58E-04	3.20E-05	7.09E-05	4.41E-04	5.29E-04		
Fe	4.68E-05	5.24E-05	8.73E-05	1.93E-04	1.34E-04	2.45E-04		
Cd	4.30E-03	4.82E-03	8.98E-03	1.99E-02	1.33E-02	2.47E-02		
Pb	4.08E-03	4.56E-03	6.06E-05	1.34E-04	4.14E-03	4.70E-03		
Mn	4.13E-03	4.62E-03	8.98E-03	1.99E-02	1.31E-02	2.45E-02		
Ni	2.91E-04	3.26E-04	1.90E-04	4.20E-04	4.81E-04	7.46E-04		
Co	5.73E-01	6.41E-01	5.98E-03	1.32E-02	5.79E-01	6.55E-01		
				HI_{total}	6.86E-01	7.97E-01		
Station-3								
Al	5.60E-03	6.28E-03	1.54E-04	3.41E-04	5.76E-03	6.62E-03		
Sb	6.85E-04	7.67E-04	1.79E-04	3.96E-04	8.64E-04	1.16E-03		
As	1.21E-02	1.36E-02	7.03E-05	1.55E-04	1.22E-02	1.38E-02	5.54E-06	6.28E-06
Cu	7.34E-04	8.22E-04	3.36E-05	7.44E-05	7.68E-04	8.96E-04		
Hg	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00		
Zn	1.18E-03	1.32E-03	9.25E-05	2.05E-04	1.27E-03	1.53E-03		
Fe	4.84E-05	5.43E-05	9.03E-05	2.00E-04	1.39E-04	2.54E-04		
Cd	8.22E-05	9.21E-05	1.72E-04	3.80E-04	2.54E-04	4.72E-04		
Pb	3.11E-03	3.49E-03	4.63E-05	1.02E-04	3.16E-03	3.59E-03		
Mn	2.52E-04	2.82E-04	5.48E-04	1.21E-03	8.00E-04	1.50E-03		
Ni	1.03E-04	1.15E-04	6.72E-05	1.49E-04	1.70E-04	2.64E-04		
Co	3.01E-01	3.38E-01	3.15E-03	6.96E-03	3.05E-01	3.44E-01		
				HI_{total}	3.30E-01	3.75E-01		
Station-4								
Al	1.12E-04	1.26E-04	3.09E-06	6.84E-06	1.16E-04	1.33E-04		
Sb	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00		
As	4.25E-02	4.76E-02	2.46E-04	5.44E-04	4.28E-02	4.82E-02	1.94E-05	2.20E-05
Cu	3.53E-03	3.96E-03	1.62E-04	3.58E-04	3.70E-03	4.32E-03		
Hg	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00		

Table 5. Continue.

Station-4								
Zn	2.24E-03	2.50E-03	1.75E-04	3.87E-04	2.41E-03	2.89E-03		
Fe	9.67E-05	1.08E-04	1.80E-04	3.99E-04	2.77E-04	5.07E-04		
Cd	3.73E-03	4.17E-03	7.78E-03	1.72E-02	1.15E-02	2.14E-02		
Pb	2.11E-03	2.36E-03	3.13E-05	6.93E-05	2.14E-03	2.43E-03		
Mn	1.72E-04	1.93E-04	3.74E-04	8.27E-04	5.46E-04	1.02E-03		
Ni	3.94E-04	4.41E-04	2.57E-04	5.69E-04	6.51E-04	1.01E-03		
Co	5.45E-01	6.11E-01	5.69E-03	1.26E-02	5.51E-01	6.23E-01		
				HI_{total}	6.15E-01	7.05E-01		
Tap Water								
Al	4.37E-04	4.90E-04	1.20E-05	2.66E-05	4.49E-04	5.16E-04		
Sb	6.85E-04	7.67E-04	1.79E-04	3.96E-04	8.64E-04	1.16E-03		
As	2.00E-02	2.23E-02	1.15E-04	2.55E-04	2.01E-02	2.26E-02	9.10E-06	1.03E-05
Cu	8.28E-04	9.27E-04	3.79E-05	8.39E-05	8.66E-04	1.01E-03		
Hg	6.39E-05	7.16E-05	6.81E-05	1.51E-04	1.32E-04	2.22E-04		
Zn	1.78E-04	2.00E-04	1.40E-05	3.09E-05	1.92E-04	2.31E-04		
Fe	1.63E-06	1.83E-06	3.04E-06	6.74E-06	4.68E-06	8.56E-06		
Cd	1.73E-03	1.93E-03	3.60E-03	7.97E-03	5.33E-03	9.91E-03		
Pb	2.93E-03	3.28E-03	4.36E-05	9.64E-05	2.97E-03	3.38E-03		
Mn	1.12E-04	1.26E-04	2.44E-04	5.41E-04	3.57E-04	6.66E-04		
Ni	1.47E-04	1.64E-04	9.58E-05	2.12E-04	2.43E-04	3.76E-04		
Co	3.16E-01	3.54E-01	3.30E-03	7.30E-03	3.19E-01	3.61E-01		
				HI_{total}	3.51E-01	4.01E-01		

HQ and HI were computed based on the following formulas (7 and 8) (US EPA, 2004):

$$HQ = \frac{ADD_{ingestion}/ADD_{dermal}}{RfD_{ingestion}/RfD_{dermal}} \quad 7$$

$$HI = \sum(ADD_{ingestion} + ADD_{dermal}) \quad 8$$

Medical disorders in humans caused by heavy metals may be observed when HI, HQ>1. In contrast, no adverse health effect is observed if HI, HQ <1. Wu & Sun (2016) report that HI (hazard index) is evaluated in 5 categories: $HI_{total} > 4$ means extreme risk, $3 < HI_{total} < 4$ means high risk, $2 < HItotal < 3$ means medium risk, $1 < HI_{total} < 2$ means low risk, and $HI_{total} < 1$ means no risk. Therefore, in this paper, low risk in association with non-carcinogenic health hazards was found for children, while no risk was calculated for adults (Table 5).

Lifetime cancer risk (LCR) may be defined as conveying possible risk because of exposure to a carcinogen throughout life, and it was calculated using formula (9) (US EPA, 2004).

$$LCR = ADD \times CSF \quad 9$$

In the present investigation, LCR was assessed due to arsenic content, since it is one of the most carcinogenic toxic metals analyzed. Based on previous articles (Gao et al. 2019; Saha et al. 2017), Cancer Slope Factor (CSF) values were utilized as 0.0015 and 0.00366 µg/kg/day for ingestion and dermal exposure, respectively. As reported by the US EPA (2004), the tolerable or acceptable carcinogenic risk ranges from 10^{-6} to 10^{-4}. From another point of view, adverse health effect is very likely by the time LCR ≥ 10^{-4}. As can be seen in Table 5, Station-1 has LCR, while other stations and tap water do not show LCR. The reason why station-1 shows poor water quality and LCR may be due to toxic materials leaking from the GDF.

Source identification

The origin identification was based on hierarchical cluster analysis (HCA) and principal component analysis (PCA) to classify clusters of water quality variables and sampling sites of similar contamination features, as described by Mishra et al. (2017). Therefore, PCA was conducted to associate the contribution source as well as providing consistent evidence through the correlation of metals (Table 6). As evidenced in Figure 2, the component plot in rotated space indicated that metals are linked with three different sources. The outcome from PCA was supported by HCA, signifying sample grouping within the dataset by three clusters (Figure 3). Since sampling stations are in the vicinity of GDF and an agricultural region where pesticides are applied, one of the most significant reasons for toxic metal pollution can be explained as anthropogenic. In addition, it is possible that geogenic contamination has an effect on water quality, as Çavuşlu Stream is also fed from groundwaters.

Pearson's correlation (PC) matrix was employed to observe whether or not metal levels in the stream water are interconnected with one another (Ustaoğlu & Islam, 2020; Mutlu, 2019). As proposed by Ali et al. (2016), strong interrelation among certain metals in riv-

Table 6. PCA Component values of metals analyzed.

	Components		
Variable	**PCA1**	**PCA2**	**PCA3**
Al	*.999*	-.007	.040
Sb	*.987*	.118	.038
Fe	*.982*	.187	-.035
As	*.940*	.308	.131
Mn	*.891*	.285	.352
Pb	*-.829*	-.147	.528
Ni	*.715*	.689	-.117
Zn	*.700*	.333	-.532
Co	.023	*.956*	.188
Ca	.228	*.940*	-.231
Cd	.299	*.925*	.208
Cu	.511	*.833*	.148
Mg	-.055	*.784*	-.615
Se	.060	-.077	*.918*
Hg	.046	.325	*.871*
Eigenvalues	8.564	3.073	2.835
% of variance	57.095	20.487	18.897
Cumulative %	57.095	77.582	96.479

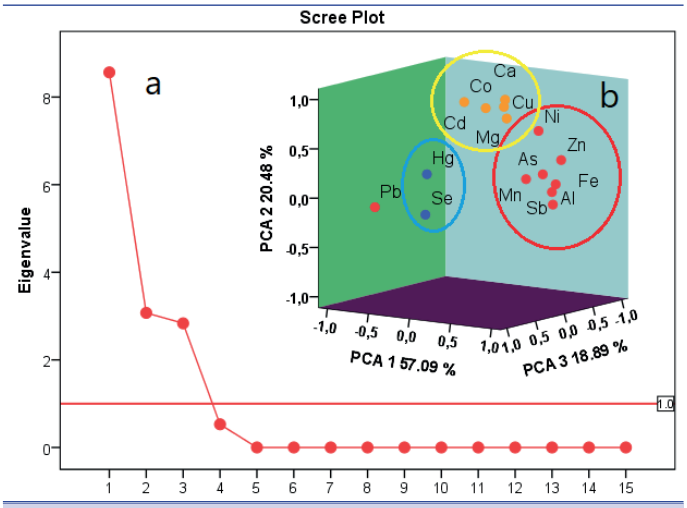

Figure 2. PCA of measured parameters by (a) scree plot of the characteristic roots (Eigen values) and (b) component plot in rotated space.).

Table 7. Correlations of metals.

Correlations															
	Al	**Sb**	**As**	**Cu**	**Hg**	**Zn**	**Fe**	**Cd**	**Pb**	**Mn**	**Ni**	**Se**	**Ca**	**Mg**	**Co**
Al	1														
Sb	.986**	1													
As	.941*	.976**	1												
Cu	.513	.595	.746	1											
Hg	.074	.153	.282	.369	1										
Zn	.680	.677	.667	.608	-.448	1									
Fe	.978**	.992**	.977**	.650	.082	.762	1								
Cd	.299	.423	.600	.938*	.535	.370	.461	1							
Pb	-.804	-.827	-.763	-.451	.332	-.870	-.861	-.287	1						
Mn	.902*	.930*	.973**	.741	.451	.521	.916*	.606	-.597	1					
Ni	.705	.781	.867	.925*	.149	.797	.834	.825	-.753	.792	1				
Se	.102	.048	.127	.160	.637	-.340	.006	.096	.490	.344	-.111	1			
Ca	.210	.338	.480	.849	.155	.557	.409	.901*	-.462	.393	.836	-.312	1		
Mg	-.086	.021	.113	.523	-.259	.526	.115	.588	-.402	-.041	.571	-.654	.873	1	
Co	.027	.121	.325	.870	.393	.311	.191	.906*	-.035	.353	.657	.187	.835	.616	1

**. Correlation is significant at the 0.01 level (2-tailed). *. Correlation is significant at the 0.05 level (2-tailed).

er water can be evidence of the same origin of the contamination. The results of the PC matrix are illustrated in Table 7. Mn has a positive correlation with Al, Sb, As, and Fe, proposing that these metals in the stream water have similar sources. Furthermore, these findings are consistent with the outcome of PCA and HCA.

CONCLUSION

For the first time, this paper has outlined the impact of the GDF on the water quality of the Çavuşlu Stream, located in Görele, Giresun, Turkey. We also developed a simple revalidation procedure employing in-house and certified reference materials, which resulted in further accurate results after instrumental analysis by ICP-MS. Station-1, in the vicinity of GDF, was classified as having poor water quality. Furthermore, LCR was significant only at station-1 both for adults and children, and non-carcinogenic health hazard was estimated only at station-1 for children. Source identification by PCA and HCA indicated that metals in the Çavuşlu Stream might primarily originate from anthropogenic and geogenic sources. Overall, the results point to the GDF decreasing the water

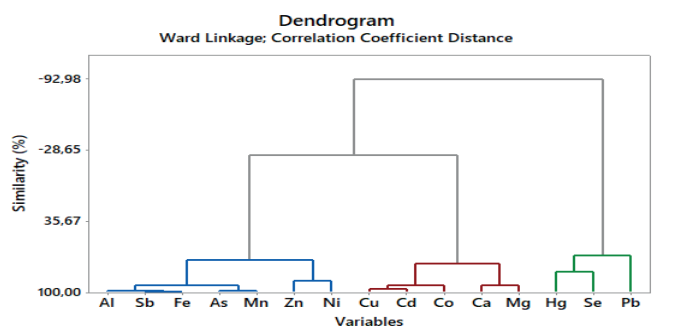

Figure 3. Hierarchical cluster analysis.

quality of the stream and possibly being responsible for LCR. Hence, we propose that the GDF should be moved somewhere else, not in the proximity of water resources and towns. In addition, as a temporary solution, new caisson wells should be constructed on the upper side of the stream where water quality is not directly affected by the GDF. However, the ecological environment and human health will be at risk as long as the activity of the GDF lasts. Environmental monitoring to assess the effect of the GDF on the ecological system should be maintained. Therefore, sediment and fish samples will be investigated to assess ecotoxicological risk in the next phase of this research project.

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Original Article

Yellow Tainting of Flesh in Pangasius (*Pangasianodon hypophthalmus*): Origin of the Color and Procedures for Removal

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ABSTRACT

Pangasius (*Pangasianodon hypophthalmus*) is a commonly farmed fish in ponds in Bangladesh but its yellow flesh color creates a barrier for export. Here, we investigated if feed ingredients and environmental parameters might impact yellow tainting of the flesh. In experimental feeds, maize (a typical ingredient in commercial feeds) was replaced by soybean meal, and frequent exchange of pond water was introduced. Chemical analyses showed that the commercial feed was high in carotenoids (lutein, zeaxanthin and β-carotene; 18.8 mg/kg) as compared to the experimental feeds (≤1 mg carotenoids/kg). After feeding the fish commercial and experimental feeds for 9 months, the content of total carotenoids in the flesh was reduced by 48% to 64% and visual yellowness by up to 47% by the experimental feeds. Weekly or biweekly water exchange in combination with the experimental feeds further reduced the yellow coloration to 22% relative to fish given commercial feed and without water exchange. Our study demonstrates that pangasius with white flesh can be produced in Bangladesh by combining feeds low in pigments with frequent water exchange.

Keywords: Pangasius, yellow flesh, water quality, feed composition, maize, carotenoids

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INTRODUCTION

Catfish are globally widespread and are also commonly produced fish in subtropical and tropical aquaculture (FAO, 2018). In south and southeastern Asia, an important catfish species in freshwater fish farming is pangasius (*Pangasianodon hypophthalmus*) (Jespersen et al., 2014). In Bangladesh, pangasius was the most produced aquaculture species in 2017 and 2018 and reached a production of 453,383 tons (DoF, 2018). Export of pangasius from Bangladesh is currently limited and the fish are mainly sold at domestic markets (Alam et al., 2019). Recently, pangasius production has exceeded local demand and this has opened an option for export, but lack of interest by international companies in the import of pangasius has hindered the establishment of a profitable export-oriented pangasius industry in Bangladesh. A main barrier for the export appears to be the yellow flesh color that occurs in some catfish species (Qiufen et al., 2012) and that has been reported as a common characteristic of pangasius produced in Bangladesh, as illustrated in Figure 2A & B and reported by Hoque et al. (2021). On the basis that the yellow color limits the export of pangasius from Bangladesh, pangasius with white flesh but produced in other Asian countries, e.g., Vietnam, has successfully been exported to Europe and other international markets (Belton et al., 2011; FAO, 2019).

Consumers tend to prefer fish with white flesh (Lovell, 1998), except for species with a known different color, such as pinkish flesh in salmon (Sheehan et al., 1998). In some countries, especially in USA and Europe, consumers are often reluctant to buy fish products with yellow pigmentation, because they suspect the fillets are

damaged, spoiled or have been stored longer than the "best before" date (Lovell, 1984; Shahidi et al., 1998).

The yellow color of catfish flesh is typically caused by the yellow-orange carotenoid pigments carotenes and their oxygenated derivatives xanthophylls (Lee, 1987; Li et al., 2007; Maoka et al., 1989). Different species of fish accumulate different kinds of carotenoids (de Carvalho & Caramujo, 2017), but the xanthophylls lutein and zeaxanthin are considered the primary yellow pigments in catfish (Lee, 1987; Li et al., 2011). Carotenoids are synthesized by microorganisms and plants and are not produced by animals. Hence, carotenoids in catfish originate from an external source. In commercial production, carotenoids may either be feed ingredients or originate from ingestion of materials or organisms in the water. Common ingredients in catfish feeds are maize, corn gluten meal and mustard oil cake due to their protein content, but these plant products may also contribute to accumulation of carotenoids in fish flesh (Lee, 1987).

Catfish are omnivore, opportunistic fish that in their wild habitats feed on algae, aquatic plants, crustaceans, as well as on other organisms in the water (Zimba et al., 2003). This means that natural, carotenoid-containing organisms in water in fish farms may also contribute carotenoids in catfish flesh after ingestion. Supporting this, a correlation between xanthophylls in catfish and microorganisms >75 µm (assumed to be mainly phytoplankton) was observed by Hu et al. (2013). Some reports mention that environmental conditions, e.g., low oxygen content and a high ammonium level, can also cause yellow discoloration in pangasius (Qiufen et al., 2012; Waycott, 2015), but no scientific evidence was given in these reports.

The yellow pigments do not influence the flavor, taste, storage capacity, or safety of catfish flesh (Lovell, 1998), and there are indications that consumption of yellow pigments might be beneficial to human health (Snodderly, 1995; Young & Lowe, 2001). However, unless consumer acceptance of yellowish color in catfish changes, yellow catfish fillets will still be opted out by most consumers.

The difference in flesh color of pangasius raised in Vietnam and Bangladesh is not related to species, since taxonomically identical pangasius species are cultured in both countries (Belton et al., 2011). Rather, fish farm practices with respect to feed ingredients, and possibly also water quality, might explain the yellowish color but so far, no experimental evidence for such effects has been given.

In this study, we fed pangasius different diets with a variable content of carotenoids and manipulated the water exchange in experimental ponds. To mimic actual conditions in commercial fish farms, earthen ponds with natural well water were used in the study. We hypothesize that changed feeds and possibly also better monitoring and control of the water quality can reduce the yellow color in pangasius flesh.

MATERIAL AND METHODS

Field experiment setup

A controlled field study was conducted for 9 months between May 2018 and January 2019 in uniformly sized soil fishponds, each with a surface area of 40.5 m^2 and a depth of 1.20 m, at the Faculty of Fisheries, Bangladesh Agricultural University (BAU), Mymensingh, Bangladesh. Before the start of the experiments, all ponds were dried out in the sun to exclude the presence of other fish species in the ponds. Separate inlet and outlet canals were built into each pond to prevent the exchange of fish between ponds, and dikes prevented the entry of other fish and the escape of stocks. The pond soil was conditioned with lime as is practiced in Bangladesh to improve the structure of the pond soil. After two weeks, the ponds were filled with underground water up to 1 m in depth, and after an additional week, healthy and uniformly sized fingerlings (average length of 20 cm and weight of 60 g) were stocked at a rate of 5/m^2. The fish for analysis were harvested in September, October and January (see below). No mortality of fish was observed in any of the treatment.

Feed formulation and feeding

Based on the hypothesis that certain ingredients in standard pangasius feed cause the yellow coloration of fish flesh, two experimental feeds were formulated and fed to the fish. A commercial pangasius feed type (Pangasius floating feed; www.megafeedbd.com) served as the control. Ingredients in commercial pangasius feed (feed type 1; FT-1) typically include fish meal, meat and bone meal, maize, soybean meal, rice bran, mustard oil cake, wheat flour, salt, and vitamin and mineral premix. The proportion of maize and mustard oil cake in commercial feeds in Bangladesh usually varies from 10-25% and 10-15% (Ali et al., 2013), respectively. We suspected that these two ingredients were mainly responsible for the yellow color in pangasius flesh. Therefore, the two experimental feed types were formulated without maize and mustard oil cake, and each feed had a content of crude protein of 30%. Feed type 2 (FT-2) contained fish meal (25%), soybean meal (25%), rice bran (24%), wheat flour (24%), salt (1%), and vitamin-mineral premix (1%). Feed type 3 (FT-3) was made from meat and bone meal as the main animal protein source (30%), while soybean meal (30%) was the main plant-based protein source. The remaining ingredients were rice bran (19%), wheat flour (19%), salt (1%), and vitamin-mineral premix (1%). The ingredients were finely ground, mixed with clean water and made into pressed pellets by a fish feed pelleting machine. The content of carotenoids varied from 18.8 mg/kg in the commercial feed to about 0.9 mg/kg in the experimental feeds (determined from published values) (Table 1). Individual proportions of the carotenoids lutein, zeaxanthin and β-carotene in the three feeds were 43, 56 and 1% (commercial feed), 81, 8 and 11% (experimental feed 2), and 83, 6 and 11% (experimental feed 3), respectively. The fish were fed the three feed types as 3.0-4.0 mm pellets at a rate of 4-5% of their average body weight twice a day and this was adjusted bi-weekly with the body weight.

Management of ponds, feeds and water exchange

Instead of small replicate ponds with few fish, single but large ponds with many fish (200 fish per pond) were used for each treatment. Pond 1 was the control pond and the fish were fed the commercial feed (FT-1) and no water exchange (NWEX) was applied. Fish in Pond 2, 3 and 4 received our own formulated feed type 2 (FT-2) and had no, 50% fortnightly, or 50% weekly water exchange (NWEX, FWEX or WWEX, respectively). Fish in Pond 5, 6 and 7 received our own formulated feed type 3 (FT-3) and had

Table 1. Different feed types and their composition.

Ingredients	Percentage dry weight (except for carotenoids)		
	Feed type 1 (FT-1)	Feed type 2 (FT-2)	Feed type 3 (FT-3)
Fish meal	10	25	-
Meat and bone meal	13	-	30
Maize	11	-	-
Soybean Meal	6	25	30
Rice bran	38	24	19
Mustard oil cake	10	-	-
Wheat flour	8	24	19
Salt	1	1	1
Vitamin-mineral premix	1	1	1
Total carotenoids* (mg/kg)	18.8	0.86	0.91

*Lutein, zeaxanthin and β-carotene. Content of carotenoids in the ingredients was based on data by Aruna & Baskaran (2010); Kim et al. (2010); Wu et al. (2016); Trono (2019).

no, 50% fortnightly, or 50% weekly water exchange (NWEX, FWEX or WWEX, respectively). For the commercial feed FT-1, no water exchange was applied to resemble real pond farming practices in which no water exchange is applied. Moreover, due to a limited availability of ponds for the study, we could not replicate different water exchange conditions for feed FT-1.

Comparisons of feed effects were done at identical water exchange conditions and with similar feeds at different water exchange conditions. Biological replicates of the fish (i.e., "parallel measurements of biologically distinct samples that capture random variation"; Blainey et al., 2014) were included in all observations to validate statistical analyses. The similar growth rates observed for fish in the different ponds, irrespective of feed type and water exchange, justify application of the large-pond strategy, relative to replicate and smaller ponds.

Analysis of water quality and sampling of fish

Water quality parameters were measured monthly from May to October in 2018, and in January 2019. On each sampling day, temperature, dissolved oxygen and pH were measured between 9:00 and 11:00 using portable devices (www.extech.com) at the sampling site, while water transparency was measured with a Secchi disk. Ammonia, nitrate and ortho-phosphate were measured immediately after return to the laboratory by application of specific reagent kits by Lovibond (www.lovibond.com). The fish were sampled at three time points (September, October and January) using nets. The fish were stunned and bled out by cutting of the gill blood vessels. Fillets of the fish were stored at -70°C until analysis.

Color analyses

The color of the fish fillets was measured using the Commission Internationale de l'Eclairage (CIE) color system by measurement of *L*, a*, b** values which are widely used to determine the color intensity of fish (Gouveia et al., 2003; Wathne et al., 1998). *L** measures the lightness on a 100- point scale, *+a** measures the redness and *−a** the greenness, *+b** measures the yellowness and *−b** the blueness. The *L*, a*, b** values were measured with a Minolta CR-400 Chroma Meter (Konica Minolta, Inc., USA) using a pulsed xenon lamp with an 8 mm diameter opening, corresponding to the measuring surface. Before analysis, the instrument was calibrated with a white ceramic plate. To ensure a uniform analysis of the entire fillet, three measuring points were defined on the surface of the flesh side of the raw fillets (Figure 2C) (Li et al., 2011). All measurements were made on the flesh side of the fillets, since it appeared more homogeneous than the skin side, and at the same locations in each fillet. The results are expressed as mean value of readings from three points from the same fillet. After the measurements, the fillets were immediately stored in aluminum foil at -70°C for subsequent carotenoid analysis by HPLC.

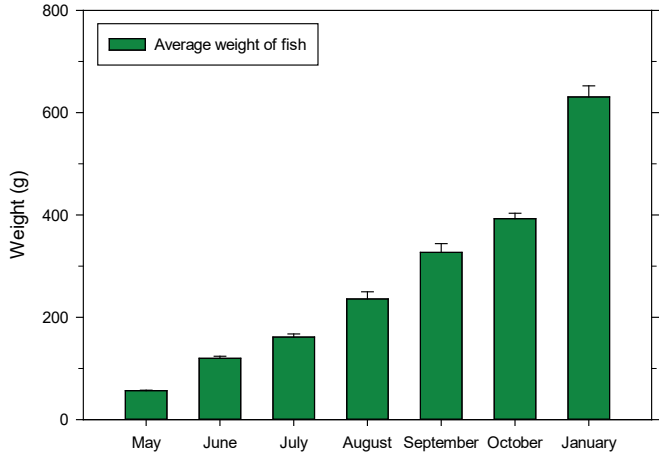

Figure 1. Weight of fish at the monthly samplings. The monthly weights were similar irrespective of feed and water exchange ($p>0.05$; n = 35 fish at each sampling time; SEM shown).

Extraction of pigments

The procedure for extraction of carotenoids was modified from the technique given by Liu et al. (2012). Samples of fish fillet of 20 g were freeze-dried and ground in a mortar before addition of 20 ml acetone. The acetone was mixed with the sample to dissolve the pigments before filtration through a glass fiber filter and transfer to a separating funnel. Four ml n-hexane and 20 ml water were added to the acetone extract and mixed and left undisturbed for 15 min for separation to occur. Next, acetone and water were discarded, and the pigment extract was washed twice with Milli-Q water. The n-hexane layer was collected and filtered through Whatman 0.45 µm nylon GMF filter paper before storage at 4°C in dark 2 ml glass vials without headspace. Feed and feed ingredients were treated similarly by extraction of 5 g samples. Finally, all pigment extracts were concentrated by evaporation of n-hexane under N_2 stream and stored at -20°C until subsequent HPLC analysis.

HPLC analysis of carotenoids

Pigments were dissolved by addition of 6 ml 95% acetone to each sample before sonication in an ice-cool sonication bath for 10 min. To improve the solution of the pigments, the samples were left for 20 h at 4°C, after which the extracts were vortex-mixed for 10 sec. In a few samples, a thin layer of whitish fat occurred at the bottom, but this layer was not included in the pigment analysis. Analysis of the carotenoids was carried out according to Schlüter et al. (2016) using the Van Heukelem and Thomas HPLC method (Van Heukelem and Thomas, 2001) with an adjusted pump gradient to optimize the resolution. The HPLC system consisted of a Shimadzu LC-10ADVP system with one pump (LC-10ADVP), photodiode array detector (SPD-M10AVP), SCL-10ADVP system controller with Lab Solution software, temperature-controlled auto sampler (SIL-10ADVP) (set at 4°C), column oven (CTO-10ASVP), and degasser (ERC 3415a). Retention times of peaks were confirmed using pigment standards from DHI Lab Products, and peak identities were routinely confirmed by online photodiode array analysis. To further assure correct pigments concentrations in the fish flesh, replicates of five fish samples were validated and confirmed by a commercial laboratory (www.eurofins.dk).

Statistics

All statistical analyses were performed with the statistical software SPSS (version 23.0, SPSS, Chicago, IL, USA). Univariate analysis of variance (ANOVA) was performed through the General Linear Model (GLM) procedure in SPSS. The GLM procedure provides univariate ANOVA for one dependent variable by one or more independent variables. In the GLM, feed type and water exchange rate were included as independent variables to test for individual and interactive effects on several dependent variables, e.g., *b**, zeaxanthin, lutein, etc. Tukey's test was applied for post hoc detection of significant pair-wise comparisons.

RESULTS AND DISCUSSION

Physicochemical parameters and growth performance

Seasonal water quality parameters in ponds receiving different feeds and with different water exchange rates are presented in Table 2. The water temperature ranged from 19.3°C in January to a maximum of 35.7°C in August. Relative to the commercial feed FT-1, the experimental feeds FT-2 and FT-3 led to a higher oxygen content, lower concentrations of phosphate and ammonia (only in FT-3) and raised pH (ANOVA test). When weekly or biweekly water exchanges were applied, oxygen level (in FT-3 only) and water transparency increased, while phosphate was reduced (in FT-3 only) (ANOVA). The water exchange had variable effects on pH, ammonia and nitrate.

Conditions for pangasius production in the ponds were representative for fish farms in Bangladesh with respect to temperature, oxygen level and inorganic nutrients (Abedin et al., 2017). However, in some periods the oxygen concentration was below the recommended level of 5 mg O_2/L (Abedin et al., 2017; Ali et al., 2013), except in the ponds given feed FT-3 and with weekly or biweekly water exchange. It has been suggested that a low oxygen content and increased ammonium concentrations can lead to yellow coloration in pangasius (Qiufen et al., 2012; Waycott, 2015), but no studies have confirmed this. Many Bangladeshi fish farmers also assume that a reduced water quality is the main reason for the yellow flesh (own personal communication). Possibly, a relation between oxygen level, metabolic activity and yellow coloration might exist, as discussed below.

During the 9 months growth period, the biomass of fish from the different treatments increased from a mean weight of 57 g in May 2018, to a mean weight of 631 g in January 2019. Weight gains at the monthly samplings were similar irrespective of feed and water exchange ($p>0.05$; Figure 1).

Color of fish flesh and effects of water exchange

Feed type and water exchange affected the yellow coloration *b** and the lightness *L**, but no changes of redness *a** of the fish fillets were observed (Table 3). Lightness and yellowness correlated negatively, meaning that less yellow color occurred when the fillets had a higher lightness.

Without water exchange, the average yellowness *b** at three sampling times (September, October and January) was 24.7 (feed FT-1), 19.7 (feed FT-2) and 15.7 (feed FT-3) (the *b** values were statistically different; $p<0.05$) (Figure 3). Thus, a significant color improvement occurred when the fish were given the

Table 2. Water quality parameters in ponds with different feeds and water exchange rates. No water exchange (NWEX), 50% fortnightly water exchange (FWEX) or 50% weekly water exchange (WWEX) were applied.

Feed	Water exchange rates	Temperature (°C) (min - max)	Dissolved oxygen (mg/l)	pH	Transparency (cm)	Ammonia (mg/l)	Nitrate (mg/l)	Phosphate (mg/l)
FT-1	NWEX	22.6-33.7	5.15^{b}±0.82	6.66^{a}±0.41	15.74±5.10	0.38^{ab}±0.32	6.74±2.6	1.50^{b}±0.38
	NWEX	21.5-33.4	4.53^{a}±0.86	7.43^{b}±0.53	16.77^{x}±4.93	$0.54^{b,y}$±0.56	9.27±4.37	$0.26^{a,x}$±0.11
FT-2	FWEX	19.3-35.7	4.83±0.87	7.02±0.67	21.25^{y}±3.06	0.16^{x}±0.18	8.37±2.26	0.24^{x}±0.19
	WWEX	20.7-34.0	4.70±0.79	7.35±0.53	27.89^{z}±3.19	0.44^{xy}±0.55	8.69±3.39	0.39^{y}±0.24
	NWEX	22.0-33.2	$5.78^{c,x}$±0.90	7.38^{b}±0.65	15.89^{x}±4.15	0.14^{a}±0.09	8.37^{x}±2.06	$1.29^{b,y}$±0.30
FT-3	FWEX	23.4-34.3	6.01^{x}±0.65	7.43±0.62	21.49^{y}±5.12	0.19±0.10	11.72^{y}±4.60	0.70^{x}±0.34
	WWEX	22.7-35.4	6.85^{y}±0.76	7.67±0.49	28.36^{z}±2.36	0.11±0.06	7.73^{x}±1.60	0.78^{x}±0.45

*Different superscripts (a,b,c) in the same column signify statistical differences in different feed types with no water exchange (NWEX) ($p<0.05$); superscripts x,y,z in the same column signify statistical differences for application of fortnightly water exchange (FWEX) and weekly water exchange (WWEX) for feed type 2 (FT-2) and feed type 3 (FT-3) ($p<0.05$). Means ± 1 STD (n = 5) shown.

low-pigmented feeds FT-2 and FT-3, relative to the high-pigmented commercial feed FT-1.

With water exchange, the effects of the two low-pigmented feeds were significantly enhanced and caused an additional reduction in the yellow color of the fillets (Figure 3). The lowest observed yellowness (*b**- value 4.69) was observed in January in fish given feed type FT-3 and with a weekly water exchange (WWEX).

Carotenoids in fish relative to carotenoids in feeds

The absence of maize and mustard oil cake in the experimental feed types was clearly reflected in the pigment content of the fish flesh. Fish which had been fed the commercial feed accumulated more zeaxanthin (187 ng/g) and lutein (213 ng/g) than fish which had been fed the experimental feeds (zeaxanthin and lutein content with FT-2 and FT-3 was 96 and 71 ng/g, and 128 and 67 ng/g, respectively) (Table 4). β-carotene contributed only a little to the pigment concentrations (content of 2-5 ng/g). Two unidentified pigments in the flesh with absorption maxima a few nm from zeaxanthin and lutein, and with retention time close to these two pigments in the HPLC chromatograms, were considered as derivatives of lutein and zeaxanthin and added to the total carotenoid content in Table 4. The sum of the carotenoids confirmed that the experimental feeds FT-2 and FT-3 led to a significantly lower content of carotenoids than feed FT-1 (summarized in Figure 5).

The co-occurrence of carotenoids in feed and fish shows that pigments in feed can be a main source for the coloring of flesh in catfish, as also observed for other catfish species (Qiufen et al., 2012). Supporting this relation, removal of carotenoids from the feed was found to reduce carotenoid pigments in fish. Li et al. (2011) fed catfish (*Ictalurus punctatus*) a diet containing zeaxanthin and lutein (total of 82 mg/kg feed) for 11

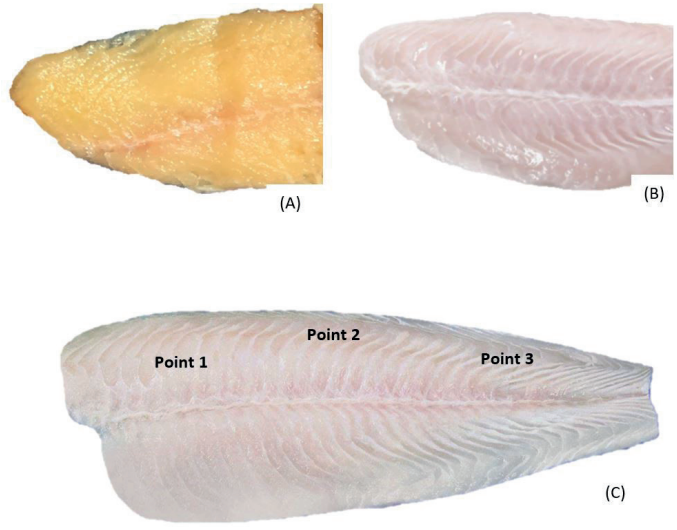

Figure 2. Intense yellow (A) and white (B) pangasius fillets from the studied ponds, and locations on pangasius fillet along the dorsal line (C) where the *L**, *a** and *b**- values were measured.

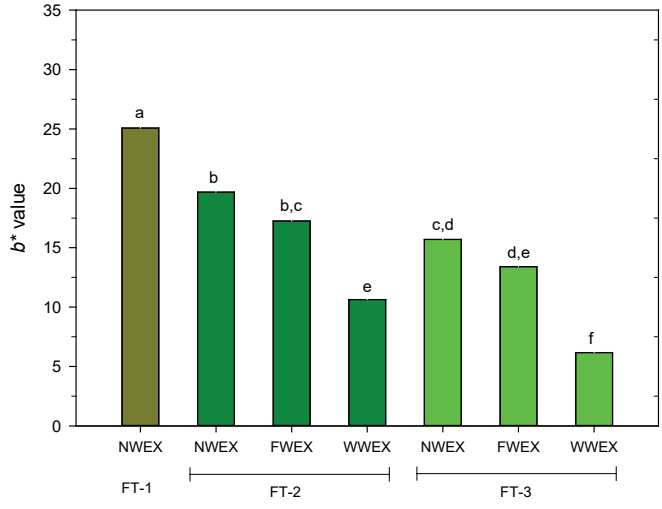

Figure 3. Visual yellow color of fillet (*b**) in different feeds (FT-1, FT-2 and FT-3) and water exchange rates (none (NWEX), fortnightly (FWEX) or weekly (WWEX) water exchange). Means of 15 fish shown. Letters above columns indicate significant differences ($p<0.05$).

Table 3. Effects of different feeds and water exchange rates on *L** (Lightness), *a** (Redness) and *b** (Yellowness) of fish fillets from three months. NWEX, FWEX and WWEX refer to none, fortnightly or weekly water exchange.

Feed	Treatment	September			October			January		
		*L**	*a**	*b**	*L**	*a**	*b**	*L**	*a**	*b**
FT-1	NWEX	$61.78^{a,1}$±1.75	5.91±0.63	26.42^{b}±2.65	$64.52^{a,12}$±2.27	5.84±0.30	26.30^{b}±6.35	66.44^{2}±3.62	5.91±0.16	21.48^{b}±5.41
	NWEX	$62.31^{a,1}$±2.77	6.65±0.92	$19.34^{a,y}$±2.51	$66.51^{a,2}$±1.37	6.01±1.21	$19.32^{ab,y}$±2.68	$68.26^{x,2}$±2.61	5.64±0.57	$20.38^{b,y}$±3.92
FT-2	FWEX	65.85±4.35	5.16±0.59	16.77^{y}±0.92	68.42±3.48	6.15±0.54	18.98^{y}±4.61	70.42^{xy}±1.75	5.93±0.74	15.99^{y}±2.14
	WWEX	66.70^{1}±1.97	5.50±1.10	10.85^{x}±2.15	70.06^{2}±2.30	6.61±0.50	11.68^{x}±5.07	$72.35^{y,2}$±2.24	6.23±0.75	9.36^{x}±3.60
	NWEX	71.10^{b}±3.27	5.82±1.48	$17.42^{a,y}$±1.82	71.79^{b}±2.15	5.87±1.14	$17.38^{a,y}$±4.47	70.86^{x}±3.28	5.22±0.39	$12.30^{a,y}$±3.20
FT-3	FWEX	72.12±3.92	6.08±0.77	14.42^{y}±2.80	72.41±2.04	6.11±0.48	13.47^{y}±2.95	73.92^{x}±3.32	5.91±0.44	12.33^{y}±1.88
	WWEX	72.46^{1}±3.98	6.29±0.64	$8.09^{x,2}$±1.08	72.74^{1}±1.85	6.66±1.37	$5.72^{x,1}$±1.72	$78.68^{y,2}$±1.68	6.04±0.98	$4.69^{x,1}$±1.47

*Different superscripts (a,b) in the same column signify statistical differences in different feed types at NWEX condition ($p<0.05$); superscripts x,y in the same column signify statistical differences in different water exchange conditions for feed type FT-2 and FT-3 ($p<0.05$); superscripts 1,2 in the same row signify statistical differences between different months ($p<0.05$). Means ± 1 STD (n = 5) shown.

Table 4. Pigment profile of fish that were fed with different feed types and without water exchange. Fish were harvested in January 2019.

Feed type	Zeaxanthin (ng/g)	Lutein (ng/g)	β-carotene (ng/g)	Unknown I (ng/g)	Unknown II (ng/g)	Total carotenoids (ng/g)
FT-1	187^{b}±82	213^{c}±80	5^{a}±6	37^{b}±15	104^{b}±44	546^{b}±212
FT-2	96^{a}±28	128^{b}±38	3^{a}±3	20^{a}±8	40^{a}±22	287^{a}±87
FT-3	71^{a}±56	67^{a}±47	2^{a}±1	19^{a}±9	39^{a}±22	197^{a}±130

*Different superscripts in the same column signify statistical differences ($p<0.05$). Means ± 1 STD (n = 10) shown.

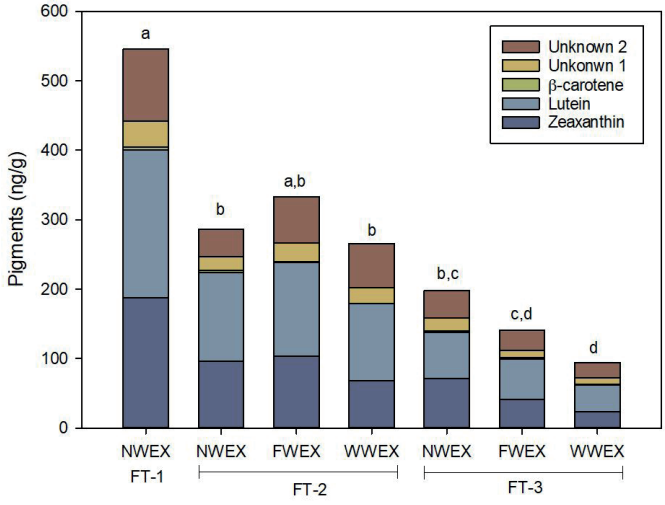

Figure 4. Carotenoids in fish fillet in different feeds (FT-1, FT-2 and FT-3) and water exchange rates (none (NWEX), fortnightly (FWEX) or weekly (WWEX) water exchange). Means of 10 fish shown. Letters above columns indicate significant differences ($p<0.05$).

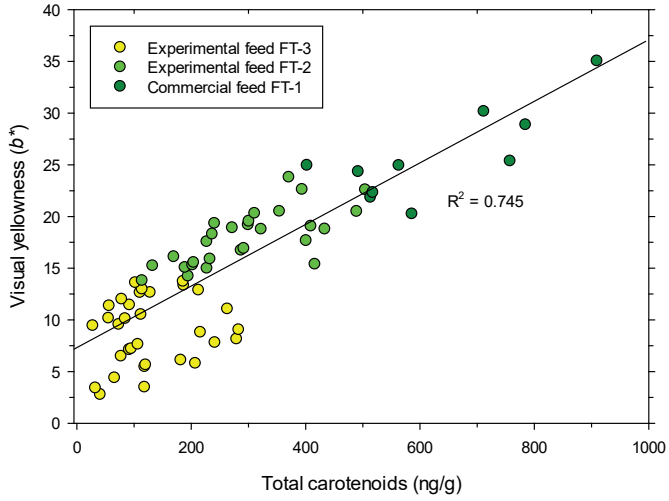

Figure 5. Relationships between visual yellow color (b^*) and total carotenoids content (ng/g) in 70 fish.

weeks, followed by 12 weeks without carotenoids in the feed, and this reduced carotenoids in the flesh from 12 µg/g to below 2 µg/g at 20°C or 30°C water temperature, while a lower reduction occurred at 10°C. The temperature effect suggests that metabolic activity of the fish promoted the release of carotenoids.

Our study indicates that the replacement of a commercial feed, containing maize and mustard oil cake, with the experimental feeds without these two ingredients, can significantly reduce the total carotenoid content and cause a lower pigment content in the flesh and a decreased yellow flesh color. Maize is often used in commercial fish feeds to reduce costs, but maize is known to introduce a general risk of yellow tainting of fish flesh (Amaya & Nickell, 2015). This was confirmed in breeding of tilapia in Chinese fish farms when using feeds rich in maize (Qiufen et al., 2012). Maize is an inexpensive ingredient that typically is mixed with mustard oil cake, fish meal, and meat and bone meal as the main ingredients in Bangladeshi fish feeds (Ali et al., 2013).

In contrast to Bangladeshi farms, maize is not used in pangasius production in Vietnam and here farm-produced pangasius is known for its white flesh color (Phan et al., 2009). In Vietnam, pangasius feed may include trash fish (usually of marine origin), fish meal powder, crushed dried fish, soybean meal, broken rice, and rice bran as the main ingredients in farm-made feed (Phan et al., 2009). The absence of maize in Vietnamese feeds and the white flesh color strongly suggest that maize is the main source of yellow tainting of pangasius flesh in Bangladesh due to the content of carotenoids, as suggested by Uddin et al. (2019). In general, it is not recommended to use feeds rich in carotenoids for catfish species, e.g., channel catfish, pangasius and African catfish, because these fish are expected to have white flesh (Amaya & Nickell, 2015).

Carotenoids in fish relative to water exchange

Water exchange also affected the carotenoid content of the pangasius fish, but a significant effect was only found for FT-3. For FT-2, the weekly and biweekly water exchange did not affect the pigment content relative to the absence of water exchange (Figure 4). When the fish were fed FT-3, the total pigment content declined in proportion to the water exchange rate, reducing the content of carotenoids in the fish from 197 ng/g (no water exchange) to 94 ng/g (weekly water exchange). Although not statistically significant, the biweekly water exchange also suggested that this reduced the carotenoid content in the fish (content of 140 ng/g).

The frequent water exchange led to a higher oxygen content in the water, relative to absence of water exchange (Table 2). We speculate if the higher oxygen level stimulated physical activity

and metabolic activity of the fish, causing a lower fat content. We did not determine the fat content of the fish, but visual inspection after slaughter showed a high accumulation of belly fat in fish which had been fed the commercial feed, as compared to fish given the experimental feeds. An increased fat content in fish given the commercial feed might also explain the higher content of carotenoid in these fish, since carotenoids are lipid soluble.

An alternative process that may have reduced the carotenoid content of the fish is a reaction with free radicals in the pond water. In waters with a high oxygen content, the level of free oxygen radicals in fish is typically also increased (Lushchak & Bagnyukova, 2006). When carotenoids react with free radicals, the pigments lose color and pigment function (Qiufen et al., 2012). In our study, when fish were fed feed FT-3 and had weekly water exchange, the highest oxygen level occurred in the ponds and coincided with the lowest content of pigments in the fish. However, more analyses are required to determine whether free oxygen radicals can cause reduction of carotenoids in pangasius.

Pigment composition of fish

Surprisingly, the proportion of the different carotenoids in the fish did not differ between feeds nor relative to the water exchange. In the analyzed fish (total of 70 fish), the proportion of zeaxanthin, lutein, β-carotene, unknown 1 and unknown 2 was on average 30.7%, 40.3%, 0.6%, 8.3% and 20.2%, respectively (Figure 4). No statistically significant differences from feed or water treatment on the composition were found. One noticeable observation, although not statistically significant, was reduction of zeaxanthin from 34% in fish fed FT-1 and without water exchange, to 25% in fish fed FT-3 with weekly water exchange.

The rather parallel variations of the five pigments suggest that changes in the matrix containing the pigments caused the different pigment contents. A changed pigment content might reflect metabolic conversion of individual pigments, as shown for astaxanthin and lutein, both of which can be converted to vitamin A in some catfish (Lee, 1987). A co-occurring removal of pigments (lutein and zeaxanthin) was also observed in catfish by Li et al. (2011) and supports that matrix containing the pigments, rather than the individual pigments, is reduced when fish lose pigments. In our study, the main matrix for the pigments was probably lipids in muscle tissues, but gonads and skin may also contain carotenoids (de Carvalho & Caramujo, 2017).

Visual yellow color vs. carotenoid content in fish

When relating the measured *b** values (indicating intensity of the yellow color) to the measured carotenoid concentrations, a strong relationship was found ($R^2 = 0.745$) (Figure 5). Thus, higher carotenoid pigment concentrations coincided with a more intense yellow color. Similar relationships between the individual carotenoids and *b** values had a weaker relationship (data not shown), suggesting that the sum of the major carotenoids gave the most precise indication of the yellow color.

In our study, temperature may also have affected the yellow color by influencing the amount of feed ingested by the fish. The lowest feeding rate occurred in the coldest month January (visual inspection showed that the fish were reluctant to eat the feed) and this probably explains the reduced yellow color observed for FT-1 (reduction in *b** from 8.09 in September to 4.69 in January). Thus, if using the commercial feed FT-1 and not applying water exchange, fish farmers should harvest the fish in January to ensure the least yellow flesh.

After slaughter, the yellow coloring of catfish flesh can also be minimized by chemical treatment, typically by dipping in sulfite solutions, as shown for channel catfish by Li et al. (2013, 2017). These authors found that the frequently observed yellow discoloration during refrigerated storage (assumed to originate from carotenoids or their degradation products) was reduced by the chemical treatment. However, from a consumer viewpoint, use of low-carotenoid feeds appears a more attractive approach than post-harvest treatment with chemicals to reduce the yellow color in fish.

Carotenoids and fish health

The absence of carotenoids in fish feed may have negative consequences to the health of the fish. Carotenoids are lipid-soluble compounds known to stimulate the immune defense and quench reactive oxygen species (Babin et al., 2015; Yujing et al., 2012). Supporting this, in yellow catfish (*Pelteobagrus fulvidraco*), supplementation of carotenoids to the feed has recently been shown to enhance the immune system by raising the lysozyme activity and the immuno-globulin level, and to reduce symptoms from high temperature stress (Liu et al., 2019). A similar immune stimulation was observed in the cold-water species rainbow trout (*Oncorhynchus mykiss*) when fed carotenoids from the marine alga *Dunaliella* (Amar et al., 2004). It remains to be determined if and how the present low-carotenoid feeds may affect fish health. During our study, no adverse effects on fish health from the absence of carotenoids were observed.

Irrespective of the positive or negative effects of carotenoids, maize and mustard cake will probably remain the preferred ingredients by the feed industry and by farmers who produce their own feed, since they are inexpensive sources of protein and carbohydrates, e.g., relative to soybean. Removal of carotenoids from maize might be a future option for feed production. Technically, carotenoids can be extracted from maize by combined supercritical fluid extraction and activated carbon treatment (Sessa et al., 2003) or by solvent extraction (Park et al., 1997). Presently, these techniques are expensive and not suited for large-scale production of maize for animal feeds.

Phytoplankton as a source of carotenoids

Pangasius is an omnivore species that may feed on algae (depending on the size of the algae) and detritus in the ponds as a supplement to added feed (The Fish Site, 2019). These additional food sources might also contribute carotenoids to farmed fish. In our study, abundance and composition of phytoplankton were monitored and showed that green algae made up the most abundant group of phytoplankton (Islam et al.; in preparation). Common pigments in green algae are lutein, β-carotene, violaxanthin, neoxanthin and zeaxanthin (Roy et al., 2011). Thus, ingestion of green algae in the ponds might have contributed carotenoids to the fish and added yellow color to the flesh.

In this study, the detection of carotenoids in pangasius that were fed the experimental feeds with a very low carotenoid content

(below 1 mg/kg) supports the view that the fish had an additional source of pigments, being phytoplankton or detritus in the ponds, but we cannot quantify this. Pigments extracted from phytoplankton are commonly used in feeds for color enrichment in fish (Shahidi et al., 1998), but it remains to be determined to which extent pigmentation of fish flesh is due to digestion of phytoplankton in aquaculture ponds.

CONCLUSION

In conclusion, this study demonstrates that pangasius with minimum yellow color can be produced in earthen ponds with natural well water in Bangladesh. The change in flesh color requires the application of low-carotenoid feeds and the introduction of regular water exchange. Replacement of maize as a common feed component by other ingredients, e.g., soybean meal, fish meal or meat and bone meal, may raise the production costs of the fish, but it may open the way for export to new markets for the benefit of the Bangladeshi aquaculture industry.

Compliance With Ethical Standard: The present study was conducted in accordance with the Ethical Standard of Research Committee of Bangladesh Agricultural Research System (BAURES), Bangladesh Agricultural University, Mymensingh.

Conflict of Interest : The authors declare that they have no conflicts of interest.

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Original Article

Determination of the Risky Microorganisms in Frozen Ready-to-Eat Seafood Sold in Istanbul Market

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ABSTRACT

Since frozen ready-to-eat seafood has the potential to cause food poisoning, this study focuses on microorganisms that have the potential to be a health hazard found in frozen ready-to-eat seafood, which is sold to highly populous communities. Therefore, the most popular frozen ready-to-eat seafood, fish balls and surimi crab legs, from Istanbul market were studied. Samples were gathered from seven different branches of four major chain market brands, twice in winter (October-March) and twice in summer (April-September). As a result of seasonal conditions summer samples had higher microbial loads compared to winter samples. The average mesophilic and psychrophilic aerobic bacteria counts of fish balls and surimi crab legs were below ≤4 log cfu/g. All samples were safe in terms of *Salmonella*, *Vibrio parahaemolyticus* and *Vibrio cholerae*; and acceptable in terms of *Staphylococcus aureus Clostridium perfringens*, and *Bacillus cereus*. The microbiological load of fish balls was higher ($p<0.05$) than the other samples. It was observed that more than 80% of them are risky in terms of coliform bacteria. It is concluded that attention should be paid especially to minced and spice added products. It is essential to pay more attention to the marketing of ready-to-eat seafood during the summer seasons for the prevention of public health.

Keywords: Pathogen, seafood, surimi crab leg, fish balls, ready to eat, frozen

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INTRODUCTION

Frozen and ready-to-eat foods are increasingly more preferred due to their fast and easy preparation, proximity to qualities of fresh produce, storability for long periods after purchase, provision of an individual, complete meal to the consumer, and widespread frozen product storage facilities (Giannakourou & Taoukis, 2005). The importance of seafood is well-known in terms of nutrition. Since many consumers do not prefer to clean and cook seafood at home, they are very important in the ready-to-eat food sector (Mol & Varlik, 2004). In addition to this, currently it is of utmost importance that the product is hygienic and safe because contamination that may occur during preparation of the product or long haul transportation and inappropriate conditions that may occur during storage or sales can cause ready-to-eat food to pose critical health risks to humans (Christison et al., 2008). In the course of transportation, storage, and sales of frozen ready-to-eat food, temperatures below -18 °C should be maintained and fluctuations in the temperature should not be allowed; otherwise, microorganisms posing health risks to humans may develop (Giannakourou & Taoukis, 2005). World Health Organization/Food and Agriculture Organization's committees of food safety experts have indicated that the most important health problem of the modern world is the diseases due to the consumption of food. Existence of risky microorganisms in ready-to-eat meals made of seafood is a great danger to public health since it may lead to mass food poisoning. However, there is very limited information on ready-to-eat seafood sold to a highly

populous community. That is why it has become necessary and essential to give useful information on bacterial load of ready-to-eat seafood sold in Istanbul where a large population inhabits.

This study aimed to determine the risky microorganisms that lead to food poisoning in widely consumed frozen seafood. Results of this study will create awareness in the seafood sector and consumers, determine the bacteriological risks of frozen ready-to-eat seafood, and reveal the potential harms to public health.

MATERIAL AND METHODS

Materials and sample collection

In this study, two of the most consumed frozen ready to eat seafood, fish-balls and surimi crab legs, were purchased from the Istanbul market. Fish-balls (n=112) are procured from seven branches of four supermarkets twice in summer (April to September) and twice in winter (October to March) periods. Sampling of surimi crab legs was conducted following the same method (n=112). Considering the possible impact of seasonal temperature changes on sales conditions, sampling was conducted both in summer and winter. Sampling locations are demonstrated in Figure 1.

Figure 1. Sampling locations in İstanbul, Turkey.

Microbiological analysis

The frozen samples were thawed at 5˚C for 12 h before analysis. Under aseptic conditions, 50g of the samples were taken and diluted in 450 ml Butterfield's phosphate buffered water for total mesophilic aerobic bacteria count, total psychrophilic bacteria count, total coliform bacteria count, *Escherichia coli* count and *Staphylococcus aureus* enumerations. For *Clostridium perfringens* and *Bacillus cereus* analysis, the sample (50 g) was diluted in 450 ml Maximum Recovery diluent and serial dilutions were prepared. After serial dilutions, samples were tested by the pour plate method (Standard Plate Count Agar, Oxoid, CM463) to determine total mesophilic aerobic and psychrophilic bacteria. For mesophilic bacteria count, incubation was at 35˚C for 24 h; for psychrophilic bacteria count, incubation time was 10 days at 7˚C (Baumgart, 1986). Total Coliform and *Escherichia coli* count were made according to Feng et al. (2020). Dilutions were plated on VRB-MUG Agar (Merck 1.04030) and typical pink colonies were counted to determine the coliform count after 18h incubation at 37˚C. Also, pink colonies were detected under UV lamp (366 nm, Merck 1.13203.0001) to define *Escherichia coli*. The colonies showing fluorescence under UV light were confirmed by indole, methyl red, Voges Proskauer and citrate tests (Feng et al., 2020). Baird Parker Agar (Himedia M043) was used for *Staphylococcus aureus* enumeration. Spread plates are incubated at 35˚C for 48 h. Typical *Staphylococcus aureus* colonies with a clear zone were confirmed using coagulase test (Tallent et al. 2016). For *Salmonella spp.* detection, 25 g of the sample was pre-enriched in Lactose Broth (Merck 1.07661) then, Rappaport–Vassiliadis Broth (Merck 1.07700) and Tetrathionate Broth (Merck 1.05285) were used for selective enrichment. Selective media used were Xylose Lysine Deoxycholate (Merck 1.05287) agar, Bismuth Sulfite Agar and Hektoen Enteric Agar (Merck 1.05418). Suspected cultures were screened by preliminary and secondary biochemical tests according to Andrews et al. (2011). The method described by Kaysner et al., (2004) was used to determine *Vibrio spp.* Each sample was examined for the presence of *Vibrio* species. After overnight incubation at 37˚C in alkaline peptone water (Oxoid CM1028), a loopful from the top culture was streaked onto Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS Agar, Merck 1.10263). Following incubation at 37˚C 24 h, the suspected colonies were purified and tested for Gram staining, catalase, oxidase, aerobic and anaerobic utilization of glucose, motility, sensitivity to 10 and 150 µg O/129, H_2S production, acid production from D-cellobiose, lactose, arabinose, D-mannose, D-mannitol and sucrose, ONPG, Voges Proskauer, arginine dihydrolase, lysine and ornithine decarboxilase, salt tolerance (0, 3, 6, 8, 10% NaCl), gelatinase, urease, indole and growth at 42˚C. For the detection of *Bacillus cereus*, Mannitol-egg Yolk-Polymyxin Agar (MYP Agar, HI-MEDIA, Kat No: M636) was used. At the end of incubation (48 hours at 28 ˚C), colonies with pink-violet centers were counted as *Bacillus cereus* (TSC Agar, MERCK, Kat No:1.11972) (Rhodehamel & Harmon, 2001a; Halkman, 2005). To determine the number of *Clostridium perfringens,* Tryptose Sulfite Cycloserine (TSC Agar, MERCK, Kat No:1.11972) Agar was used. Black colonies were detected as *Clostridium perfringens* in petri dishes, which were incubated anaerobically for 24-48 hours in TSC Agar medium (Rhodehamel & Harmon, 2001b).

Statistical analysis

Statistical evaluation (means, standard deviations, t-test) of the data obtained from microbiological analysis (in log CFU g/ml) was made using SPSS 16.0. The study was duplicated. T- test was applied to evaluate the differences between the mean results of the groups. Differences were accepted as significant when $p<0.05$.

RESULTS AND DISCUSSION

Mesophilic aerobic bacteria count is determinative for freshness of seafood. According to International Commission on Microbiological Specifications for Foods (ICMSF), the maximum total mesophilic aerobic bacteria value for breaded and precooked seafood is 7 log cfu/g (ICMSF, 1986). On the other hand, Mol et al. (2007) reported that spoilage is observed when the psychrophilic aerobic bacteria load exceeds 6 log cfu/g in fish. It is reported that high levels of total psychrophilic bacteria count indicate that proper temperature was not maintained during storage (Alvarez-Astoga et al., 2002). The above-mentioned values were not

exceeded in any of the samples in our study (Table 1). This result suggests that products did not lose their freshness; storage and sale conditions were satisfactory.

Coliform bacteria are the frequently used indicators for determining sanitation conditions (Kala, 2006). High levels of total coliform load indicate lack of hygiene and post-processing contamination (Gonzalez et al., 2003). In our study, especially fish ball samples contained a higher amount of coliform bacteria (Table 1). Spice addition and mincing was considered the cause of high levels of coliform bacteria in the fish balls. As a matter of fact, total coliform load of fish-ball mince was detected to be 3.30 log cfu/g (Suvanish et al., 2000). Since it is known that frozen storage does not have a detrimental effect on coliform bacteria load and these bacteria can grow well under low temperatures as well (Suvanish et al., 2000; Umoafia & Okoro, 2018), high levels of coliform load in our samples is of great importance.

It was observed that fish-balls have statistically significantly higher ($p<0.05$) *E. coli* load than Surimi crab legs (Table 1). On the other hand, 12% of Surimi crab legs and 39% of fish-balls exceeded the limits (2 log cfu/g) suggested by Forsythe (2010) for ready-to-eat foods (Table 2, 3). Yalçın (2020) studied the microbiological quality of surimi sold in the markets and reported *E. coli* counts between $3.2 * 10^2$ - $9 * 10^2$ cfu / g. Adebayo-Tayo et al, (2012) sampled frozen fish samples from three different markets and reported 20% of them positive for *E.coli*. As freezing does not destroy *E. coli*, it is important to make sure food is safe before freezing (Oranusi et al., 2014)

The limit value for *Staphylococcus aureus* was given to be 4 log cfu/g (Anon, 2011; Forsythe, 2010; ICMSF, 1986) (Table 3). None of the frozen ready-to-eat seafood samples exceeded these limits in this study. High levels of *Staphylococcus aureus* in the food indicates poor hygienic conditions in the preparation of the product, especially due to personnel (Karaboz & Dinçer, 2002). Our findings demonstrated that conditions in the preparation of these products were generally appropriate.

Clostridium perfringens and *Bacillus cereus* are spore-forming pathogens and it is known that they can be easily isolated from spices as well (Iurlina et al., 2006). As a matter of fact, we ob-

Table 1. Minimum, Maximum and Average bacterial load results (log cfu/g) of the frozen fish balls and surimi crab leg in different seasons.

		Surimi crab legs			Fish-balls		
		Min	**Max**	**Average**	**Min**	**Max**	**Average**
Total mesophilic bacteria	Winter	2.26	4.26	2.94 ± 0.42^{x}	2.49	5.33	3.47 ± 0.56^{x}
	Summer	2.36	5.4	3.62 ± 0.65^{y}	2.42	5.59	4.53 ± 0.76^{y}
	All samples			**3.28 ± 0.64^{a}**			**4.00 ± 0.85^{b}**
Total Psychrophilic Bacteria	Winter	1.93	4.13	2.85 ± 0.37^{x}	2.54	4.48	3.39 ± 0.54^{x}
	Summer	2.45	4.98	3.62 ± 0.58^{y}	2.73	5.39	4.40 ± 0.64^{y}
	All samples			**3.23 ± 0.62^{a}**			**$3.90\pm0,78^{b}$**
Total Coliform	Winter	0	3.73	1.99 ± 1.23^{x}	0	4.27	2.40 ± 1.33^{x}
	Summer	0	3.45	2.58 ± 1.04^{y}	0	3.79	3.11 ± 0.80^{y}
	All samples			**2.28 ± 1.17^{a}**			**2.75 ± 1.15^{b}**
Escherichia coli	Winter	0	2.7	0.09 ± 0.50^{x}	0	3.32	0.43 ± 0.95^{x}
	Summer	0	2.93	0.54 ± 1.01^{y}	0	3.27	1.63 ± 1.23^{y}
	All samples			**0.31 ± 0.82^{a}**			**1.03 ± 1.25^{b}**
Staphylococcus aureus	Winter	0	3.46	1.77 ± 0.85^{x}	1.18	3.14	2.12 ± 0.54^{x}
	Summer	0	3.54	2.38 ± 0.52^{y}	1.88	3.3	2.68 ± 0.47^{y}
	All samples			**2.08 ± 0.77^{a}**			**2.40 ± 0.57^{b}**
Bacillus cereus	Winter	0	2.3	0.17 ± 0.56^{x}	0	3.44	1.16 ± 1.30^{x}
	Summer	0	3.27	1.46 ± 1.23^{y}	0	3.44	2.33 ± 1.17^{y}
	All samples			**0.81 ± 1.15^{a}**			**1.74 ± 1.36^{b}**
Clostridium perfringens	Winter	0	3.51	0.14 ± 0.30^{x}	0	2.48	0.41 ± 0.86^{x}
	Summer	0	3.27	0.81 ± 1.06^{y}	0	3.58	2.04 ± 1.32^{y}
	All samples			**0.48 ± 0.93^{a}**			**1.23 ± 1.38^{b}**

x, y: the difference between lines; a, b: the difference between columns ($p<0.05$).

Table 2. Exceeding the limit percentages of samples and number of samples exceeded limit/total number of samples.

	Surimi Crab leg		Fish Ball	
	E. coli	***S. aureus***	***E. coli***	***S. aureus***
Anon 2011		0%		0%
ICMSF 1986	4% (4/112)	0%	16% (18/112)	6% (7/112)
Forsythe 2010	12% (10/112)	0%	39% (44/112)	0%

Table 3. Microbiological Limits (log cfu/g).

	E. Coli	***S. aereus***	***B. cereus***	***C. perfringens***
Anon 2011	No limit	4	4	No limit
ICMSF 1986	2.69	4	No limit	No limit
Forsythe 2010	2	4	4	5

served that both bacteria species were present at significantly higher levels ($p<0.05$) in the fish-balls which contained spices (Table 1). *C. perfringens* can be found in raw ingredients like spices used in food processing. *C. perfringens* outbreaks usually occur from improper handling, such as insufficient cooling at the home, retail, or during food service (Juneja et al., 2009). However, there are no official criteria established by the European Commission (EC) for *Bacillus cereus* and *Clostridium perfringens*. But according to Anon (2011) the *Bacillus cereus* limit is 4 log cfu/g. Also Forsythe, (2010) reported 5 log cfu/g and 4 log cfu/g as inappropriate values for *Bacillus cereus* and *Clostridium perfringens*, respectively. In our study, these bacteria were higher in the summer period ($p<0.05$).

Food codex urges that 25 g food should not have any *Salmonella* spp. and in the case of their presence these products should not be offered for human consumption (ICMSF, 1988), because *Salmonella* spp. is the most widely spread bacteria that causes food poisoning in the world (Gonowiak, 1990). Similarly, *Vibrio parahaemolyticus* and *Vibrio cholerae* should not be present in the 25 g of seafood (ICMSF, 1988). In our study, *Salmonella spp., Vibrio parahaemolyticus* and *Vibrio cholerae* were not detected in the Surimi crab legs and fish-ball samples.

As a result of our study, we observed that fish-balls have statistically significantly higher microbial load than Surimi crab legs ($p<0.05$). The mincing operation in the preparation of fish-balls leads to extension of the surface and microorganisms spread all over this surface (Vural & Yesilmen, 2003). Moreover, it is known that spices are contamination sources (Little et al., 2003) and it is concluded that spices present in fish-balls are effective at producing this result. As a matter of fact, an increase in the bacterial load of fish was reported after mixing with spices.

It was observed that there are differences between samples of both Surimi crab legs and fish-balls taken in summer and winter in terms of all bacteria ($p<0.05$) and bacterial load of samples taken in summer were always higher (Table 1). It was also reported as a result of a study conducted on shrimps stored at different temperatures that increasing temperature led to increased microbial activity and quality of the food degraded rapidly (Umoh & Odoba, 1999). Likewise, the microbial load of foods is estimated to be higher in summer by Vural & Yesilmen (2003).

CONCLUSION

It was concluded in our study that samples of Surimi crab legs and fish-balls were marketed without losing their freshness and they were safe in terms of *Salmonella* spp., *Vibrio parahaemolyticus*, and *Vibrio cholerae*, and acceptable in terms of *Staphylococcus aureus Clostridium perfringens* and *Bacillus cereus* which are generally observed due to inappropriate processing conditions. In general, fish-balls have a higher microbiological load. It was also detected that bacterial load of samples taken in summer time is always higher ($p<0.05$). The fact that microbial load of fish-balls is higher than surimi crab legs indicates that this product can pose hazard on public health if the conditions of storage and sale are inappropriate. The minimum and maximum values recorded for each parameter demonstrated variability within samples. This variation can be defined by the fact that the samples were collected from various sellers where the selling conditions are very important. According to the findings of our study, careful action should be taken in terms of working conditions and raw material procurement in the production of especially minced food with spice addition; special attention should be paid to the sales of ready-to-eat frozen seafood in terms of public health especially in summer.

Conflict of Interests: The authors have no conflicts of interest to declare.

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Original Article

Effect of Different Shaking Systems on the Growth of Marine Diatom *Phaeodactylum Tricornutum*

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ABSTRACT

In aquatic ecosystems, the fact of encountering fluctuations is vital for the survival of phytoplankton, in terms of remaining in the euphotic zone and reaching the necessary nutrients for their growth. The existence and the abundance of the phytoplankton are also vital for the other living things in indirect or direct ways, due to being the fundamental components of the food chain and webs, in addition to their usage in several industries like fuel, pharmacy, or cosmetics. However, particularly for the energy industry, the production cost of biofuels by using phytoplankton is relatively higher than the cost of conventional fossil fuels. Thus, the need of increasing the phytoplankton biomass in artificial environments has emerged to reduce the biofuel production cost. For this purpose, the correlation of turbulence and growth rate has been investigated through various experimental studies. In addition to the previous studies, this study focuses on the turbulence effects at a small scale in respect of the movement directions. Fixed, axial, and orbital movements were performed and quantified in terms of the specific growth rate, doubling time and the productivity of biomass for each system. The frequencies of the axial and orbital systems were set to 40 and 80 rpm, respectively and the specific growth rates were quantified as 0.38, 0.43 and 0.42 μ day^{-1} whereas the doubling times were calculated as 1.84, 1.62 and 1.63 day. In conclusion, it was observed that the frequency of the movement is more influential rather than the type of the movement.

Keywords: Marine diatom, *Phaeodactylum tricornutum*, shaking conditions, growth rate, algae cultivation

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INTRODUCTION

Both fresh and marine aquatic ecosystems are sustained by the phytoplankton directly or indirectly due to its property of being the main element of the food chain and food web in these environments (Marra 1980). It is stated that almost half of the global primary productivity depends on the phytoplankton both in the areas of coastal and open ocean (Friend et al. 2009). In a shallow marine environment like coastal zones, the presence and the abundance of the phytoplankton are highly affected by the wind mixing; wind speed, and the prevailing wind direction (de Souza Cardoso and da Motta Marques 2009; Moreno-Ostos et al. 2009; Zhou et al. 2015). In the ocean, the interaction of the water surface with the ambient air temperature and the surface winds generate a mixing environment and turbulence that affects the physical parameters and the nutrient supply of the marine environment and consequently change the environmental conditions for the residing phytoplankton (Hemer et al. 2013a, b; Fan et al. 2013; Rumyantseva et al. 2015; Burns 2017). As seen, fortuitously oscillations of velocity in water flow, namely turbulence, can exist in all environments due to winds, tidal currents, or breaking winds with a broad range of turbulence dissipation rate changing from 10^{-2} to 10^{-10} m^2s^{-3}. The smallest values of this range were stated as

the result of the stratification in the water column in the oceans formed due to the weak near-surface wind mixing (Gargett 1989, 1997; MacKenzie et al. 1994; Johnson et al. 1994; Terray et al. 1996). Small-scale turbulence is mostly seen as the driving force of plankton trophodynamics (Saiz et al. 1992; MacKenzie et al. 1994) and interaction between the organisms and the surrounding particles (Rothschild and Osborn 1988). This condition is also pointed out with the different impacts of the large- and small-scale turbulent processes on the phytoplankton, individually or collectively. It was explained that individual physiology and population growth are directly altered by the large-scale turbulent processes, due to its impact on the primary physical factors like light, temperature, and nutrient availability, whereas only individuals are affected by the small-scale turbulent processes due to its scale of millimeters in terms of shear and strain forces (Sullivan and Swift 2003). The availability of nutrients directly affects the trophic state and community structure of the phytoplankton (Hecky and Kilham 1988). With the increase of turbulence, the diffusion boundary layer surrounding the phytoplankton cells decrease and a nutrient concentration boundary layer forms around the cells due to the rate difference of the absorption and diffusion processes which supports the uptake of the nutrients in the surrounding water and the removal of the photosynthetic wastes (Koch 1971; Jumars 1993; KarpBoss, L; Boss, E; Jumars 1996; Köhler 1997a; Barton et al. 2014). Moreover, the positive correlation of the small-scale turbulence and the growth rate has been observed in several experimental studies (Thomas and Gibson 1990; Beauvais et al. 2006; Mari and Robert 2008; Hondzo and Wüest 2009; Rokkan Iversen et al. 2010), specifically below the Kolmogorov length scale which states the length of the smallest turbulent eddies (Kolmogorov 1962), and in the estuarine incubation due to the sedimentation (Burns 2017).

Phytoplankton is one of the main players of global CO_2 budget and aquatic ecosystems due to its capability of fixing around half of the global biogenic carbon (Field, C. B.; Behrenfeld, M. J.; Randerson 1998), its role in the mechanism of the biological pump (DeVries et al. 2012) and being an essential carbon source to aquatic food cycles (Köhler et al. 2018). Besides, phytoplankton has many uses in today's world in the field of nutritional source, prevention of environmental pollution, and fuel industry, in addition to their usage in cosmetics, pharmaceuticals, and aquaculture (Schenk et al. 2008; Mata et al. 2010; Makareviciene et al. 2011, 2013). Phytoplankton can be considered as an alternative source to renewable fuels due to its production potential as methane (Spolaore et al. 2006), electricity (Becker 1994), hydrogen (Ghirardi, M.L.; Zhang, J.P.; Lee, J.W.; Flynn, T.; Seibert, M.; Greenbaum, E.; Melis 2000) and biodiesel (Chisti 2007; Patil et al. 2008). Thus, increasing the biomass of the phytoplankton has been a current interest particularly for biodiesel production because the production cost is still much higher than the production of fossil fuels. Even though biodiesel from microalgae is much more environmentally friendly and contributes to the solution for global warming, the production cost is the barrier to overcome. The solution can be the increase of the biomass production, the increase of the microalgae lipid content under stress conditions, the combination of the water treatment process with the biofuel production or the increased number of the end products while treating microalgae. For the optimization of the phytoplankton biomass increase in artificial environments, there are several factors to adjust such as light, temperature, pH, dissolved oxygen, dissolved CO_2, photosynthesis rate, nutrient and turbulence as a result of mixing (Fernández et al. 2012; Lucker et al. 2014; Shriwastav et al. 2017). Productivity of the phytoplankton and its relation with the ambient turbulence intensity in natural waters has been shown in many studies (Hondzo and Lyn 1999; Zhong 2004; Hondzo and Al-Homoud 2007; Wang et al. 2016). It has been also stated that mixing has many impacts on the production of phytoplankton such as transporting them by advection between the light and dark conditions, intensifying the air-water interface interaction in terms of dissolved oxygen and carbon, enhancing the nutrient transfer as a result of decrement on the boundary layers around the cells and providing them to stay in the water column as suspended material (Marshall and Huang 2010).

Taking into account the turbulent pulsation for the phytoplankton productivity, it is stated that moderate turbulent pulsation with frequencies of 0.5 and 1.0 Hz displays better results in terms of biomass quantity (San et al. 2017). On the contrary, the same study expresses that strong turbulent pulsation with a frequency of 1.5 Hz or more, conduce to lower biomass than stationary conditions by damaging the cells and reducing the growth rate of the phytoplankton. It is also stated that immoderate turbulence causes phytoplankton cell damages (Thomas and Gibson 1990; Bałdyga and Pohorecki 1998) and consequently a decrement in the growth rate (Marshall and Huang 2010). The quantification of the growth rate is a required step of monitoring the increase of phytoplankton cultivation and, counting the cells, measuring the optical density and determination of chemicals can be given as examples of the quantitative methods (Caspers 1970; Schwartz 1975; Hallegraeff 1977). Altering in the number of cells (Rhee and Gotham 1981; Kagami and Urabe 2001) or chlorophyll pigment (Bienfang and Takahashi 1983; Landry et al. 1995; Calbet and Landry 2004) has been considered as the indicator of the growth rate.

Cultivation of the phytoplankton is mostly performed in batch cultures which can be defined as enclosed circuits with finite volume and nutrient sources that will eventually encounter the diverse limiting factors (Barsanti and Gualtieri 2014). Thus, cultivation of the phytoplankton needs mixing to provide a homogeneous medium in terms of nutrient, light, temperature, and gas exchange distribution and to prevent the sedimentation of the organisms. It is remarked that the growth of the phytoplankton is affected by the parameters such as mixing, light intensity, and CO_2 concentration (Sforza et al. 2012), and the biomass production of the phytoplankton increases with efficient mixing due to the frequent exposure of the cells with essential nutrients and necessary physical parameters like light (Kunjapur and Eldridge 2010). A way to provide a small-scale mixing medium is continuous shaking the cultivation flasks (Barsanti and Gualtieri 2014). Thus, shaking the flasks also provides aeration which potentially increases the growth rate of phytoplankton due to the efficient uptake of nutrients and proper illumination medium following the increase of the photosynthesis process (Zhao et al. 2011). Diverse methods are used for the cultivation of phytoplankton in

flasks, for example, manual mixing once or twice a day (Makareviciene et al. 2011), mixing with the help of a shaker (Han et al. 2012) or through aeration (Barsanti and Gualtieri 2014). The positive effect of aeration on the growth rate of phytoplankton is shown through the experimental studies consisting of three different treatments; aeration, manual shake, and no shake (Harun et al. 2018). It is also shown through the experimental studies that shaking and aeration are efficient parameters of the phytoplankton cultivation in order to increase biomass productivity and concentration due to their impacts on the light intensity and CO_2 concentration (Vanags et al. 2015). Another experimental study which compares the effects of still and turbulent conditions has concluded that the growth rates of the phytoplankton are higher in turbulent waters than in still waters, independently of the phytoplankton size. In the same experimental study, an orbital shaker table oscillating with a velocity of 130 rpm has been used to form turbulent conditions. As a result, they add that the reason of this fact is due to the slowness of the nutrient flux towards the cells whereas the flux increases under turbulent conditions, and consequently increases the opportunity of absorbing nutrients and the growth rate of the phytoplankton cells (Peters et al. 2006). In the light of these information, this study is aimed to investigate the effect of three different shaking conditions on the growth rate of a marine diatom, *P. tricornutum*.

MATERIALS AND METHODS

Algae cultivation

Seawater from the upper layer of Tarabya coast in the Istanbul Strait with approximately 17-18 psu was collected for algae medium and filtered with active carbon and filters of different pore sizes Whatman™ GF6 filter and 0.45 um pore sized Sartorius cellulose nitrate filter, respectively. Totally 12 flasks, with four parallels for each system, were filled with 400 mL modified f/2 nutrient solution, the conventional nutrient mixture of diatoms, which is given in Table 1 (Guillard and Ryther 1962; Hao et al. 2020). The temperature of the incubation environment was set about 21±1°C in a temperature-controlled room under a continuous light intensity of about 3150-3200 lux. Prior to the experiment, the diatom species *P. tircornutum* of approximately 10 µm length, has been kept in the same temperature-controlled room under continuous light intensity, and the cultivation was performed using the batch of the species in exponential growth phase.

Table 1. Modified f/2 medium for microalgae cultivation.

Chemical	Concentration (mg L^{-1} seawater)
$NaNO_3$	75
$NaH_2PO_4.H_2O$	5
$Na_2SiO_3.5H_2O$	12.9
$CuSO_4.5H_2O$	0.005
$ZnSO_4.7H_2O$	0.011
$CoCl_2.6H_2O$	0.005
$MnCl_2.4H_2O$	0.090
$FeCl_3.6H_2O$	0.909

Experimental setup

The experiment was set on three different conditions: Manual shaking, once vigorous shaking by hand every day, and constant automatic shaking with axial, 80 rpm, and orbital, 40 rpm, movements. The frequencies of the systems were selected as stated because the movements of the systems were almost identical with these frequencies. Orbital and axial movements were provided by ISOLAB 3D Shaker and IKA HS 260 Horizontal Shaker, respectively.

Analyzing data

During the experiment, cell numbers were quantified by means of Beckman Coulter Z2 Coulter® Particle Count and Size Analyzer, and the fluorescence intensity of the cells were measured by using PerkinElmer LS- 55 fluorescence spectrophotometer. Cell numbers were utilized to measure the rate of the diatom growth and the productivity of the diatom biomass over time and chlorophyll-a measurements with fluorescence spectrophotometer were used to control the correction of the cell number measurements. The wavelengths of excitation and emission were adjusted as 430 nm and 663 nm respectively for chlorophyll-a pigment (Yentsch and Menzel 1963). The measurements were performed for 26 days. All measurements that are given as results are the averages of 4 parallels mentioned in the methods section.

All the graphs were plotted and the statistical analysis was performed with SigmaPlot. In addition to the graphical representation of the growth, numerical calculations were performed to quantify the specific growth rate, doubling time and biomass productivity of each system by using the data of the log phase in the equations of 1, 2, and 3, respectively with the representation of cell density and time by N and t (Issarapayup et al. 2009; Wang et al. 2010; Asmare et al. 2013; Zhu et al. 2013; Komolafe et al. 2014; Harun et al. 2018).

$$\text{Specific growth rate (u day}^{-1}) = \frac{ln\left(N_2/N_1\right)}{t_2 - t_1} \quad (1)$$

$$\text{Doubling time (day)} = \frac{\ln 2}{specific\ growth\ rate} \quad (2)$$

$$\text{Biomass productivity} = \frac{N_2 - N_1}{t_2 - t_1} \quad (3)$$

RESULTS AND DISCUSSION

First of all, the obtained results will be given for each type of movement separately, then they will be compared with each other in the context of the growth rate of the selected diatom. For each type of movement, the change of cell numbers and fluorescence intensity over time were counted and measured during the whole experiment period. Fluorescence intensity is considered as the indicator of vitality due to its decreasing intensity level in senescent cells (Gielen et al., 2007; Subhash, Wenzel, & Lichtenthaler, 1999). On the other hand, any discrimination like that cannot be achieved by counting the cell numbers with the instrument used in the experiment. That could be the reason for seeing possible correlation differences of the two measuring methods towards the end of the experiment.

The first type of movement which is still and has no constant velocity or motion was chosen as the control system of the whole experiment. The increase in the cell numbers and the fluorescence intensity during the experiment period is given in Figure 1. It is seen that the diatom culture is in the lag phase of its growth in the time of the first two days of the experiment. This lag phase is followed by the log phase which represents the exponential increase of the cell numbers. The log phase continues about a week, and then the rate of the growth lessens its effect, meaning that the log phase is followed by a declining growth rate for a few days. About 12 days later, the stationary phase is reached. Correlatively, the change in the fluorescence intensity during the experiment period follows the same trend of the cell numbers. The declining trend observed during the last days of the experiment is probably because of the death of the cells, therefore it can be said that after about 22 days, the death phase arises. This trendline difference in the cell number and the fluorescence intensity is possibly due to the difference in the two measurement methods as mentioned before.

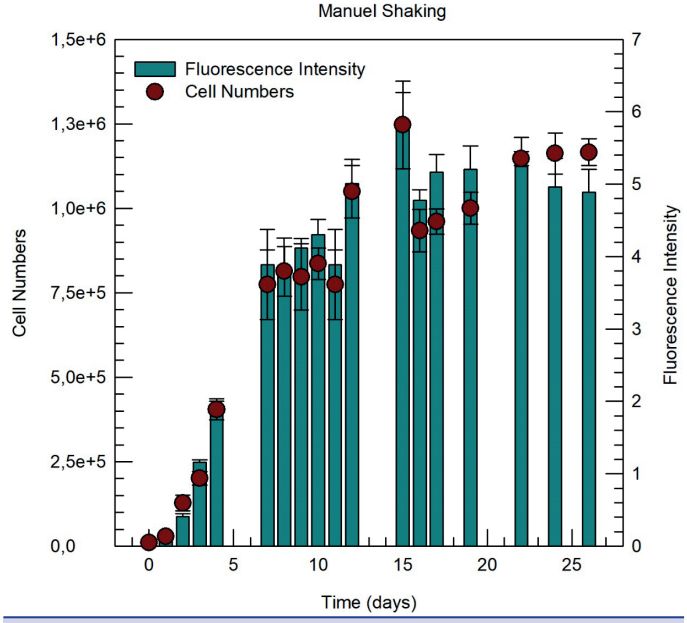

Figure 1. The growth trend of *Phaeodactylum tricornutum* with manual shaking during a 26-day experiment period, by means of the average cell number and fluorescence intensity of 4 parallels.

The second type of movement was axial shaking with a frequency of 80 rpm. Similar to the findings shown in Figure 1, the increase of the cell numbers and the fluorescence intensity in the axial shaking system is given in Figure 2. The first two days show a similar pattern with the control system and are considered as the lag phase, which is followed by the log phase until the 9th day. Then the growth slope declines gradually and a stationary phase is reached on the 17th day.

The third type of movement was 3D orbital shaking with a frequency of 40 rpm. Although it seems that there is a numerical difference in the shaking frequency of the axial and orbital shakers,

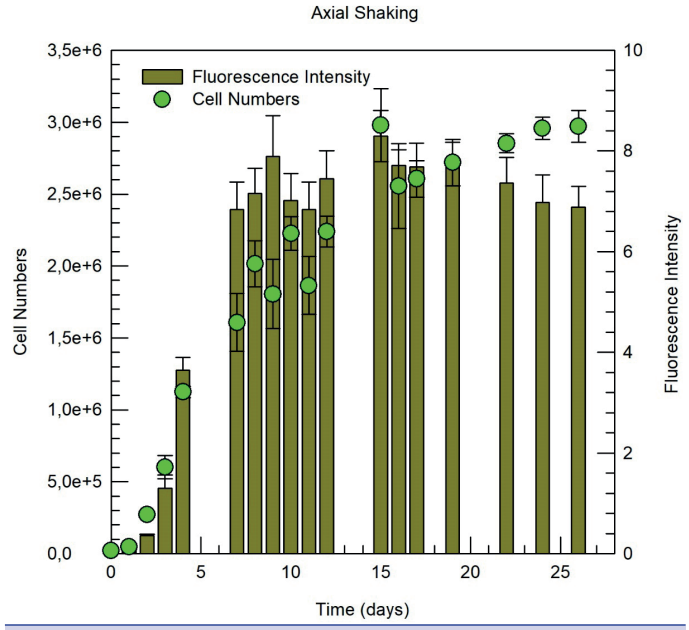

Figure 2. The growth trend of *Phaeodactylum tricornutum* in an axial shaking environment with a frequency of 80 rpm during a 26-day experiment period, by means of the average cell number and fluorescence intensity of 4 parallels.

these frequencies were chosen to equalize the shaking impact on the flasks as mentioned before. The variation of the cell numbers and the fluorescence intensity in the orbital shaking system is plotted with respect to the experiment duration and is shown

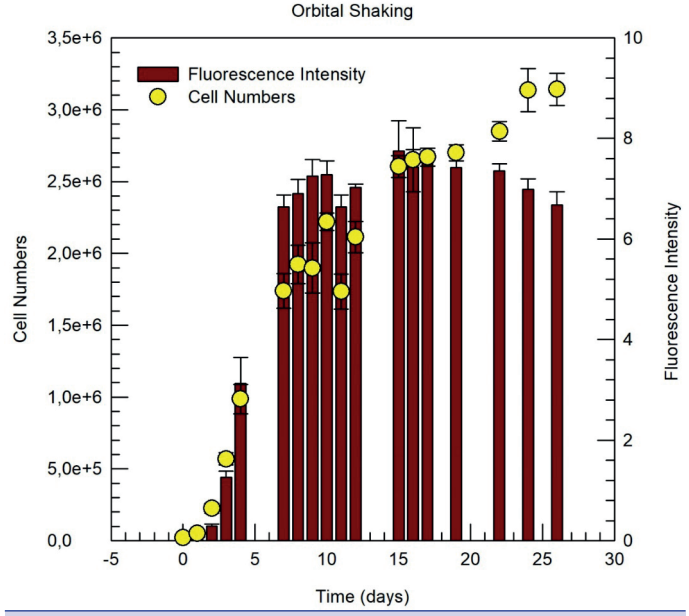

Figure 3. The growth trend of *Phaeodactylum tricornutum* in an orbital shaking environment with a frequency of 40 rpm during a 26-day experiment period, by means of the average cell number and fluorescence intensity of 4 parallels.

in Figure 3. Again, the first two days show a similar pattern with the control system and are named as the lag phase. Then it is followed by the log phase until the 12th day. Later, a gradual decline is observed until the 16th day, and then the stationary phase is reached.

After separate observations of each type of movement and their impact on cell growth, an overview of the systems in terms of cell number and fluorescence intensity will be useful. Therefore, a comparison of the observations is seen in Figure 4. For all of the systems, the lag phase seems identical for the first two days. After the lag phase, a log phase with almost the same increasing slope is seen for the first 10 days of the experiment duration. Later, the separation of the systems emerges, this case is obvious especially for the still system. Although all the systems show a similar pattern, the cell numbers in the still system reach the stationary phase at a lower level. This separation and level difference are also apparent in fluorescence intensity measurements shown in Figure 4(b), in addition to the early separation of the still system seen in the log phase.

The positive correlation between the cell number and the fluorescence intensity, which can also be named as optical density, has been previously stated in several studies for decades (Toennies and Gallant 1949; Butterwick et al. 1982; Sandnes et al. 2006; Myers et al. 2013; Sivakumar, R; Rajendran 2013), in addition to the usage of optical density as an indirect measure of the growth (Harun et al. 2018). Thus, the parallelism in the trends of the two measuring methods is normally expected in the present study.

Specific growth rate, doubling time, and biomass productivity of each system were calculated from the data of cell numbers and given in Table 2. The inverse ratio of the specific growth rate and the doubling time is apparent due to the nature of their formulizations as given in Eq. 1 and 2. Rapidly growing cells represent a lower doubling time and vice versa. The calculations of Eq. 1, 2, and 3 were performed taking into account the interval between the first and the 10th days. The reason for choosing this period is its correspondence to the log phase for each type of movement.

The results point out the effectiveness of shaking, independent of its type, whether the movement is in the axial or orbital direction. Previous studies also emphasize the difference of shaking type by quantifying the rate of the phytoplankton growth as 0.26 and 0.23 μ day^{-1} for the manual and no shake treatments, respectively (Harun et al. 2018). Except for discrete manual movement, the growth rates of the cells subjected to continuous vertical

Table 2. The rate of the specific growth (μ day^{-1}), doubling time (day) and the productivity of the biomass of still, axial, and orbital type of movement (average of 4 parallels).

	Specific growth rate (μ day^{-1})	Doubling time (day)	Biomass productivity (cell day^{-1})
Fixed	0.38	1.84	184557
Axial	0.43	1.62	249024
Orbital	0.42	1.63	248092

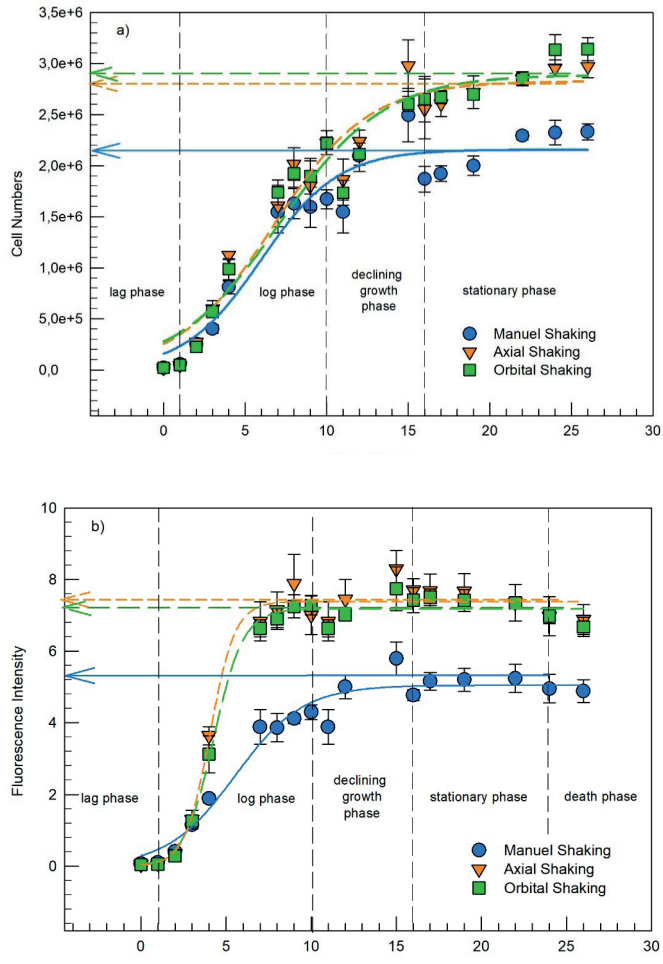

Figure 4. The trend of *Phaeodactylum tricornutum* growth in three types of movement; still, axial and orbital shaking from the perspective of a) cell number b) fluorescence intensity (average of 4 parallels).

movements with the frequencies of 2, 1.5, and 1 Hz, corresponding to 120, 90, and 60 rpm, the growth rates were observed to be varying according to the species. In detail, the findings can be summarized as shown in Table 3 (Burns 2017).

Although the species used in the present study is a marine diatom, it is worth mentioning a previous study that is about the effects of small-scale turbulence on the growth rates of ten different flagellates. The experimental observations of the study emphasize that the degree of the turbulence can show insignificant change, a reduction, or an increase in the net growth rate. The authors conclude that this variation of the effect of turbulence in the growth rate highly depends on the physical parameters such as the degree of light, nutrients, or temperature in addition to the phytoplankton species (Sullivan and Swift 2003). In addition, a mean growth rate of 0.42 day^{-1} was measured in a field study performed in the adjacent mainstream of River Severn in the UK for a composition of chlorophytes, centric diatoms, pennate diatoms, and cryptophytes (Köhler 1997b)therefore natural mixing conditions should be simulated as closely as possible during the

Table 3. The growth rates of phytoplankton species changing with different frequencies (Burns, 2017).

Species	Cell length (µm)	Growth rate (day-1)	Frequency (Hz)
Thalassiosira	18–20	0.38	2
Thalassiosira	18–20	0.41	1.5
Thalassiosira	18–20	0.42	1
Chaetocero	14–16	0.97	2
Chaetocero	14–16	0.94	1
Thalassiosira pseudonana	6–8	1.02	2
Thalassiosira pseudonana	6–8	1.02	1
Thalassiosira pseudonana	6–8	0.99	0.5
Phaeocystis globosa	4–7	0.79	2
Phaeocystis globosa	4–7	0.8	1
Phaeocystis globosa	4–7	0.75	0.5

incubation. A new device is described here which combines the advantages of a dialysis chamber with a programmable vertical mixing regime. Realistic phytoplankton growth rates can thus be measured in situ under conditions of vertical mixing and small-scale turbulence. The chamber made of transparent, UV-transmitting acrylic glass was fitted at both ends with permeable polycarbonate membranes. It was moved vertically through the water column by a pocket-sized lift and rotated simulataneously on its central axis. The method was applied to two types of experiments on growth and losses of phytoplankton in the River Severn, UK. The first one compared changes in biovolume of phytoplankton in a water parcel flowing downstream (6% h^{-1} decline). Taking into consideration all these findings, a remarkable point of the present study can be put forward as the frequency of the movement is more influential on the growth characteristics rather than the type of movement. Thus, the observed data of the axial and orbital types of movements that are close to each other seems reasonable.

CONCLUSION

An experimental study with a duration of 26 days was performed to observe the outcomes of the turbulence on a small scale in the context of the growth process of *P. tricornutum*. Three separate movement systems were set in a temperature-controlled and well-lighted room for the incubation of the selected diatom phytoplankton species. These were fixed, axial, and orbital shaking systems with four replicates of each. Two measurement methods were utilized to follow the growth trend of the species; cell counting and optical density (fluorescence intensity). The fixed system with no constant motion gave rise to lower growth rates compared with the other dynamic systems, as expected. The frequency of the axial and orbital systems was set almost at the same rate by checking visually, and the growth rate of the axial and orbital shaking systems was observed similarly. Therefore, it is concluded that the frequency of the turbulence on a small scale is more impactful on the rate of the phytoplankton growth rather than the direction of the turbulence source. Optimization of the biomass production of the microalgae is a highly focused topic because replacement of the fossil fuels by biodiesel from microalgae is still not feasible because of the high production cost. Presented study and further studies may contribute to the advancement in this field to increase biomass production while reducing production costs.

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Data Set Statement: The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

Conflict of interests: The authors declare that they have no conflict of interest.

Ethics committee approval: -

Funding: -

Acknowledgements: -

Disclosure: -

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Short Communication

The First Record of *Geryonia proboscidalis* (Forskål, 1775), (Cnidaria: Hydrozoa) on the Coasts of Gökçeada, the Aegean Sea, Turkey

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ABSTRACT

The present study represents the first record of Hydrozoan medusae *Geryonia proboscidalis* off of Gökçeada island in June 2021. Although this species is found in the Mediterranean, it is the first record specifically in the coastal areas of Gökçeada island. Holidaymakers are extremely irritated by the sting of *Geryonia proboscidalis*, especially with its sudden accumulation in the coastal areas of Gökçeada island.

Keywords: *Geryonia proboscidalis*, Hydrozoan, Gökçeada, Aegean Sea, holidaymaker

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INTRODUCTION

The island of Gökçeada, with a coastline of 92 km and a surface area of 279 km^2, is the largest island of Turkey and is located in the northeastern Aegean Sea. Especially in summer, the northeast and southwest winds are dominant (Kocataş & Bilecik, 1992). Also, due to the presence of many nearby islands, water movements in the northern Aegean Sea around Gökçeada show great variation. For example, the Black Sea waters coming from the Çanakkale Strait are directed north in winter and south in summer (Kocataş & Bilecik, 1992). The water body surrounding the island is enriched with nutrients from the Meriç River (Ulutürk, 1987) and is affected by the colder and less salty water from the Marmara Sea (Kocataş & Bilecik, 1992).

The class Hydrozoa is a diverse taxon with a wide variety of prey preferences and with more than 3800 species, the majority of which are pelagic and benthic predators (Bouillon, Gravili, Pagès, Gili, & Boero, 2006). Hence, they are an important component of marine ecosystems, and their bloom may cause significant effects. Regular monitoring may help awareness of new species introductions and/or unpredicted blooms over time (e.g., İşinibilir, Ulucam, & Yüksel, 2019; Marambio et al., 2021). The jellyfish *Geryonia proboscidalis* (Forskål, 1775) is a holoplanktonic species belonging to the order Trachymedusae. *G. proboscidalis* is distributed in the Atlantic, Indo-Pacific, and Mediterranean and prefers warm waters, living in the surface areas of tropical and subtropical seas (Vanucci, 1957). *G. proboscidalis* has previously been recorded in Boncuk Bay, (Aegean Sea), in Iskenderun Bay (Mediterrenean Sea) and in Sığacık Bay (Aegean Sea) respectively on the coasts of Turkey (Gülşahin, Tarkan, & Bilge, 2013; Ergüden, Turan, Çevik, & Uygur, 2014; Akçınar, 2017). The present study indicates that the distribution of *G. proboscidalis* is spreading northerly. In this study, we report on an exceptionally high density of *G. proboscidalis* off the coasts of Gökçeada, in the north Aegean Sea. The study aimed to report the first record of *G. proboscidalis,* with the associated impact on holidaymakers on the Gökçeada island.

MATERIALS AND METHODS

Geryonia proboscidalis (Forskål, 1775) were observed and sampled on 24th, 26th and 29th June 2021 in different areas (Yıldız Bay (Yıldızkoy),

Kaleköy, Kuzulimanı, Mavi Bay (Mavikoy) and Balık Çiftliği respectively) in the northeastern part of the island (Figure 1) while snorkeling and then they were preserved in formaldehyde for identification in the laboratory. The jellyfish were identified referencing the proper literature (Bouillon et al., 2004). Physico-chemical parameters were measured with a multi-probe system in surface water.

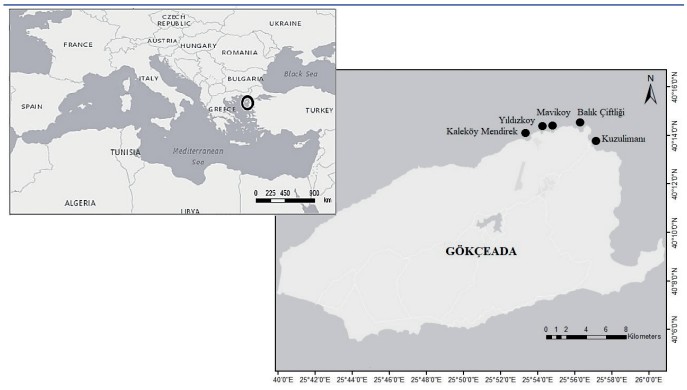

Figure 1. The sampling and observation locations of *Geryonia proboscidalis* in Gökçeada island.

RESULTS AND DISCUSSION

The presence of this jellyfish was first discovered in Yıldız Bay during the afternoon of the 24th of June 2021 across the island thanks to the complaints of holidaymakers. They stated that they had swum in Yıldız Bay on the morning of the same day, but they had not experienced any burning or itching. The first theory was that it was caused by dense mucilage aggregation, which was observed in the Marmara Sea in January 2021 and on the coast of Gökçeada island in May 2021. However, when the water samples were taken and examined in the laboratories of Gökçeada Marine Research Unit, it was identified as trachymedusae *Geryonia proboscidalis*. In the following days, similar complaints came from holidaymakers who went in the sea on the beaches of Kuzulimanı and Kaleköy. Samples were also taken from these regions and underwater photography was taken of the jellyfish accumulation (Figure 2B). Approximately 30 people were hospitalized in Gökçeada island with complaints of redness, itching, and burning all over the body, especially on the arms and neck (Dr. Özlem Çırpan, personal communication). Similar disturbances and events were also occurring in Küçükkuyu, Edremit Bay between 17-26th June 2021 (Yazgülü Turan, personal communication). The presence of this species in the northern part of the Aegean Sea may be caused by warmer seawater because of climate change or transportation via water currents.

The highest water temperature in the sampling areas was observed as 30.6°C and salinity was 34.8 ppt. The umbrella diameters of the *Geryonia proboscidalis* (Figure 2A) varied between 3-9 mm wide and were hemispherical with a tiny manubrium on a long, conical gastric peduncle. The mouth has 6 lips; there are up to seven centripetal canals between the radial canals; and the gonads are heart-shaped and quite broad above. It has 12 statocysts, 6 long perradial hollow tentacles with cnidocyst rings, and 6 little solid interradial tentacles with adaxial cnidocyst clusters.

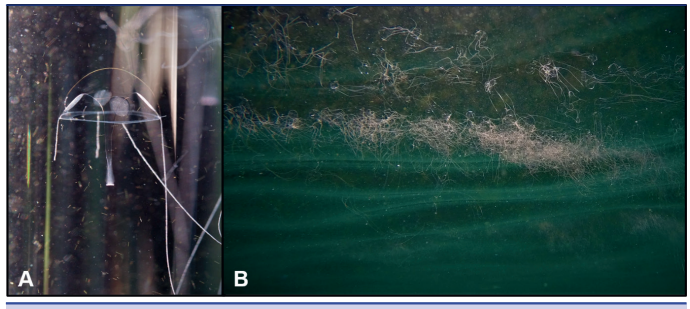

Figure 2. Live specimen (A) and the accumulation (B) of *Geryonia proboscidalis* in Yıldız Bay, Gökçeada (Photograph: Saadettin Aşkın).

Consequently, the aggregation of *G. proboscidalis* observed in the different coastal areas of Gökçeada was an apparent bloom and was concentrated at one coastal location due to strong winds/currents, transporting them to the coast. The number of new records of Hydrozoa species in the Aegean Sea coast of Turkey has increased in recent years (Gülşahin et al., 2013; Isinibilir, Martell, Topçu, Yilmaz, & Piraino, 2015; Yilmaz, Martell, Topcu, & Isinibilir, 2020). The hydrozoa species diversity in Gökçeada was found to include 48 species (Isinibilir et al., 2015). *G. proboscidalis* is a new record from the coasts of Gökçeada island. The species has an important ecological role due to its general ability to feed upon fish eggs and larvae and to feed on the plankton that fish larvae consume. They are therefore potential predators and competitors of commercial species. Furthermore, holidaymakers are extremely irritated by the sting of *G. proboscidalis* (Castellani & Edwards, 2017). Therefore, the occurrence of *G. proboscidalis*, may cause severe effects on public health and tourism in Gökçeada island.

Conflicts of interest: The authors have no conflicts of interest to declare.

Ethics committee approval: The authors declare that this study does not include any experiments with human or animal subjects.

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Disclosure: -

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Short Communication: This type of manuscript discusses important parts, overlooked aspects, or lacking parts of a previously published article. Articles on subjects within the scope of the journal that might attract the readers' attention, particularly educative cases, may also be submitted in the form of a "Short Communication" Readers can also present their comments on the published manuscripts in the form of a "Short Communication". The main text should contain Introduction, "Materials and Methods", "Result and Discussion", "Conclusion" and "References" sections.

After the Conclusion section and before references list, information regarding conflict of interest, financial disclosure, ethics committee approval and acknowledgement are given. These information are to be provided in the author form which must be submitted togather with the manuscript.

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Tables should be included in the main document, presented after the reference list, and they should be numbered consecutively in the order they are referred to within the main text. A descriptive title must be placed above the tables. Abbreviations used in the tables should be defined below the tables by footnotes (even if they are defined within the main text). Tables should be created using the "insert table" command of the word processing software and they should be arranged clearly to provide easy reading. Data presented in the tables should not be a repetition of the data presented within the main text but should be supporting the main text.

Table 1. Limitations for each manuscript type

Type of manuscript	Page	Abstract word limit	Reference limit
Original Article	≤20	250	40
Review Article	≤25	250	60
Short Communication	≤5	250	20

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Figures, graphics, and photographs should be submitted as separate files (in TIFF or JPEG format) through the submission system. The files should not be embedded in a Word document or the main document. When there are figure subunits, the subunits should not be merged to form a single image. Each subunit should be submitted separately through the submission system. Images should not be labeled (a, b, c, etc.) to indicate figure subunits. Thick and thin arrows, arrowheads, stars, asterisks, and similar marks can be used on the images to support figure legends. Like the rest of the submission, the figures too should be blind. Any information within the images that may indicate an individual or institution should be blinded. The minimum resolution of each submitted figure should be 300 DPI. To prevent delays in the evaluation process, all submitted figures should be clear in resolution and large in size (minimum dimensions: 100 × 100 mm). Figure legends should be listed at the end of the main document.

All acronyms and abbreviations used in the manuscript should be defined at first use, both in the abstract and in the main text. The abbreviation should be provided in parentheses following the definition.

When a drug, product, hardware, or software program is mentioned within the main text, product information, including the name of the product, the producer of the product, and city and the country of the company (including the state if in USA), should be provided in parentheses in the following format: "Discovery St PET/CT scanner (General Electric, Milwaukee, WI, USA)"

All references, tables, and figures should be referred to within the main text, and they should be numbered consecutively in the order they are referred to within the main text.

Limitations, drawbacks, and the shortcomings of original articles should be mentioned in the Discussion section before the conclusion paragraph.

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While citing publications, preference should be given to the latest, most up-to-date publications. If an ahead-of-print publication is cited, the DOI number should be provided. Authors are responsible for the accuracy of references. List references in alphabetical order. Each listed reference should be cited in text, and each text citation should be listed in the References section. The reference styles for different types of publications are presented in the following examples.

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Aquatic Sciences and Engineering complies with APA (American Psychological Association) style 6th Edition for referencing and quoting. For more information:

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Citations must be indicated with the author surname and publication year within the parenthesis.

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Samples:

More than one citation;
(Esin et al., 2002; Karasar, 1995)
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(Akyolcu, 2007)
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(Sayıner & Demirci, 2007)
Citation with three, four, five authors;
First citation in the text: (Ailen, Ciambrune, & Welch, 2000)
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- **A book chapter, print version:** Haybron, D. M. (2008). Philosophy and the science of subjective well-being. In M. Eid & R. J. Larsen (Eds.), *The science of subjective well-being* (pp. 17-43). New York, NY: Guilford Press. ISBN 4546469999
- **An eBook:** Millbower, L. (2003). *Show biz training: Fun and effective business training techniques from the worlds of stage, screen, and song.* Retrieved from http://www.amacombooks.org/ (accessed 10.10.15)
- **An article in a print journal:** Carter, S. & Dunbar-Odom, D. (2009). The converging literacies center: An integrated model for writing programs. *Kairos: A Journal of Rhetoric, Technology, and Pedagogy, 14*(1), 38-48.
- **An article with DOI:** Gaudio, J. L. & Snowdon, C. T. (2008). Spatial cues more salient than color cues in cotton-top tamarins (saguinus oedipus) reversal learning. *Journal of Comparative Psychology,* https://doi.org/10.1037/0735-7036.122.4.441
- **Websites - professional or personal sites:** The World Famous Hot Dog Site. (1999, July 7). Retrieved January 5, 2008, from http://www.xroads.com/~tcs/hotdog/hotdog.html (accessed 10.10.15)
- **Websites - online government publications:** U.S. Department of Justice. (2006, September 10). Trends in violent victimization by age, 1973-2005. Retrieved from http://www.ojp.usdoj.gov/bjs/glance/vage.htm (accessed 10.10.15)
- **Photograph (from book, magazine or webpage):** Close, C. (2002). *Ronald.* [photograph]. Museum of Modern Art, New York, NY. Retrieved from http://www.moma.org/collection/object.php?object_id=108890 (accessed 10.10.15)
- **Artwork - from library database:** Clark, L. (c.a. 1960's). *Man with Baby.* [photograph]. George Eastman House, Rochester, NY. Retrieved from ARTstor
- **Artwork - from website:** Close, C. (2002). *Ronald.* [photograph]. Museum of Modern Art, New York. Retrieved from http://www.moma.org/collection/browse_results.php?object_id=108890 (accessed 10.10.15)

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When submitting a revised version of a paper, the author must submit a detailed "Response to the reviewers" that states point by point how each issue raised by the reviewers has been covered and where it can be found (each reviewer's comment, followed by the author's reply and line numbers where the changes have been made) as well as an annotated copy of the main document. Revised manuscripts must be submitted within 30 days from the date of the decision letter. If the revised version of the manuscript is not submitted within the allocated time, the revision option may be canceled. If the submitting author(s) believe that additional time is required, they should request this extension before the initial 30-day period is over.

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