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Correspondence Address

Istanbul University Faculty of Aquatic Sciences,
Ordu Caddesi No: 8 34134 Laleli-Fatih / İstanbul, Turkiye
E-mail: ase@istanbul.edu.tr
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Address: İstanbul Üniversitesi Su Bilimleri Fakültesi Yetiştiricilik Anabilim Dalı Kalenderhane Mah. 16 Mart Şehitleri caddesi No: 2 Vezneciler / İstanbul, Türkiye
Phone: +90 212 4555700/16448
Fax: +90 212 5140379
E-mail: mdevrim@istanbul.edu.tr

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Contents/İçindekiler

Research Articles

Short Communication

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Research Article

Investigation of the Impact of Can-filling Medium on the DNA Quality of Canned Tuna Sold in Supermarkets

Elif Tugce Aksun Tümerkan[1,2]

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ABSTRACT

Canned tuna is one of the most commonly consumed food products globally. Due to its high profitability and the increasing demand for it, fraudulent canned tuna products have become a serious problem. The traceability of fish species in packaged material and, in the case of highly processed forms, in canned products, has become impossible; therefore, canned tuna is on the list of the top ten food items affected by fraud. These fraudulent actions cause not only unfair trade in the commercial market and fishing industry, but also cause health damage (such as allergies and poisoning) to the public. Complex food matrices also affect the extracted DNA quality when the main food products are served with another medium. Brine solutions, different kind of oil, and several types of sauce are used as filling medium in the canned tuna production process. These filling medium can cause contamination depending on whether they include oil, salt or other ingredients during DNA extraction from main products. DNA-based protocols have become popular due to their higher reliability rate compared to other protocols. This research investigates the potential impact of can-filling medium on DNA quality, which is a key factor for food traceability research. With this aim, canned tuna from various brands in different can-filling medium such as olive oil, sunflower oil and different kinds of sauces, were obtained from a Turkish supermarket. The quality properties, such as yield and purity, affected the traceability analyses. This study was designed to investigate the potential effect of the filling medium on DNA quality. The results revealed that different kinds of sauce utilization as a can-filling medium cause a reduction in the DNA quality of canned tuna compared to other canned tuna samples that contain olive oil and sunflower oil. The purity of extracted DNA in canned tuna where olive oil was used was found to be relatively higher than other tuna groups with different can-filling medium. Melting curve analyses revealed that sunflower oil causes relatively lower degradation than olive oil and different types of sauce used as filling medium. These results could be beneficial for further seafood traceability research, especially in complex matrices.

Keywords: Canned tuna, filling medium, DNA quality, DNA yield, traceability

ORCID IDs of the author:
E.T.A.T. 0000-0003-1993-0569

[1]Ankara Yıldırım Beyazıt University, Vocational School of Health Services, Department of Food Processing-Food Technology, Ankara, Turkiye

[2]Ankara Yıldırım Beyazıt University, Central Research Laboratory, Application and Research Center, Ankara, Turkiye

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Correspondence:
Elif Tugce Aksun Tümerkan
E-mail:
etaksun.tumerkan@aybu.edu.tr

INTRODUCTION

Due to its health benefits, seafood consumption has increased dramatically over recent years and has reached around 20 kg per capita. Seafood products are considered one of the most traded food items (Asche, Bellemare, Roheim, Smith, & Tveteras, 2015; FAO, 2018). The rising trend of seafood consumption and the reduction of fish stocks have caused a significant increase in fraudulent actions in the seafood industry (Tamm, Schiller, & Hanner, 2016). Fish species are considered the third-highest risk group for fraudulent actions among other foods (Reilly, 2018). The most common types of fraudulent actions in seafood production can be classified as intentional species substitution, species adulteration and mislabelling (Fox, Mitchell, Dean, Elliott, & Campbell, 2018). Substitution or intentional mislabelling of a species

generally appear when replacing the high-value species with a cheaper or less-desirable species for illegal economic gain. In addition, unintentional mislabelling may arise because of misidentification or doubts in the naming of closely related species along the seafood supply chain (Barendse et al., 2019). All these fraudulent actions not only cause unfair financial gain and pose a serious threat to public health but may also have ecological impacts, such as affecting biodiversity and further fisheries activities (Pardo & Jimenez, 2020).

Tuna species, a large group of important fishes that belong to members of Scombridae family, are classified into three genera (Katsuwonus, Sarda, and Euthynnus) and these species have different economic and ecological value (Abdullah & Rehbein, 2015). The over-consumption of the most desirable species, challenges regarding raw material sustainability for the tuna canning industry and mostly unregulated economic incentives could cause an increase in substitution and mislabelling in the canned tuna industry (Sotelo et al., 2018). Fraudulent actions in the tuna industry differ depending on the country, local market demand, consumer preference and regional tuna catch annually. Gordoa, Carreras, Sanz, & Viñas (2017) highlighted that the mislabelling and substitution rate changed from 37% to 48% in the Spanish tuna processing chain, and the mislabelling of tuna products rate was found to be much higher (95%) in Brussels restaurants (Europe, 2015). These results revealed that the importancet of monitoring of traceability in tuna products globally. While reaching the higher-yield DNA of unprocessed raw material is relatively easy, it becomes a complex problem with DNA extracted from processed and mixed seafood due to thermal treatment, acidic application and pressure. In the canned tuna industry, several can-filling mediums consisting of sauce, spices and other ingredients are used for increasing consumer acceptance and product variability. Despite their benefits, the filling medium decrease the quality and yield of DNA due to the variations in thermal conductivity and acidity. These cause degradation of DNA from canned tuna. Sunflower oil, olive oil and brine solutions are the most commonly used can filling mediums in the canning industry. Sunflower oil offers more palatable tuna with a relatively lower cost, and olive oil extends shelf-life by retarding the oxidation of tuna and leads to more acceptable colorimetric characteristics (Boughattas, Le Fur,& Karoui, 2019). Recently, usage of various sauces, spices and slices of vegetables as filling medium has become more common due to increasing consumer preference. These can-filling mediums seem to benefit the consumer, however, different spices have not only been used as flavouring and colouring agents in the food items, but also in fraudulent actions such as masking undesirable colour and rancid taste (Julien-David & Marcic, 2020). In light of increasing fraudulent actions in the seafood industry, usage of these risky components in canned tuna products should be controlled through authorized methods. The quality and yield of DNA from food items does not depend on the initial material characterization. DNA extraction technique is another essential factor impacting DNA quality. In addition to several confirmed chemical, lyses and enzymatic methods, different commercial kits have also been used for the DNA extraction process (Barbosa, Nogueira, Gadanho & Chaves, 2016; Sajali et al., 2018).

MATERIAL AND METHODS

Twenty-one commercial canned tuna products (Table 1) obtained from Turkish supermarkets were purchased in April 2021 and analysed. The 21 products were chosen to represent all the main cannery brands available on the Turkish market (12) with various can-filling media (e.g., oils, spices and/or sauces). Commercial samples representing the three product categories considered in this study (canned tuna with sunflower oil (SO), canned tuna in olive oil (OO) and canned tuna with sauces (SA) including different ingredients such as pepper, tomato or mustard, etc.) were extracted with the same protocol. Commercial canned tuna samples were dried with sterile filter paper to eliminate the oils, spices and sauces, and then 15-g samples of tuna were transferred into 50 mL Falcon tubes and stored at -80° C. DNA was extracted from all samples with the same extraction methods: 20-mg sample, 250 µl Buffer ATL and 20 µl Proteinase K were mixed. Thermal processing was applied to the mixture until the mixture was completely lysed. The lysed mixture was centrifuged at 12000 g for 30 seconds and then the supernatant was directly transferred to the sterile tube. An extractions buffer (250 µl) was added to the spin column and heated at 56 °C for 10 minutes in order to reach a better yield of DNA. Then 250 µl of a binding buffer (BF) was added and the total mixture was vigorously mixed in a vortex for 15 seconds. Afterward, the mixture was applied to the mini-spin columns for binding DNA. Then the spin column was washed with AW1(650 µl) and AW2 (500 µl) and centrifuged. Finally, pre-heated buffer AE (200 µL) was used for the elution of the purified DNA and then thr purified DNA was stored at -20°C until further analysis.

Determination of DNA quality and amplificability

The quality of DNA from canned tuna in terms of concentration, purity and presence of any contaminants was investigated with a NanoDrop 1000 spectrophotometer (Thermo Scientific, Pittsburgh, PA, USA). The amplificability of the gDNA was then determined by amplifying a 655 bp region from the COI which was targeted teleo primer (COI F: 5′ TCGACTAATCATAAAGATATCGGCAC 3′ and COI R: 3′ ACTTCAGGGTGACCGAAGAATCAGAA 5′) (Ward, Zemlak, Innes, Last, & Hebert, 2005). The reaction mixtures were prepared as follows: 2 µL of template gDNA, 2 µL of forward and reverse primer, 10 µL Master Mix (Thermo Scientific™ Maxima SYBR Green/ROX qPCR Master Mix (2X) and 6 µl DNA free water. The PCR was run and analysed on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) according to the following cycling protocol: denaturation at 95°C for 2 minutes, 35 cycles of 30 s at 94 °C, 30 s at 53 °C, and 60 s at 72 °C, and the final extension at 72°C for 10 minutes. Generally, real-time PCR and melting curve analysis (MCA) are performed in combination to better understand the kinetics of denaturation and offer detailed knowledge about the following sequence, which differs depending on main food items and different components found in food matrices. The melting curve analysis (MCA) was carried out between 65°C and 95°C.

Statistical analysis

For comparison of the yield, purity and contamination level of DNA extracted from all commercial canned tuna samples (SO, OO, SA), a two-way cross-classification analysis of variance

Table 1. Canned tuna sample description.

Sample	Fish type[a]	Canning Matrix[b]	Brand[c]	Exp.Date
1	Tuna	Olive oil	Brand 1	22.06.2024
2	Tuna	Tomato sauce	Brand 1	22.12.2024
3	Tuna	Mustard sauce	Brand 1	20.01.2024
4	Tuna	Olive oil	Brand 2	20.01.2026
5	Tuna	Sunflower oil	Brand 2	19.11.2024
6	Tuna	Olive oil	Brand 2	19.11.2025
7	Tuna	Sunflower oil	Brand 3	11.07.2024
8	Tuna	Pepper sauce	Brand 3	11.07.2026
9	Yellowfin tuna	Olive oil	Brand 4	05.12.2025
10	Yellowfin tuna	Sunflower oil	Brand 4	05.12.2026
11	Skipjack tuna	Sunflower oil	Brand 5	15.11.2025
12	Skipjack tuna	Pepper sauce	Brand 5	15.11.2025
13	Tuna	Sunflower oil	Brand 6	22.09.2025
14	Tuna	Olive oil	Brand 6	22.09.2025
15	Skipjack tuna	Sunflower oil	Brand 7	19.11.2024
16	Skipjack tuna	Olive oil	Brand 8	19.11.2025
17	Tuna	Pepper sauce	Brand 8	25.10.2025
18	Tuna	Sunflower oil	Brand 9	25.10.2026
19	Skipjack tuna	Tomato sauce	Brand 10	21.12.2025
20	Skipjack tuna	Tomato-pepper sauce	Brand 11	23.12.2025
21	Yellowfin tuna	Olive oil	Brand 12	19.09.2024

[a] fish variety declared in the product label; [b] canned tuna packaged with different filling medium; [C]For privacy reasons, brands are not reported and are listed numerically.

(ANOVA) was performed. All the statistical significances were determined by SPSS software version 19 (Chicago, Illinois, USA). Statically important differences were evaluated at a level of 5% ($P< 0.05$). All the DNA quality analyses were performed in triplicate assays for each canned tuna sample group.

RESULTS AND DISCUSSION

Determination of DNA quality

The purity of the gDNA is a key parameter that can powerfully affect the success of PCR amplification and sequencing processes and thereof the traceability analyses. The purity of the DNA was calculated with the A260/A280 ratio. Significant differences were observed among the canned tuna samples with different can filling medium (Table 2). While the highest values were obtained in canned tuna with sunflower oil (with 2.26), the other groups (filling medium of olive oil and sauce) have 1.95 and 1.90. The optimal purity value is between 1.8-2.0 (Piskata, Pospisilova, & Borilova, 2017). These differences could be related to the thermal integrity of sunflower oil and components that differ from sauce. Treatment with different compounds cause differentiation of the purity of DNA, which is accepted as an indicator for the DNA yield. Another key parameter for the molecular analyses performed for food traceability is the A260/A230 ratio, which is accepted as a sign of the presence of organic contaminants (from carbohydrates to salts) in the extracted DNA. There were no significant differences in terms of the presence of contaminants observed in canned tuna products with olive oil and sunflower oil filling medium canned tuna products. The highest contamination rate was determined to be in the tuna soaked in a sauce filling medium. These differences could be explained by treatment with different ingredients present in sauce, which reduces the purity of extracted DNA. As stated by Lucena-Aguilar et al (2016), the optimal value for the presence of any contaminant in extracted DNA ranges from 2.0 to 2.2. The highest contaminant values observed from canned tuna samples with sauce was 2.31. This significant higher value may be related to the presence of more than one medium in the sauces used for canned tuna.

The results of the extracted DNA from canned tuna samples with different can-filling medium are given in Figure 1 and Table 2. Calculation of the DNA yield was performed depending on DNA concentration, initial weighted tuna muscle and the final volume obtained. As stated in Table 2 and Figure 1, significant differences in terms of DNA yield were found among canned tuna with various can-filling medium ($P<0.05$). The highest DNA yield was determined in the SO group (916.1 (ng/ul) and the lowest DNA yield, which was found to be significantly lower than the other groups, was determined in the SA group (211.9 (ng/ul). This is similar to other quality parameters. As a filling medium, sunflower oil and olive oil lead to better DNA yield, DNA purity and the absence of organic contaminants than does sauce. Chapela et al. (2007) performed a comparative study for canned tuna with different filling medium such as vinegar, brine tomato sauce and oil by various extraction techniques; they reported comparatively higher DNA concentrations obtained in tuna in oil groups in all extraction methods. Due to can-filling sauce made with several ingredients that could have different sensitivities to thermal process and acidity, the quality and yield of DNA is not stable. This is consistent with what was highlighted by Chapela et al. (2007)

and Elsanhoty, Ramadan & Jany (2011), who stated that a significant reduction in DNA purity for those canned tuna samples with filling sauce may be related to the acidity causing the hydrolytic degradation mechanism of DNA.

radation of DNA is an important problem that can cause disruptions in species control and protection against food fraud. The thermal process is accepted as the main reason for the DNA degradation. Because of the variation in consumer consumption

Table 2. DNA quality assessment of canned tuna sample.

Canned tuna with can-filling medium	Quality assessment		
	DNA Yield (ug/uL)	Purity (A260/A280)	Chemical Contamination (A260/A230)
SO	916.1±0.03^{c}	2.26±0.10^{b}	2.13±0.11^{b}
OO	578.10±0.01^{c}	1.95±0.16^{ab}	2.19±0.07^{b}
SA	211.9±0.10^{b}	1.90±0.05^{a}	2.31±0.09^{a}

Groups: Canned tuna with sunflower oil (SO), canned tuna in olive oil (OO), canned tuna with sauces (SA).
Values are expressed as average ± standard deviation (n =3).
Values in the same column followed by different numbers show significant difference ($P < 0.05$) between canned tuna groups.

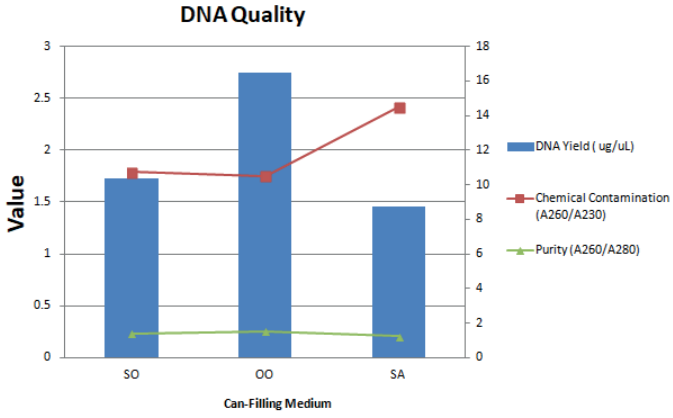

Figure 1. DNA quality variation of canned tuna sample.
Groups: (SO): canned tuna with sunflower oil, (OO): canned tuna in olive oil, (SA): canned tuna with sauces

The quality characteristics of DNA directly impact the amplification, sequencing and therefore the achievement of molecular methods for food authentication, especially in complex food matrices. The canning process includes both thermal treatment and high pressure, which have an impact on the stability of DNA. Different filling medium has also cause variation in DNA yield, quality and organic contaminant presence in extracted DNA, which can directly impact the further step of traceability analyses. The achievements of food traceability research are mainly driven by the extracted DNA quality and purity.

Determination of DNA degradation

DNA degradation among canned tuna samples with different can-filling medium determined by threshold cycle (Ct) value. The statically significant differences in terms of melting curves among the groups are shown in Figure 2. These variations could be explained by the impact of heat treatment on the filling medium during the canning and sterilization process. Ballari and Martin (2013) also reported that the variations in DNA fragmentation and amplificability observed resulted from autoclaving. Regardless of the aim of sequencing in DNA-based methods, the degtendency toward rapidly consumed food and technological improvements, the ready-to-eat food market has grown rapidly and several processed food products are consumed globally. This case also causes challenges in the traceability of food products, which is very important for public health and fair trade. Fraud detection analyses depend on quality, yield and degradation level.

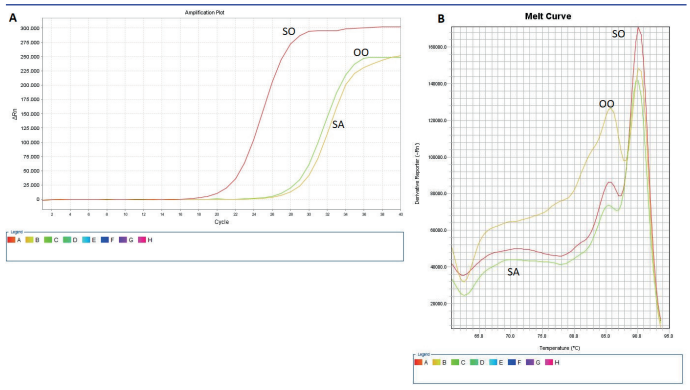

Figure 2. Amplicon plots (A) and Melting Curve (B) of tuna sample with different can-filling medium.
Groups: (SO): canned tuna with sunflower oil, (OO): canned tuna in olive oil, (SA): canned tuna with sauces

CONCLUSION

The effects of can-filling medium on the quality of DNA and the level of DNA degradation in canned tuna were compared with the same extraction methods and the same amplification procedures. In total, 21 different commercial canned tuna products with different can-filling medium examined in terms of DNA quality (DNA yield, DNA purity and presence of contaminants) and DNA degradation. The results revealed that different filling medium caused variance in DNA degradation and quality parameters in canned tuna, which could be related to the thermal integrity of different compounds used as a filling medium, such as oil or sauce ingredients. These findings could be useful for other thermally processed products, especially seafood products

which are highly perishable without acidic or thermal treatment. The results of this research are also valuable for other complex food matrices. As part of the increased demand for well-organized analyses methods for food authentication, better quality and low DNA degradation are accepted as the initial step within molecular-based methods. The results revealed that monitoring of DNA quality and yield are essential for the food traceability research in thefor other seafood , especially in seafood products that have complex food matrices.

Note: This study was presented as an online presentation at the 2nd Aquatic Biotechnology Symposium.

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Research Article

Sensory, Chemical and Microbiological Properties of Trout Sausage (Fermented Sucuk)

Dilek Kahraman Yılmaz[1], Nermin Berik[2]

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ABSTRACT

This study aimed to produce fish sucuk using rainbow trout (*Oncorhynchus mykiss*) fillet and investigate its suitability for consumption. Sensory, nutrient composition, fatty acid and amino acid composition analyses were carried out in the sucuk prepared with a commercial spice mix. Total aerobic mesophilic bacteria, total coliform, total *Enterobacteriaceae*, Lactic acid bacteria, yeast and mould counts were found to be smaller than 1 log cfu g^{-1}. It was found that the ratio of water, protein, fat, ash, total saturated FA, total mono-unsaturated FA and total poly-unsaturated FA were 48.63%, 17.27%, 22.00%, 2.52%, 50.17%, 43.23% and 6.6%, respectively. Glutamic acid (19.1 g 100 $^{g-1}$), aspartic acid (17.17 g 100g-1), leucine (9.45 g 100 g^{-1}) and lysine (8.05 g 100 g^{-1}) were the main amino acids in fish sucuk. In addition, the sensory analysis results of the sucuk produced entirely from trout meat showed that it was acceptable. This study concluded that a sucuk product suitable for consumption could be produced using trout meat entirely, with the appropriate spice mixture, heat treatment and air conditions.

Keywords: Rainbow trout, fermented products, fatty acids, amino acids

ORCID IDs of the author:
D.K.Y. 0000-0002-9626-5446;
N.B. 0000-0003-3015-8688

[1]Onsekiz Mart University, Department of Marine Biology, Faculty of Marine Sciences and Technology, Çanakkale Çanakkale, Turkiye

[2]Çanakkale Onsekiz Mart University, Department of Fisheries and Processing Technology, Faculty of Marine Sciences and Technology, Çanakkale, Turkiye

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Correspondence:
Nermin BERİK
E-mail:
nberik@yahoo.com

INTRODUCTION

Aquatic products are one of the most important food sources to meet the animal protein needs in human nutrition. Fish, an aquatic product, meets the basic nutritional needs because of the essential amino acids, mineral matter, vitamins and polyunsaturated fatty acids (Pal et al., 2018; Khalili Tilami & Sampels, 2018). In addition to nutrition, aquatic products assume an effective role in strengthening immunity and treating some diseases (Kaya, Duyar, & Erdem 2004; Turan, Yalçın, & Sönmez, 2006; Cicero, Ertek, & Borghiet, 2009). Thus, it is among the important nutrients in maintaining both physiological and metabolic activities (Kaya et al., 2004). Since fish consumption is very important for human health, consumption of aquatic products ranks first in human nutrition in many countries in the world, while annual aquatic product consumption per capita in Turkey is 6.3 kg (TUIK, 2020). Aquatic product consumption in Turkey is very low per capita. Traditional consumer habits in Turkey generally tend toward consumption of fresh products. This consumption preference results from the fact that fresh products are easy to access and processed products suit people's tastes. Promotional studies on the consumption of processed seafood products in Turkey need to be increased. Thus, the consumption of aquatic products will be boosted in regions far from coastal areas and in seasons outside of hunting season.

There has always been a demand for delicatessen products in Turkey. Traditional sucuk products are among the most consumed ones. Beef sucuk produced in different ways since the Middle Ages are mainly consumed in the Balkans, Caucasia, Middle East, Central Asia, Southeast

and Northern Europe (Ercoşkun & Özkal 2011; Sarıışık & Tagmanov 2020). Sucuk is a fermented meat product prepared with a mixture containing different spices. The mixture is filled in natural or artificial dried casings, shaped in different ways (coil, baton) and ripened under certain conditions (TS 1070, 2002; TGK, 2019). While the meat of cattle, buffalo and camel is mostly used in sucuk production, sucuk is also produced using bovine sheep and goat (TGK, 2019). This study investigated whether the sucuk obtained from rainbow trout (*Oncorhynchus mykiss*), which is cultivated most in Turkey and is a fish species with high nutritional content, can be an alternative to the sucuk produced from red meat.

MATERIAL AND METHODS

Fish material

15 kg rainbow trout (*Oncorhynchus mykiss*), obtained fresh after harvest from farm conditions, was used as raw material in sucuk production. The average length of the fish was 28.25 ± 0.5 cm, and the weight was 246.2 ± 16.59 g. They were purchased from a private enterprise located in the Çanakkale region. The fish were brought to the Faculty Processing Technology Laboratory without impeding cold chain conditions.

Sucuk mixture

After cleaning the internal organs of the rainbow trout used in the study, the fillets were removed. At the end of this process, a total of 4.984 kg fish meat was obtained. Fish fillets were minced by means of an electric meat grinder (Tefal 1800w). Two types of sucuk pastes were obtained from the minced fish using two different spice mixtures, in accordance with sucuk production technology (Table 1).

Table 1. Sucuk mixture formulation.

Traditional		Commercial	
Ingredients (g 100g^{-1})		Ingredients (g 100g^{-1})	
Fish Meat	70	Fish Meat	70
Fat (1/2 sunflower oil+1/2 beef fat)	20	Fat (1/2 sunflower oil+1/2 beef fat)	20
Starch	2.4	Food salt	
Salt	1.75	Ground red pepper	
Ginger	0.05	Ground cumin	
Cinnamon	0.05	Dried ground garlic	
Allspice	0.08	Ground black pepper	
Sweet Paprika	1.70	Stabilisers (E450, E451, E452)	10
Hot Paprika	0.09	Maltodextrin	
Garlic	1.40	Dextrosemonohydrate	
Granulated sugar	0.52	Anti-oxidant: sodium erythorbate	
Black pepper	0.30	Yeast extract	
		Spice extracts	

The first type of sucuk mixture was obtained from a mixture of minced fish and various spices. The second type of sucuk mixture was obtained by adding a spice mixture from a private company to the minced fish (Table 1). Both types of sucuk mixture were stored for 24 hours at +4°C under refrigerated conditions for fermentation.

Filling and ripening of trout sucuk

The fermented trout sucuk mixture was filled into intestinal casings that were kept in cold water (7%) with vinegar beforehand. The filled sucuk were grouped according to ripening conditions and pre-treatment conditions and left to ripen (Table 2). After the fermented sucuk were kept in the refrigerator at +4°C for one day, their sensory, physicochemical and microbiological analyses were carried out. However, analyses were not performed on the experimental groups (1, 3, 4, 5, 6, 8, 9, 11, 13, 14 and 16) that showed rancid odour and were in an inedible condition.

Sensory analysis

An academic panellist group consisting of 10 people was formed for the sensory analysis. The sensory analysis was performed using the sensory test criterion of Fernández-Fernández, Vázquez-Odériz & Romero-Rodríguez, (2002a). Appearance, colour, odour, texture and flavour parameters of sucuk were evaluated using the scoring 5=Very good, 4=Good, 3=

Table 2. Different ripening conditions of different sucuk groups.

Spice Types			
Traditional		Commercial	
Sucuk Group	Ripening Conditions	Sucuk Group	Ripening Conditions
1	1st day heat treatment * + room conditions **	9	1st day heat treatment * + room conditions **
2	room conditions **	10	room conditions **
3	1st day heat treatment * + air conditioning cabinet conditions **	11	1st day heat treatment * + air conditioning cabinet conditions ***
4	air conditioning cabinet conditions ***	12	air conditioning cabinet conditions ***
5	room conditions ** + 3rd day heat treatment *	13	room conditions ** + 3rd day heat treatment *
6	air conditioning cabinet conditions *** + 3rd day heat treatment *	14	air conditioning cabinet conditions *** + 3rd day heat treatment *

Table 2. Continue.

Spice Types			
Traditional		Commercial	
Sucuk Group	Ripening Conditions	Sucuk Group	Ripening Conditions
7	1st day heat treatment * + air conditioning cabinet conditions *** + daily heat treatment for 6 days *	15	1st day heat treatment * + air conditioning cabinet conditions *** + daily heat treatment for 6 days *
8	1st day heat treatment * + air conditioning cabinet conditions *** + 3rd day heat treatment *	16	1st day heat treatment * + air conditioning cabinet conditions *** + 3rd day heat treatment *

Note: *Heat treatment: 20 min at 70°C. **Room conditions: 18-20 °C *** Air conditioning cabinet conditions: 18-20 °C

Moderate, 2=Bad and 1=Very bad (Fernández-Fernández, Vázquez-Odériz, & Romero-Rodríguez, 2002b; Fernández-Fernández et al., 2002b).

pH analysis
Measurements were performed on ripened fish sucuk and cooked sucuk using a pH meter (HANNA / HI 2211) (Landvogt & Fischer 1991).

Proximate composition
Nutrient composition analyses were performed in three parallel procedures. Moisture, protein and ash analysis were carried out to AOAC standards (AOAC, 2005). Crude protein (factor: 6.38) was determined by the Kjeldahl method. Crude fat was determined by the methanol–chloroform extraction method (Folch, Lees, & Sladane-Stanley, 1957).

Fatty acid methyl ester analysis (FAME)
Lipid extraction of sucuk samples was carried out according to Folch et al. (1957). For fatty acid analysis, 0.1 g sample was treated with 10 mL of n-hexane and 0.5 mL of 2N methanolic KOH solution was added (I.U.P.A.C., 1987). Gas chromatograph analysis was performed on a Shimadzu GCMS QP 2010 ULTRA instrument equipped with a RTX-2300 capillar column (60 m; 0.25 mm; 0.2 μm). Each fatty acid peak was determined by comparing the retention times in a mixture of known standard fatty acids United States Pharmacopeia (USP) Fame Mix Reference Standard (US Pharmacopeia, Maryland, USA) run under the same operating conditions. Fatty acids were presented as a percentage of total methylated fatty acids.

Amino acid composition
For amino acid analysis, 0.5 grams of sucuk sample was weighed and burned with 20 mL of HCl at 110°C for 18-24 hours. 20 mL of distilled water was added and dried in an evaporator at 70°C. The volumetric flask was set to 25 mL with pure water. Amino acid samples were analysed on a Shimadzu LC-MS/MS 8040 and determined using a Zorbax Eclipse AAA column (4.6 X 150 mm, 3.5 µm). Mobile phase consisted of 1‰ formic acid in ultrapure water (eluent A) and 1‰ formic acid in methanol (v/v) (eluent B). The column temperature was set at 40 °C, the injected sample volume was 0.2 µL and the flow rate was 1 mL min^{-1}.

Microbiological analysis
10 g of sucuk samples were taken for microbiological analysis. The samples were homogenised for three minutes in 90 mL of peptone water using a blender (Seward Stomacher 400). In the total viable count of sucuk samples, Tryptic Soy Agar medium (TSA at 36 °C for 48 h) (Merck 1.05458), (FDA, 2001); for lactobacillus count, DeMan, Rogosa and Sharpe (MRS at 36 °C for 120 h) Agar (Merck 1.10660) medium (Jokovic et al., 2008); for coliform group microorganisms count, MacConkey (MAC at 36 °C for 48 h) Agar (Merck 1.05465); for enterobacteria count, Violet Red Bile Dextrose (VRBD at 36 °C for 48 h) Agar (Merck 1.10275) medium; for yeasts and moulds count, Potato Dextrose Agar (PDA at 22°C for 120 h) (Merck, 1.10130) were used.

Statistical analysis
Statistical analyses of the data obtained as a result of the study were performed using the SPSS package program. SPSS 17.0 package program was used for statistical evaluation. In order to detect differences in the study, Oneway analysis of variance (ANOVA) and Tukey tests were run on the data. Differences were evaluated at the 0.05 significance level.

RESULTS AND DISCUSSION

Microbiological results
Table 3 presents the microbiological analysis findings of the sucuk mixture and different sucuk groups. Microbiological analyses were not performed on the experimental groups that showed rancid odour and were in an inedible condition. When the production was completed, the yeast-mould counts of group 7, group 2 and group 10 sucuk exceeded the limit (2 log cfu/g) specified in the Communiqué on Turkish Food Codex Meat and Meat Products (TGK, 2010). This study aimed to produce Turkish-type "fish sucuk" suitable for consumption by using fish meat entirely instead of red meat. 16 groups of sucuk were produced under different ripening conditions, including traditional spice mixture and commercial spice mixture. Sucuk that was not suitable for consumption due to rancid odour and sucuk that failed to meet the microbiological limits as per the Communiqué on Turkish Food Codex Meat and Meat Products (TGK, 2010) was excluded from the analysis. For this reason, among 16 different sucuk groups, Group 15 was found to be suitable for consumption in terms of microbiology, and other analyses were performed on this group.

Total aerobic mesophilic bacteria (TAMB), lactic acid bacteria (LAB), total coliform counts (TC), total *Enterobacteriaceae* counts

(TE) and yeast and mould counts (YM) of sucuk mixture prepared for both spice mixtures were found to be similar to the previous shark sucuk study (Kahraman, 2010). While TC was 2 log cfu/g and YM 3.81 log cfu/g on the first day in carp sucuk, it was reported as TC < 1 log cfu/g and YM 4.23 log cfu/g in the ripened sucuk (7th day) (Arslan, Dinçoğlu, & Gönülalan, 2001a). While TC was 0 log cfu/g on day 7 in silverside sucuk, YM was found to be 4.55 log cfu/g (Arslan, Dinçoğlu, & Gönülalan, 2001b).

Kılınç & Çaklı (2021) kept the traditional spice-mixed sucuk they produced using trout and sea bass fillets in the refrigerator, and as of the 10th day for trout sucuk and 30th day for sea bass sucuk, YM limit value was exceeded (2 log cfu/g). Similarly, in our study, YM exceeded the limit value after fermentation in traditional spice blended groups. In addition, On the other hand, this study determined that trout sucuk can be made suitable for consumption in cases where a commercial spice mixture is used and suitable air conditioning conditions are provided.

Sensory properties and pH values

Table 4 presents the sensory analysis findings in raw and cooked sucuk samples. The general like rate of the sucuk sample, which was evaluated out of five, was found to be 4.4 in raw sucuk and 4.54 in cooked sucuk. While the pH value of raw sucuk was 5.6, it was found to be 5.8 in cooked sucuk. In all sensory analysis results of trout sucuk, it was seen that the panellists liked the sucuk produced from fish meat. Similar results were obtained in sucuk produced before using only fish fillets. For example, it has been reported that the overall tastes of sucuk produced entirely with fish meat using carp (Arslan et al., 2001a), mullet (Berik & Kahraman, 2010) and shark (Kahraman, 2010) fillets were good or very good. While determining the quality of fish and similar products, pH measurement is also carried out during production and subsequent stages. Among the control parameters, the pH value plays an important role. As per the "Communiqué on Turkish Food Codex Meat and Meat Products" in the Turkish Food Codex, it is stated that the pH value of sucuk should be 5.4 at the highest (TGK, 2019). In TS 1070/T4, the Turkish Standards Institute reported the pH value of Turkish sucuk to be at most 5.8 and at least 5.4 (TSE, 2019). In our study, it was observed that the pH findings were also in parallel with the literature. In grey mullet sucuk, while the pH value was 5.34 in ripened raw sucuk, it was determined to be 5.30 in cooked sucuk (Berik & Kahraman 2010). pH was reported to be 5.48 in ripened raw carp sucuk (Arslan et al., 2001a) and 5.96 in silverside fish sucuk (Arslan et al., 2001b).

Table 3. Microbiological analysis findings of sucuk mixture.

	TAMB	TC	TE	LAB	YM
Traditional Sucuk Mixture	4.6	2.5	2.0	1.9	1.3
Commercial Sucuk Mixture	4.6	3.1	3.0	2.7	1.2
Group 15	<1	<1	<1	<1	<1
Group 7	3.7	<1	<1	<1	2.3
Group 2	4.3	<1	<1	4.1	4.0
Group 10	4.6	2.0	2.0	5.2	3.3
Other Groups*	-	-	-	-	-

Note: * Microbiological parameters were not analysed because other sucuk groups showed a high level of rancid odour. CFU: Colony forming unit, TAMB: Total aerobic mesophilic bacteria, TC: total coliform counts, TE: Total *Enterobacteriaceae* counts, LAB: Lactic acid bacteria, YM: Yeast and mould counts

Proximate composition

For the trout sucuk (Group 15), water was determined as 48.63%, protein as 17.27%, fat as 22.00% and ash as 2.52% (Table 5). In accordance with the Communiqué on Turkish Food Codex Meat and Meat Products (TGK, 2019), the total meat protein value of sucuk produced using trout meat was found to be at least 14% by mass, the ratio of moisture content to total meat protein was found to be below 3.6 and the ratio of fat to total meat protein was found to be below 2.5. Previous studies show that the water, protein, fat and ash were between 50.35% to 37.62%, 20.05% to 45.21%, 11.52 to 31.47% and 3.64 to 5.33% respectively, in Turkish type sucuk produced using carp (Arslan et al., 2001a; Çiltaş, 2009), silverside (Arslan et al., 2001b), sea bass (Çiltaş, 2009), mullet (Berik & Kahraman 2010) and shark (Kahraman, 2010). These different results might be associated with differences in fish species, mixture formulation, heat treatment and/or air conditioning conditions.

Fatty acid composition

In rainbow trout sucuk (Table 2), a high oleic acid (C18:1) concentration was found, which was 38.68% of total fatty acids, followed by palmitic acid (C16:0; 27.18%), stearic acid (C18:0; 14.32%), myristic acid (C14:0; 5.30%), linoleic acid (C18:2; 3.95%), palmitoleic acid (C16:1; 4.50%), margaric acid (C17:0; 1.74%) and doco-

Table 5. Proximate compositions of rainbow trout sucuk.

	Mean ± SD
Moisture (%)	48.63±0.08
Crude Protein (%)	17.27±0.09
Crude Fat (%)	22.00±0.17
Crude Ash (%)	2.52±0.26

Table 4. Sensory properties of fish sucuk.

	Appearance	Colour	Texture	Odour	Flavour	Overall acceptability	pH
Raw Sucuk	4.6	4.1	4.6	4.3	-	4.40	5.6
Cooked Sucuk	4.7	4.2	4.7	4.5	4.6	4.54	5.8

Note: The assessment test is 1–5 indicator value levels. 5 = Very good, 4 = Good, 3 = Fair, 2 = Bad, 1 = Very bad

Table 6. Fatty acid composition (%) of the rainbow trout sucuk.

Fatty acid	
Saturated fatty acids	**Mean**
C8:0	0.01
C10:0	0.38
C12:0	0.95
C13:0	0.05
C14:0	5.30
C16:0	27.18
C17:0	1.74
C18:0	14.32
C20:0	0.18
C21:0	0.02
C22:0	0.04
Mono-unsaturated fatty acids	
C16:1	4.50
C18:1	38.68
C20:1	0.01
C24:1	0.04
Poly-unsaturated fatty acids	
C18:3 (n-3)	0.9
C18:2 (n-6)	3.95
C20:4 (n-6)	0.08
C20:3 (n-3)	0.03
C20:5 (n-3)	0.08
C21:5 (n-3)	0.14
C22:5 (n-3)	0.4
C22:6 (n-3)	1.02
Total saturated FA (SFA)	50.17
Total mono-unsaturated FA (MUFA)	43.23
Total poly-unsaturated FA (PUFA)	6.6
(n-6) / (n-3)	1.57

Table 7. Amino acid composition of the rainbow trout sucuk (g/100g protein).

	Rainbow trout sucuk	Preschool Childs* (2 to 5 years)	Adult*
Amino acid			
Leucine	9.45	6.6	1.9
Lysine	8.05	5.8	1.6
Histidine	2.61	1.9	1.6
Isoleucine	3.07		
Methionine	3.51	2.8	1.3
Phenylalanine	2.48		
Threonine	3.47	3.4	0.9
Valine	3.53	3.5	1.3
Arginine	3.3		
Tryptophan[2]	n.d	1.1	0.5
Total essential amino acids	39.47		
Cysteine	0.70		
Glutamine	1.11		
Glutamic Acid	19.1		
Proline	1.67		
Alanine	5.49		
Serine	5.24		
Trosine	3.88		
Aspartic Acid	17.17		
Total non-essential amino acids	54.36		
Total amino acids	93.83		
Methionine + Cysteine	4.21	2.5	1.7
Phenylalanine + Trosine	6.36	6.3	1.9

Note: * Expert Consultations, 1985. (Amino acid requirements) [2]n.d. – no determined

sahexaenoic acid (C22:6; 1.02%). There is a limited number of studies on the fatty acid composition of fish sucuk in the literature. Only one study reported that the fatty acids in shark sucuk were C18:1>C16:0>C22:6>C18:0>C18:2>C22:5>C14:0 from the highest concentration to the lowest (Kahraman, 2010). This order was found to be C18:1>C16:0>C18:0>C14:0> C18:2>C16:1> C17:0> C22:6 in rainbow trout. Differences between studies may result from factors such as fish species, habitat and diet. The (n-6)/(n-3) ratio we obtained for trout sucuk is 1.57. This rate was reported to be 6.22 for beef and 2.95 for camel meat in Turkish-type sucuk produced using different types of meat (Kargozari et al., 2014). In our study, we determined the PUFA concentration to be 6.6%. In different studies, PUFA concentrations were reported to be 0.6% (Yıldız-Turp & Serdaroğlu 2008a), 2.6% (Yıldız-Turp & Serdaroğlu 2008b) for beef and 4.38% (Kargozari et al., 2014) and 2.76% for camel meat (Kargozari et al., 2014). Omega-3 (ω-3) polyunsaturated fatty acids (PUFAs) obtained from fish and fish oils are considered to have a protective effect against coronary heart disease (Cicero et al., 2009; Abdelhamid et al., 2018; Ajith & Jayakumar 2019). Compared to Turkish type sucuk produced using red meat, the lower (n-6)/(n-3) ratio, higher PUFA concentration and nutritional and health benefits of the sucuk produced from trout meat increase the value of the product.

Amino acid composition

Amino acid composition of the rainbow trout sucuk is given in Table 7. In this study, it was determined that among the fish sucuk aspartic acid, glutamic acid, leucine, and lysine were in higher concentrations. In the literature, only one study (Kahraman, 2010) reported the amino acid composition of Turkish type fish sucuk. Present findings are similar to the results of Kahraman (2010), who found that the main amino acids for spiny dogfish sucuk were glutamic acid, aspartic acid, lysine and leucine. In addition, a similar amino acid composition was also reported in sausages made from tilapia fillet scraps (Oliveira Filho, Maria Netto, Ramos, Trindade, & Viegas, 2010) and

sausages produced using silver carp roe protein hydrolysate (Hajfathalian, Jorjani & Ghelichiet, 2020). Moreover, it was observed that fish sucuk contains a balanced ratio of essential amino acids except for tryptophan, and it meets the amino acid requirement standards (Expert Consultations, 1985) for both adults and especially children aged 2-5.

CONCLUSION

This study concluded that when the appropriate spice mix, heat treatment and air conditioning conditions are provided, sucuk suitable for consumption can be produced using trout meat. The low (n-6)/(n-3) ratio and high PUFA concentration in the sucuk produced using fish meat increased the product's value in the healthy food category. From now on, studies should be carried out to increase the shelf life of fish sucuk, which is produced as a qualified and safe food.

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Research Article

Effects of Adding Laurel (*Laurus nobilis*) Essential Oil to the Diet of Tilapia Fish on Growth and Intestinal Histology

Metin Yazıcı[1], Yavuz Mazlum[1], Mehmet Naz[1], Çiğdem Ürkü[2], Musa Türkmen[3], Tülay Akaylı[2]

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ABSTRACT

The effects of adding laurel oil to the experimental diet on growth performance, biochemical compositions of fish and feeds, sand liver and intestine histology in Nile tilapia (*Oreochromis niloticus*) juveniles were evaluated. 180 fish (12±0.02 g) were used in the study. They were randomly placed in 12 tanks with a volume of 500 liters, with 15 fish per tank. The commercial laurel oil was added to the diets at 0, 0.3, 0.6, and 1.2%. The fish were fed with experimental diets twice a day as apparent satiation for 60 days. In the current study, weight gain (WG), feed conversion ratio (FCR), specific growth rate (SGR) and survival rates (SR) were statistically similar ($p>0.05$). While no difference was observed between protein and ash values in the biochemical analysis of fish, lipid values were found to be lower in the 0.3% and 0.6 supplemented groups compared to the control and 1.2% supplemented groups. In addition, there was no statistical difference in protein, lipid, and ash values in the biochemical composition of the feeds. In the study, essential oil components of *Laurus nobilis* oil such as Linalool, Elemene, Trans-Caryophyllene, Cis-α-Bisabolene, A-Terpinyl Acetate, Methyleugenol, β-Eudesmol were determined in low levels. The addition of 0.3% laurel oil to the diet did not cause histopathological findings, and it was found to improve liver and intestinal tissues. In conclusion, it is suggested that 0.3% laurel oil addition can be used as a feed additive in tilapia culture, especially considering the data obtained from growth and histological analyzes. Further studies are deserved need to examine the effects of laurel oil on immunity and resistance to various stress factors in other fish.

Keywords: Laurel oil, *Oreochromis niloticus, Laurus nobilis*, growth parameter,biochemical composition

ORCID IDs of the author:
M.Y. 0000-0002-7011-886X;
Y.M. 0000-0002-9547-0966;
M.N. 0000-0002-5129-8498;
Ç.Ü. 0000-0003-0381-9321;
M.T. 0000-0001-9914-9523;
T.A. 0000-0003-2375-2224

[1]Iskenderun Technical University, Faculty of Marine Sciences and Technology, Department of Aquaculture, Iskenderun, Hatay, Turkiye

[2]Istanbul University, Faculty of Aquatic Sciences, Department of Aquaculture and Fish Diseases, Istanbul, Turkiye

[3]Hatay Mustafa Kemal University, Institute of Natural and Applied Sciences, Department of Field Crops, Hatay, Turkiye

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Correspondence:
Metin YAZICI
E-mail:
metin.yazici@iste.edu.tr

INTRODUCTION

The number of fish caught in the world has reached the maximum level. Although aquaculture accounts for half of the total global production, it has grown rapidly over the past 30 years to meet people's food and nutrition security targets due to the increasing world population (Stratev et al., 2018). As aquaculture is an economical, quality, and healthy protein source, it has become a commercial sector that has an important role in meeting the global food demand and in contributing to the national economy in the future (Cottrell et al., 2021; Reverter et al., 2021). Due to these potentials, there are important expectations for the growth of aquaculture (Brugere et al., 2021). However, there are many challenges hindering the development of aquaculture (Reverter et al., 2021).

Aquatic organisms are under stress due to overstocking, deterioration of water quality, and malnutrition, weakening their immune systems and becoming more susceptible to diseases (Stratev et al., 2018; Dinardo et al., 2020; Lieke et al., 2020). This facilitates the emergence and spread of more virulent pathogens

in aquaculture systems (Reverter et al., 2021). In addition, inadequate hygiene facilitates the spread of pathogenic microorganisms to different regions by fish and equipment and leads to high mortality rates (Stratev et al., 2018; Lieke et al., 2020). Despite all efforts, economic losses from a disease outbreak in the aquaculture sector are estimated to be over $9.5 billion per year (Reverter et al., 2021).

The unconscious and continuous use of antibiotics for treatment and prevention of infectious diseases in aquaculture leads to the development of antibiotic-resistant bacteria, pollution of the aquatic environment, and accumulation of toxic residues in fish. This situation causes a global health problem among humans (Stratev et al., 2018; Reverter et al., 2021).Therefore, various alternative strategies such as the use of functional feed additives and vaccination have been suggested to prevent disease outbreaks and minimize the use of antibiotics in aquaculture (Stratev et al., 2018; Reverter et al., 2021). Studies in recent years have revealed that safer, easier to produce, biodegradable, and economical functional plant feed additives come to the fore (Awad & Awaad, 2017; Abdel-Latif et al., 2020; Dinardo et al., 2020; Hoseinifar et al., 2020).

The World Health Organization encourages the use of phytogenic substances (plantderivatives), their extracts, and phytochemicals (Kaur & Shah, 2017).

Although medicinal plants have been widely used to treat diseases in humans for thousands of years, studies of their effects on growth performance and protection of fish diseases have not yet reached the desired levels. Medicinal plants have an important potential in aquaculture, not only as a therapeutic tool but also in promoting growth and preventing stress and infectious diseases (Stratev et al., 2018; Hoseinifar et al., 2020; Dawood et al., 2020).

One of the plants with this potential is laurel, a medical and aromatic plant. *Laurus nobilis* is a tree belonging to the Lauraceae family, which is usually evergreen and always green in winter, and can reach 2-10 m in height. Being one of the richest countries of 600 medicinal and aromatic countries, Turkey is the largest supplier, accounting for 90% of the world's laurel production with a production of 32600 tons (Yilmaz & Deniz, 2017; Yilmaz & Ciftci, 2021).

Laurel leaves and their products are increasing every year depending on their use in many areas such as medicine, food, chemistry, and cosmetics (Yilmaz & Ciftci, 2021)). Dried leaves of laurel are used in many countries as a culinary seasoning, aromatic flavoring, and food preservative (Kara et al., 2020; Molina et al., 2020; Yilmaz & Ciftci, 2021). Soap, perfume, and body lotion are produced from the oil of laurel leaves (Yilmaz & Ciftci, 2021). In addition, to its use in the food, spice, and cosmetic sectors, it also meets the needs of many sectors with the essential oil (1-3%) obtained from its leaves and the fixed oil derived from its seeds (Yilmaz & Ciftci, 2021). It has been reported that non-toxic bay extracts act as a natural antibiotic in preventing food contamination by affecting the biofilm formation and virulence of Gram (+) and Gram (-) bacteria (Molina et al., 2020).

Laurel plant with its anthocyanin content in its fruit, is also used as a natural dye in the cosmetics, pharmaceutical, and food industries (Celik & Gul, 2020; Yilmaz & Ciftci, 2021). It is thought that the antioxidant, antiseptic, anti-inflammatory, anticonvulsant, antifungal, and immune-modulating properties in bay leaves are caused by 1.8 cineole and eugenol derivatives (Yilmaz & Deniz, 2017; Yilmaz & Ciftci, 2021). It has been reported that, in addition to methyl eugenol and 1.8-cineol, compounds such as camphor, β-caryophyllene, myrcene, eugenol, p-cymene, α-pinene, and β-pinene are found in essential oils obtained frombay leaves.

Due to the large number of plants grown in Turkey and especially endemic species, the medical evaluation of species has gained importance in recent years (Kara et al., 2020). Laurel (*Laurus nobilis*) is also a plant grown in Turkey and used medicinally (Karik et al., 2015; Kivrak et al., 2017; Celik & Gul, 2020). Apart from its beneficial pharmacological properties, the usage area of bay leaf essential oil is gradually expanding due to its many advantages such as thermal stability, and non-phototoxic effect (Yilmaz & Deniz, 2017). However, there are very limited studies on the use of laurel in aquaculture (Cagiltay et al., 2011; Turan et al., 2016; Dernekbasi et al., 2017). Although these medicinal plants have been tested as an immune mechanism in trout (Bilen & Bilen, 2012). Until now, there is no study to assess the effects of this plant oil on growth performance in tilapia. This is the first research to investigate the growth, biochemical composition, and histological effects of laurel oil use in tilapia (*Oreochromis niloticus*) which is the second most produced fish group worldwide after carp, which makes a very important contribution to global food security.

MATERIALS AND METHODS

Experimental diet and experimental design

The present study was carried out at Iskenderun Technical University Fisheries Application and Research Unit. Tilapia (*Oreochromis niloticus*) fingerlings used in the current study were provided by the Aquaculture Unit of Faculty. Fish were adapted for a week before the study. During the adaptation period, the fish were fed with the control diet as *ad libitum* 3 times a day. Trout feed supplied from a commercial enterprise was used as control feed (Table 1). 180 fish (12.0 ± 0.02 g) were randomly distributed to 12 tanks with a 500-liter capacity, 15 fish per tank. In the study, a semi-open recirculating system supported by air stones was used. The experiment was performed in a Completely Randomized Design with three replications. The fish were fed with experimental diets twice a day at 9:00 am and 17:30 pm as apparent satiation. During the experiment, the water temperature varied between 19-24 °C, and the pH was 7.5-8.5.

Natural photoperiod application was applied (12 D: 12L). The dissolved oxygen level in the tanks was measured as approximately 5 mg L^{-1}. The commercial *Laurus nobilis* oil used in the study was supplied from laurel leaves in Hatay.

Experimental feeds were prepared by mixing 0% (Control), 0.3%, 0.6% and 1.2% laurel oil with 8 ml distilled water for 100 g feed. To ensure a homogeneous mixture of laurel, oil was added to the

feed, Alphi 1 (Hexagon Product Development Pvt. Ltd. India) was mixed in a multi- dimensional mixer at 80 revolutions for 10 minutes. Before adding the laurel oil, and distilled water mixture to the feed, the continuity of homogeneity between oil and water was ensured by continuously mixing them. After mixing, the pellets were dried for a certain period and then kept at 4°C until use.

Table 1. Ingredients (%) and chemical composition (%) of the control diets.

Moisture - %Max	10
Crude Protein - % (Min.)	48
Crude Oil - % (Min.)	18
Crude ash -% (Max.)	10
Celulose -% (Max.)	2
Gross Energy- (Kcal/kg.) (Min.)	4800
Digestable Energy-(Kcal/kg.) (Min.)	4300
Metabolic Energy (Kcal/kg. (Min.)	4000
OMEGA-3 (Gr./kg.) (Min.)	10
OMEGA-6 (Gr./kg.)W3/W6	3
Ca % (Min./max.	2.5

Raw materials:Fish Meal, Fish Oil, Soybean meal, Soybean oil, Chicken meal, Wheat Gluten, Corn Gluten, Vitamin and Mineral.

Growth parameters

Fish were weighed and sampled after 24 hours of fasting. The growth performance parameters of *Oreochromis niloticus* were performed at 20-day intervals using the following formulas (Yazici et al., 2021).

(WG, g) = (final weight (fw) − initial weight (iw)),

(SGR, % day−1) = (ln fw − ln iw) / times (days) × 100,

(FCR, %)= [feed intake (g) / weight gain (g) and

(SR, %) = (final number of fish / initial number of fish) * 100 were calculated.

WG: Weight gain; SGR: Spesific Growth Rate; FCR: Feed Conversion Ratio; SR: Survival Rate

Biochemical composition of fish and experimental diet

After the trial was completed, it was carried out according to the AOAC (1997) procedures to determine the crude protein content of the control and treatment group fish and the experimental feed. Bligh & Dyer (1959) method was used to evaluate the crude lipid content, and the method defined by Vollenweider et al. (2011). It was used to determine the raw ash amount. Body proximate analysis of tilapias and experimental diets were applied in triplicate.

Fatty acid contents and Essential oil components of *Laurus nobilis* oil

Fatty acid methyl esters (FAMEs) of *Laurus nobilis* oil were prepared using the protocol reported by Parry et al., 2005.

Essential oil components of laurel oil were determined with Gas Chromatography equipped with a 5% Phenyl Polysylphenylene siloxane column (0.25 mm diameter*60 m long column with 0.25 µm thickness. A Flow rate of helium was used as carrier gas was 1mL min -1. MS transfer line, ionization, injection, and column temperatures were 250 °C, 220 °C, 220 °C, and 50 °C, respectively. Column temperature increased 3°C per minute from 50 °C to 220 °C. Each compound was determined with the X Calibur Program and Mas Spectra (Kocer et al., 2022).

Histological examination

At the end of the 60th day of the study, the digestive tracts, and livers of 5 fish were taken randomly from each treatment group for histological examination and fixed with 10% PBF, (phosphate buffered formaldehyde). Following fixation, samples of the tissue were covered with embedding material and then placed in paraffin blocks. Tissue sample sections of 4-5 µm thickness were examined under a light microscope after staining with the hematoxylin-eosin (H&E) staining method (Yazici et al., 2021).

Statistical analysis

Data on the Nile tilapia of *Oreochromis niloticus* growth performance parameters of different levels of laurel oil were subjected to One-way ANOVA. Normality and homogeneity were tested by using Kolmogorov Smirnov and Levene tests, respectively. All statistical analyses were done by using SPSS 17.0 software according to Duncan's New Multiple Range Test to identify the 5% level of significance of variance among the treatment groups's means. All experimental data were expressed as mean ± standard deviation (SD).

RESULTS AND DISCUSSIONS

Growth performance

In this study, the effects on growth performance were investigated, body composition, and histology of Nile tilapia (*Oreochromis niloticus*) of *Laurus nobilis* oil supplemented with feeds. In this study, the addition of laurel oil to the feeds did not affect the growth parameters of tilapias such as WG, SGR, FCR, and SR ($p>0.05$). Ning et al. (2021) reported that when Genetically Improved Farmed Tilapia (GIFT) added 300 mg kg-1 Blend Essential Oil (EO) to tilapia feeds, did not cause any change in growth parameters such as WG, SGR, FCR, and SR in parallel with our study (Table 2). On the other hand, studies on different essential oils and fish species obtained variable results in growth performance. According to Ghafarifarsani et al. (2022) reported that no change was observed in SGR in carp fed with 1-2% Thyme EO added feed, and their results were consistent with our results. In contrast, they suggested that 1-2% Thyme EO addition to the subject caused an increase in WG, SR, and a decrease in FCR. Yousefi et al. (2022) found that WG, SGR, and SR values were higher than the control, while the FCR values were found to be lower than the control in the study they carried out with Thyme EO and Immunogen prebiotic added feeds in trout. Like our study, Abdel Latif et al. (2021), when they added Origano EO (1-2%) to carp feeds, they did not detect any change in FCR and SR values, but they reported an increase in WG and SGR, unlike our study. Heluy et al. (2020) obtained variable results at different rates in their study with Origano EO on trout. WG value was highest and FCR was lowest in fish fed with 0.75 g kg-1

OEO, while WG was lowest and FCR was highest in 1.5% supplemented groups. No changes were observed in SGR and SR in parallel with our current study.

Table 2. Growth performance of Nile tilapia fed with different *Laurus nobilis* oil level for 60 days. Data expressed as mean ± standard deviation (SD).

Parameter	Control	0.3%	0.6%	1.2%
IW (g)	12.77±0.18[a]	12.75±0.14[a]	12.77±0.18[a]	12.75±0.22[a]
FW (g)	35.86±1.94[a]	32.28±0.41[a]	30.07±1.57[a]	33.37±2.43[a]
WG (g)	23.08±1.88[a]	19.53±0.54[a]	17.30±1.40[a]	20.62±2.65[a]
FCR	1.01±0.11[a]	1.14±0.02[a]	1.38±0.17[a]	1.05±0.19[a]
SGR (%)	0.84±0.05[a]	0.75±0.02[a]	0.66±0.09[a]	0.90±0.16[a]
SR (%)	100	100	100	100

Lines assigned with the same letter show no significant difference (p>0.05).

Body composition

The fish body composition is important in terms of reflecting the nutritional status and health of the generally farmed species (Hoang et al., 2019). It is also important for consumers who prefer to consume and buy fish with lower lipids and higher protein content (Safavi et al., 2019). Recently, the effects of some essential oils on body composition have been investigated. For instance, it has been reported that the addition of Origanum EO (Heluy et al., 2020) and Blend EO (Ning et al. 2021) to tilapia feeds, and Thyme EO to carp feeds (Abdel-Latif et al., 2021; Ghafarifarsani et al., 2022) did not affect body composition. In this study, in line with the studies mentioned, the addition of laurel oil did not affect the biochemical composition of the fish except for lipids (Table 3). The addition of 0.3% and 0.6% laurel oil was found to be lower than both the control group and the 1.2% group. Lipids are well-known as one important dietary component, which tend to show greater fluctuations than other carcass components (Peng et al., 2008). Rasoarahona et al. (2005) revealed that the reasons for the differences observed in body lipid levels of tilapia species may be related to species, season, age, sexual maturity, geographical origin, and diet composition. It has been suggested that optimum dietary lipid content is important, as low, and excess dietary lipids can have adverse effects on fish growth, feed consumption, health, and immunity (Rahimnejad et al., 2015).

Table 3. Body composition of Nie tilapia fed diet supplemented with different proportions of *Laurus nobilis* levels. Data expressed as mean ± SD.

Biochemical compositions of fish			
Treatment groups	**Protein**	**Lipid**	**Ash**
Control	76.63±0.09	4.71±0.26[a]	4.26±0.46
0.3%	69.30±3.93	3.49±0.21[b]	4.38±0.79
0.6%	74.08±1.67	3.61±0.37[b]	3.61±0.29
1.2%	68.43±5.47	4.26±0.06[a]	3.40±0.21

Values in a row with different superscripts indicate significant difference (p<0.05)

Table 4 shows the biochemical composition of the feed.

Table 4. Biochemical composition of Commercial diet supplemented with different proportions of *Laurus nobilis* levels. Data expressed as mean ± SD.

Biochemical compositions of feed			
Treatment groups	**Protein**	**Lipid**	**Ash**
Control	59.62±0.57	19.31±0.69[a]	6.94±0.98
0.3%	59.99±0.20	21.14±0.16[b]	7.01±0.13
0.6%	59.11±0.39	21.83±1.17[ab]	6.86±0.18
1.2%	59.12±0.41	22.60±1.18[ab]	7.16±0.22

Values in a row with different superscripts indicate significant difference (p<0.05)

Fatty Acid Contents and Essential Oil Components of *Laurus nobilis*

Fatty acid values of *Laurus nobilis* were given in Table 5. Saturated and unsaturated fatty acids of *Laurus nobilis* values were 36.2% and 62.89%, respectively. The highest values of saturated and unsaturated fatty acids were observed in 18.34% (Palmitic Acid-C16:0) and 35.72% (Oleic Acid-C18:1n9), respectively. The level of Lauric acid (C12:0) from saturated fatty acids was 15.95%. The amount of linoleic acid (C18:2n6) obtained in the study was 25.04%.

Table 5. Fatty Acids of *Laurus nobilis* oil (%).

Fatty Acids	%
C10:0	0.31
C12:0	15.95
C13:0	0.01
C14:0	0.75
C15:0	0.02
C16:0	18.34
C17:0	0.04
C18:0	0.78
Saturated Fatty Acids	36.2
C16:1n7	0.6
C16:2n4	0.01
C18:1n9	35.72
C18:2n6	25.04
C18:3n3	1.06
C20:1n9	0.42
C20:2n6	0.01
C20:4n6	0.03
Unsaturated Fatty Acids	62.89
Total Fatty Acids	99.09

Table 6 shows the essential oil components of *Laurus nobilis*. In the study, essential oil components of *Laurus nobilis* oil such as Linalool, Elemene, Trans-Caryophyllene, Cis-α- Bisabolene, A-Terpinyl Acetate, Methyleugenol, β-Eudesmol were determined in low levels.

Table 6. Essential oil components of *Laurus nobilis* oil (%)

Chemical Components	%
Linalool	0.01
Elemene	0.2
Trans-Caryophyllene	0.06
Cis-α-Bisabolene	0.06
A-Terpinyl Acetate	0.16
Methyleugenol	0.01
β-Eudesmol	0.02

Fatty acid and essential oil components

In the current study, lauric acid, palmitic acid, oleic acid, and linoleic acid levels were obtained at 15.95%, 18.34%, 35.72%, and 25.04%, respectively. Marzouki et al. (2008) found that the most common fatty acids of the *Lycium barbarum* (whole berry oil) were lauric acid (27.6%), Oleic acid (27.1%), Linoleic acid (21.4%), and Palmitic acid (17.1%). The study results of Ayanoglu et al. (2018) revealed that the major fatty acids of the *Laurus nobilis* species were lauric acid, oleic acid, palmitic acid, and linoleic acid. Yilmaz & Deniz (2018) showed that the most plentiful fatty acids for fleshy parts of *Laurus nobilis* berries were oleic acid (27.06% - 48.93%) and linoleic acid (29.18%-34.39%) from unsaturated fatty acids and palmitic acid (20.70%-33.07%) from saturated fatty acids. Petkova et al. (2019) indicated that the main fatty acids (FAs) in the lipid fractions were oleic, palmitic, and linoleic. The results obtained from fatty acid levels in the study are consistent with the literature.

Previous studies have shown that around 270 essential oil components can be found in bay leaves. Among them, 1,8-cineol (22-66.90%), sabinene (4.5–12.7%), α-terpinyl acetate (4.09-22.60%), α-pinene (2.2–15.9%), linalool (0.9-26.9%), α-terpineol (0.9%–12.0%), β-pinene (1.9%) –15.3%, terpineol-4 (0.9–4.1%) constitute the major part (Santos et al., 2014; Karik et al., 2015; Kivrak et al., 2017; Fidan et al., 2019; Stefanova et al., 2020; Kizak et al., 2020). Laurel oils have a variety of pharmacological effects, including antimicrobial, cytotoxic, and immune-modulating. The properties and compositions of the essential oil vary according to the harvested region, altitude, sun exposure time, and harvest conditions. The results in this study were found at lower levels than those reported in the literature.

Histological Examination

The effects of adding laurel oil at various rates on tilapia liver tissue are shown in Fig. 1, 2, 3. The liver, which is a primary organ in the metabolizing of nutrients, can reflect deteriorations or effects in nutritional status with changes in its histological structure (Caballero et al., 2004). In this study, large fat vacuoles were observed around the exocrine pancreas in tilapia fish fed with 0.6% and 1.2% laurel oil. In parallel with the present study, dense vacuoles have been reported to develop around the exocrine pancreas of the liver of tilapia fish fed with menthol oil added to the diets of tilapia fish kept at high stocking density (Dawood et al., 2020).

The nuclei of hepatocyte cells were localized at the cell margin. In addition, the presence of dense zygmogen granules was detected between the acinar cells located in the exocrine part of the pancreas (Fig. 1a, b). It was observed that the nuclei of hepatocyte cells, in which lipid vacuoles decreased in the liver tissue of fish fed with 0.3% laurel oil, were more centrally located related to the control group. Zygmogen granules were also found in the acinar cells of the pancreatic tissue (Fig. 2a, b). Although the number of lipid vacuoles increased in fish fed 0.6% laurel oil compared to the 0.3% group, the nuclei of hepatocyte cells were found to be centrally located. While the formation of large fat vacuoles was observed on and around the outer surface of the exocrine pancreas, the presence of zygmogen granules in the exocrine pancreas was not detected (Fig. 3a, b). Fish fed with 1.2% laurel oil had an increase in fat vacuoles in the liver, as well as swelling of large blood vessels, and large fat vacuoles increased significantly in exocrine tissue (Fig. 4a, b).

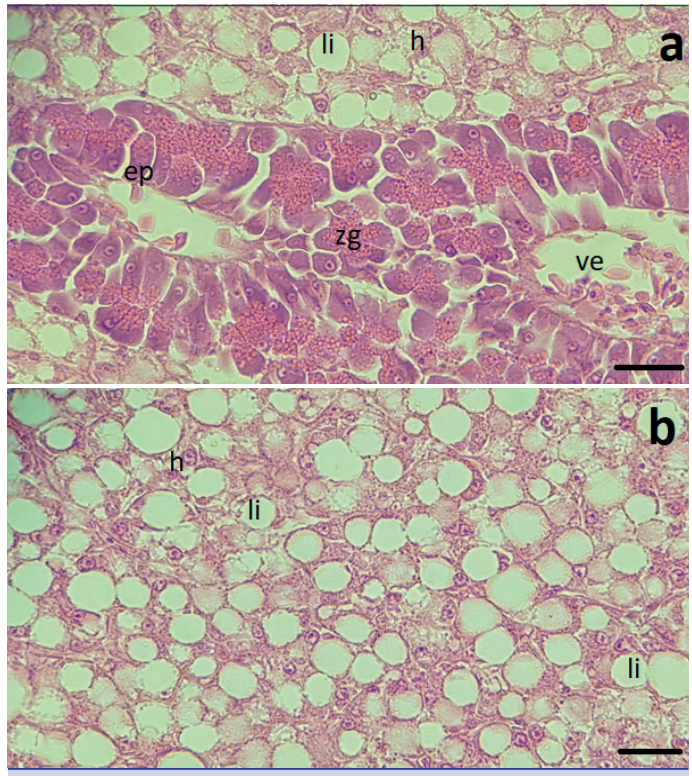

Figure 1. Liver tissue of fish in the control group bar: 15μm (H&E). zg: zymogen granules, ep: exocrine pancreas, h: hepatocytes, li: lipid vacuoles, ve: vein

In our study, vacuolization was observed in hepatocyte cells with the addition of laurel oil. Similar to our study, it was observed in tambaqui fry-fed 1.0 mL kg $^{-1}$ginger EO (Chung et al., 2021) and rainbow trout fed with *Origanum onites* essential oil (Yigit et al., 2017). However, in the tilapias examined, no pathological picture such as necrosis was found in hepatocyte cells reported by these researchers.

Intestinal tissue

The effects of laurel oil on the foregut (Fig. 5a, b, c, d) midgut (Fig. 6a, b, c, d) and hindgut (Fig. 7a, b, c, d) have been shown.

It was determined that the nuclei of enterocyte cells in the foregut tissue of the control group were centrally located and the number of

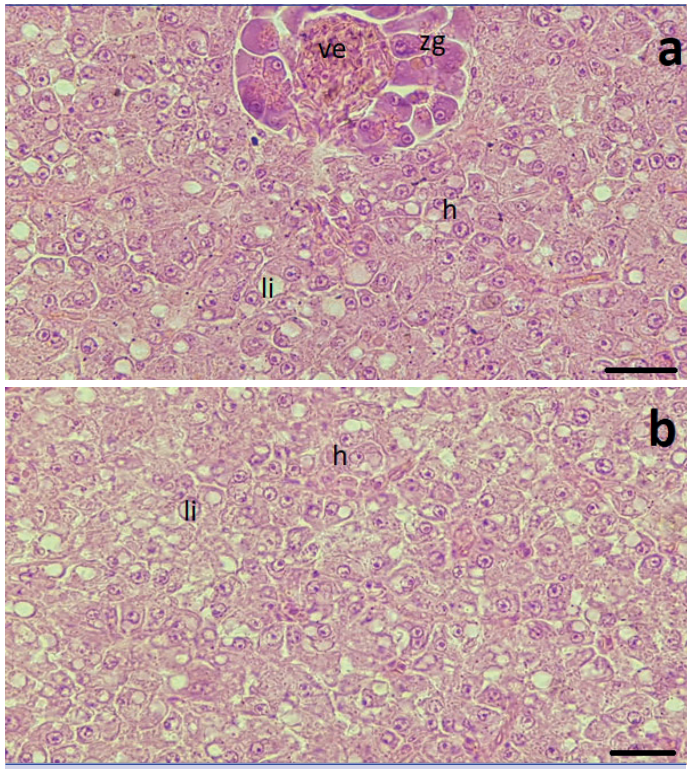

Figure 2. Liver tissue of tilapia fish fed with 0.3% laurel oil supplement bar: 20µm. zg: zymogen granules, h: hepatocytes, li: lipid vacuoles, ve: vein

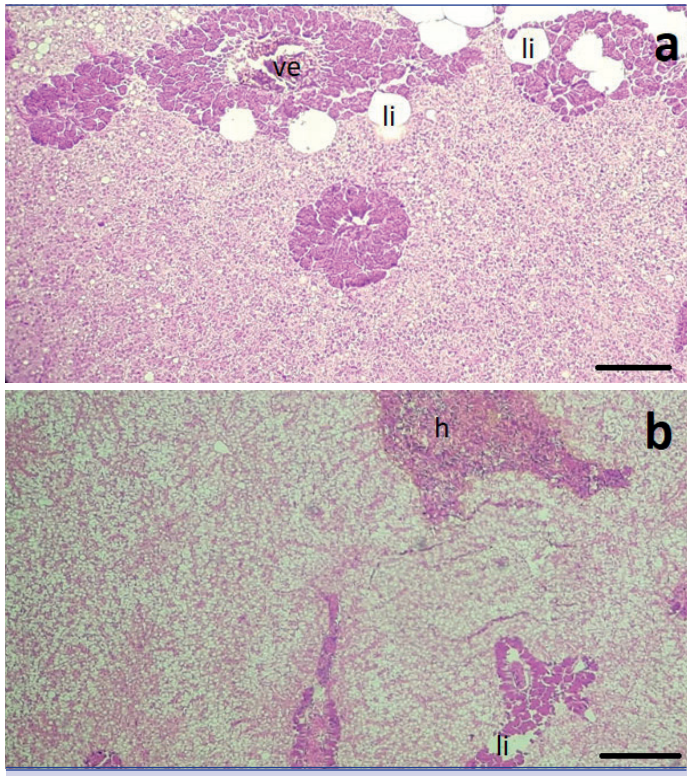

Figure 4. Liver tissue of tilapia fish fed with 1.2% laurel oil supplement bar: 100µm (H&E). zg: zymogen granules, h: hepatocytes, li: lipid vacuoles, ve: vein

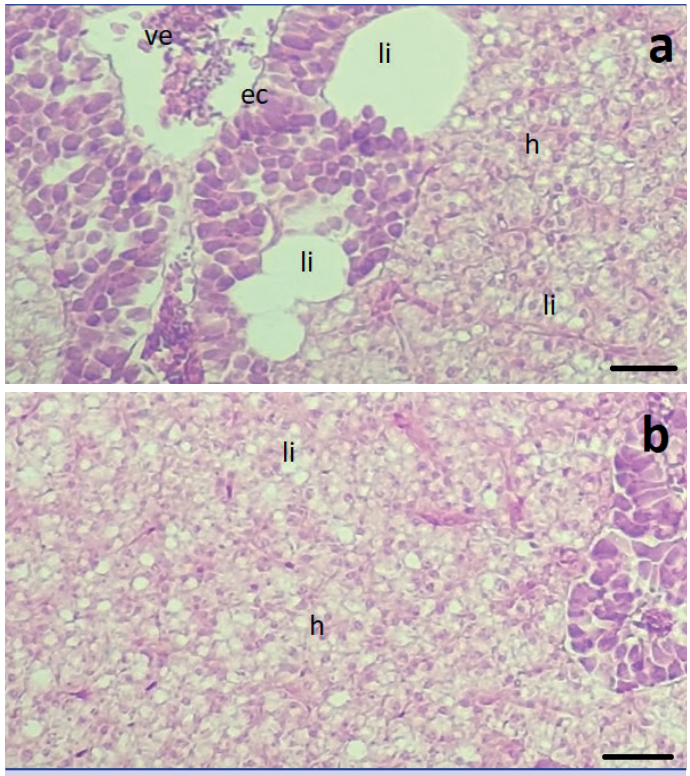

Figure 3. Liver tissue of tilapia fish fed with 0.6% laurel oil supplement bar: 20µm (H&E). zg: zymogen granules, ec: exocrine pancreas, h: hepatocytes, li: lipid vacuoles, ve: vein

goblet cells was low (Fig. 5a). It was observed that goblet cells in the intestinal tissue of fish fed 0.3% laurel oil was localized towards the ends of the villi (Fig. 5b). Fish fed with 0.6% laurel oil showed an increase in the number of goblets cells as well as thickening and branching formations in the villi compared to the control group (Fig. 5c). In fish fed 1.2% laurel oil, on the other hand, branching of the villi and enlargementof the lamina propria were observed (Fig. 5d).

EOs given at appropriate rates provide improvement in intestinal histology, while other rates either causes histological disorders or shows no effect (Valladao et al., 2017; Brum et al., 2018).In this study, it was determined that the surface area increased with the thickening of the villi rather than the longitudinal growth in the hindgut of the fish fed with laurel oil.

Branching was detected in the midgut villi of the fish belonging to the control group (Fig. 6a). In the midgut tissue of fish fed 0.3% laurel oil, it was observed that these branches increased, the lamina propria expanded, and there was a serious increment in the number of goblet cells (Fig. 6b). However, it was determined that the short villi enlarged and the lamina propria thickened in the midgut of the fish fed with 0.6% laurel oil (Fig. 6c). It was noted that this thickening was much wider in fish fed 1.2% laurel oil, and there were also thickenings in the mucosal layer of the midgut tissue (Fig. 6d).

Goblet cells play a very important role in protecting intestinal barriers against pathogenic microorganisms by producing mucus and antimicrobial substances. They also stimulate intestinal local immunity by secreting chemokines and cytokines (Knoop

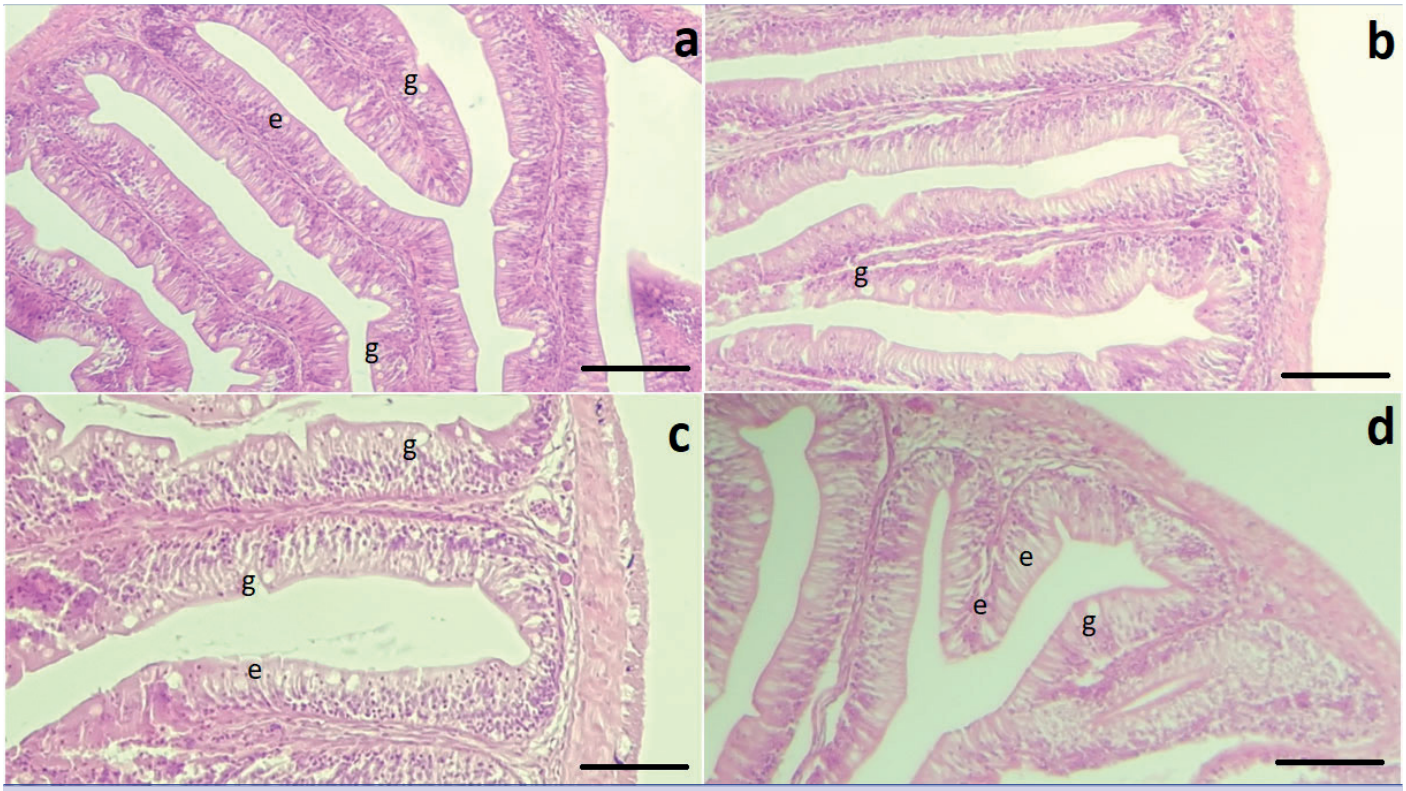

Figure 5. Foregut histology of fish fed control group (a), 0.3% (b), 0.6% (c), and 1.2% (d) with laurel oil (a,b, and d bar: 100µm, c bar 60µm) (H&E). e: enterocyte, g: goblet cells

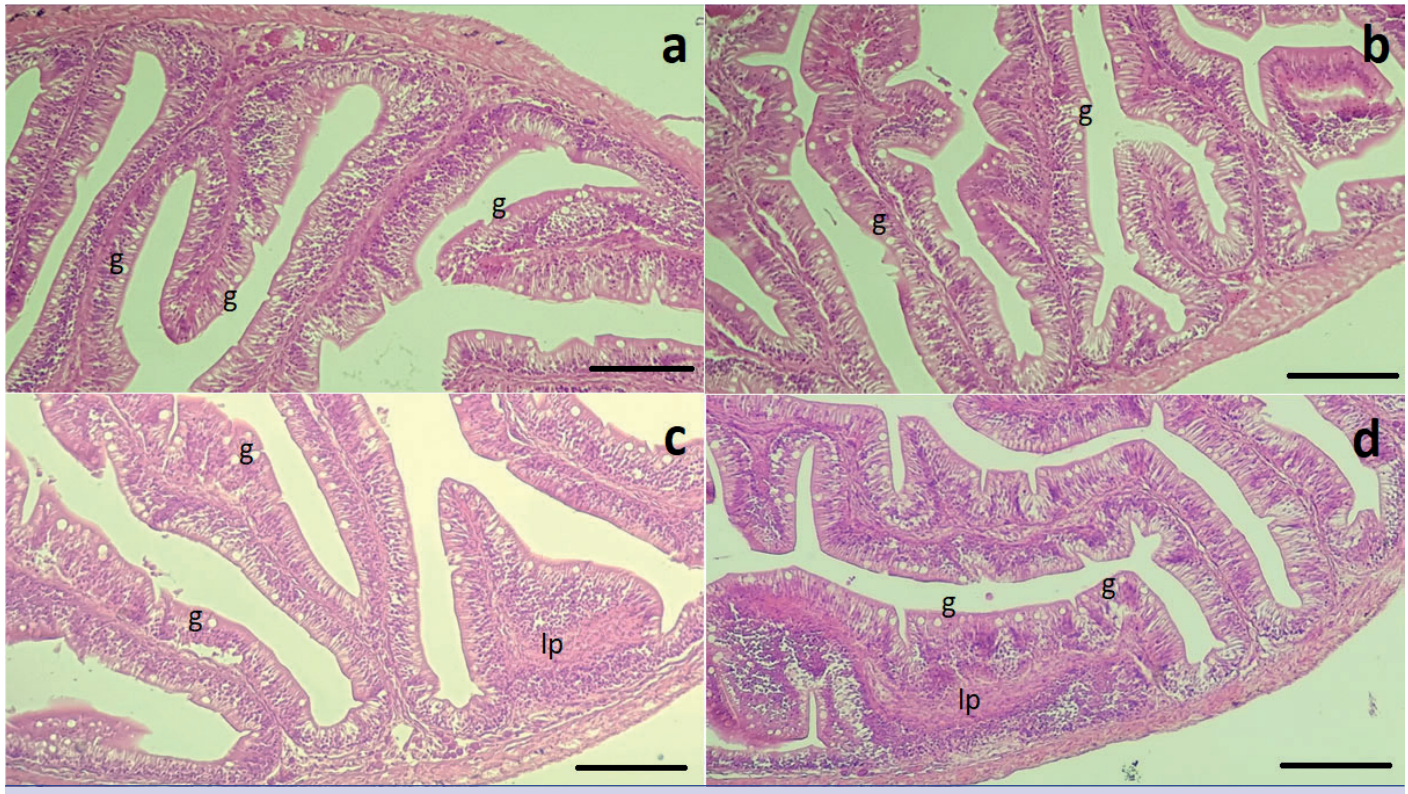

Figure 6. Histological sections of midgut tissues of tilapia fish fed with control (a), 0.3% (b), 0.6% (c), and 1.2% (d) laurel oil diet. (bar 100 µm) (H&E). g: goblet cells, lp: lamina propria

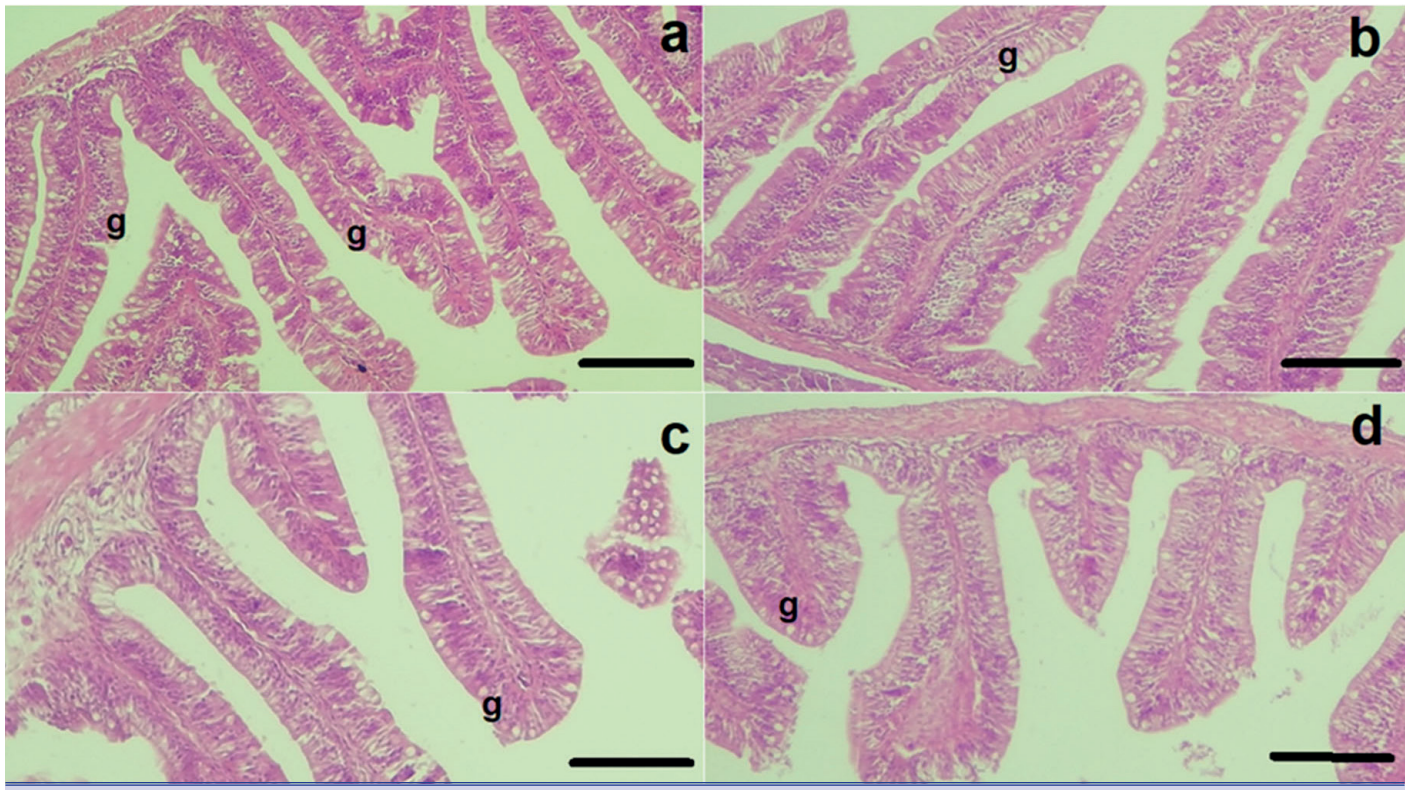

Figure 7. Histological sections of hindgut tissues of tilapia fish fed with control (a), 0.3% (b), 0.6% (c), and 1.2% (d) laurel oil diet (bar 100 µm).

& Newberry, 2018; Dawood, 2021). In the present study, an increase in the number of goblet cells was observed in the midgut of tilapia fed with laurel oil-added feeds. In contrast, Chung et al. (2021) reported that essential oils obtained from ginger reduced the height of villi in the intestines and reduced goblet cells. In addition, it is suggested that 0.6% and 1.2% laurel oil in the midgut, which is important for digestion, causes cell infiltration in the lamina propria, which may cause a decrease in the absorption of nutrients by reducing the contact between villi and nutrients, as reported by other researchers (Valladao et al., 2017; Chung et al., 2021).

Branching was detected in the hindgut villi of the fish of the fed control diet (Fig. 7a). It was determined that these branches were decreased, and the villi enlarged in the hindgut tissue of fish fed with 0.3% laurel oil (Fig. 7b). Thickening of the lamina propria and a decrease in the number of goblet cells were detected in the hindgut of the fish fed with 0.6% laurel oil (Fig. 7c). It was noted that the villi shortened and thickened and there was a serious decrease in the number of goblet cells in fish fed 1.2% laurel oil (Fig. 7d).

Intestinal anatomy and histology are very important for nutritional evaluation and immunological functions in fish health. Therefore, histological examination of the gut can be considered a potential indicator of the overall health of the fish and the efficacy of feed additives (Yigit et al., 2017; Abdel-Latif et al., 2021; Acar et al., 2021).

CONCLUSION

As a result of the study, the addition of laurel oil to the feed did not show negative results in growth performance. In addition, unlike other groups, histopathological findings were not found in the intestines and livers of fish fed 0.3% bay oil. In addition, it has shown positive effects such as branching of villi in the intestines, expanding the food use surface area in the midgut, increasing goblet cells, and reducing the fat vacuoles in the liver. Therefore, it is recommended to use 0.3% laurel oil as an additive to feeds. More experiments should be designed to determine the efficacy of laurel oils in different fish species farmed as well.

Conflict of Interest: The authors declare that there are no conflicts of interest.

Ethical Approval: Animal care and experiments were carried out considering national and /or international guidelines.

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Research Article

Seasonal Length-Weight Relationships and Condition Factors of *Mystus tengara* (Hamilton, 1822) in Two Habitats

Arun Jana[1], Godhuli Sit[1], Purnachandra Das[1], Angsuman Chanda[1], Sanjat Kumar Sahu[2]

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ABSTRACT

The aim of this study is to examine the relationship between length, weight, condition factor (K), and relative condition factor (Kn) of *Mystus tengara* (Hamilton, 1822), with special emphasis on seasonal variation of growth patterns, productivity, stocks, and conservation in two distinct ecosystems: pond and river. The results demonstrate that the species did not strictly adhere to the predicted cube law and had allometric growth patterns throughout the season.The condition factor (K) ranged from 0.33 to 1.49, the relative condition factor (Kn) varied from 0.44 to 1.77, and the 'b' values ranged from 2.00 to 3.29. The post-monsoon season saw the greatest average values for the regression parameter (b), condition factor (K), and relative condition factor (Kn). The r^2 values represent a significant relation between length and weight in two habitats and throughout all seasons. The Pearson's correlation indicates some positive and negative significant correlations among season, length, weight, condition factor, and relative condition factor in the study area ($P<0.01$ and $P<0.05$). The Post Hoc test indicates no significant habitat-based association between the same characteristics. However, there is a significant ($P<0.05$) seasonal link between relative condition factor, condition factor, weight, and length. As a result, these current findings will assist fisheries managers in creating efficient strategies for the long-term management of *M. tengara* in its habitats.

Keywords: Condition factor, Length, *Mystus tengara*, Season, Weight

ORCID IDs of the author:
A.J. 0000-0003-3547-6247;
G.S. 0000-0002-5821-7937;
P.D. 0000-0003-4331-1143;
A.C. 0000-0003-1781-5057;
S.K.S. 0000-0003-2907-9253

[1]Natural and Applied Science Research Center, Post Graduate Department of Zoology, Raja Narendra Lal Khan Women's College (Autonomous), Midnapore, Paschim Medinipur, West Bengal, India

[2]Department of Environmental Science, Sambalpur University, Jyoti Vihar, Burla, Sambalpur, Odisha, India

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Correspondence:
Angsuman CHANDA
E-mail:
chandaangsuman182@gmail.com

INTRODUCTION

Length-weight interactions are important for both practical and fundamental comparative growth studies in fishery management (Moutopoulos & Sterigiou, 2002). Fish length-weight relationship (LWR) data are significant for assessing and managing the fish population (Martin-Smith, 1996). Fish biology focuses on the connection between length and weight for a number of reasons, such as finding out the yield, finding out the standing crop biomass, estimating the age of a fish based on its weight, determining the age structure and function of a fish population, observing growth, stock differentiation, ecological modelling, and doing acoustic surveys (Eduardo et al., 2019; Siddique et al., 2016; Ozaydin et al., 2007; Froese, 2006; Haimovici & Velasco, 2000). The length and weight relationship was selected to gather data on the condition of the fish and to assess if the somatic growth is allometric or isometric (Ujjania et al., 2012). According to the Cube law ($W=L^3$), fish frequently grow isometrically (Lagler, 1952). However, a deviation from Cube's rule in the length-weight relation is always viable due to the many factors of the environment that affect the physicochemical properties of the water in which individual fish species live. LeCren (1951) revised the Cube equation as $W=aL^b$ to calculate the data from weight and length measurements for their connection throughout the life cycle phases of fish.

The condition factor, a quantitative index of health for fish that affects growth, reproduction, and viability, will decide the favourable outcome of current and future populations. The condition of a fish changes based on a complex interaction of physiological parameters, parasite diseases, and dietary conditions.The relative condition factor (Kn) can be used to determine the fitness of fish. A fish farmer can access the weight of fish to a calculated standard to decide whether the fish are in better or worse condition than the standard. Fish can be compared in terms of their general health, body mass index, and gonad development using the relative condition factor (Thomas, 1969). *Mystus tengara* (Hamilton, 1832) is a significant catfish belonging to the family Bagridae of the order Siluriformes, with strong nutritional and decorative characteristics. Prior to our studies, few scientists had investigated the length and weight relations and the condition factor in this species. The relation between length, weight and relative condition factor of *M. tengara* were researched by Kalita et al. (2017) in the Lechia-Pavomaribeel wetland of Dhemaji, Assam, India. Length-Weight relationships of *M. tengara* were reported by Gupta and Banerjee (2015) from Baruipur, South 24 Paraganas, West Bengal. However, no thorough investigation into the season and habitat effects on length, weight, condition factor and relative condition factor of *M. tengara* has yet been conducted. Therefore, this present study is the first to report the seasonally habitat-wise relationship among relative condition factor, condition factor, weight, and length of *M. tengara* in the Jhargram and Paschim Medinipur districts of West Bengal, India.

MATERIALS AND METHODS

Specimen collection & identification

Between June 2019 and May 2021, specimens were collected from two habitats (rivers and ponds) in two districts, Paschim Medinipur and Jhargram, throughout the pre-monsoon (March to June), monsoon (July to October)and post-monsoon (November to October) seasons. In total 64 specimens were collected from the study area during those seasons and from thetwo habitats. The specimens were identified based on existing literature (Jayaram, 2010).

Length (L) and weight (W) measurement

After identification, the length of each specimen was measured with a digital slide caliper (0.01 mm accuracy) and the weight by digital balance (0.01g accuracy) throughout different seasons and habitats.

Length and weight relation

Length and weight relationship was calculated using the formula **$W=aL^b$** (Le Cren, 1951)

Here,

W= Weight (g)

L= Length (cm)

a= Intercept or the initial growth index

b= Slope/ growth coefficient/ an exponent.

The logarithmic conformation of this equation is **LogW= Loga+ bLogL**

Condition factor (K)

The following formula was used to calculate Fulton condition factor of the species (**$K =100\times(W/L^3)$**) (Fulton, 1904)

Here,

W= Weight(g)

L= Length (cm)

Relative condition factor (Kn)

The following formula was used to estimate the Relative condition factor.

$Kn= W/aL^b$ (Le Cren, 1951)

Here,

W= weight (g)

L= Length (cm)

a & b=regression parameter

Data analysis

Finally, data were analysed with Pearson's Correlation, Regression, Post Hoc test used by Microsoft Excel-2019, SPSS-2021 and Originpro-2022 software system.

RESULT AND DISCUSSION

In the current study, the length and weight range of *M. tengara* was from 7.1 to 17.3 cm and 2.12 to 22.44 gm, respectively. The descriptive statistics of length, weight, 'K' and 'Kn'are presented in Table 1 and Figure 1. In contrast to the pre-monsoon and post-monsoon seasons, the monsoon season saw the maximum increases in length and weight (Figure 2). In the current study, table 6 shows the logarithmic and parabolic equations for the length-weight relationship. In the pond, the values 'b' and 'r^2' range from 2.28 to 3.29 and from 0.73 to 0.79, similarly from 2.01 to 2.69 and from 0.83 to 0.87 in the river, respectively (Table 2). The minimum 'b' value was seen in both habitats during the monsoon season, and the greatest was seen throughout the post- and pre-monsoon periods in the pond and river respectively. Pearson's correlation represents a significant correlation ($p<0.01$ & $p<0.05$) among season, length, relative condition factor, and condition factor; length has only a significant negative correlation with 'K', although weight has a positive correlation with 'Kn' ($p<0.01$) (Table 5 and Fig 4). The results of the Post Hoc test show that, except for the post monsoon and monsoon seasons, there is a seasonally significant ($p<0.05$) connection between length and condition factor but not between relative condition factor and weight (Table 6). The r^2 results show that length and weight are positively and strongly correlated throughout the year in both habitats (Table 2 and Figure 3). The value of 'b' only exhibits positive allometric growth in post-monsoon ponds and negative allometric growth in rivers and ponds throughout the other seasons. If the fish are not feeding well enough or if their environment, such as their physicochemical characteristics and/or their breeding season, is not conducive to their growth, negative allometric growth was observed (Deka &Bura Gohain,

Table 1. Descriptive statistics of length, weight of *M. tengara*.

Habitat	Season	Total Length (cm)				Total Weight (g)			
		Min.	Max.	Mean	SD	Min.	Max.	Mean	SD
POND	PREMONSOON	7.6	16.2	11.7797	±2.22198	3.96	22.31	14.1583	±5.25713
	MONSOON	8.1	17.1	12.8844	±2.0623	3.42	21.12	14.763	±4.63694
	POSTMONSOON	7.2	15.2	10.6688	±1.8253	2.12	19.08	11.8275	±5.42513
RIVER	PREMONSOON	7.8	16.9	11.6266	±2.43514	3.21	21.22	13.0022	±6.05971
	MONSOON	8.4	17.3	12.6625	±2.21076	3.8	22.44	15.2413	±4.49056
	POSTMONSOON	7.1	15.5	11.25	±2.17248	2.97	19.37	13.1564	±5.09321

N=64; Min=Minimum; Max=Maximum; SD=Standard Deviation

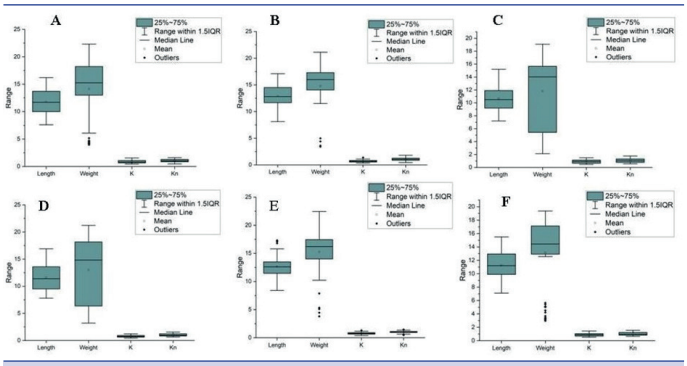

Figure 1. Length, Weight, K and Kn of *M. tengara*.
A-C= Pond; D-F= River; A & D= Pre-monsoon; B & E= Monsoon; C & F= Post-monsoon

Table 2. Regression parameters of *M. tengara*.

Habitat	Season	a	b	r^2
POND	PREMONSOON	0.0468	2.289146494	0.7855
	MONSOON	0.0296	2.407828679	0.7385
	POSTMONSOON	0.0043	3.293539607	0.7826
RIVER	PREMONSOON	0.0157	2.699501283	0.8798
	MONSOON	0.0905	2.005843224	0.8383
	POSTMONSOON	0.0248	2.56160902	0.8482

a=intercept; b=slope, R=coefficient correlation

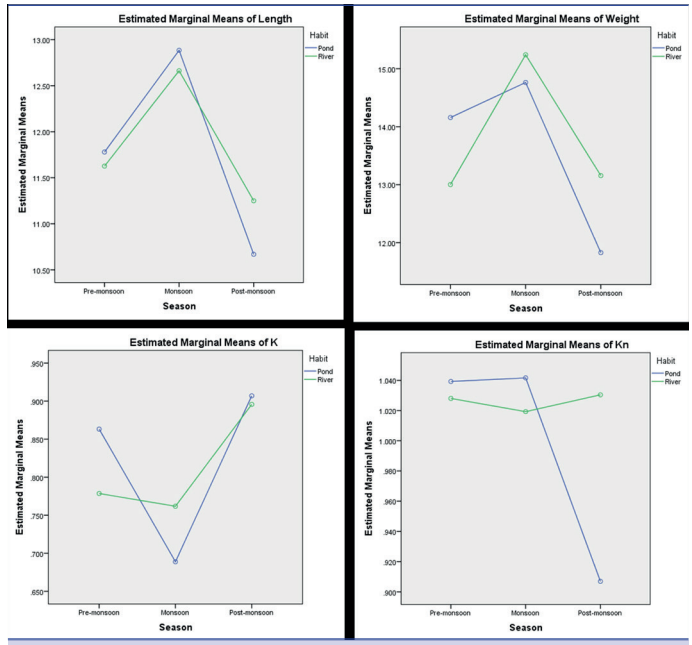

Figure 2. Seasonally changes length, weight, K, Kn of *M. tengara*.

2015; Weatherly, 1972; Soni & Kathal, 1953; Le Cren, 1951). Kalita et al. (2017) reported that the 'b' value was 2.07, and the Kn value ranged from 0.74 to 1.39 with 1.00±0.125 in *M. tengara* from Assam, India. Gupta and Banerjee (2015) reported that the 'b' values were 3.071, 3.119 and 2.941 for males, females, and mixed sex in the *M. tengara* from West Bengal, India. Hossain et al. (2006) represented b values of 2.96, 3.13, and 3.05 and also Victor et al. (2014) cited the same values of 2.732, 2.873 and 2.405 in

Table 3. Habitat-wise, seasonally parabolic and logarithmic values of *M. tengara*.

Habitat	Season	Parabolic Equation	Logarithmic equation
POND	PREMONSOON	W=0.0468L^2.289	LogW=-1.329+2.289logL
	MONSOON	W=0.0296L^2.407	LogW=-1.527+2.407logL
	POSTMONSOON	W=0.0043L^3.293	LogW=-2.363+3.293logL
RIVER	PREMONSOON	W=0.0157L^2.699	LogW=-1.803+2.699logL
	MONSOON	W=0.0905L^2.005	LogW=-1.043+2.005logL
	POSTMONSOON	W=0.0248L^2.561	LogW=-1.605+2.561logL

W= weight; L=length

combined sex, female, and male of the *M. vitatus*. For the combined sex of *M. vittatus*, Srivastava et al. (2013) and Hossain et al. (2009) recorded values of 'b' to be 2.88 and 3.27, respectively. For the combined sex of *M. cavasius*, 'b' values of 2.83, 2.91, 3.21, and 3.009 were recorded by Karna and Panda (2012), Hossain et al. (2012), Sani et al. (2010), Krishna Rao (2007), whereas Venkateshwarlu et al. (2007) had listed 'b' values of 2.7402 and 2.493 for the female and male in the same species. While Begum et al.

Table 4. Habitat-wise, seasonally condition factors of *M. tengara*.

Habitat	Season	Condition factor (Fulton, 1904)				Relative Condition factor (Le Cren, 1951)			
		Min.	Max.	Mean	SD	Min.	Max.	Mean	SD
POND	PREMONSOON	0.44	1.56	0.8632	±0.27968	0.47	1.61	1.0393	±0.28107
	MONSOON	0.33	1.38	0.6888	±0.211	0.44	1.81	1.0416	±0.27624
	POSTMONSOON	0.47	1.49	0.9069	±0.27901	0.56	1.77	1.0482	±0.32389
RIVER	PREMONSOON	0.43	1.21	0.7785	±0.18885	0.65	1.56	1.028	±0.24416
	MONSOON	0.4	1.31	0.7618	±0.19177	0.47	1.45	1.0193	±0.18244
	POSTMONSOON	0.52	1.44	0.8958	±0.2384	0.65	1.56	1.028	±0.24416

N=64; Min=Minimum; Max=Maximum; SD=Standard Deviation

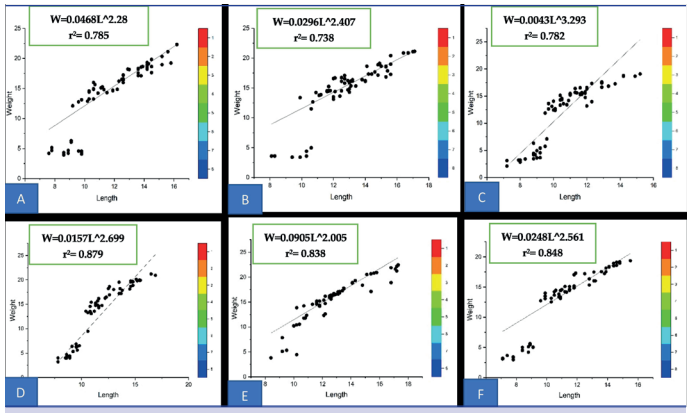

Figure 3. Length-Weight relationship of *M. tengara*.
A-C= Pond; D-F= River; A & D= Pre-monsoon; B & E= Monsoon; C & F= Post-monsoon

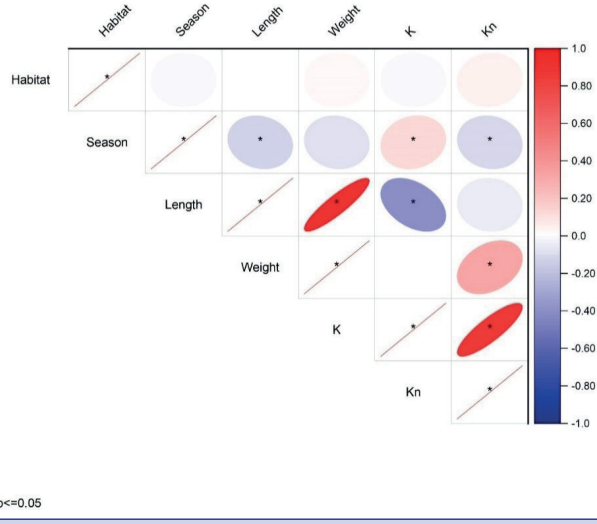

Figure 4. Correlation of *M. tengara*. (*p≤0.05).

Table 5. Pearson's correlation of *M. tengara*.

		Habitat	Season	Length	Weight	K	Kn
Habit	Pearson Correlation	1	.000	.015	.021	-.016	.058
	Sig. (2-tailed)		1.000	.768	.688	.762	.256
Season	Pearson Correlation	.000	1	-.133**	-.084	.134**	-.103*
	Sig. (2-tailed)	1.000		.009	.099	.009	.044
Length	Pearson Correlation	.015	-.133**	1	.895**	-.415**	-.058
	Sig. (2-tailed)	.768	.009		.000	.000	.257
Weight	Pearson Correlation	.021	-.084	.895**	1	.012	.336**
	Sig. (2-tailed)	.688	.099	.000		.814	.000
K	Pearson Correlation	-.016	.134**	-.415**	.012	1	.848**
	Sig. (2-tailed)	.762	.009	.000	.814		.000
Kn	Pearson Correlation	.058	-.103*	-.058	.336**	.848**	1
	Sig. (2-tailed)	.256	.044	.257	.000	.000	
	N	384					

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

Table 6. Post Hoc test seasonally of *M. tengara*.

Dependent Variable	(I) Season	(J) Season	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Length	Pre-monsoon	Monsoon	-1.0703*	.27032	.000	-1.7064	-.4343
		Post-monsoon	.7438*	.27032	.017	.1077	1.3798
	Monsoon	Pre-monsoon	1.0703*	.27032	.000	.4343	1.7064
		Post-monsoon	1.8141*	.27032	.000	1.1780	2.4501
	Post-monsoon	Pre-monsoon	-.7438*	.27032	.017	-1.3798	-.1077
		Monsoon	-1.8141*	.27032	.000	-2.4501	-1.1780
Weight	Pre-monsoon	Monsoon	-1.4219	.64831	.074	-2.9473	.1036
		Post-monsoon	1.0883	.64831	.215	-.4372	2.6137
	Monsoon	Pre-monsoon	1.4219	.64831	.074	-.1036	2.9473
		Post-monsoon	2.5102*	.64831	.000	.9847	4.0356
	Post-monsoon	Pre-monsoon	-1.0883	.64831	.215	-2.6137	.4372
		Monsoon	-2.5102*	.64831	.000	-4.0356	-.9847
K	Pre-monsoon	Monsoon	.09555*	.029309	.003	.02659	.16452
		Post-monsoon	-.08048*	.029309	.017	-.14945	-.01152
	Monsoon	Pre-monsoon	-.09555*	.029309	.003	-.16452	-.02659
		Post-monsoon	-.17604*	.029309	.000	-.24500	-.10707
	Post-monsoon	Pre-monsoon	.08048*	.029309	.017	.01152	.14945
		Monsoon	.17604*	.029309	.000	.10707	.24500
Kn	Pre-monsoon	Monsoon	.00320	.031928	.994	-.07193	.07833
		Post-monsoon	.06496	.031928	.105	-.01016	.14009
	Monsoon	Pre-monsoon	-.00320	.031928	.994	-.07833	.07193
		Post-monsoon	.06176	.031928	.130	-.01336	.13689
	Post-monsoon	Pre-monsoon	-.06496	.031928	.105	-.14009	.01016
		Monsoon	-.06176	.031928	.130	-.13689	.01336

(2010) showed 'b'values of 1.468 and 1.388 for female and male in the same species, Karna and Panda (2012) reported the 'b' value of 3.032 for mixed sex of *M. gulio*. According to Naeem et al. (2012), the "b" values for *M. bleekeri's* combined sexes, female, and male were 2.62, 2.70, and 2.64, respectively. The 'b' values (ranging from 2.00 to 3.29) in the present study are closer to the previous studies of Kalita et al. (2017) and Gupta & Banerjee (2015). Till now, in seasonal and habitat-wise studies, no information has been found about the length, weight, 'K', and 'Kn' relationship of *M. tengara*. Therefore, it is impossible to comprehensively contrast the present result with previous data. In the current study, the 'K' and 'Kn' values were 0.33-1.56 and 0.92-1.17, respectively (Table 4). The peak average 'K' and 'Kn' values for this species in both environments occur post-monsoon. Kalita et al. (2017) reported values of 0.74-1.39 with a mean SD of 1.00±0.125; this result shows 'Kn' values greater than the present study. The majority of the fish had 'Kn' values greater than 1, indicating that they were in good health. The relative condition component, however, was seen to be more or less unchanging from lighter to heavier fish, plainly indicating the well-being and status of the fish to be healthy. In their investigation, Bhatta & Goswami (2014) noted a reversal situation where the high'Kn' value was found in the medium-sized *Channa aurantimaculata* fish. Rahaman et al. (2015) and Das et al. (2015) reported that male-*Heteropneustes fossilis* and female *Anabas testudineus* showed a trend where 'Kn' decreased from smaller fish exhibiting the small value at medium fish and then fixedly increased to get the peak value in bigger fishes, but the current study indicates that the highest 'Kn' value occurs during the post-monsoon season and that during this season the weightand length of the species were less than during the monsoon and pre-monsoon season.

CONCLUSION

According to the current study, *M. tengara* from both habitats (pond and river) in the Jhargram and Paschim Medinipur districts of West Bengal exhibit an allometric growth pattern with a 'b' value less than 3. Therefore, it might be said that this species did not strictly abide by the intended cube law. The species show a strong relationship between length and weight in both habitats in all seasons. There is a significant seasonal relationship among weight, length, 'K' and 'Kn'of *M. tengara*, but there is no significant habitat-based relationship between the same parameters. Seasonally, the 'K' and 'Kn' were discovered to be in a typical position to maintain the health of the fish species. This study

achieved its aims, and the data acquired may be helpful in advising on how to design subsequent biometric studies for fish taken from the study area. As a result, the current findings will assist fisheries managers in creating efficient strategies for the long-term management of *M. tengara* in its habitats.

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Research Article

Using The Thick-Shelled River Mussel (*Unio crassus*) Filtering Ability for Water Treatment Process in Aquaculture Systems: an In Vitro Study on Removal of the Bacteria from The Water

Didem Demircan[1], Aygül Ekici[2], Gökhan Tunçelli[2], Merve Tınkır[2], İlker Keskin[2], Devrim Memiş[2]

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ABSTRACT

The thick-shelled river mussel (*Unio crassus*) is listed as 'Endangered' on the IUCN Red List of Threatened Species and it is important to culture them for the conservation of natural stocks. Integrating mussels into the freshwater aquaculture system could be an efficient method, because of their filtering ability. In this study, it was aimed to determine the bacteria filtering ability of the thick-shelled river mussel on an aquaculture system to determine if the water quality got better in terms of bacteriology. Depuration, disinfection, and antibiotic treatments were applied to reduce the bacterial load in the mussels' bodies. Disinfection was made using $NaCl_2$, 2-Phenoxyethanol, Formalin, Virkon® S and Chloramine T. Antibiotic treatment was performed using Oxytetracycline and Florfenicol. The best result was obtained in the group to which 5 mg L^{-1} Oxytetracycline was added. However, since mussels can uptake the same bacteria into own bodies with their own pseudofeces, it was found that it is appropriate to use antibiotic treatment and depuration applications together. In the experiment of keeping them in the same environment with the pathogens (*Staphylococcus epidermidis, Aeromonas caviae*), intense growth of bacteria was inoculated into water. Thus, it has been determined that mussels clean the water by removing bacteria from the environment within 48 hours, so river mussels can be adapted to aquaculture systems to reduce aquatic bacteria.

Keywords: Unionidae, Filtering, Aquatic Bacteria, Aquaculture

ORCID IDs of the author:
D.D. 0000-0002-3571-4682;
A.E. 0000-0003-1285-0304;
G.T. 0000-0003-1708-7272;
M.T. 0000-0003-2807-2789;
İ.K. 0000-0002-1896-9069;
D.M. 0000-0001-7378-0165

[1]İstanbul University Faculty of Aquatic Sciences, Department of Fish Diseases, Istanbul, Turkiye

[2]İstanbul University Faculty of Aquatic Sciences, Department of Aquaculture, Istanbul, Turkiye

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Correspondence:
Devrim MEMIŞ
E-mail:
mdevrim@istanbul.edu.tr

INTRODUCTION

Members of the Unionidae occur on all the continents except for Antarctica. *Unio crassus*, a thick shelled river mussel, has been classified as an endangered species in the IUCN Red list of threatened species since 1994. There was insufficient data found for taxonomic problems from these mussels in 2014 (Lopes-Lima et al., 2014). In Turkiye *U. crassus* has detected in Lake Sapanca basin, Sakarya (Ercan et al., 2013a), the Aras river, Erzurum (İşlıyen, 2017), Tersakan Stream, Muğla (Bahrioğlu, 2017), Karasu stream, Sinop (Coşkun et al., 2019), Çine Creek, and the Aydın (Serdar et al., 2019). In Particular, *U. crassus* was found to be more abundant than the other Unionids in the Maşukiye stream (Ercan et al. 2013a).

Unionids are highly endemic and sensitive to human impact (Strayer, 2008). Studies have shown that glochidia and juvenile mussels are sensitive to some chemicals (Gillis et al., 2008; Ingersoll et al., 2006). Also, since the breathing and exhalant mouths of freshwater mussels are positioned adjacent to each other, continuously flowing waters are suitable habitats for freshwater mussels. Since freshwater mussels can retain toxicants in their tissues and pseudo feces, they play an important role in maintaining water quality (Bauer & Wachtler, 2001).

Since the breathing and exhalant mouths of freshwater mussels are positioned adjacent to each other, continuously flowing waters are suitable habitats for freshwater mussels. Since mussels are fed by filtering the water in the environment, they assimilate suspended particles such as bacteria and plankton in their environment (Grizzle & Brunner, 2009; Starliper, 2001). This feature allows them to live symbiotically with bacteria as well as cause them to become infected (Antunes et al. 2010). Freshwater mussels may store toxicants in their tissues and pseudofeces (Bauer & Wachtler 2001) and the pseudofeces also contains bacteria (Ercan et al., 2013a), therefore it may play a key role in maintaining water quality (Bauer & Wachtler 2001). The pathogen bacteria species of the unionid mussels are also an undefined subject (Carella et al., 2016). Researchers recently found 47 bacterial genera from the hemolymph of freshwater mussels (Leis et al., 2019). The microbial biota is still unclear which of the species are endosymbionts and which are accidentally siphoned into the body. In addition, the microorganisms present in the body varied by collection locality, season, or density of contaminants (Grizzle & Brunner, 2009; Starliper et al., 2008).

The amount of particulate matter filtered from the water column by bivalves may be important, and freshwater mussels can be useful in the rehabilitation of organically contaminated waters, especially those associated with aquaculture (Ercan, 2009). There are studies in which mussels are used as filters for the outlet water, the purpose of re-use, instead of leaving the water to nature in the land aquaculture systems. Additionally, mussels are integrated into fish ponds and co-cultured with fish species (Zheng et al., 2017). Cultivation experiments were performed in order to recover thick-shelled mussels from endangerment (Serdar et al., 2018). Therefore, the depuration process is recommended before the replacement of mussels in hatcheries (Leis et al., 2019). This process is conducted to remove the bacteria that mussels take from the environment to their bodies. In addition, it is recommended to do quarantine and disinfection procedures to prevent mussels from carrying pathogens to new places during their relocation (Augspurger et al., 2003; Waller & Cope, 2019).

The depuration process of marine bivalves is applied to remove low and medium level contaminants from their intestines for a few hours to a few days by taking the bivalves into clean seawater tanks with normal water pumping movements (Lee et al., 2008). Few studies have been done on this process for freshwater mussels (Starliper, 2001). In addition, there is no research about disinfection with chemical or antibiotic treatment.

Although Unionid mussels are said to be endangered due to habitat destruction, pollution, and poor water quality, the large-scale death of mussels in streams with good quality water cannot be explained (Waller & Cope, 2019). Adult Unionids can tolerate low oxygen levels (Strayer, 2008). They can maintain their normal metabolism even at dissolved oxygen levels as low as 1mg L^{-1} and can even tolerate full anoxia for several weeks by simply covering their shells (McMahon & Bogan, 2001).

Many studies have been conducted on the health of mussels, and the scarcity of studies on the role of pathogens in the decrease in mussel populations draws attention (Waller & Cope, 2019). The most important step in determining whether these species are healthy or not requires knowledge about pathogens. Reported pathogens have the potential to disrupt the health conditions of Unionid mussels, but their role in disease is not well established (Carella et al., 2016).

Mussels are living communities that are involved in the removal of inorganic substances such as nutrient salts and/or heavy metals that cause accumulation in the aquatic ecosystem with their filtration functions and therefore in the improvement of water quality (Cummings & Graf, 2010; Lei et al., 1996; Strayer, 2008). During the nutritional activities of mussels, filtering and accumulating pollutants in their bodies is highly effective in improving water quality. The aim of this study was to determine the bacteria filtering ability of mussels before being adapted to fishponds in the aquaculture system.

MATERIALS AND METHODS

Collection and adaptation of mussels

Unio crassus were collected by hand and with a rake from the area of the Maşukiye stream with a depth of 10-80 cm and a length of 50 m, and 700 m away from the Sapanca Lake (40° 43′ 1.542″, 30° 8′ 29.0292″) in June 2019 in Sakarya city. The Maşukiye stream has high nutrient water and a sandy-muddy bottom. The water temperature was 16°C at the sampling time. Mussels were determined to be from the sampling area of the Maşukiye Stream, all experiments were done with a total of 28 specimens for three different treatments. They were transported to the "Aquatic Vertebrate Living Experiment Unit" of the Sapanca Inland Fisheries Production Research and Application Unit in a dry environment with net bags in 20 minutes.

They were quarantined and acclimated for 30 days at an enclosed holding facility. Accordingly, they were placed indoors, in a 200L circular polypropylene tank until used for experimental purposes. During the adaptation period, the tank was supplied with 16°C well water continuously which has between 7-8mg L^{-1} dissolved oxygen, 6pH and 5L min^{-1} flow. Mussels were fed daily at a rate of 1% body weight with dried algae (Algome, MarinBio). The algae suspension was mixed homogeneously in 1L water and added to the tanks (Bahrioğlu, 2017). The artificial lighting was adjusted to the summer photoperiod (12h light: 12h dark). After the adaptation period, 28 specimens were measured using digital calipers and electronic balance and grouped for the experiments. The mussels were measured as the mean length of 49.44 ± 2.29mm, a width of 27.72 ± 1.11mm, a height of 17.21 ± 0.82mm, and a weight of 15.30 ± 1.8g.

Firstly, three different methods were used; depuration, disinfection, and antibiotic treatment, to eliminate or reduce the natural bacterial biota of *Unio crassus*. After these steps, the pathogenicity of *A. caviae* and *S. epidermidis* in *U. crassus* and filtering ability for elimination of these bacteria from waters were investigated. During the experiment, the water parameters were adjusted as water was renewed. The constancy of the water temperature was provided by the air conditioner that controls the room temperature.

Detection of the bacterial density in mussels and water
At the beginning of the experiments, the bacterial density was determined in the body of the mussels and the water where the mussels were held in experimental tanks. One mussel was sampled and examined under aseptic conditions to determine the bacterial biota. After that the shells were disinfected with 70% alcohol, the internal organs of the mussel were separated from the shell with the scalpel and taken into sterile sampling bags. The mussel organs were homogenized in an equal amount (w/v; 1:2 dilution) of sterile peptone water for 10 minutes by Stomacher. Homogenate was diluted 10-fold serially with peptone water. 0.1mL of each dilution was streaked onto TSA plates with the drigalski. The plates were incubated for 24-48 hours at 22°C and the total bacteria were enumerated and recorded as CFU g^{-1} (Whitman, 2004).

For determination of the bacterial density in water, the water samples (0.1mL) were streaked onto tryptic soy agar (TSA) plates. Plates were incubated for 24-48 hours at 22°C and the total bacteria were enumerated and recorded as CFU mL^{-1} (Whitman, 2004). Bacterial observation was done only by macroscopically examining the colonial morphology. Additionally, water samples were streaked onto Baird Parker Agar (w/RPF Supplement) and Blood Agar to determine presence of *Staphylococcus* spp. and *Aeromonas* spp.

Elimination of the bacterial biota of mussels
After the detection of bacterial biota in the mussels, depuration, disinfection, and antibiotic treatment experiments were made for the elimination of bacterial density. Measurement of bacterial density was the same in all the applications. Water samples were taken by micropipette from each group. To measure the bacterial density, the water samples (0.1mL) were streaked onto tryptic soy agar (TSA) plates at 0-2-24 hours and 24-hour intervals for all experiments. Plates were incubated for 24-48 hours at 22°C and the total bacteria were enumerated and recorded as CFU mL^{-1} (Whitman, 2004). The removal degree of total bacteria was calculated as a percentage according to Lekang (Verdegem, 2007).

Depuration experiment
The mussels were subjected to the depuration process using ultra-pure water (Bighiu et al., 2019). Each mussel was held in different beakers. A total of 9 mussels were used for the depuration experiment. To sustain the filtration process, aeration was continued during depuration. To prevent contamination from an external source, the beakers in which the mussels were kept were covered with aluminum foil. The water in each beaker was renewed by using ultra-pure water at 24-hour intervals for 168 hours (7 days), and 0.1mL of water samples was taken from the containers before the water change. Water samples were spread onto TSA plates in duplicate. The survival rate during the experiment was 100%.

Disinfection experiments
Three mussels in each beaker were treated with three different disinfectants. In the first application, three different groups of mussels were held in ultra-pure water with $NaCl_2$ (20g L^{-1}), 2-Phenoxyethanol (1.5mL L^{-1}) and Chloramine T (0.5g L^{-1}) for one hour then taken into ultra-pure water. In the second application, one mussel was held in ultra-pure water with NaCl2 (20g L^{-1}) for 24 hours then taken into ultra-pure water (Stockton & Moffitt, 2013; Garcia et al., 2014). In the third application, the shells were brushed with toothbrushes soaked in solutions of Formalin (10%), Virkon® S (1:100) and Chloramine T (5 g L^{-1}) separately. After the mussels were brushed for 30 minutes then they were washed and taken into ultra-pure water. They were observed for 120 hours (5 days). In the experiments, the used water was renewed at 24-hour intervals with ultra-pure water. Water samples were spread onto TSA plates in duplicate.

Antibiotic treatment experiment
An antibiogram test was performed on bacteria taken from water samples with fish antibiotic discs on Mueller Hinton Agar. As a result of this experiment, the use of oxytetracycline and florfenicol was found appropriate in the treatment of mussels. Three different doses of Oxytetracycline (0.5, 1, and 5mg L^{-1}) and Florfenicol (0.05, 0.1, and 0.5mg L^{-1}) were added to the ultra-pure water every day for 5 days. During this experiment, the water was not changed. After the experiment ended, the mussels were held in the same beakers for a week without any manipulation. Afterward, the water samples were taken from each beaker for control purposes. Water samples were spread onto TSA plates in duplicate.

Preparation of bacterial inoculum
The strain of *Aeromonas caviae* reference strain ATCC 15468 was taken from ATCC and *Staphylococcus epidermidis* was originally isolated from fish in 2017 (Çanak & Timur, 2020).

The bacteria were cultured in nutrient broth (NB) at 23°C on a 150-rpm shaker for 48 hours. The culture was centrifuged for 10 minutes at 3000g, the pellet was washed with phosphate buffered saline (PBS, Oxoid) and adjusted to an optical density of 1.0 at 600nm. Tenfold dilutions of the cells were prepared in PBS for determination of viable colony-forming units (CFU). 0.1mL of each dilution were placed on the surface of Tryptic Soy Agar (TSA) (Paniagua et al., 1990; Zepeda-Velázquez et al., 2017).

Elimination of *A. caviae* and *S. epidermidis* by mussels
According to results of earlier experiments, oxytetracycline was used at a dose 0.5mg L^{-1} for 5 days before beginning the exposure experiment. Then, the water was changed with autoclaved distilled water before the inoculation of bacteria.

The growth bacterial colonies were enumerated, and prepared dilutions inoculated into 1L water. Mussels in experimental groups formed in 5L beakers were exposed to two bacterial species at a final concentration 10^8 CFU mL^{-1} or 10^7 CFU mL^{-1} *S. epidermidis*, 10^7 CFU mL^{-1} or 10^6 CFU mL^{-1} *A. caviae*. An aliquot of PBS was added to the control group. Water samples were taken by micropipette from beakers to measure the bacterial density. The water samples (0.1ml) were spread onto TSA at 0, 2, 4, 6, 24, 48, 336 hours after bacterial inoculation. Growth colonies were enumerated and recorded.

Statistical analysis
An assessment of the correlation between the experiment groups for elimination of bacteria was determined using SPSS version 28, and an analysis by bivariate Pearson correlation coefficient ($p<0.01$).

RESULTS AND DISCUSSION

At the beginning of the experiment, all mussels were kept in the same tank. Total bacteria were determined 1.1x10^5 CFU mL^{-1} in the water where the mussels were kept. This number was used as the initial (I) amount of bacterial density for all experiments. These colonies had very high concentrations and some of them were swarm type colonies.

It was observed that the bacterial endobiota contained different types of bacteria and very intense growth on TSA. Total bacterial recovery from the tissues was found as 4.1 x10^7 CFU g^{-1}. Considering the morphological features, five different types of bacterial colonies

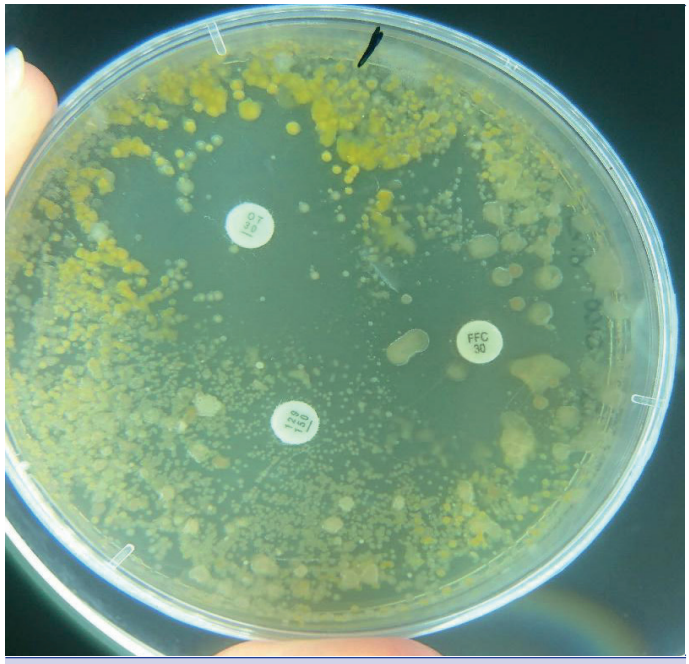

Figure 1. Antibiogram test on TSA plate (OT, FFC) and microbial variation in the water at the beginning of the study

were observed on TSA, but the species were not identified (Fig. 1).

As a result of the 7-days depuration process at 22 °C performed to reduce the natural bacterial biota, it was determined that 68.2 % of the total bacteria were removed and decreased to 3.5 x10^4 CFU mL^{-1} (Fig. 2).

In the groups kept for 1 hour in $NaCl_2$ (20g L^{-1}, NCL), phenoxyethanol (1.5mL L^{-1}, PHN) and Chloramine T (0.5g L^{-1}, CHL1), a decrease in bacterial load was observed after 120 hours but could not be eliminated. The total amount of bacteria was found to be 5.5 x10^1 CFU mL^{-1}, 4.3 x10^2 CFU mL^{-1} and 8.2 x10^2 CFU mL^{-1} at 2 hours and 1.15 x10^3 CFU mL^{-1}, 3.59 x10^3 CFU mL^{-1} and 2.83 x10^3 CFU mL^{-1} at 120 hours, respectively (Fig. 2). Removal of bacteria was determined as 91.36%, 67.72% and 73.72% respectively. However, the total bacteria amount was found 10-fold higher in all groups after 144 hours when the experiment was terminated.

Although the bacterial density decreased to almost 3 x10^4 CFU mL^{-1} after 24 hours in all groups where the mussel shells were brushed with solutions of formalin (7%, FRM), Virkon® S (1:100, VRN) and Chloramine T (5 g L^{-1}, CHL2) (Fig. 2), all mussels were dead at the 48th hour.

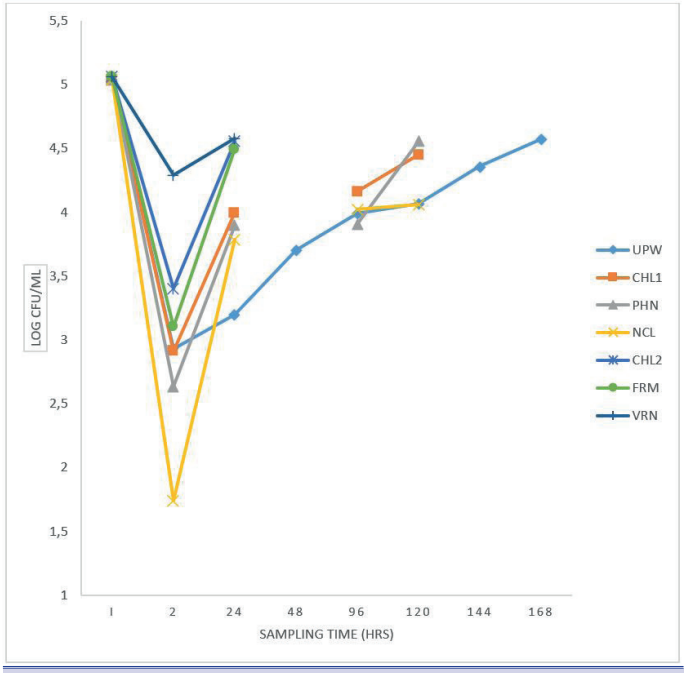

Figure 2. Time-dependent variation of total bacterial load (log CFU mL^{-1}) in the water in which the mussels were kept during different treatments. UPW: depuration with ultra-pure water change daily; CHL1: Chloramine T (0.5g L^{-1}); PHN: Phenoxyethanol (1.5mL L^{-1}); NCL: NaCl2 (20g L^{-1}); CHL2: Chloramine T (5g/L); FRM: Formalin (%7); VRN: Virkon (1:100).

In the antibiotic treatment groups (Oxytetracycline and Florfenicol), after 5 days, the best results were obtained as 5 x10^2 CFU mL^{-1} from the group in which 5mg L^{-1} oxytetracycline was added (Fig. 3).

Oxytetracycline reduced both the number and types of bacteria. While five types of colonies were grown at the beginning of the study (Fig. 1), two types of bacteria were grown at the end in the oxytetracycline treatment group. However, after a week with no treatment, bacterial concentration increased again.

According to results of the bacterial elimination experiment; Bacterial density decreased 10 times in the group that added 10^8 and 10^7 CFU mL^{-1} of *Staphylococcus epidermidis* bacteria to the water. 10^6 CFU mL^{-1} *Aeromonas caviae* (ATCC 15468) bacteria in the water was filtered from the water after 24 hours and 10^7 CFU mL^{-1} *Aeromonas caviae* (ATCC 15468) bacteria was reduced to 10^1 CFU mL^{-1}. No death was observed during the experiments with both bacteria.

It has been found that the natural biota of mussels has very dense bacteria. Although sterile water was used, it destroyed patho-

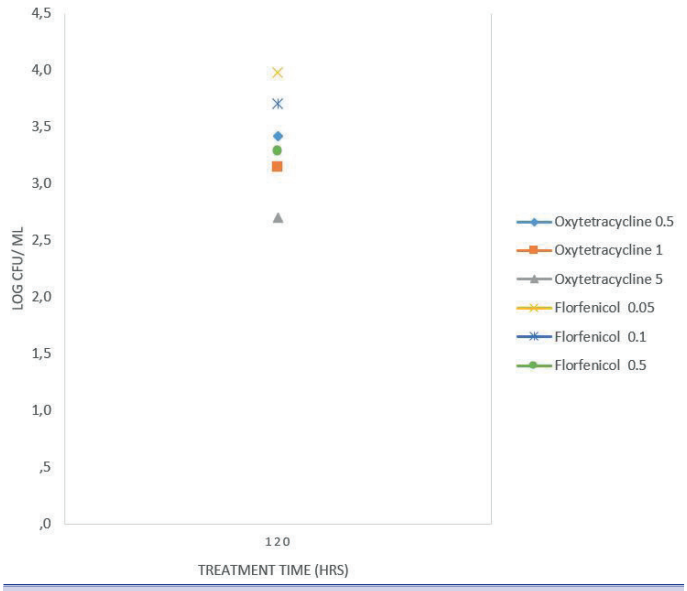

Figure 3. The total bacteria amount in water after 120 hours of the antibiotic treatments; Oxytetracycline and Florfenicol with different doses (mg L^{-1}).

genic bacteria but reproduced other bacteria within itself. Different from *A. caviae*, yellow to orange colonies grew on the TSA of the control group 2 hours later and yellow colonies were seen on the TSA of 10^6 CFU mL^{-1} *A. caviae* inoculated group after 24 hours. After 48 hours, *A. caviae* was eliminated in this group while yellow colonies increased on the TSA. After 14 days, all groups of the *A. caviae* experiment and control were checked again and it was found that all contained the same number of bacteria (Fig. 4). The control with 10^7 CFU mL^{-1} *A. caviae* and with 10^6 CFU mL^{-1} *A. caviae* added groups were found with a negative correlation (r= -.439, p=0.01 and r= -.444, p=0.01, respectively). But between the two groups, 10^7 CFU mL^{-1} *A. caviae* and 10^6 CFU mL^{-1} *A. caviae*, was found to be a strong positive correlation (r=1, p=0.01) by SPSS version 28, and an analysis by bivariate Pearson correlation coefficient.

Also, in this study we observed that, mussels could have cleaned the water of 10^6 CFU mL^{-1} *A. caviae* inoculated group and 10^7

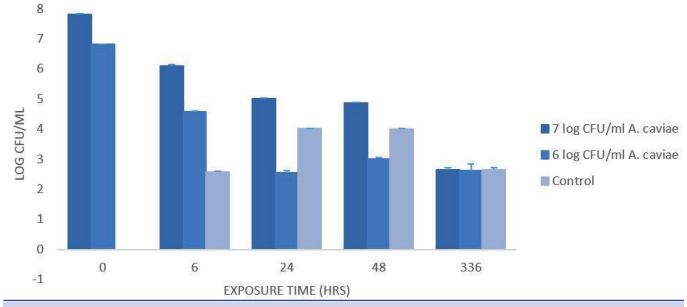

Figure 4. Time-dependent variation of total bacterial load (log CFU mL-1) in water, during the exposure of different concentration for *A. caviae* (The bars showed SD).

CFU mL^{-1} of *Staphylococcus epidermidis* inoculated group (Fig. 5) and made pseudo feces.

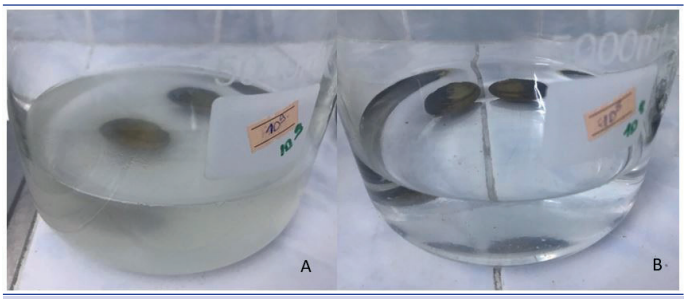

Figure 5. Mussels filter bacteria from water; a view of water at the beginning of experiment (A) and view of cleared water after 48 hours (B).

In this study, it was determined that there were a wide variety of bacteria in the biota of mussels sampled from the Maşukiye stream. The sample taken from the water of mussels placed in sterile water was streaked on selective media and it was found to contain *Staphylococcus* spp. and *Aeromonas* spp. in that water sample. Therefore, experiments were made to reduce the natural bacterial load from the environment so that bacteria, which are present in the mussel, do not mix with *Staphylococcus epidermidis* and *Aeromonas caviae*, which were required to be filtered in this study. For this reason, depuration was the first step, and after this process, different disinfectants and antibiotics were applied because the bacterial density was still high. Despite all the interventions to destroy bacteria found in the natural biota it was achieved only to a limited extent. But a significant reduction was achieved for a short time at 48 hours.

Two bacterial species that can be pathogenic for fish, *A. caviae* and *S. epidermidis*, were selected and mussels were expected to be able to filter and eliminate them from water. As a result of the study, a significant difference was found between the control group and 10^6 CFU mL^{-1} *A. caviae* and with 10^7 CFU mL^{-1} *A. caviae* groups. However, no difference was found between 10^6 CFU mL^{-1} *A. caviae* and 10^7 CFU mL^{-1} *A. caviae* groups in terms of the bacterial filtration. The previous studies showed that mussels assimilated the various bacterial genera by siphoning contaminated water in the tank or natural habitats (Ercan et al., 2013b; Leis et al., 2019; Starliper, 2001; Starliper et al., 2008). According to our results, mussels can be used for elimination of *A. caviae,* but it is not appropriate for *S. epidermidis*. In other words, the two different bacterial species were assimilated at different rates. In previous studies mussels assimilated bacteria at different rates, even if they belonged to the same genus (Bighiu et al., 2019).

According to the results, there was a high concentration of bacteria in the whole-body of the homogenate sample, and the biota on the shell was thought to be strong. It is known that bacteria make a biofilm layer on hard substances. The possibility of formation of a biofilm layer on the shell was considered, and the shell was brushed to remove bacteria. The shell brushing method was not successful because even if the shell is resistant to

chemicals, the mussel may have absorbed these chemicals during the purification from the chemical process. Or, the destruction of bacteria on the shell and the normal life cycle of the mussel, which can feed on bacteria, may have been adversely affected.

Some studies showed that glochidia and juvenile mussels were sensitive to certain chemicals compared to cladoceran, amphipod and different fish species (Waller and Fisher 1998; Ingersoll et al., 2006; Gillis et al., 2008). Furthermore, the sensitivity of different mussel species to chemicals was also found to be different in a report (Waller & Fisher, 1998). In their study, high mortality was observed in some mussel species in the groups that were kept in water with 2% $NaCl_2$ added for 6 hours, while no mortality was observed in others. In our study, we kept *U. crassus* in water with 2% $NaCl_2$ for 1 hour, and no deaths were observed during the experiment (approximately 1 week). While no death was observed in the group that held 1 hour with 0.5g L^{-1} Chloramine T in the study, it is thought that the reason for death by brushed shell with 5g L^{-1} Chloramine T may be due to the increased chemical concentration or the deterioration of bacterial biota on the shell.

Starliper et al., (2001) found 1.88 x10^5 CFU g^{-1} bacteria in soft tissues of *Amblema plicata*, which was less than our findings. But a mussel's body fluid was separated from organs and tissues and were rinsed with sodium hypochlorite in that study. In another study, researchers found a maximum of 1.52 x10^7 CFU g^{-1} in soft tissues of *Villosa iris* (Starliper et al., 2008). The fact that we sampled all tissues and body fluids together and the tissues were not washed with sodium hypochlorite caused the bacterial load to be a bit higher as 4.1 x10^7 CFU g^{-1} in all soft tissues. Different mussel species may contain different concentrations of bacteria in their body depending on the living habitat. Although the bacterial density was high in the soft tissues, the number of mussels was not low in the sampling area of the stream. This situation showed that thick shelled river mussels could tolerate this high density of bacteria.

The antibiotic dose selection was chosen as similar as the doses applied in fish but was concentrated in the water and accumulated in the mussel tissue as there was no water change, therefore the dose was not chosen too high. According to our results, oxytetracycline treatment was found as the best method to reduce bacterial biota of mussels. However, it is thought that this method alone is not sufficient, and it would be more effective when used together with the depuration method. Because mussels can retake the same bacteria with the feces that they leave in the water. The daily cleaning would change the quality of the water and the biota of the mussel. All species of bacteria need different antibiotic treatment, so the antibiotic treatments would be decided according to the result of the antibiotic susceptibility test following the determination of the mussel bacterial biota.

While fish pathogen bacteria, *Flavobacterium columnare*, could be destroyed in one day by the depuration of mussels (Starliper et al., 1998), *Aeromonas salmonicida* was only reduced by 70% in another study by the same researcher (Starliper, 2001). Starliper (2001) also mentioned that the results of the 1 day and 5 days' depuration process were not different. Based on this, different bacterial species can give different responses to the depuration process in mussels. According to this study, a limited decrease in total bacterial load with depuration was due to the presence of different species in the bacterial biota of mussels. Some species still existed, and some were eliminated after the depuration process. Moreover, a rapid decrease was observed within 24 hours, and an increase in the total number of bacteria was observed in the following days. For this reason, it suggested that 24 and 48 hours were sufficient for the depuration process, and it should not be forgotten that different reductions can be obtained for different bacterial species. Furthermore, unculturable bacteria cannot be cultured in artificial media and some bacteria prevent the growth of other bacteria in the same media, the mussel microbiota, which can be found in many more bacterial species, can be revealed in more detail by metagenomics studies.

The symbiotic life of mussels with bacteria suggests that there may be an important relationship to protecting the ecosystem. Because the number of bacteria decreased in the first days of the applications and even though the contaminated water was replaced with ultrapure water every day, the bacterial load continued to increase day by day in the following days. This might be an effort to create a food for the mussel since there is no food in its environment. Although it has been reported in previous studies that bacteria were eliminated by depuration (Lee et al., 2008), in this study, it was observed that depuration was not valid for many bacteria in *U. crassus,* and individual experiments should be made for each bacteria.

Leis et al., (2019) suggested that future studies should investigate associations between *Aeromonas* spp. and unionid health and disease. Sicuro et al., (2020) worked on the use of freshwater bivalves in rainbow trout (*Oncorhynchus mykiss*) farm wastewater filtering. In this research Unionid species (*Sinanodonta woodiana*) were used. They found that the efficiency of freshwater bivalves in reducing the bacterial load, against *A. hydrophila* was successfully done. In this study, we investigated the association between *Aeromonas caviae* and *Unio crassus*. According to our results, a high concentration of (10^8 CFU ml^{-1}) *Aeromonas caviae* was added to the water of mussels, and after 21 days, these bacteria were found not to be associated with the disease of *Unio crassus*. During the 21-day observation period, there was no mortality in all mussel groups and no signs of disease of the mussel organs. It was determined that this bacterium, which is a fish pathogen, did not show a pathogenic effect for mussels. Additionally, mussels survived six months more at around 20°C without any addition of food or air in the beakers covered with aluminum foil after the study. These findings are similar to those reported in previous reports (McMahon & Bogan, 2001). This event suggests that the balance between oxygen production and consumption can be established with the help of endosymbiont bacteria. Besides, it was known that they could use bacteria as food sources (Silverman et al., 1997; Leis et al., 2019). More detailed work needs to be done to understand the complex relationship between bacteria and with these mussels.

Unionids and other freshwater bivalves are important components of the freshwater ecosystem. Several species of potentially pathogenic bacteria have been isolated from freshwater bi-

valves, but their role in diseases of bivalves has not been established (Grizzle & Brunner, 2009). According to some reports, the bacterial biota in mussels changes very quickly (Starliper et al., 1998; Nichols et al., 2001). Starliper et al., (1998) demonstrated that the bacterial biota in mussels changed significantly within 24 hrs of a change in water supply. The finding that the bacterial biota rapidly responds to changing water supplies could be used favorably to minimize the risk for introduction of pathogens. These findings should be supported by further studies.

As Waller and Cope (2019) mentioned earlier, there are many questions that need to be answered in order to protect mussel health. Comparisons can be made with the data in healthy populations by examining the water and mussel tissues, especially in regions with intense mussel death. In this way, it can give us information about the main factor that causes death for mussels. In addition, by detecting the presence of pollutants in regions with concentrated deaths, it can be determined whether the cause of death is due to organic pollutants or chemical pollutants.

One of the aims of the study was to remove any potentially harmful bacterial biota before placing mussels at the bottom of a fish pond within aquaculture conditions. It has not been found appropriate to be kept in the same environment with fish before antibiotic treatment and depuration, due to its symbiotic life with motile *Aeromonas* spp. For this reason, it is thought that mussels collected from a stream can be adapted to fish ponds after investigating the natural bacterial biota and treating them with appropriate antibiotic.

This is the first study carry out in the application of disinfection and antibiotic therapy for reducing the bacterial load of river mussels. According to our experience, it was concluded that mussels increase the bacterial load consciously and can keep the normal biota in balance. There is a symbiotic lifestyle with some bacteria. Moreover, mussels have a complex bacterial biota. Despite all the used interventions, it was impossible to eliminate the bacteria in the mussels' microbiota. It is an original finding that mussels regenerate their bacterial biota continually. It was concluded that mussels can filter and digest some bacterium types (Fig. 5), and some cannot or willingly do not, so it was suggested that if this feature is to be used in rearing conditions, it is necessary to conduct separate trials with each type of bacteria.

CONCLUSION

Mussels can be adapted to mussel-fish integrated culture systems, as it minimizes the amount of *A. caviae* polluting the water in the culture tanks after 48 hours, although it is not highly effective for *S. epidermidis* contamination in culture tanks. To successfully use freshwater mussels to filter bacteria in the water, further studies are needed with mechanical and UV filtration systems.

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Research Article

Multi-species Fish Identification using Hybrid DeepCNN with Refined Squeeze and Excitation Architecture

Jansi Rani Sella Veluswami[1], Nivetha Panneerselvam[1]

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ABSTRACT

Fish play a prominent role in the food web and fish farming has value for both human consumption and tourist attractions. Due to the increasing importance of marine biodiversity, recognition of fish species has become a prominent task in monitoring the mislabelling of seafood and extinct species. This problem can be solved using traditional manual annotation on the images. To reduce manpower, cost, and tremendous time, deep learning approaches are used which always require large datasets. Therefore, fish species identification is a challenging task using disproportionately small data sets. In this research, we develop a new method by refining the squeeze and excitation network for the automatic fish species classification model to identify 23 different types of fish species. To achieve this, a hybrid framework using deep learning is proposed on a large-scale dataset and implemented transfer learning for a small-scale dataset. Deep learning methods can be used to identify fish in underwater images. In this study, we have proposed a new method of hybrid Deep Convolutional Neural Network (CNN) along with a Support Vector Machine (SVM) for classification. Additionally, the Squeeze and Excitation (SE) block has been improved for improved feature extraction. The proposed method achieved an accuracy of 97.90%. Then post-training with the small-scale dataset (Croatian) achieved an accuracy of 94.99% with an 11% improvement compared to Bilinear CNN (B-CNN) (Qui et al., 2018) and can be used in any underwater applications to identify fish species and avoid mislabelling of seafood.

Keywords: Deep Learning, Convolutional Neural Network, Squeeze and Excitation, Fish species, Fish4knowledge dataset

ORCID IDs of the author:
J.R.S.V. 0000-0003-2863-5465;
N.P. 0000-0002-4556-1034

[1]Sri Sivasubramaniya Nadar College of Engineering, Chennai, Tamil Nadu, India

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Correspondence:
Jansi Rani SELLA VELUSWAMI
E-mail:
svjansi@ssn.edu.in

INTRODUCTION

The average fish consumption boosted from 9.0 kg per capita in 1961 to 20.5 kg in 2018. The common fish intake extended from 9kg consistent with capita in 1961 to 20.5 kg in 2018 (Dagoudo, Qiang, & Solevo., 2022). Climatic changes, excess fishing and other human activities are the factors that affect the marine ecosystem and the fisheries. They also pressure the fish and their habitats. This increases the need for monitoring and managing the population of fish. But it is difficult in coastal areas where humans get directly involved. Failing to do so will result in the degradation of marine ecosystems and extinction of a specific fish species. For example, there is a huge shrinkage of salmon species in the Northwest Pacific which contributes to a major part of fisheries (Crozier et al.,2019). Therefore, a warning should be imposed by the government and aquatic management to preserve the endangered species. Fish identification helps biologists, academic researchers, and ocean scientists to determine the geological changes and the biomass level in oceans due to its prominence in marine science. Secondly, people buy seafood by believing the selling person or the label on the food packet. But often, people are cheated by seafood mislabelling (Chen et al.,2020). Often, Tilapia is

mislabelled/ substituted as Snapper (Naaum, Warner, Mariani, Hanner & Carolin.,2016). Many fish species are similar in taste and texture. Hence several retailers sell low-market value fish as high-market value fish. There arises an imbalance in prices due to mislabelling (Pollack et al.,2018) since seafood is a highly traded food commodity (Kroetz et al.,2020). Deep learning approaches can be used to fix these problems instead of the manual fish annotation in the images collected through sea divers.

With the advancement in internet technology, fish species classification uses computer vision technology. In the inception, machine learning algorithms (Fouad et al.,2013) were widely used where feature selection was done manually. Now, many research works are carried out using deep learning models (Villon et al.,2020) where feature selection is done automatically. Xu et al. (2021) proposed a method for small-scale unbalanced fish species identification in which they implemented Transfer learning and SE-ResNet152 on the Fish Pak dataset which has 915 images. The SE-ResNet152 network was employed in this study to extract fish image features of higher quality and improve fish species identification. The body, head, and scale datasets have classification accuracy ratings of 98.80%, 96.67%, and 91.25%, respectively. This study was able to solve the problem of small-scale and unbalanced datasets using their class-balanced focal loss function. The environment is varied and diversified in the actual development and processing, and even specific aspects of the fish are blocked, resulting in fish images with fewer feature information. Their approach still must be optimised for this situation. The suggested approach, on the other hand, does not take into account the impact of a complex environment on fish species identification.

For fish recognition and species identification in underwater habitats, Jalal et al. (2020) used a combination of GMM-YOLO and optical flow-YOLO. For the purpose of automatically identifying fish species present in coral reefs, Villon et al. (2018) analysed the performance of four models developed with the same CNN architecture. For underwater fish detection in the wild, Labao & Naval. (2019) proposed a cascaded deep network system with linked ensemble components for 18 underwater videos using R-CNN. Santos & Goncalves. (2019) proposed a CNN pretrained model Inception which classifies fish species, family, and order of a pantanal image dataset. In another study (Allken et al.,2019) they implemented fish species recognition using InceptionNet which pretrained on the ImageNet Classification dataset. Ovalle et al. (2022) used iObserver (Vilas et al.2020) for the input data collection and these images were annotated manually with the species name and size using Mask R-CNN.

For Morphological based fish species identification on a small-scale dataset (FishPak dataset with 915 images) HT, Rauf et al. (2019) used 32-Layer CNN architecture enhancing the network's extensive feature extraction capabilities using ResNet-50, GoogleNet, AlexNet, and LeNet-5. For fish recognition using an underwater drone with a Panoramic camera that is automated using Deep learning algorithms, Meng, Hirayama & Oyanagi. (2018) analysed the performance of three networks: AlexNet, GoogleNet, and LeNet. Prasetyo et al., (2021) proposed a new residual network strategy called MLR (Multi-level residual) by combining low-level features with high-level features using depth-wise separable convolution (DSC) for the FishKnowledge dataset. Zhang et al. (2021) extracted texture features after reducing the noise from the fish4knowledge dataset and implemented a Deep Neural Network (DNN). In Jin et al.'s (2022) study, they proposed an integrated two-stage spatial pooling method in the squeeze part of the SE Block which consists of a rich descriptor extraction by fusing these descriptors into a C-dimensional channel feature. An accurate re-weight score can be returned for channel attention. Using this method, we can get both local and global informative features, but computational cost is additional in the squeeze part.

For fine-grained fish species identification on small-scale data sets (Croatian fish dataset with 794 images), Qiu et al. (2018) suggested an enhanced transfer learning algorithm with refined SENet. This paper compared the experiments on B-CNNs, B-CNNs plus SE blocks, and B-CNNs plus refined SE blocks, and the highest accuracy reached was BCNN+SE - 71.80%. This paper managed to work on a small data set, but they didn't achieve results with great accuracy. This can be improved by using DeepCNN techniques. Our method outperforms their results without pre-processing for the same dataset i.e., the Croatian dataset.

To summarize, though much work has been done, there are still challenges in improving the accuracy of the classification of different species in a large, unbalanced dataset. The objective is to preserve the aqua ecological species. And hence we have worked with the large dataset Fish4Knowledge(F4K) (Phoenix, Huang & Fishera., 2013) where there are 23 different species. The proposed system aims at developing an automated system to identify fish species with less computation time.

The main contribution of this paper can be summarized as follows:

1. The proposed hybrid framework of the CNN model is combined with refined Squeeze Excitation (SE) and SVM to improve the overall performance of the model
2. This develops an automatic fish species classification method to identify 23 different fish species
3. The hybrid CNN-refined SE-SVM model achieves good classification performance for a large-scale unbalanced dataset using augmentation and also for a small-scale dataset
4. The experimental result comparison with existing works

The rest of the paper is organised as follows: Section 2 describes the methodology and the algorithm used, section 3 talks about the experimental results with comparisons, and section 4 concludes the article with the findings.

MATERIALS AND METHODS

Dataset

The Fish4Knowledge dataset consists of 27,370 images of fish that were generated from underwater fish videos that were taken off the coast of Taiwan. This dataset includes images of 23 spe-

cies. As part of the Fish4Knowledge project, the initial dataset developed includes the snapshots of fish underwater and uses binary masks that separate the fish from their backgrounds.

Proposed methodology

The proposed methodology aims at developing an automated fish species classification for a large dataset using the hybrid framework of CNN with SE and SVM. Approaches based on deep learning can be applied without having feature extraction. By regularly changing the weights, the model automatically learns the features. When there are more layers in a deep learning model, it is referred to as being deeper. In essence, deep CNN is just CNN with more layers.

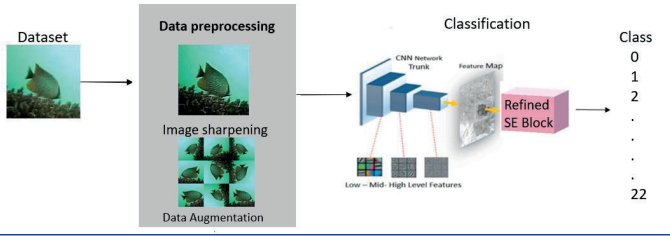


Figure 1. Proposed Architecture

Image preprocessing

As shown in Fig.1, the images from the dataset are subjected to image sharpening in the preprocessing stage. Before sharpening, the resolution of the image is slightly improved using a pre-trained model called LapSRN (Laplacian Pyramid Super-Resolution Network). LapSRN is a sequential super-resolution method that incorporates a coarse-to-fine Laplacian pyramid framework to super-resolve low-resolution images. LapSRN uses the Charbonnier loss function in Equ.1 instead of MSE loss. This loss function is robust enough to handle outliers. rs is assumed to be the s-level pyramid residual image and x_s denotes the image after up-sampling. The corresponding high-resolution (HR) image is $y_s = r_s + x_s$ and Y_s equivalent pyramid level is generated from the high-resolution images after down-sampling and the bicubic interpolation and loss function is Equ.1 and Equ.2.

$$\text{Loss}(Y, y; \theta) = \frac{1}{N}\sum_{i=1}^{i=N} \sum_{S=1}^{S=L} \delta(Y_s - y_s) \quad (1)$$

$$\text{Loss}(Y, y; \theta) = \frac{1}{N}\sum_{i=1}^{i=N} \sum_{S=1}^{S=L} \delta((Y - x_s) - r_s)) \quad (2)$$

where $\delta()$ in Equ.2 is $\delta(x) = \sqrt{X^2 + \varepsilon^2}$; X denotes $\delta((Y - x_s) - r_s))$; ε is a penalty which is a very small value. L represents the number of layer levels in the pyramid (L = 1, 2, 3); i is the image pixels, N denotes the number of pixels in the image. Usually, image sharpening is done using a Laplacian kernel where the sum of elements is 0 giving a binary image. So, we have used a modified kernel whose sum of elements is 1 giving a coloured image. Then, the sharpened images in the classes with a count less than 2000 is augmented. Since the dataset is unbalanced, image augmentation is performed. Images are randomly rotated to an angle of 90°,180° and 270° and flipped horizontally and vertically. After augmenting 1000 images per species, the total number of images increased from 27,370 to 45,360 images. The pre-processed images are sent to the deep convolutional neural network and Refined SENet for feature extraction. Then, classification of the species is done using SVM classifiers.

Feature extraction

Convolutional neural networks (CNN) can handle both feature extraction and classification methods. The performance enhancement with CNN for image classification, as illustrated in Fig.3, was remarkable. Convolution Filters use convolution to combine a kernel with the input image to produce feature maps. The pooling layer handles the down sampling procedure and can either employ Max pooling or Average pooling. Dense layers are utilised to link every neuron from the previous levels to the ones after them.

CNN architecture

A Convolutional Neural Network (CNN) is a Deep Learning algorithm which consists of multiple hidden layers where the convolution layers consist of image maps and filters. A CNN has multiple hidden layers to extract low-level features from the input image. The important layers in a CNN are: (1) Input layer, (2) Convolutional layer, ReLU layer & Pooling layer which are feature extracting layers, (3) Fully Connected layer which is the classification layer.

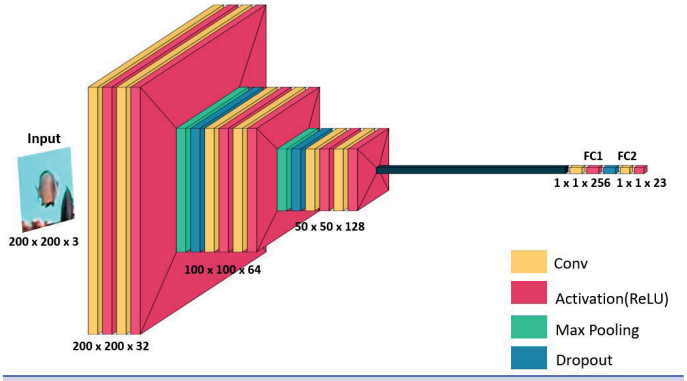


Figure 2. CNN Architecture

1. An RGB image is nothing more than a three-dimensional matrix of pixel values which is the input layer.
2. Convolution Filters use convolution to combine a kernel with the input image to produce feature maps. Lower-level features include edges or colour arrangement whereas higher-level features can recognise distinct fish shapes. The activation function, ReLU, performs an element-wise operation by setting all negative pixel values to zero. The pooling layers reduce the dimension of the feature maps. In the fish species identification, highlighted features are crucial in the images, as a result, we employed the max pooling operation, which chooses the largest element from the feature map region enclosed by the filter.
3. Dropout is an operation that ignores randomly selected neurons during training. It is a computationally cheap operation that prevents overfitting.
4. The pooled feature map is flattened to convert all the resultant 2-D arrays into a single linear vector and fed to a fully

connected layer to classify the image and get the final output.

Refined squeeze and excitation

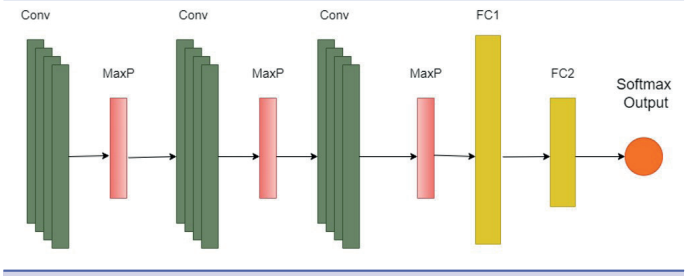


Figure 3. DeepCNN

The Squeeze-and-Excitation Block shown in Fig.5 is an architectural block that allows a network to implement dynamic channel-wise feature recalibration to improve its representational power. The SE block comes after every block of the Baselines and it can be used with any network. To generate an explicit channel relationship using global spatial features, the authors introduced a lightweight module called a squeeze and excitation block or an SE block which consists of a squeeze step and an excitation step (Hu, Shen & Son,2018).To improve the squeeze operation further we refined the squeeze operation by adding global average pooling, Equ.3, and global max-pooling layers, Equ.4, in order to get the benefits from both the layers

$$SQ^{c}_{avg} = \frac{1}{S' \times S'} \sum_{a=1}^{S'} \sum_{b=1}^{S'} (f_{a,b,c}) \quad (3)$$

$$SQ^{c}_{max} = \max_{a,b=1}^{S',S'} (f_{a,b,c}) \quad (4)$$

where S' denotes the modified dimensions and f ∈ RS' ×S' ×B' is the input feature map to the SE block, fa,b,c is the feature at (a,b) position. SQ^{c}_{avg} and SQ^{c}_{max} are the cth channel's squeezed values applying the global average and maximum pooling. The squeeze technique fundamentally extracts the channel-specific information. Further, the maximum pooling will keep the information in a local context, whereas the global pooling will retain the knowledge in a global context. The matrix is aggregated into a Squeeze-and-excite operation to produce a matrix that can emphasise information features and suppress less useful information channel-wise, and it is also proven to improve the image classification performance. For current state-of-the-art CNNs, SE blocks greatly enhance performance at a small additional computational cost (Hu et al., 2018). In order to get high performance and accuracy, we integrated CNN and a Refined SE (Squeeze and Excitation) Block.

Proposed CNN-SE architecture

The architecture of the CNN-SENet is depicted above in Fig.4. The input image of size 200 x 200 with 3 RGB channels is fed as input to the convolution block. The first and second iterations of this block have 32 filters in 3 x 3 each, followed by a ReLU activation function and then max pooling, followed by a dropout layer. The third and fourth iterations have 64 filters in 3 x 3 each, followed by a ReLU activation function and then max pooling, followed by a dropout layer. The fifth iteration has a convolution layer containing 128 filters in 3 x 3 followed by a ReLU and then a final iteration with 128 filters. This output is batch normalized and then enters the refined SE Block which is depicted in Fig.6

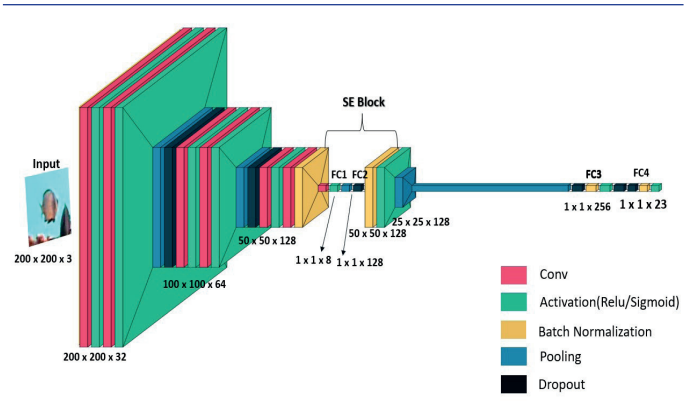


Figure 4. CNN-SE Architecture

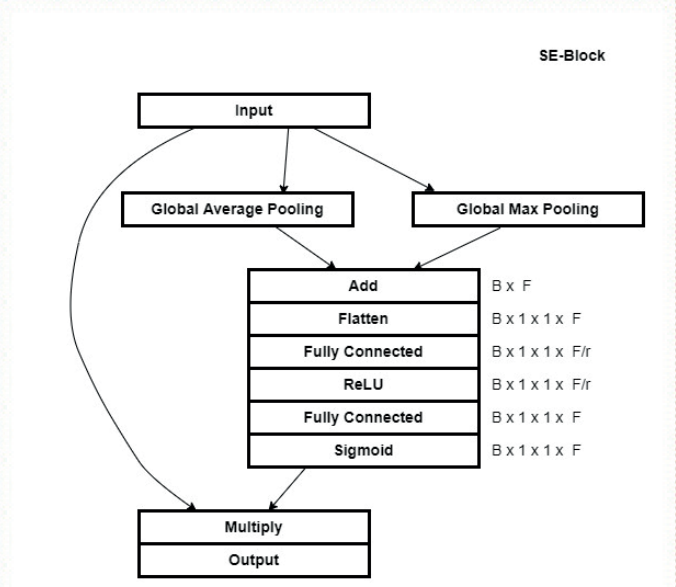


Figure 5. Refined SE Block

1. The input for the Refined SE block in Fig. 5 is a convolutional block.
2. Using global average pooling and global max pooling, each channel is "squeezed" into a single numeric value.
3. Two squeezing channels are taken into consideration to produce the excitation scores through the addition of global average pooling and global max pooling respectively.
4. Two fully connected layers with ReLU and Sigmoid activation are used:

- We use two fully connected layers to get non-linear relations
- We use independent sigmoid activation to get non-mutually exclusive channel relations
- The intermediate layers' node size is reduced for better generalization and to reduce computation overhead (F / r)

5. The output of the SE block (channel attention) is multiplied channel-wise to the original input (Hu et al.,2018)

This offers a CNN building block, shown in Fig.2, that improves network dependencies at nearly no cost in terms of computation. With the dimension size unchanged, this multiplied outcome is then extended to a rectified linear layer that performs element-wise activation. The result is then dimensionally reduced and resized by passing it through a Max Pooling layer (2x2). The network also comprises two fully connected (FC) levels. The first FC layer, which has 256 neurons, is flattened after max pooling. After batch normalising, the output from this fully connected layer, a reduction function, is applied. Before the final fully connected layer is executed, a 20% dropout layer is employed, which has 23 neurons. Softmax is the final layer, which uses a classifier function to calculate the probability distribution for each class. The Adam optimizer applies a categorical cross-entropy to the data.

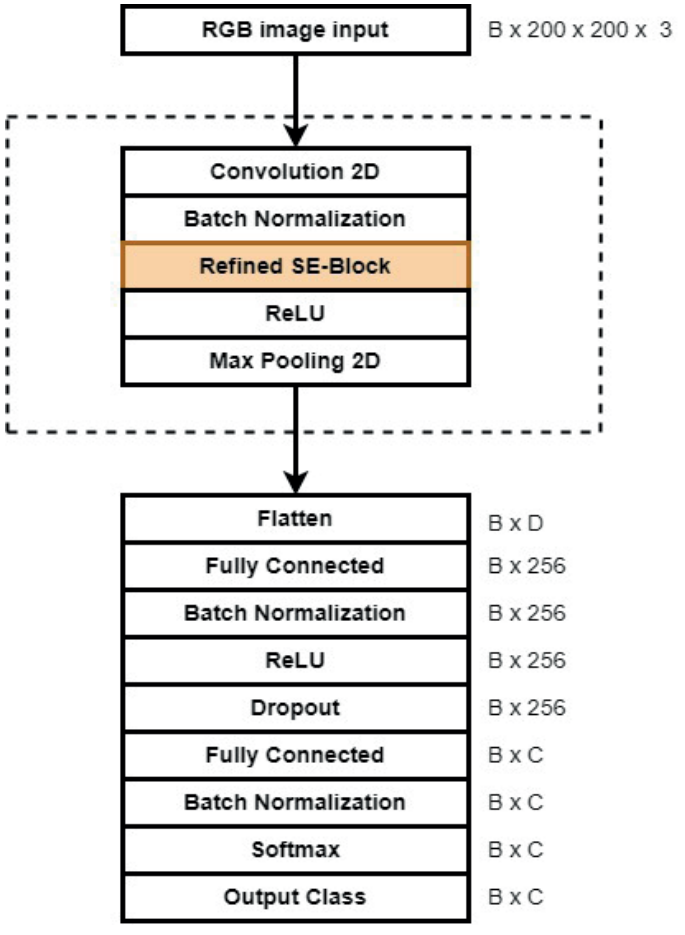


Figure 6. CNN SE Block

Training procedure

Adam was used as the optimiser and a batch size of 24 on various epochs with a default learning rate of 0.001. We divide the training set of images into batches of 24. It takes 1276 iterations to complete 1 epoch. Experiments are run on a system with an AMD Ryzen 3250U processor running at 260 GHz using 4 GB of RAM, running Windows version 10. The Keras and TensorFlow frameworks are used to implement the proposed work. The platform we employed for the execution was Google Collaboratory in which a GPU hardware accelerator is enabled.

Performance evaluation parameters

Accuracy, precision, f1-score and recall are the performance metrics used for performance comparison.

Accuracy: Accuracy is a popular metric in multi-class classification that may be calculated straight from the confusion matrix. Accuracy is a metric that indicates how well the model predicts the whole collection of data accurately. To validate the proposed hybrid model, training and testing is carried out using the Fish-4Knowledge dataset (Phoenix et al., 2013).

$$Accuracy = \frac{TP + TN}{TP + FP + TN + FN}$$

True Negative (TN): the actual is not fish and predicted is also not fish
True Positive (TP): the actual is fish and predicted is also fish
False Negative (FN): the actual is fish and predicted is not fish
False Positive (FP): the actual is not fish and predicted is fish

Precision: A model's precision describes how many of the identified items are relevant. It is defined as the ratio of true positives to the sum of true positives for each class.

$$Precision = \frac{TP}{TP + FP}$$

Recall/Sensitivity: The number of positive class predictions made from all positive cases in the dataset is calculated.

$$Recall = \frac{TP}{TP + FN}$$

F1 score: The F1 score, which ranges from 0.0 to 1.0, is a weighted harmonic mean of recall and precision. The scores for each class indicate how accurate the classifier was in classifying the data points in that class in comparison to all other classes.

$$F1\ score = 2 * \frac{Precision * Recall}{Precision + Recall}$$

Support: Support is the total number of actual count of each class in the test set. For example, in Fig.8, under the support column, fish_01 has 1197 test images.

Confusion Matrix: A confusion matrix, Fig.7, is a table that lists how many predictions a classifier made correctly and incorrectly. It is employed to evaluate a classification model's effectiveness. There are 23 classes in the reported study and hence the confusion matrix shown in Fig.7 has 23* 23 values.

RESULTS AND DISCUSSION

Experimental Results for the proposed method using the Fish4Knowledge dataset

After training the model through 50 epochs, we achieved sufficient accuracy and further training did not improve accuracy on the validation set. The model was then evaluated on the test dataset and the results are shown in Fig.8. The performance values of all 23 classes are listed. The classes, 10, 16, 19, 20, and 21, have an f1 score of 100%, and all others range from 93% to 99% except for class 8. The accuracy obtained is 98%.

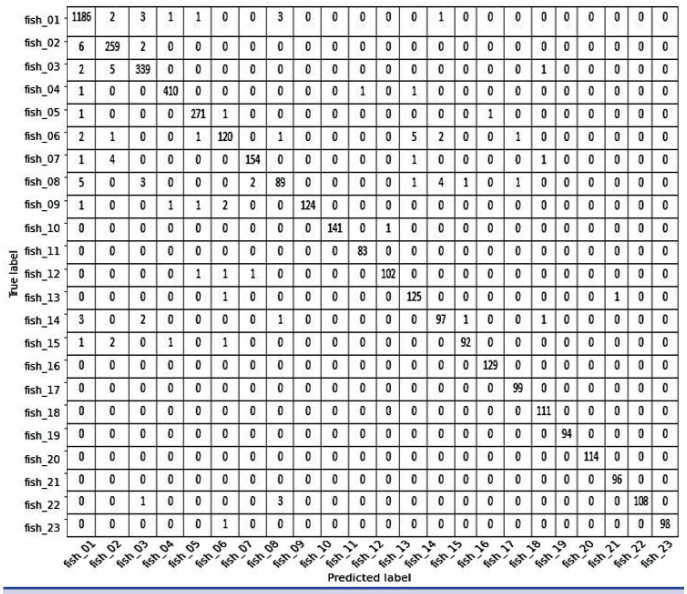


Figure 7. Confusion Matrix for the deepCNN-SENet with SVM

Table 1. Parameter sizes at different r for SE Block and Refined SE Block

r	'p' size with SE	'p' size with Ref-SE
8	22.778M	22.778M
16	22.776M	22.776M

where 'r' denotes the reduction ratio and 'p' denotes the parameter size. As we can see from Table.1, increasing the r value has decreased the parameter size slightly.

As we train the model through multiple epochs, we can see in Fig.9a the train set accuracy (blue) is steadily increasing, and the validation set accuracy also increases along with it. Since the validation set accuracy has not decreased compared to the training set, we find that variance is low, and the model has not overfit the training set. The final validation accuracy is 97.36%. Since the validation set accuracy is high, we find that bias is also low.

The training loss steadily reduces to below 1.0 in Fig.9b throughout the span of 8 training epochs and a lower loss value on the validation set indicates that the model successfully fit the training data. As a result, the model's overall performance is satisfactory.

	precision	recall	f1-score	support
fish_01	0.98	0.99	0.99	1197
fish_02	0.95	0.97	0.96	267
fish_03	0.97	0.98	0.97	347
fish_04	0.99	0.99	0.99	413
fish_05	0.99	1.00	0.99	272
fish_06	0.95	0.91	0.93	132
fish_07	0.98	0.96	0.97	161
fish_08	0.92	0.84	0.88	106
fish_09	1.00	0.96	0.98	129
fish_10	1.00	0.99	1.00	142
fish_11	0.99	1.00	0.99	83
fish_12	0.99	0.97	0.98	105
fish_13	0.94	0.97	0.95	129
fish_14	0.93	0.94	0.94	103
fish_15	0.98	0.96	0.97	96
fish_16	1.00	1.00	1.00	129
fish_17	0.99	1.00	0.99	99
fish_18	0.97	1.00	0.99	111
fish_19	1.00	1.00	1.00	94
fish_20	1.00	1.00	1.00	114
fish_21	1.00	1.00	1.00	96
fish_22	1.00	0.96	0.98	112
fish_23	1.00	0.99	0.99	99
accuracy			0.98	4536
macro avg	0.98	0.97	0.98	4536
weighted avg	0.98	0.98	0.98	4536

Figure 8. Performance evaluation of proposed frameworks

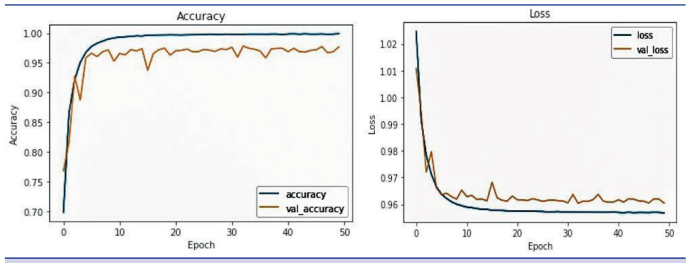


Figure 9. Accuracy and Loss plot of CNN-Refined SENet-SVM on the Fish4knowlege dataset

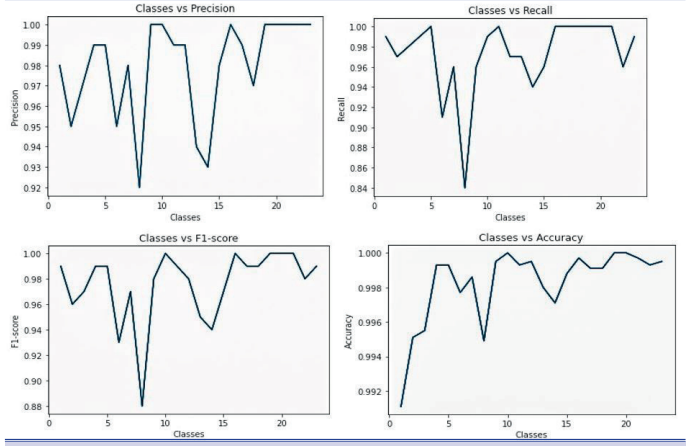


Figure 10. Metrics vs Classes

Fig.10 demonstrates the results of our model under different evaluation metrics. In Fig.10d we can see that the performance is particularly outstanding for species 22 and 23 with an accuracy of 99.95%. Fig.7 shows, for species 22, that 111 images out of 112 are correctly identified, and for species 23, all the images from the test data are correctly identified.

In Table.2, we can see that the CNN with SE model using the Fish4Knowledge dataset with 15 epochs achieved an accuracy of 97.15%. The best results are highlighted. CNN's TPE (Time per epoch) for 5 epochs is 144 sec and CNN+SENet took 157 sec for the same. This slight increase in the computational cost can be justified by its model's performance improvement (accuracy of CNN is 89% and CNN plus SE is 96.25% for 5 epochs).

In Table.3, we can see that CNN with SE model using the Fish4Knowledge dataset after pre-processing the images with 50 epochs achieved an accuracy of 97.77%. The hybrid CNN+SE model with SVM Classifier for the same dataset with squared-hinge loss has achieved an accuracy of 97.83% for 50 epochs. Finally, an accuracy of 97.90% for 50 epochs for the Refined SE model is recorded. This proves that refined SE improves the performance of the hybrid model.

The Cross-entropy loss is used for CNN-SENet and squared-hinge loss for CNN-SE with the SVM classifier. Table.4 proves that CNN-SE with SVM classifier has better performance.

Table 5 shows the comparison of the proposed model with the existing works. The proposed model has an improved accuracy of 97.9%, but Ensemble of Google InceptionNet and SVM achieved 95.37%, DNN achieved 96% and MLR_VGG19 achieved 97.09%. The proposed model has improved by 0.89% when compared with MLR_VGG19.

Experimental Results for the proposed method using small

Table 2. Experimental Result for Fish4Knowledge

Methodology	Epochs	Accuracy	Precision	Recall	F1-score
CNN	5 epochs	89%	0.85	0.60	0.68
	10 epochs	92.06%	0.80	0.60	0.66
	50 epochs	94.70%	0.94	0.95	0.64
CNN+SE	5 epochs	96.25%	0.93	0.82	0.85
	10 epochs	97.08%	0.94	0.83	0.87
	50 epochs	**97.15%**	**0.92**	**0.83**	**0.88**

Table 3. Experiment & Result for Fish4Knowledge after Preprocessing

Methodology	Epochs	Accuracy	Precision	Recall	F1-score
CNN+SE	15 epochs	97.46%	0.97	0.98	0.97
	50 epochs	97.77%	0.98	0.98	0.98
CNN+SE+SVM	15 epochs	97.28%	0.97	0.97	0.97
	50 epochs	97.83%	0.98	0.98	0.98
CNN+Refined SE+SVM	15 epochs	97.70%	0.98	0.97	0.97
	50 epochs	**97.90%**	**0.98**	**0.97**	**0.98**

Table 4. Metrics vs Loss

Metrics	Cross Entropy Loss	Squared Hinge Loss
Accuracy(%)	97.77	97.83
Recall(%)	97.78	97.78
Precision (%)	97.56	97.52
F1-score	97.69	97.73

Table 5. Comparison with existing state of art for Fish4Knowledge Dataset

Reference	Model	Accuracy
(Murugaiyan et al.,2021)	Ensemble of Google InceptionNet and SVM	95.37%
(Zhang et al.,2021)	DNN	96%
(Prasetyo et al.,2021)	MLR _VGG19	97.09%
Proposed model	**CNN-Refined SE-SVM**	**97.90%**

scale Croatian dataset

In a paper (Qiu et al.,2018), they used a small-scale Croatian dataset for post-training and achieved an accuracy of 83.56% for B-CNN with SE. They primarily pre-trained the model on the ImageNet dataset, then on the Fish4Knowledge dataset (Phoenix et al.,2013), and finally on a Croatian dataset to fine-tune it (small-scale fine-grained dataset). The Croatian Fish Dataset has a total of 794 images with 12 classes and after augmenting 500/1000 images per species, the total number of images increased to 10,794 images. The results are tabulated below by applying the proposed model on this small-scale dataset with and without augmentation.

the model, so it is a good fit for the training data. After 20 epochs, it maintains a uniform value in the validation accuracy.

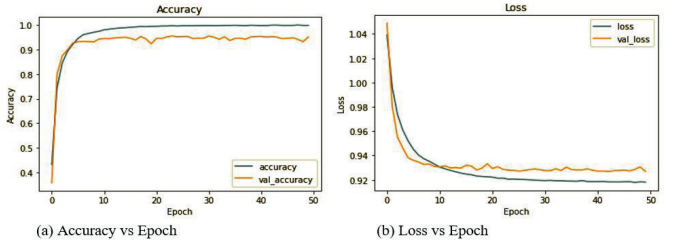
Table.7 shows the accuracy achieved by Qui et al. (2018), where they pre-processed the Croatian dataset with SRGAN, and augmentation was also performed. They also employed pre-training with ImageNet and the Fish4Knowledge dataset in their B-CNN with the refined SENet model, which consists of 10 convolution blocks, while the proposed model consists of 3 convolution blocks. The maximum accuracy produced by Qui et al. is 83.92%. However, the accuracy of the proposed model is 94.99%. It is well proven that there is a performance improvement of 11% when compared to BCNN-SE.

Table 7. Comparison with existing state of the art for Croatian Dataset using transfer learning

Method	Model	Accuracy
(Qui et al.,2018)	BCNN	83.52%
	BCNNs-SE blocks	83.78%
	Improved BCNNs-SE blocks	83.92%
Proposed model	**CNN-refined SE-SVM**	**94.99%**

Table 6. Experimental Result for Croatian Dataset after Preprocessing (Wo_A(Without Augmentation),W_A(With Augmentation)

Methodology	Epoch	Accuracy
CNN (Wo_A)	50 epochs	31.5%
CNN+SE(Wo_A)	50 epochs	56.6%
CNN+SE+SVM (W_A)	30 epochs	79.2%
CNN+SE+SVM (W_A)	**50 epochs**	**94.99%**



(a) Accuracy vs Epoch (b) Loss vs Epoch

Figure 11. Accuracy and Loss plot of CNN-SENet-SVM on Croatian dataset

From Table 6, it is seen that the CNN with SENet model using the Croatian dataset with 30 epochs achieved an accuracy of 71.37%, and then the hybrid CNN integrated with the SVM classifier achieved an accuracy of 79.2%. The lower accuracy could be due to the dataset's limited size and the lack of pre-training. Therefore, the weights from the pretraining step (CNN-SE-SVM) with the Fish4Knowledge dataset are loaded before initiating post-training with this small-scale dataset (Croatian), and the accuracy of the model improves from 79.2% to 94.99% in comparison with the previous result. Hence, transfer learning has a greater impact on performance. From Fig.11 we can see that the accuracy and loss plots are stable, therefore the transfer learning process has not overfit

CONCLUSION

In this study, we have proposed a hybrid framework comprising of CNN, Refined SE, and SVM for identifying 23 different fish species and increased model performance on the Fish4knowledge dataset and achieved an accuracy of 97.90%. It can work on small-scale and large-scale datasets by enhancing transfer learning and squeeze-and-excitation networks for fish image classification on small-scale datasets due to the non-informative channel suppression property of SE blocks, and enhanced classification using SVM. By post-training this model on the Croatian small-scale dataset, we achieved 94.99% accuracy. Thus, the proposed method, CNN-Refined SE with SVM, shows an 11% improvement over the existing method BCNN-SE. This model has achieved better generalisation and distinguishes the fish species well. In future work, this model will be modified to identify the absence of fish and some super-resolution techniques can be used to handle ocean images with varying lighting conditions.

Conflict of interest: There is no conflict of interest.

Ethics committee approval: This study does not need ethical approval.

Financial disclosure: NIL

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Research Article

Application of Hypothetical Ecological Risk Analysis to Sustainable Usage of Possible Winter Recreation Areas in Seyhan Basin (Türkiye)

Okan Yeler[1], Gazel Burcu Aydın[2], Belgin Çamur-Elipek[2], Süha Berberoğlu[3]

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ABSTRACT

In this study, the long-term suitability of the area proposals for winter recreation activities in the Seyhan Basin (Türkiye), which is located in the Mediterranean and Central Anatolia regions and includes a large part of the Taurus Mountains, were examined ecologically. For this purpose, the predicted global warming scenarios in the basin and the anthropogenic impacts arising from the planned recreation areas were evaluated for the upper basin (recreation areas) and lower basin (water resources, agricultural lands, and settlements) using a hypothetical risk analysis. For this purpose, multispectral images were obtained by using Landsat 8 Oli Multispectral images of the snow areas in the region in January-February-March 2019, and a hypothetical ecological risk analysis was created considering a total of 5 pressure factors originating from global climate change and anthropogenic effects. These possible factors were determined as flood (S1), drought (S2), sedimentation (S3), aquatic nutrients (S4), and tourist density (S5). The effects of these factors on a total of four features (C1: water quality, C2: fauna-flora, C3: agricultural areas, and C4: settlements) in the region were evaluated by hypothetical grading based on the literature. According to the hypothesis results obtained by the formula and statistical calculations, it was determined that the flood factor (S1) that will occur due to possible snow melt due to global climate change in the winter recreation areas in the studied region is the most significant factor limiting the sustainable usage of the Basin. For this reason, it has been emphasized in this study that the possibility of regions being exposed to the effects of climate change in the future should be taken into account, especially when planning for winter recreation areas. At the end of this study, it was concluded that the ecological balance analysis of basins is important, especially in terms of ensuring the long-term sustainable use of winter recreation areas.

Keywords: Climate change, Ecology, Environmental parameters, Hypothetical risk, Sustainability

ORCID IDs of the author:
O.Y. 0000-0002-0405-4829;
G.B.A. 0000-0002-9131-240X;
B.Ç.E. 0000-0002-0954-8967;
S.B. 0000-0002-1547-6680

[1]Van Yüzüncü Yıl University, Architecture and Design Faculty, Landscape Architecture Department, Van, Turkiye

[2]Trakya University, Faculty of Science, Department of Biology, Edirne, Turkiye

[3]Çukurova University, Faculty of Architecture, Adana, Turkiye

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Correspondence:
Gazel Burcu AYDIN
E-mail:
gburcuaydin@trakya.edu.tr

INTRODUCTION

Considering the basic needs and the balance of protection/use of recreational areas due to the increase in the population living in the cities and the acceleration of urbanization activities, the decrease in the quality of life due to the lack of recreational activities emerges as the most important problem (Değerliyurt & Çubuk, 2015). Although Turkiye has recreational areas that can be created in many different regions, from ecosystems at sea level to ecosystems in high mountain areas, it is important that the usability in these areas is sustainable. For this reason, deficiencies in the analysis of the relationship between natural/cultural features and systems in planning and design processes leads to the deterioration of the natural balance and the inability to meet the needs.

One of the recreational activities that people prefer is undoubtedly winter sports activities. The suitability of the areas where these activities will be planned is related to meteorological

events and it can be said that it will be one of the areas most affected by global climate change. However, in order to determine the recreational areas suitable for winter sports, many factors, especially the potential snow amounts in the region and the effects of global warming, should be evaluated together. In addition, it should not be forgotten that the ecological changes that these effects will create in the upper basin will also affect the lower basin.

Turkiye hosts many areas which are potentially suitable for all kinds of recreational activities. There are many suitable areas in Turkiye for winter sports, which is one of the recreational activities that people prefer, especially in winter. However, it is reported that even in the first priority centers for winter sports in Türkiye, there are tourism investments that are not compatible with the environment based on incorrect land use (Akın & Erdoğan, 2017). For this reason, the adaptation of the activity to the ecosystems is as important as the suitability of the site for the activity in the selection of recreational areas.

Snow, which is an important component of mountainous ecosystems, in particular, will undoubtedly exhibit a very sensitive structure against temperature increases due to global warming, which has become more important in recent years. This situation will not only affect the areas planned for winter recreation but also cause changes in the ecosystem balance. Because it is inevitable that the snowmelt in the upper basin will put pressure on other ecosystems, especially the freshwater resources in the -basin (Dönmez, Berberoğlu & Çilek, 2016). In addition, anthropogenic effects (such as aquatic nutrient salt and pollution) in the upper basin will be carried to the lower basin by the melting of snow. For this reason, it is also important to determine the ecological risks by taking into account the global warming scenarios as well as the amount of snow in winter recreation areas when planning.

Physical and chemical components of the ecosystem, climate changes, and anthropogenic effects are among the main factors that cause pressure on ecosystems. Determining the probability of adverse effects due to these physical, chemical, and biological pressures is called "ecological risk analysis" (USEPA, 1998; Salihoğlu & Karaer, 2004; Erdoğan, 2012). When performing an ecological risk analysis, it is important to identify "ecological risk factors" (ERF), which represent possible and potential pressures on the preservation and long-term sustainability of the ecosystem structure. Thus, the sustainability of the ecosystem can be ensured by predicting the long-term effects of the factors causing ecosystem degradation and determining the measures that can be taken.

There are a lot of analyses on Ecological Risk Assessment (ERA), which can be used for different ecosystems: Harris et al. (1994) developed a methodology based on anthropogenic stressors affecting an ecosystem and a set of impaired use criteria; Håkanson (1980) used an ecological risk index for aquatic pollution control based on a sedimentological approach; Elias et al. (2014) and Ilie et al. (2017) evaluated heavy metal contamination by ecological risk index for freshwater ecosystems.

ERA analysis is one of the important environmental management tools applicable to various ecosystems and is characterized as an essential process for developing environmental management decisions, and compiling and presenting scientific information (Lemly, 1997; Serveiss, 2002). In addition, studies on the application of ERA by using Geographic Information Systems (GIS) and ecology are becoming increasingly common (Lemly, 1997; Preston, 2002; Solomon & Sibley, 2002).

In Türkiye, the Seyhan Basin is reported to be one of the areas that will be significantly affected by global climate change (Özfidaner, Şapolyo & Topaloğlu, 2018). Researches indicate that the average monthly temperatures in the Seyhan Basin (especially in the months of January, April, October, November, and December) will increase by 3^0C and the annual precipitation will decrease by 25% in the near future (Özfidaner et al., 2018). It is also reported that these climate changes will adversely affect water resources, snow storage, and groundwater potential by up to 30% in many basins (Kimura, Kitoh, Sumi, Asanuma & Tatagai, 2006; Tezcan et al., 2007).

Although there are studies on the seasonal snow dynamics of some areas in the Seyhan Basin and the proposal for suitable recreation areas in the region, no comprehensive study has been found that considers the basin as a whole and evaluates the ecological risks together. In this study, the suitability of the potential recreation areas for winter sports in the mountainous regions of the Seyhan Basin was examined by considering the future global climate change scenarios and the possible ecological effects of the settlements to be located in these regions on ecosystems. For this purpose, ecological risk factors determined based on global climate change scenarios and the literature were evaluated with a hypothetical ecological risk analysis and it was aimed to make some suggestions on the sustainability of winter recreation areas to be planned.

MATERIALS AND METHODS

Study area

The Seyhan Basin, located in Central Anatolia and the Mediterranean Region, is located between the coordinates 36° 33'- 39° 12 'North and 34° 24' - 36° 56 'East. Seyhan, Göksu, and Zamantı Rivers are located in the basin (Figure 1). In addition, most of the northeastern extensions of the Taurus Mountains are located within the basin, and the Seyhan Basin has very favorable conditions for both mountain and water recreation areas.

A large part of the basin is located in the Mediterranean climate zone with an average rainfall of 700 mm in winter, and it is an important food production area for Turkiye and Europe as well as being a common agricultural and pasture potential (Özfidaner et al., 2018). Therefore, water resources and management in the basin are very important for the agricultural production, ecosystem productivity, food safety, and biodiversity future of the basin.

Methods

In this study, image bands of areas suitable for winter recreation, using Landsat 8 Oli Multispectral images, were combined and a Red Green Blue (RGB) color code was set as 5-6-4. Thus, images of snow areas covering the months of January-February-March 2019 were obtained. Then, considering the effects of global warming scenarios and the effects that may develop depending on the tourist density in the region, a total of 5 different ecological Impact Factors (IF) were determined. These hypothetical IFs are grouped

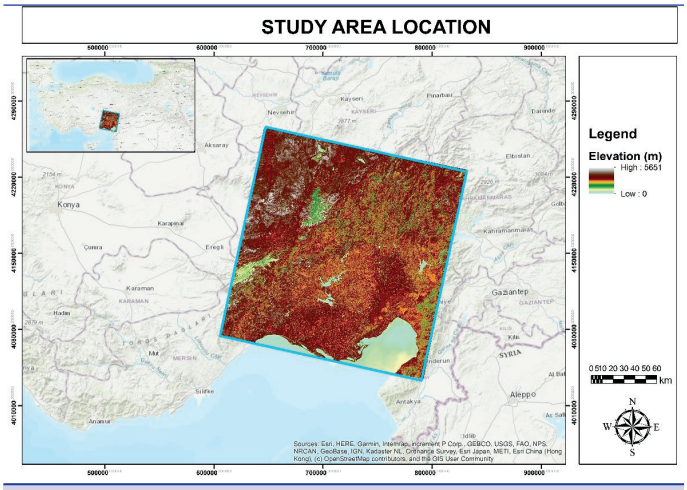

Figure 1. The location of study area

into flood (S1), drought (S2), sedimentation (S3) due to global warming, aquatic nutrient salt increase (S4), and tourist density (S5) due to anthropogenic use (Table 1). Also, for the hypothetical ecological risk assessment, a total of 4 main factors (MF) to be affected by IFs were identified: water quality (C1), fauna-flora (C2), agricultural lands (C3), and settlement areas (C4). A conceptual model developed by Harris et al. (1994) was applied to the identified factors (Figure 2). This conceptual model is used as a multi-criteria decision-making technique derived from fuzzy logic theory and allows the comparison of environmental risks with the risks presented to the ecosystem (Harris et al., 1994). The relevant literature was used to determine the degree of effect in the formula (Salihoğlu & Karaer, 2004). Accordingly, the main of "0 degree" was determined as "ineffective", "1 degree" as "mild effect", "2 degrees" as "effective", "3 degrees" as "severe effect", hypothetically. In the formula, the "k" value was taken as 1, "m" value was taken as 5, and the effect of each IF on each MF was determined as Matrix R.

$$D_k(i,j) = X_{ik} - X_{jk}$$

$$\text{Matrix R} = r_{ij} = \sum_{k}^{n} D_k(i,j);\ j=1,2,\ldots,m$$

Figure 2. The Formula of Hypothetical Ecological Risk Analysis (Harris et al., 1994)

RESULTS AND DISCUSSION

In this study, the future ecological risks of the areas to be proposed for winter recreation in the mountainous regions in the north of the Seyhan Basin were evaluated based on global climate change scenarios in the literature and the effects that will occur due to anthropogenic use. For this purpose, a hypothetical ecological risk analysis based on the literature was conducted. Thus, it is aimed to make suggestions for the long-term sustainable use of the areas to be proposed as a winter recreation area and to be invested in the basin.

Firstly, satellite images of areas with snow dynamics suitable for winter recreation in the mountainous regions of the Seyhan Basin between January-February-March 2019 were obtained and are presented in Figure 3. When the maps were evaluated, it was observed that the snow dynamics, especially in January and February, were suitable for winter recreation, but there were rapid area contractions due to the melting of snow with the onset of the spring season. This observation was evaluated as an indication that the snow melting at the beginning of spring from the areas to be planned for recreation will affect the downstream. Thus, the situations that may occur due to global warming in the Region (flood, drought, sedimentation) and the situations that may occur due to anthropogenic use in recreation areas (increased aquatic nutrient salt and tourist density) were considered as pressure factors (IF). In addition, the impact values of these IFs especially on the sub-basin (water quality, flora and fauna, agricultural lands, and residential areas) were also determined using the literature and formulated hypothetically. Accordingly, obtained results of hypothetical impact situations are presented in Table 2. According to the hypothetical impact matrix results, it was determined that the most influential factor was flood (S1=22), followed by drought (S2=17), sedimentation (S3= -3), nutrients (S4= -8),

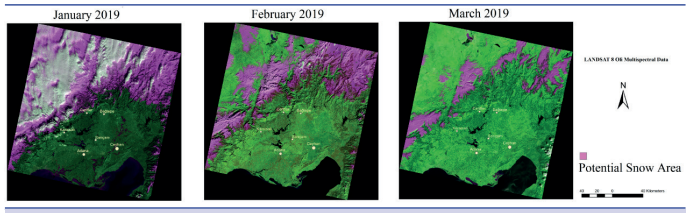

Figure 3. Satellite Images of Snow/Water Dynamics in Seyhan Basin (LANDSAT 8 Oli Multispectral Data)

Table 1. Hypothetical ERA pressure profile for Seyhan Basin Winter Recreation Areas

	Criteria			
Pressure Elements	**Water quality (C1)**	**Fauna-Flora (C2)**	**Agricultural land (C3)**	**Settlement area (C4)**
Flood (S1)	3	3	3	3
Drought (S2)	3	3	3	2
Sedimentation (S3)	2	2	1	2
Nutrients (S4)	2	2	2	0
Tourist population (S5)	1	1	0	0

0: No effect, 1: Slight effect, 2: Considerable effect, 3: Severe effect

Table 2. Hypothetical Impact Matrix Results

Stressors Assessment	S1	S2	S3	S4	S5	Row Sum (Impact Assessment)
S1	0	1	5	6	10	**22**
S2	-1	0	4	5	9	**17**
S3	-5	-4	0	1	5	**-3**
S4	-6	-5	-1	0	4	**-8**
S5	-10	-9	-5	-4	0	**-28**

and tourist density (S5= -28) (Table 2).

Flood and drought are among the leading effects of global warming, and it is estimated that this situation will have negative consequences both in the upper basin recommended for winter recreation and in the lower basin due to the resulting effects. It was predicted that this situation will especially affect water resources, agricultural areas, and settlements in the lower basin, affecting both the upper and lower basin fauna and flora with the creatures they drag. On the other hand, the runoff of melting snow due to global warming can be beneficial by bringing the nutrient salts from the water to downstream agricultural fields. However, erosion material in flood sediment can cause turbidity of the water and degradation of the fauna/flora ecosystem. It should also be noted that the negative effects of melting and drought on water quality will be the most important factors that will affect the lower basin rather than the upper basin. In addition, the human population in winter recreation areas will also affect the upper basin and indirectly will contribute to the negative impacts lower basin.

Although snow dynamics is an important component for winter recreation activities planned in mountainous areas and plays an important role, especially in the establishment of winter tourism, many factors such as land appearance, elevation, slope, soil groups, and hydrological structure are also taken into account. However, evaluating the effects of global warming in these areas is also very important in terms of the sustainability of the areas to be determined and ensuring the balance between ecosystems.

Snow cover plays an important role in mountainous hydrological balance, and flow water stored in the form of snow in the higher parts of a basin is a potential freshwater reservoir that can be used when the snow begins to melt (Dönmez et al., 2016). In addition to providing seasonal freshwater potential in the upper and lower basin interactions, snow can be a potential hazard for rapid melting and cause major flooding problems in lowland areas. With these characteristics, the snow process is considered a critical component of hydrological processes and dynamics of many basins, especially in mountainous regions. Estimates of the temporal and terrestrial distribution of natural snow cover in snow processes are among sustainable planning objectives and issues, particularly in terms of landscape planning for the selection and suitability of potential runway areas. However, in such studies, the analysis of ecological balance and the effect of snow water quality on lower-basin water quality can be neglected. Also, with the melting of the snow, the waters mixed with the surface resources will be effective in the areas fed by snow water. Especially, the snow thickness measured in spring plays an important role in estimating the entry of snow water into regional hydrological systems (Yücel & Güventürk, 2012). Therefore, monitoring seasonal snow thickness is of great importance for climatological, hydrometeorological, and water-based issues (Tekeli, Akyürek, Şorman, Şensoy, & Şorman, 2005). However, it should not be forgotten that the thickness of snow and the presence of snow can be affected by global warming and this can directly or indirectly affect the aquatic ecosystems in the lower basin, and therefore agriculture and settlement areas.

The Seyhan Basin, which is considered within the scope of the study, is located in the Central Anatolia and Mediterranean region, and it is a suitable region for both mountainous and aquatic recreation areas as it includes a part of the Taurus Mountains. In this study, the results of previous studies were also taken into account while evaluating the long-term use of the regions to be designated as winter recreation areas in the basin.

In a study to determine suitable areas for winter sports in Aladağlar, located in the Seyhan Basin, it was emphasized that environmental sensitivity should also be taken into consideration (Akın & Erdoğan, 2017). Dönmez et al. (2016) analyzed the seasonal snow dynamics in the Eğribük sub-basin in the Seyhan Basin and they reported that the main expected effect of temperature increase will be on the change of stream flows. Gönençgil and İçel (2010) examined the precipitation changes in the Eastern Mediterranean coasts and reported that precipitation decreases in this region will have negative effects on human life, and natural geographical elements and processes. Gürkan (2005) evaluated the effects of climate change on precipitation, drought, agricultural yield, and water resources in the Seyhan Basin. Bayer-Altın & Barak (2012) investigated the temperature and precipitation rates of the Seyhan basin between 1970 and 2009, and reported that they observed significant increases in temperature and a significant decrease in precipitation. Similar results were also reported in the studies by Türkeş, Koç & Sarış (2007) and Özfidaner et al. (2018). Similarly, the possible effects of climate change on Catalan dam flows in the Seyhan basin were examined by Malkoç, Arslan, Diren & Sargın (2013), and significant flood events were reported in the basin, especially due to snowmelt in spring. In a study conducted by Kantarcı (2012), flood events and the effects of Çakıt sub-basin in Seyhan Basin were examined and it was reported that floods are more frequent in this region due to drought. By evaluating the flow data of Seyhan Basin by Özfidaner et al. (2018), drought analysis was made and it was emphasized that drought would affect the natural life. Yüce, Ercan, Equal, Ünsal & Yüce (2018) analyzed the precipitation data of Seyhan basin and reported significant decreases in some. It is stated that the temperature changes com-

pared to the 1971-2000 reference period are approximately 10 ^{0}C in the north of the Seyhan Basin, 20 ^{0}C in the south, and an average of 13 ^{0}C in the inner parts of the basin (Tezcan et al., 2007). In Tezcan's study, it is stated that the temperature increase in the region will jump after the 2021-2030 period, and the temperature will be slightly higher in regions far from the sea (Tezcan et al., 2007). In addition, in the study conducted by Bayer-Altın and Barak (2012), they examined the changes in precipitation and air temperature in the Seyhan Basin between 1970 and 2009 and determined that the temperature increase occurred in all seasons and there was a tendency towards arid conditions. In a modeling study conducted by Askar and Başıbüyük (2020), the impact of climate change on the water resources of the basin was evaluated and it was reported that negative effects due to temperature increase are expected in the 2045-2080 period. As it can be understood from all these studies it should be taken into consideration that the Seyhan Basin will be adversely affected by global climate change. So, it is suggested that snow and water-based recreation area planning, scenarios that may develop due to global climate change, and ecosystem balances should also be taken into consideration in order to make more robust recommendations for the determination of suitable recreation areas.

CONCLUSION

In this study, some possible predictions for the long-term sustainable use of winter recreation areas to be proposed in the mountainous parts of the Seyhan Basin are emphasized. Especially, it was recommended to consider the possible ecological effects that may occur in the basin due to global climate change. In addition to the problem of decreasing water resources due to drought, there is the discharge of sediment and waste from settlements in recreation areas to the lower basin due to the early melting of snow waters. This situation may cause changes in water quality that will develop with sedimentation, may affect the fauna and flora depending on water quality, and may cause an increase of nutrient salts in the lower basin.

While determining the suitable area for winter recreation, it is recommended to carry out comprehensive ecological analyses as well as an assessment of the geographical features and snow potential of the area. Thus, long-term use of these areas can be ensured.

Conflict of Interests: The authors declare that they have no financial interests or personal relationships that could affect this work.

Ethics committee approval: This study does not need ethical approval.

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Short Communication

Microplastic Occurrence in the Gastrointestinal Tract of a Risso's Dolphin *Grampus griseus* in the Northeastern Mediterranean Sea

Nebil Yücel[1], Ece Kılıç[1], Cemal Turan[1], Sefa Ayhan Demirhan[1]

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ABSTRACT

Risso's dolphin *Grampus griseus* was stranded on the coast of Arsuz-Uluçınar, Iskenderun Bay, Turkey in the northeastern Mediterranean on 29 March 2022. This study was conducted to reveal the relationship between *G. griseus* and microplastic debris in the northeastern Mediterranean Sea. The gastrointestinal tract (GIT) of one stranding *G. griseus* was examined, and 454 microplastics particles were extracted. Of all, the majority of them were fibers (96%), black in colour (62%), and 0.5-1 mm in size (38%). This paper represents the first data indicating the microplastic abundance in *G. griseus* from the northeastern Mediterranean Sea. Also, it creates a baseline to understand the relationship between cetaceans and microplastics in this region.

Keywords: Stranding dolphin, microplastic pollution, Iskenderun Bay, marine litter

ORCID IDs of the author:
N.Y. 0000-0003-2531-0198;
E.K. 0000-0003-1953-5008;
C.T. 0000-0001-9584-0261;
S.A.D. 0000-0002-5789-926X

[1]İskenderun Technical University, Faculty of Marine Science and Technology, Hatay, Turkiye

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Correspondence:
Nebil Yücel
E-mail:
nebil.yucel@iste.edu.tr

INTRODUCTION

As a consequence of unstoppable growth in plastic production, waste plastic materials reaching to marine environments are increasing day by day. The interaction between marine animals and plastic waste materials poses a threat to their well-being and causes many problems including mortality (Sharma, Sharma, & Chatterjee, 2021). For example, marine mammals were entangled in the fishing gear (Frantzis, 2007; Gomeric et al., 2009) or they try to swallow the fishing nets with the aim of feeding on trapped prey in the net (Levy et al., 2009). Similarly, some cases where large quantity of plastic materials mainly plastic bags, plastic bottles and their caps extracted from the stomach of cetaceans were reported (Simmonds, 2012).

Plastic materials found in the marine litter break up into smaller plastic particles as a result of photo degradation, oxidation, and mechanical abrasion (Andrady, 2011). Sometimes, microplastics (MPs) particles reach the marine environments from wastewater treatment plant effluents (Sun, Dai, Wang, van Loosdrect & Ni, 2019), and riverine effluents (Pojar et al., 2021). These small size plastic particles are called as MPs (less than 5 mm in size) (Arthur, Baker & Bamford, 2009), and they endanger the health of marine animals. To date, MPs ingestion has been reported in many marine animals from different trophic levels such as zooplankton (Sun, Liang, Zhu, Zhao & Zhang, 2018), bivalve (Yozukmaz, 2021), crustacea (Wu et al., 2020), fish (Kılıç & Yücel, 2022), sea birds, and cetaceans (Poeta, Staffieri, Acosta & Battisti, 2017; Fossi, Panti, Baini & Lavers, 2018).

The presence of MPs particles in the gastrointestinal tract (GIT) of cetacean species may result from direct ingestion and/or trophic transfer (Nelms et al., 2019; Novillo, Raga & Tomas, 2020). Even though, MPs found in the GIT of cetacean species may not cause congestion in the digestive system, MPs provide sorption sides for chemical contaminants which lead to the entrance of dangerous polluters into their body (Tien, Wang & Chen, 2020) that pose a threat to species well-being.

Risso's dolphin *Grampus griseus* (G. Cuvier, 1812) was considered as a regular cetacean inhabitant of the Mediterranean Sea (Bearzi, Reeves, Remonato, Pierantonio & Airoldi, 2011; Lanfredi et al., 2021). Sighting, bycatch, and stranding records of this species from Turkish waters were reported from the Mediterranean Sea, Aegean Sea, and Marmara Sea (Öztürk, Öztürk & Dede, 2001; Tonay, Dede, Öztürk & Öztürk, 2009; Öztürk, Tonay & Dede , 2011; Altuğ et al., 2011; Dede, Saad, Fakhri & Öztürk, 2012; Dede, Tonay, Bayar & Öztürk, 2013; Kesici et al., 2021). Even though this species were continuously observed in the Mediterranean Sea, a recent study indicated there is a significant decrease in their subpopulations, and they have been under the IUCN Red list of endangered species since 2020 (Lanfredi et al., 2021). In this study, a dead stranded *G. griseus* was analyzed in terms of MPs content in the stomach and intestines to understand the danger of MPs in the feeding behaviour of this species as marine mammals.

MATERIAL AND METHODS

Sampling

Risso's dolphin *Grampus griseus* was stranded on the coast of Arsuz-Uluçınar, Iskenderun Bay, Turkey in the northeastern Mediterranean Sea on 29 March 2022 [Latitude: 36.409264° Longitude: 35.875059°] (Figure 1). The sex of *G. griseus* was identified, and all morphometric characters of *G. griseus* were measured. The weight of the species was estimated with weighbridge, while the body was transported to the faculty. The mouth was checked for the existence of any macroplastic materials before digestion. Then, the specimen was dissected, and all gastrointestinal track (GIT) from the pharynx to anus was removed and transported to the laboratory in a metal bucket. The outer surface of GIT was cleaned with distilled water. The inside of the intestines was first emptied by compression, and then washed with distilled water, and the contents were transferred to jars.

Microplastic extraction

At the beginning of all processes, the equipment and tools used in laboratory as well as laboratory surfaces were cleaned with filtered ethanol and distilled water twice. The stomach and intestine content was filtered through steel sieves with different mesh sizes (2000 µm, 1000 µm, 750 µm, 200 µm) and mesh filters with 50 µm in size. The filters were covered with tin foil and kept closed to avoid contamination until the microscopic examination. In this study, chemical digestion was not applied ,but stomach and intestine content was directly filtered due to the high organic load in the GIT.

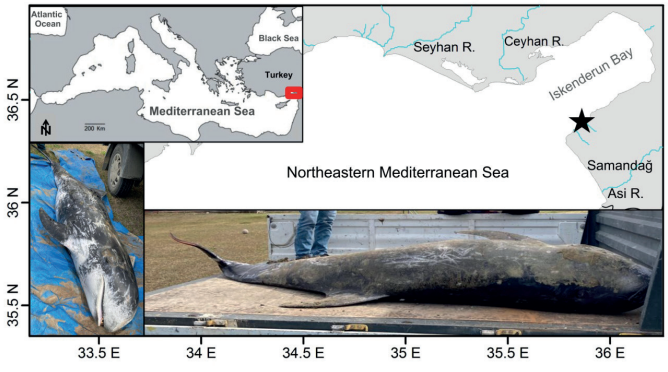

Figure 1. Pictures of stranded Risso's dolphin *Grampus griseus* with the stranding location.

Microscopic examination

The filters were investigated for the presence of MPs under Olympus SZX7 microscope with an attached Olympus DP 20 digital camera. Particles with no cellular or organic structures, fibers with the equally thick end, coloured particles and twisted flat ribbons were considered as MPs like particles (Nor & Obbard, 2014). Observed particles were exposed to a hot needle to check whether they are plastic in nature (Hanke et al., 2013). The number of MPs, size, colour and MP type (fiber, pellet and fragment) were recorded.

It is important to underline that during microscopic examination, each identified MPs like particles was checked with hot needle to validate the plastic nature. Also, organic remaining was carefully examined to reveal the trapped MPs inside of organic structures.

Contamination prevention

All steps of MPs extraction were carried out in restricted laboratories (Bessa et al., 2019), and all doors and windows were shut down (Torre, Digka, Anastasopoulou, Tsangaris, & Mytilineou, 2016). To eliminate contamination, personnel always wore cotton aprons and nitrile gloves throughout the procedure. Finally, triplicate wet filters were inserted into petri dishes for quality check. There is not any MP particle detected in the blank filters.

RESULTS & DISCUSSION

Plastics are intensely preferred in many industries due to their benefits over metal and wooden materials. However, they have become an alarming pollutant in marine environments since MP particles may easily be ingested by marine animals due to their intense and widespread presence. Also, its small size allows them to transfer to upper trophic levels via the food chain. MPs act like pollution vectors and create a gate for chemical pollutants to enter the body of marine animals (Koelmans, Bakir, Burton, & Janssen, 2016; Tien et al., 2020; Koelmans, Diepens & Mohamed Nor, 2021), which leads to health problems and even death.

Top predators like marine mammals are at higher risk in terms of MPs toxicity since as the size of the animal increases, the amount of MPs increases due to the connection with upper trophic levels (Rebelein, Int-Veen, Kammann, & Scharsack, 2021; Müller, 2021; Moore et al., 2022). Lusher, Hernandez-Milian, Berrow, Rogan & O'Connor (2018) examined the 528 stranded and caught cetacean species from Ireland, and MP and/or macroplastic particles were detected at the 8.5% of examined species. Among them, they examined the digestive tract of 8(+1) *G. griseus* specimens, and they reported the presence of macro debris in 2 specimens. However, the presence of micro debris was not reported. Nelms et al. (2019) examined the MPs existence of 5 different marine mammals (n=50), including 1 *G. griseus* individual, and MPs were detected in all examined animals. Novillo et al. (2020) examined the MPs existence in the stomach of striped dolphins (*Stenella coeruleoalba*) (n=30) from the Western Mediterranean Sea, and MPs were detected in the 90% of examined cetacean species. Al-

though there are some previously published articles reporting plastic ingestions by Risso's dolphin (Shoham-Frider, Amiel, Roditi-Elesar & Kress, 2002; Baini et al., 2017; Lusher et al.,2018; Alexiadou, Foskolos, & Frantzis, 2019; Nelms et al., 2019), this is the first report presenting the MPs existence in the GIT of a Risso's dolphin *Grampus griseus,* a top predator from the Mediterranean Sea. Also, this incident is the most eastern stranding records of this species from Turkish waters.

According to the first visual examination of *G. griseus,* the specimen was freshly dead, and there was no deformation or injury in the external appearance, which may explain the cause of death. The specimen was female, and the length and the weight were measured as 294 cm and 370 kg, respectively. Detailed information regarding the morphological measurements of studied specimen was given in Table 1.

During the visual examination of stomach content in the laboratory, nine different sized squid beaks (undigested or partially digested tissue were found) were found in the fore stomach ,but other stomachs were empty. While the first part of the intestines contained greenish fluid, the remaining part was brown.

Table 1. External measurement of Risso's dolphin *Grampus griseus*.

Character	Length (cm)
Body length	294
Distance between tip of rostrum to mid-point of anus	240
Distance between tip of rostrum to mid-point of genital slit	195
Distance between tip of rostrum to mid-point of umbilicus	161
Distance between tip of rostrum to tip of dorsal fin	180
Distance between tip of rostrum to font of dorsal fin, at anterior insertion of dorsal fin	127
Distance between tip of rostrum to centre of blowhole	31
Distance between tip of rostrum to end of gape	27
Distance between tip of rostrum to centre of eye	33
Distance between tip of rostrum to front of pectoral fin	51
Length of left fluke	38
Length of right fluke	38
Total fluke span, tip to tip	76
Basal length of dorsal fin	37
Height of dorsal fin	31
Maximum width of pectoral fin	25
Outer length of pectoral fin, from anterior insertion to tip	60
Inner length of pectoral fin, from posterior insertion to tip	43
Girth behind pectoral fin	60
Girth in front of dorsal fin,	70
Girth behind the dorsal fin,	59
Girth at the genital slit	45
Girth at anus	25

Cephalopods are the major prey of *G. griseus* (Clarke, 1996), which explains the existence of squids in the stomach content. For example, Öztürk, Salman, Öztürk & Tonay (2007) extracted cephalopod remains from the *G. griseus* in the eastern Mediterranean Sea. Also, similar to findings of this study, Blanco, Raduan & Raga (2006) extracted squid species in the stomach of *G. griseus* sampled from the Mediterranean Sea.

A total of 484 MPs particles were extracted from the GIT of *G. griseus* (Figure 2). Eastern Mediterranean Sea, especially the region between Turkey and Cyprus, was categorized as the hotspot of the plastic debris (Liubartseva, Coppini, Lecci, Clementi, 2018). When the stranding location of *G. griseus* was taken into consideration, this high MPs amount is most probably related with the contamination status of the surrounding environment.

In terms of plastic type, majority of the identified particles were fibers in shaped (96%). Minor portion of identified MPs were pellets and fragments from unidentifiable larger objects (Figure 3). In the literature, the percentage of fibers among extracted MPs was reported as 73.6% in the Western Mediterranean Sea (Novillo et al., 2020), 84% in the British coast (Nelms et al., 2018), and 83.6% in Ireland (Lusher et al., 2018).

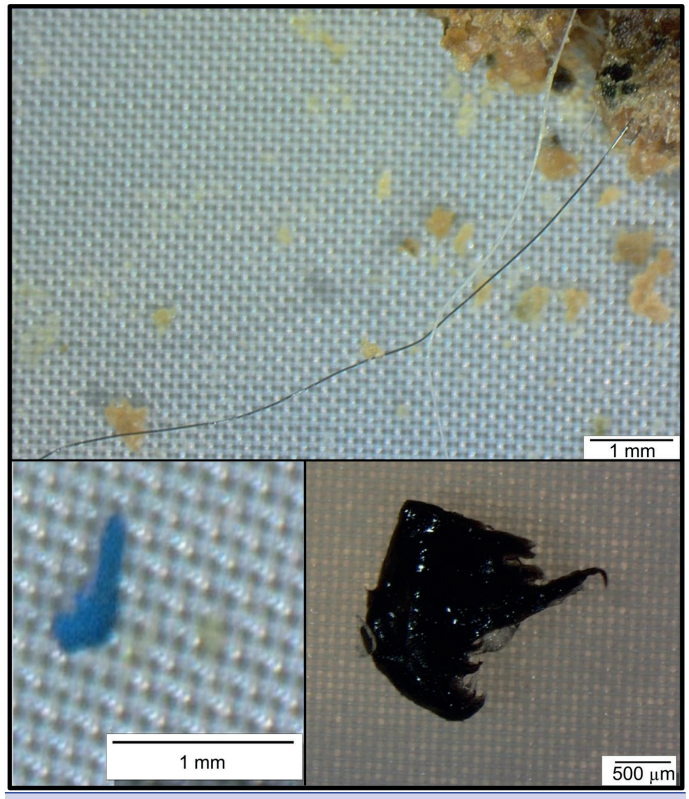

Figure 2. Some examples of extracted MPs from gastrointestinal track of Risso's dolphin *Grampus griseus*.

In terms of the size, 4% of extracted MPs belong to mesoplastics size class (5-20 mm); whereas the rest belong to microplastic size glass (<5 mm). Majority of the extracted particles were within the range of 0.5-1.0 mm size class (Figure 3), and the mean size of extracted fibers was 1.3±1.6 mm.

In terms of colour, white, black, blue, green, and red particles were identified. In this context, transparent and white MPs were categorized as white; blue and purple MPs were categorized as blue; red and pink MPs were categorized as red. Majority of the extracted MPs were black in colour, which is consistent with the previous studies (Lusher et al., 2018; Nelms et al., 2018; Novillo et al., 2020) (Figure 3).

The amounf of MPs in the stomach of common dolphin *Delphinus delphis* was found as 12 average ind^{-1} from the Spanish coast (Hernández-González et al., 2018), striped dolphins (*Stenella coeruleoalba)* was found as 14.9 average ind^{-1} from the Western Mediterranean Sea (Navillo et al. 2020). The amount of MPs extracted from Risso's dolphin *Grampus griseus* was significantly higher than that of the previous reports. There are many possible reasons for these variations. Firstly, previous studies focused on the stomach content whereas in this study, both intestine and stomach content was examined. Secondly, the amount of MPs was found to be variable between species (Nelms et al., 2020) and location (Novillo et al., 2018). Thirdly, the straightening of the MPs particles was checked with the hot needle method, advanced validation methods Fourier transform infrared spectroscopy could not be used. This may cause an overestimation of the examined MPs particles.

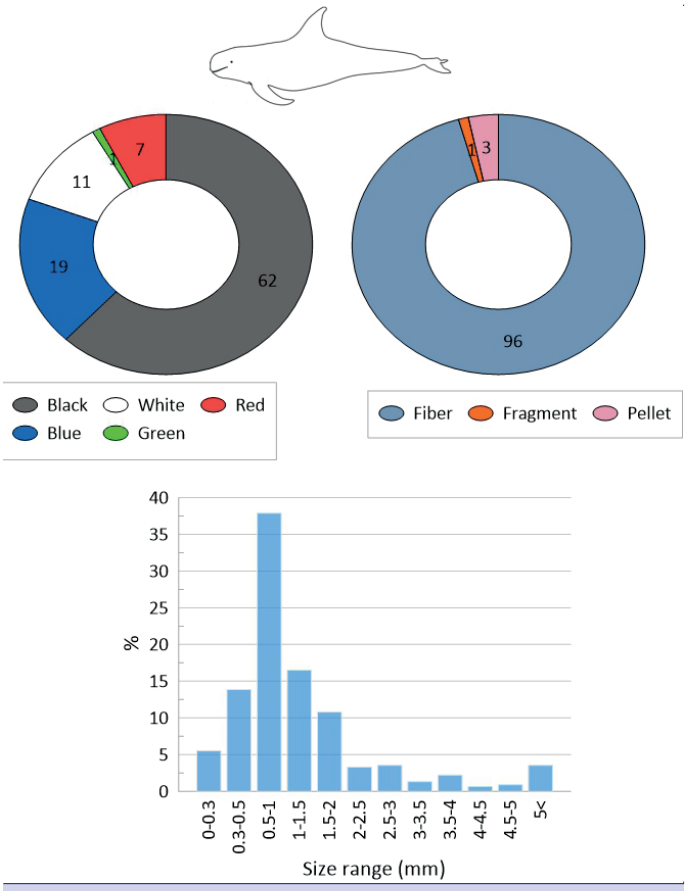

Figure 3. Characterization of extracted microplastics by colour (%), shape (%) and size (mm).

CONCLUSION

In this study, 454 MPs particles were extracted from the gastrointestinal tract of Risso's dolphin *Grampus griseus* from the northeastern Mediterranean Sea. Majority of the extracted particles were fibers in shape, black in colour, and in the 0.5-1 mm size range. The high amount of MPs particles in the GIT of Risso's dolphin *Grampus griseus* shows the intensity of MPs contamination risk of marine mammals considering their role as pollutant vectors. Also, this high presence proves the significant MPs occurrence in the Mediterranean Sea pelagic waters. More comprehensive studies need to be conducted to evaluate the threat of MPs on marine mammals.

Compliance with Ethical Standard: Authors declare that ethical approval is not require for this type of study.

Conflict of Interests: Authors declare that there is no conflict of interest.

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