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Contents/İçindekiler

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Original Article

Contribution of Passive Sampling Devices on the Determination of Hydrophobic Organic Contaminant Bioaccumulation in Marine Organisms

Sevil Deniz Yakan[1]

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ABSTRACT

Hydrophobic Organic Contaminants (HOC) are a group of chemicals needed to determine the health of marine ecosystems, and passive sampling devices are promising tools that offer a convenient monitoring opportunity. Traditional biomonitoring studies involved different types of marine organisms, and it appeared that simultaneous deployment of passive samplers with biomonitoring organisms provided the necessary information for the calculation of the aquatic organisms' bioaccumulation factors (BAF). There was not any other parameter than BAF, that could be used to determine the biomagnification and fate of contaminants in the upper trophic levels, which eventually affect all marine and terrestrial ecosystem health. In the light of the essence of BAF, this study applied a modified version of BAF estimation dependent on the contaminant concentrations both in the passive and active samplers. Thus, BAF parameters could be calculated properly without any need of a contaminant concentration in the surrounding water environment. For this purpose, the HOC concentration detected from the anthropogenic settlements in the coastal regions of Turkey were collocated, evaluated, and represented for different HOC groups. It was concluded that the present method is appropriate and applicable for BAF calculations of different groups of HOCs, where there are simultaneous deployments of both active and passive samplers in the process of biomonitoring studies.

Keywords: Passive sampling, HOC, bioaccumulation, BAF, concentration ratio model

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INTRODUCTION

Polyaromatic hydrocarbons (PAH), polychlorinated biphenyls (PCB) and organochloride pesticides all belong to the group of hydrophobic organic contaminants (HOCs). The determination of concentrations of hydrophobic organic contaminants (HOCs) in water samples is difficult because of chemical properties like low water solubility, high lipophilicity, strong adsorption tendency to suspended materials and tendency to accumulate in organisms (David et al., 2010). In order to overcome this obstacle, alternative methods such as biomonitoring and passive sampling have been used for determination of the sources and the fate of HOCs in different matrices of the marine environment (Blasco & Picó, 2009; Bourgeault & Gourlay-Francé, 2013). Bivalve mollusks and Semi-Permeable Membrane Devices (SPMD) are widely used examples of biomonitoring and passive sampling studies for the determination of HOC concentration in a water environment (André Lourenço et al., 2015; Fontenelle, Taniguchi, da Silva, & Lourenço, 2019; Lance, Matz, Reeves, & Verbrugge, 2012; Lourenço et al., 2016; Peven, Uhler, & Querzoli, 1996). A wide geographic distribution and the sessile nature of bivalves makes them the preferable organisms in biomonitoring studies, in addition to the accumulation tendency of HOCs in bivalves (Bayne, 1976). On the other hand, passive samplers have the advantage of not being affected

by the poor water quality in contrast to the organisms that need to survive to be used in biomonitoring studies. Thus, a type of passive samplers, SPMDs, have become a popular instrument of detecting HOCs in water bodies (Huckins, J.N; Petty, J.D.; Booij, 2006; Huckins, J.N.; Tubergen, M.W.; Manuweera, 1990).

Diffusion and partitioning are the main pathways of PAH accumulation in SPMDs, thus freely dissolved contaminants in water can be detected precisely over a specific time interval (Gourlay, Tusseau-Vuillemin, Garric, & Mouchel, 2003; Greenwood, Mills, & Roig, 2007). The deployment of SPMD during a specific time interval has many advantages compared with a spot analysis of water contamination. For example, the decrement of detection limits due to an accumulation tendency of HOCs into the material led eventually to a higher concentration of contaminants accumulated in SPMDs. In addition, the average measurement of a time period instead of a measurement of a moment provides more reliable results of the target zone (Uher, Mirande-Bret, & Gourlay-Francé, 2016). SPMDs are not designed to mimic all metabolic activities of aquatic organisms, but instead to mimic the pathways of diffusion and partitioning in the process of contaminant bioaccumulation through adsorption to the surfaces of the target organism like algae, mussel, fish etc. (Gourlay et al., 2005; Mayer, Philipp; Tolls, Johannes; Hermens, Joop L.M.; Mackay, 2003; Uher et al., 2016). Thus, simultaneous deployment of passive and active sampling devices (for example, SPMDs and bivalves) is promising for more sensitive and reliable measurements as applied in many studies (Amdany et al., 2014; Bourgeault & Gourlay-Francé, 2013; David et al., 2010; Verweij, Booij, Satumalay, Van Der Molen, & Van Der Oost, 2004; Vrana et al., 2014).

The main advantage of simultaneous deployment of active and passive sampling methods is to be able to determine the bioaccumulation tendency of HOCs in selected marine organisms without any need of ambient water concentration data (Booij, Smedes, Van Weerlee, & Honkoop, 2006). The similar uptake processes of HOCs in both bivalves and passive samplers enable researchers to obtain the bioaccumulation data by means of a comparison of accumulated HOC concentrations in both organisms and samplers (Harman, Brooks, Sundt, Meier, & Grung, 2011). The assessment of HOCs in different matrices of the marine environment was performed using this approach in several studies (e.g. Bourgeault & Gourlay-Francé, 2013; David et al., 2010). This study aimed to determine the bioaccumulation factors in marine organisms using the concentrations of HOCs detected in the western and north-western regions of Turkey by means of this simultaneous monitoring type of approach. Within the scope of this study, bioaccumulation of HOCs in selected marine organisms were determined without the necessity of additional HOCS data, like its presence and concentration in the surrounding water environment. Consequently, the results of the simultaneous deployment of the Mediterranean mussel species, *Mytilus galloprovincialis,* and SPMDs were evaluated using a published concentration ratio model (Booij et al., 2006) that was taken as the basis of a BAF determination, and adapted for evaluation of HOCs in determining bioaccumulation factors of selected marine organisms for PAH, PCB and OCP.

MATERIALS AND METHODS

The concentration data of HOCs (PAHs, PCBs and OCPs) in mussels and SPMDs were collocated from three previously published papers (Karacik, Okay, Henkelmann, Pfister, & Schramm, 2013; O. S. Okay et al., 2014; Oya S. Okay et al., 2017). The data in the referenced papers was reorganized into Excel sheets and used as inputs for the present study. The field studies in the papers were accomplished in total at 13 different stations: four stations along the Istanbul Strait with a deployment time of seven and 21 days; and nine stations in anthropogenic settlements like shipyards and marinas with a deployment time of 30 and 60 days, which are located in the west and north-west coast of Turkey (three marinas in the west, three shipyards and three marinas in the north-west coast of the country). Because the detailed locations of sampling stations were given in the referenced papers (Karacik et al., 2013; O. S. Okay et al., 2014; O.S. Okay et al., 2017) and the choice of sampling stations were out of the scope of the present study, the sampling stations are only shown in general as seen in Figure 1.

Figure 1. The locations of the sampling stations are marked with the red frame: four stations along the Istanbul Strait; nine stations at the west and north-west coast of Turkey. Detailed maps were given in the papers of (Karacik et al., 2013; O. S. Okay et al., 2014; O.S. Okay et al., 2017).

The variety of stations enhanced the results of this study. For example, sampling stations at the north-west coast of the country are in the most populated city of the country (Istanbul) with about 17 million inhabitants. Marinas and shipyards in this region are on different sides of the city. The east part of the south entrance of the Istanbul Strait is filled with marinas while the east border of the city is filled with several shipyard companies consisting of a higher number of workers. On the other hand, sampling stations at the west coast of the country are located in a less populated area which is popular with summer tourism. As mentioned in the referenced papers, the Mediterranean mussel, *Mytilus galloprovincialis,* was used as the selected mussel species, and simultaneously deployed with a commercial type of passive sampler, SPMD. The data from these two different types of samplers, named as active and passive samplers, was used as inputs in the present study in order to determine the bioaccumulation potential of HOCs.

The analyzed results of HOCs in mussels and SPMDs were used in the slightly modified version of the mussel/SPMD concentration ratio model (Booij et al., 2006). The modification included the parameter of initial contaminant concentration in transplanted mussels, which is not a zero value naturally, at the time of deployment. The fitting of field data was implemented using the NonlinearModelFit method in Mathematica (Wolfram Research, version 10.0). The kinetic rate constants were matched with the results of mussel and SPMD analysis. Not all the results were used in the NonlinearModelFit method because of the detection limitations of some HOCs. Among all HOCs, the data of 14 PAHs and 11 PCBs were used for the implementation of the NMF method. Regression equations presented in the referenced publication were used for determination of the SPMD-water partitioning coefficient (K_{SW}, L kg^{-1}) and elimination rate constant of the SPMD (k_e, d^{-1}) (Booij et al., 2006). The bioaccumulation factors (BAF, L kg^{-1}) were obtained by replacing the determined coefficients and constants in Equation (1).

$$C_M = \frac{\rho_S C_S\left(BAF\left(1-e^{-k_2 t}\right)\right)+C_{M,0}e^{-k_2 t}}{K_{SW}\left(1-e^{-k_e t}\right)} \quad (1)$$

In Equation (1), C_M (ng g^{-1}), $C_{M,0}$ (ng g^{-1}), C_S (ng g^{-1} triolein), ρ_S (g mL^{-1}) represents the mussel concentration at any day, the mussel concentration at the initial day (day 0), the SPMD concentration and its density, respectively. Because the initial concentrations of mussels are not always zero, Equation (1) consists of an additional term compared with the original equation in reference (Booij et al., 2006). After fitting Equation (1), the parameters of BAF were evaluated with respect to their octanol-water partition coefficient (K_{OW}) dependency. Thus, BAFs (L kg^{-1}) of mussels for a series of HOCs were obtained without any need of ambient water concentration.

RESULTS AND DISCUSSION

Log K_{ow} values for a series of HOCs range within 3.37 and 7.41; specifically, between 3.37 – 6.9 for PAHs, 5.66 – 7.41 for PCBs and 3.7 – 7.13 for OCPs. This variation brought about inherently different patterns of accumulation. It was seen that Log K_{ow} values positively correlate with the accumulation of PAH and OCP both in the passive and active samplers. On the contrary, the accumulation of PCBs followed a different trend. This difference was probably the result of having comparatively higher Log K_{ow} values. The different nature of HOCs reflected their accumulation patterns.

The activities that originated from human actions were the main source of PAHs. The field monitoring results in the areas of the marina and shipyard were evidence of this fact (O. S. Okay et al., 2014; Oya S. Okay et al., 2017; Yilmaz et al., 2014). In addition, the products of industrial waste were the sources of PCBs (Cardellicchio et al., 2007; Cetin et al., 2017; Helou, Harmouche-Karaki, Karake, & Narbonne, 2019; Hong, Yim, Shim, Li, & Oh, 2006), and this was seen clearly in the comparison between the shipyard and marina zones. The machinery and equipment used in the construction, maintenance, and repair of ships in shipyards seemed to directly affect this pattern difference. The main source of OCPs are the products related to agriculture (Ahmad, Salem, & Estaitieh, 2010; Fenik, Tankiewicz, & Biziuk, 2011; Helou et al., 2019), thus they are generally detected at low rates.

The results of HOCs were grouped into two groups, depending on their deployment times. The reason for this grouping was to help in the evaluation of different features at the sampling stations. The first group deployment time was 7 and 21 days, and the second group was 30 and 60 days. Therefore, NonlinearModelFit function was run separately for the two groups, and the output was represented as separate figures. Figures 2 and 3 are results of the first group while Figure 5 represents the output of the second group.

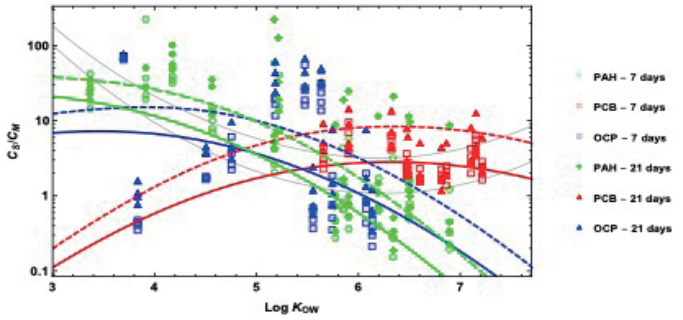

Figure 2. The ratio of contaminant concentrations in SPMD (C_S) and transplanted mussel (C_M) versus octanol-water coefficients (Log K_{ow}) of related contaminants is shown. Each contaminant group is represented with a different color: PAH in green, PCB in red and OCP in blue for the deployment durations of 7 and 21 days, formed with open and closed symbols, respectively. Fitting models of each contaminant group are shown in their assigned colors in addition to the combined PAH-PCB-OCP data in grey color.

The relationship between the ratios of SPMD and mussel concentrations (C_S/C_M) and Log K_{OW} values are shown in Figure 2 for the first group. An inverse trend was observed for the group of PAHs. The accumulation of PAHs decreased with the increase of Log K_{OW} values. In contrast, the group of PCBs, which have higher K_{OW} values, show the opposite trend. The trend of the data is represented with Equation (1) for PAH (in green), PCB (in red) and OCP (in blue) separately, as shown in Figure 2. The combined evaluation of PAH, PCB and OCP data (black spline in Figure 2) shows a plateau, which could be explained by the steric hindrance of higher molecular weight HOCs of Log K_{ow} > 5.0, especially during the accumulation of PAHs through the pores of SPMDs (Luellen & Shea, 2002). This fact of uptake restriction due to the morphology was also mentioned in several observations (Huckins et al., 1999; Luellen & Shea, 2002).

Bioaccumulation factors (BAFs) were evaluated versus Log K_{OW} values of contaminants. The data are matched separately and together for the groups of PAHs, PCBs and OCPs, as shown in Figure 3. Separate PAH and PCB data were matched well with linear regressions. Although the data of OCPs were scattered, they were also matched and represented with linear regression. Moreover, the combined data of PAH, PCB and OCP were fitted by a hyperbolic function. The characteristics of a fitted hyperbol-

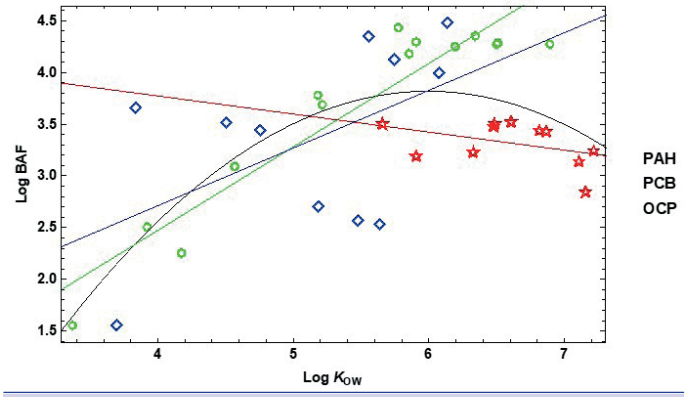

Figure 3. Log BAF versus Log K_{OW} values and model fits for PAH, PCB and OCP separately (in green, red, and blue) and for the combined data set (in black).

ic function was not affected by the scattered data of OCPs. However, the data of PCBs was the main factor affecting the characteristics of the fitted function due to its decreasing trend with the increasing Log K_{OW} values. This fact can also be considered as a probable inhibition of high Log K_{OW} valued PCB accumulation (Baskaran, Armitage, & Wania, 2019; Qiu, Qiu, Zhang, & Li, 2019).

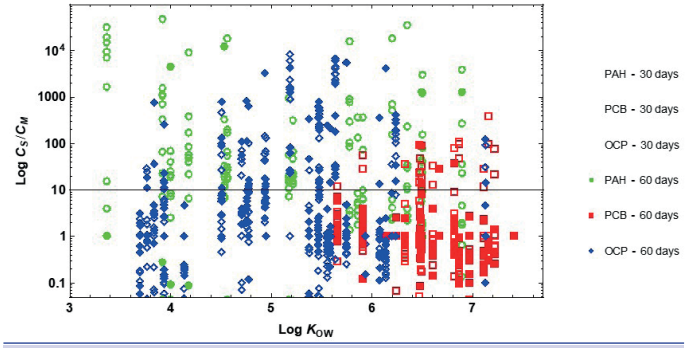

Figure 4. The ratio of contaminant concentrations in SPMD (C_S) and transplanted mussels (C_M) versus octanol-water coefficients (Log K_{ow}) of related contaminants is shown. Each contaminant group is represented with a different color: PAH in green, PCB in red and OCP in blue for the deployment durations of 30 and 60 days, formed with open and closed symbols, respectively. The black vertical line at the y-scale (Log 10=1) indicates the equality of SPMD and mussel concentrations.

In Figure 4, y-scale of Log C_S/C_M enabled researchers to observe the different accumulation trends of the active and passive samplers, and the black line at Log 10 indicates the equality of their concentrations. The upper part of the black line signifies that the metabolization of HOCs in the selected marine organism is higher, whereas the lower part of the black line indicates that the contaminants in the particular phase were higher than their dissolved forms, as a reminder that the dissolved phase of contaminants accumulated in the passive sampler (Bourgeault & Gourlay-Francé, 2013; Gourlay-Francé, Lorgeoux, & Tusseau-Vuillemin, 2008; Kim, Kim, Alvarez, Lee, & Oh, 2014; Lance et al., 2012; Luellen & Shea, 2002; Taylor, Fones, Vrana, & Mills, 2019; Zhao et al., 2018).

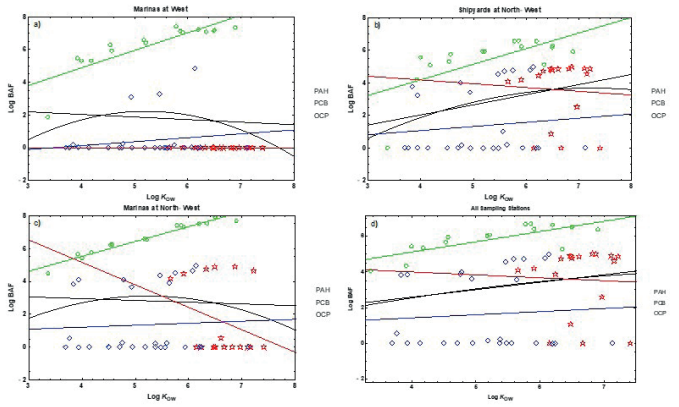

Figure 5. Log BAF versus Log K_{OW} values and model fits for PAH, PCB and OCP data separately (in green, red, and blue) and for the combined linear and non-linear fittings (in black). Each graph in the Figure represents different sampling stations: a) Marinas at West; b) Shipyards at North-West; c) Marinas at North-West; d) All the sampling stations together.

The stations in group 2 were differentiated according to their type (marinas and shipyards) and locations (west and north-west). Bioaccumulation factors were calculated by using Equation (1) and represented separately for the marinas at the west, shipyards at the north-west and marinas at the north-west region of the country as shown in Figure 5. In addition, combination of all data belonging to all stations with a deployment time of 30 and 60 days were also evaluated and shown in Figure 5, using different colors and shapes for different type of contaminants (green circles for PAH, red stars for PCB and blue diamonds for OCP).

It is clearly seen from Figure 5 that PAHs were abundant in all stations, whether the sampling stations were in an industrialized zone, a highly populated city or a low-populated district famous for its summer tourism. On the contrary, the difference in the distribution of PCBs is clearly seen in Figure 5, with the abundance of PCBs especially in the sampling stations located in the highly industrialized areas like the zones of shipyards compared with sampling stations located in the zones of marinas. Apart from this fact, the observed presence of PCBs in marinas located in the north-west region of the country can be explained because of its proximity to the industrialized and higher populated areas. In addition, the presence of OCPs at all stations can be explained due to its persistent nature, previous usage, and potential run-offs from the observed regions, although its usage has been banned for a long time (Ozcan & Aydin, 2009).

Furthermore, another evaluation was performed using a different deployment period of time for the samplers. It is clearly seen from Figure 6 that deployment time affects the range of results although the data trend remains the same. Different times of deployment were selected for this purpose. As a reference for the

deployment duration, the necessity and importance of the exposure time is a minimum of five days for SPMDs as stated in previous studies (Luellen & Shea, 2002). The outputs represented in Figure 6, range from seven days to an unlimited period of time. The results of different deployment times (7, 21, 30, 60, 360 and unlimited days), as shown in Figure 6, could be used for the evaluation and estimation of an efficient deployment time for prospective field monitoring studies. The importance of deployment time should not be undervalued for the design of field studies in an effort to reduce expenses such as disposables and chemicals used both in the field and laboratory and the addition of travel and transportation expenses in distance sampling.

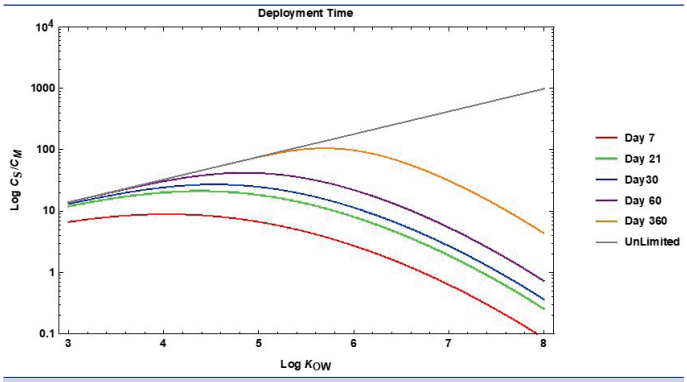

Figure 6. Predictions of deployment time, for different periods of time, ranging from seven days to infinity.

CONCLUSION

The deployment of passive sampling devices in coordination with the field studies of marine organisms is a promising tool for the determination of bioaccumulation factors. The simultaneous usage of both active and passive sampling devices is increasing worldwide for the monitoring of hydrophobic organic contaminants in coastal zones. This study points out an additional feature of this simultaneous deployment: Determination of bioaccumulation factors in marine organisms without any need of HOC concentration data from the surrounding water environment. For this purpose, the concentration ratio model was modified successfully in the implementation of field data for Turkish coastal zones. Additionally, this same model equation was used for the comparison of different periods of deployment time, that could be a guide for an efficient planning of field studies with fewer expenses in consumables and transportation.

Conflict of interests: The author declares no financial, commercial and legal conflict of interest.

Ethics committee approval: In this study, the mathematical evaluation of a number of biomonitoring studies has been performed. Thus, the author declares that it does not need any ethics committee approval.

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Disclosure: -

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Original Article

The Length – Weight Relationship and maximum length of *Umbrina cirrosa* (Linnaeus, 1758)

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ABSTRACT

In this study, length-weight relationship of *Umbrina cirrosa* was determined in the Black Sea while giving a maximum length and weight record for this species. A total of 102 *U. cirrosa* were sampled by using trammel nets between 2018 and 2019 in the southern Black Sea. The mean length and the mean weight of the specimens were estimated as 32.4 cm ± 15.02 (4.8-94) and 613.1 g ± 962.69 (1.0-7051.1), respectively. One of the sampled species was measured as 94 cm in total length, weighed 7051.1 g, and was found to be 5 years old. These measurements prove this specimen to be the largest individual for the Turkish coasts. The length-weight relationship was described as $W=0.009L^{3.0541}$ ($R^2=0.9962$) with positive allometric growth for all individuals. This is the first study conducted for *U. cirrosa* in the Black Sea while the maximum size record is given for all Turkish coastal waters.

Keywords: *Umbrina cirrosa*, length-weight parameters, maximum weight, maximum total length, Black Sea, Turkey

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INTRODUCTION

The Shi drum, *Umbrina cirrosa* (Linnaeus 1758), is a member of the Sciaenidae family (Fischer et al., 1987) and has a great economic value in commercial fishing (Fabi and Fiorentini, 1993; Mylonas et al., 2000). It is a demersal fish largely spread from the Eastern Atlantic to the Mediterranean and the Black Sea living within a depth range of 0 to 100 m (Fischer et al., 1987). The Shi drum is a bottom-dwelling species, mainly feeding on invertebrates and fish (Abellan and Basurco 1999; Lobry et al., 2003). Smaller individuals generally prefer muddy habitats of coastal zones and estuaries (Fischer et al., 1987). When the sea has a heavy condition, wave breaking action stirs up the benthic in coastal areas and reveals the creatures that the Shi drum feeds on. They generally approach the coastal area where the waves break for feeding purpose (personal observation).

Fischer et al., (1987) stated that they are usually between 30-80 cm, but they can grow up to 100 cm. Chao and Trewavas (1990) measured a 40 cm of common length with a maximum size of 73 cm. According to FishBase (2020), this species can grow up to a maximum size of 73 cm and 3.1 kg. In Turkish territorial waters, *U. cirrosa* are fished using trammel nets and trawls by fishermen in the Mediterranean Sea, Aegean Sea, Sea of Marmara, and Black Sea.

The total average production of *U. cirrosa* was 32.1 tons in Turkey between the years 2008-2018 (TUIK, 2019). It has an average price of $ 10 per kg. Due to its rapid growth, it has been used as a new species in aquaculture in the Mediterranean since the 2000s (Melotti et al., 1995; Mylonas et al., 2000; Barbaro et al., 2002; Mylonas et al., 2004; Koumoundouros et al., 2005).

There are no studies in the literature focusing

on the population structure of the natural stocks of this species in Turkish territorial waters. In this study, the length-weight relationship together with the maximum total length, total weight and age determination are given for this species.

MATERIALS AND METHODS

A total of 102 specimens of *U. cirrosa* were caught by using trammel nets (40-60-80 mm mesh size) between 2018 and 2019 in the Southern Black Sea region (between 41°12′20.94″N – 37°.18′.43.57″E and 41°.28′.02.52″N – 41°27′53.86″E) (Figure 1). It can only be sampled efficiently when the tide is high because the catching success is very low when the sea is calm.

Caught fish samples were transported in iceboxes to the Laboratory of Fatsa Faculty of Marine Sciences, Ordu University. The length and weight of the samples were measured with the accuracy of 1 cm and 0.1 g respectively for all specimens (Table 1). Sex determinations were made through macroscopic observation of the gonad. Chi-square (χ^2) analysis was used to test for significant differences between the sex ratio.

The total length-weight relationship (LWR) of the species was estimated by applying the exponential regression model, $W = aTL^b$, where a and b are constants (Ricker, 1975). LWR relationship was performed for three groups; by using only males, only females, and all samples. The Pauly's t-test was used to compare the "b" values (Pauly, 1984) to determine whether there is any significant difference or not. Sagitta otolith was used for the age determination of the maximum sized fish.

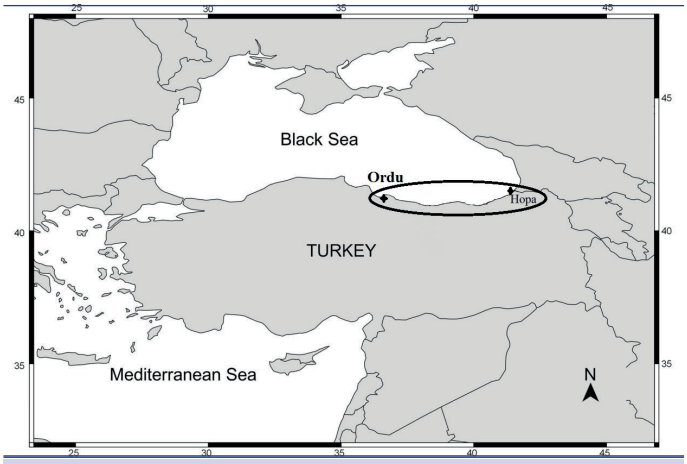

Figure 1. The map showing the sampling area.

RESULTS AND DISCUSSION

A total of 102 (52 female, 46 male and 4 immature) specimens were examined. The ratio of female to male specimens was found as 1:1.13. The difference between the sex ratios was not found to be statistically significant (χ^2=0.51, df=1, p>0.05).

The mean total length and the mean weight of the specimens were estimated as 32.4 cm ± 15.02 (4.8 - 94) and 613.1 g ± 962.69 (1.0-7051.1) respectively (Table 1).

One of the female individuals sampled from Hopa was measured as 94 cm in total length (Figure 2), weighed 7051.1 g (gonad weight: 190 g) and found to be 5 years old (Figure 3). These numbers make it the largest individual for Turkish waters.

When the *U. cirrosa* population in the Black Sea examined, the highest percentage was observed for 35-40 cm TL (21 %) length group (Figure 4).

Figure 2. The largest individual from the Black Sea.

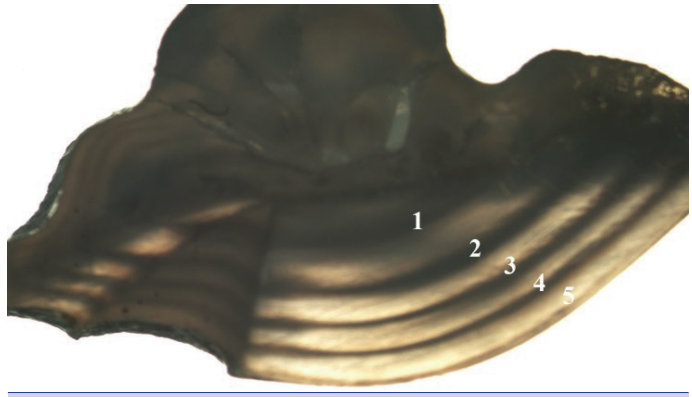

Figure 3. A thin stained otolith section of *Umbrina cirrosa* (total length 94 cm, weight 7051.1 g) aged 5 years old.

Table 1. Mean and standard deviation, maximum, minimum values for length (L) and weight (W) parameters of each sex of *Umbrina cirrosa*.

S	L (cm)					W (g)				
	Avg.	±	SD	Min.	Max.	Avg.	±	SD	Min.	Max.
Σ	32.4	±	15.02	4.80	94.00	613.10	±	962.69	1.00	7051.10
♀	34.80	±	17.03	14.00	94.00	792.20	±	1281.00	25.05	7051.10
♂	32.00	±	10.29	14.60	50.30	463.80	±	356.72	28.96	1330.00

Σ: All, ♂: Male, ♀: Female, S: Sex, N: Number of individuals, Min: Minimum, Max: Maximum, SD: Standard Deviation

The length-weight relationship for all samples (males and females) was estimated as W = 0.009 $L^{3.0541}$ (R^2 = 0.996) with a positive allometric growth. The length-weight relationship for the female, male groups, and all individuals are given in Figure 5.

The "b" value estimations from the length-weight relationship of *U. cirrosa* are 3.054 for all individuals, 2.995 for females and 3.029 for males. The value of "b" is only slightly different than 3 for all individuals and is not significantly different than 3 for the female and male groups with p> 0.05 (Table 2). Positive allometric growth values were observed for *U. cirrosa* for males and all individuals, while the females showed an isometric growth (Figure 5).

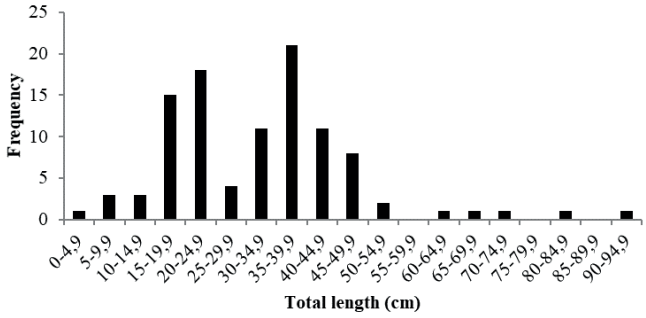

Figure 4. Length frequency distributions of *Umbrina cirrosa* collected from the southern Black Sea region.

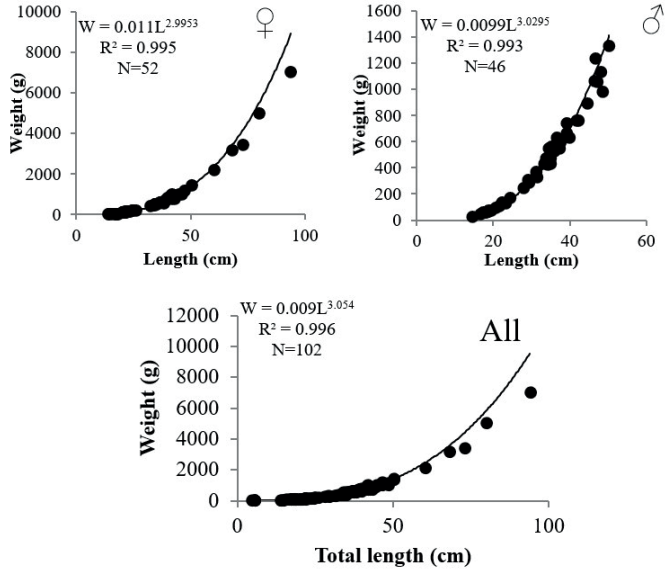

Figure 5. The Length-weight relationship for *Umbrina cirrosa* collected from the Southern Black Sea region.

U. cirrosa is a species with a high rate of catching when it approaches the coastal areas for feeding purpose, especially when the sea is heavy with big waves stirring up the bottom (Dr. Mehmet AYDIN observations). To sample the species, sampling should be done under this type of weather conditions, when fishing is quite difficult as expected.

In addition to this, due to the high preference of this fish in restaurants, this species is sold directly to the restaurants by commercial fishers, and only a very small portion of the samples can be obtained from commercial fishers. Therefore, the number of samples in this study is lower than the number of samples for the researches focusing on other similar species. If we look at the previous studies about this species, we see that the sample numbers are either very low or the size of the samples has a narrow range including mostly juvenile individuals (Table 3). Considering the size distribution of the samples for this study together with the smallest and the largest individuals of the samples, it can be said that the size structure of the natural population is more realistically represented in this study compared with the previous researches of the literature.

In the catalogue book of Fischer et al. (1987), it is stated that this species theoretically has the potential of growing up to 100 cm length. Additionally, based on the study Chao and Trewavas (1990) carried out, it grows to 73 cm in length, and a record was given in FishBase (2020) with the size of 73 cm length and 3.1 kg weight. The maximum length (94 cm) and weight (7051.1 g) values given in this study is the largest length and weight record for this species in the Black Sea region. The length-weight relationship was described as W = 0.009 $L^{3.0541}$ for all samples. In previous studies, the smallest and the largest "b" values were found to be 2.917 (Bolognini et al., 2013) and 3.419 (Başusta et al., 2019, see Table 3) respectively for this species. In this study, the b value was estimated as 3.054, which is within the limits of the smallest and largest "b" range given by the previous studies. Additionally, a relatively high "b" value by Başusta et al., (2019) conflicting with our results can be due to the juvenile profile of the sampled individuals or due to the fishing gear used in the sampling of that research. The age of the maximum-sized individual sampled in the study was determined to be 5 years old (94 cm, 7051.1 g), which is comparable with the values given by Arneri et al. (1998) for the individuals aged 3 years old (length 67 cm and weight 3200 g).

Table 2. The Length-weight relationship parameters for male, female groups and all individuals for *Umbrina cirrosa* sampled from the Southern Black Sea region.

S	N	a	b	95% Confidence Interval (+SD)	R^2	Pauly t-test	p
Σ	98+4	0.009	3.054	3.016-3.091 (+0.018)	0.996	2.870	>0.05
♀	52	0.011	2.995	2.993-3.057 (+0.030)	0.995	0.152	<0.05
♂	46	0.009	3.029	2.952-3.106 (+0.038)	0.993	0.769	<0.05

Σ: All, ♂: Male, ♀: Female, S: Sex, N: Number of individuals, SD: Standard deviation, a: Intercept, b:Slope, R^2: Determination coefficient, P: Probability of the t-test (H_0: b=3) (4 immature).

Table 3. The length- weight relationships of *Umbrina cirrosa* from different areas.

N	L_{min}-L_{max} (cm)	Mean (cm)	W_{min}-W_{max} (g)	Mean (g)	a	b	R^2	Region	References
41	36.2- 66.5	42.20±0.918	508-2915	794.8±68.3	-	-	-	Eastern Adriatic Sea	(Dulčić and Kraljević, 1996)
	11-16	13.5	-	24.1	-	-	-	Abu-Qir Bay (Egypt)	(Faltas et al., 1998)
9	6.5-24.7	-	-	-	0.0119	2.985	0.997	Porto-Lagos (NE Aegean)	(Koutrakis and Tsikliras, 2003)
10	11-54	-	-	-	-	-	-	-	(Cruz and Lombarte, 2004)
44	33.1-47	-	-	-	0.0115	3.060	0.977	River Mirna, northern Adriatic	(Dulčić and Glamuzina, 2006)
537	18.5-49.5	27.1±4.0	93-1281	240.9±133.6	0.015	2.917	0.963	Adriatic Sea	(Bolognini et al., 2013)
2	2-42	31±2.89	200-817	508.5±81.02	-	-	-	Sinop (Black Sea)	(Bat et al., 2018)
218	13.5-26.8	-	19.12-214.04	-	0.0028	3.419	0.989	Mersin Bay (Mediterranean)	(Başusta et al., 2019)
102	4.8-94	32.4±15.02	1.0-7051.1	613.1 g±962.6	0.009	3.054	0.996	Southern Black Sea	**Present study**

CONCLUSION

In conclusion, this study is very important in terms of being the first study with a sample group covering all length groups of this species while giving a weight-height relationship similar to the natural population. Nevertheless, age determination of an individual of this size both in weight and length had never been done before. The population of this species is spread over a wide area covering the Black Sea, the Mediterranean Sea and the Atlantic Ocean and has a high commercial value, therefore demans further studies in detail.

Conflict of interests: The author declares that they have no conflict of interest.

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Original Article

New Localities and Length-Weight Relationship for *Alburnus caeruleus* in the Euphrates and Tigris River Basins (Turkey)

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ABSTRACT

The aim of the present study was to contribute to the geographic distribution and length-weight relationship of *Alburnus caeruleus* Heckel, 1843 living in the Euphrates and Tigris River basins (Turkey). For this purpose, fish specimens were investigated from the collection preserved in the Istanbul University Science Faculty Hydrobiology Museum (IUSHM). The length–weight relationship was calculated using the equation: $W=aL^b$. Through sample examinations, *A. caeruleus* was identified from one new locality (Eğri Stream) in the Euphrates River basin and with this contribution, the distribution range of the fish has extended to the tributaries of the Atatürk Dam Lake in the north. The results also contributed to the literature with two new localities of *A. caeruleus* species in the Savur Stream and River Tigris, both in the Tigris River basin. The *b* values calculated for the Euphrates and Tigris populations were 3.243±0.139 (n=56) and 3.340±0.329 (n=30) respectively, which both indicated positive allometric growth. In addition, the *b* value was calculated for *A. caeruleus* from the Tigris River basin is the first finding. This study also reported a new maximum length (TL) for *A. caeruleus* (13.0 cm).

Keywords: Freshwater fish, biogeography, distribution area, *b* value, allometric growth

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INTRODUCTION

Turkey, lying at the nexus of three biodiversity hotspots (the Caucasus, Irano-Anatolian, and Mediterranean), has a high freshwater fish diversity (Myers, Mittermeier, Mittermeier, Gustavo da Fonseca, & Kent, 2000; Şekercioğlu et al., 2011) and hosts approximately a total of 368 freshwater fish species (Çiçek, Birecikligil, & Fricke, 2015). The genus *Alburnus* (Family: Leuciscidae) is one of the species-rich fish groups, which includes approximately 14% of all freshwater fish species existing in Turkey (Çiçek et al., 2015). Anatolia is the center of biodiversity and endemism of the genus *Alburnus*. However, the genus has long been accepted as complex with a number of subspecies and synonyms, which had very short descriptions and no type materials (Özuluğ & Freyhof, 2007a). Recently, in order to clarify the taxonomy of the genus, certain species have been reviewed and 26 species belonging to the genus *Alburnus* have been recognized as valid from the inland waters of Turkey (Bogutskaya, Küçük, & Ünlü, 2000; Freyhof & Kottelat, 2007a, 2007b; Özuluğ & Freyhof, 2007a, 2007b; Elp, Özuluğ, Şen, & Freyhof, 2013; Elp Şen, & Özuluğ 2015; Özuluğ, Geiger, & Freyhof, 2018; Freyhof & Turan, 2019; Freyhof, Kaya, Bayçelebi, Geiger, & Turan, 2019; Fricke, Eschmeyer, & Van der Laan, 2020).

The Euphrates and Tigris rivers, originating in Turkey, have important main drainage basins in the Southern Anatolia and the Middle East. They flow south-westward through Syria and Iraq and discharge into the Persian Gulf (El-Fadel, El Sayegh, Abou Ibrahim, Jamali, El-Fadl., 2002; Şekercioğlu et al., 2011). In total, six species be-

longing to the genus *Alburnus* (*A. caeruleus, A. heckeli, A. kurui, A. mossulensis, A. selcuklui,* and *A. sellal*) were reported from these two basins (Fricke, Bilecenoğlu, & Sarı, 2007; Elp et al., 2015; Fricke et al., 2020). The black spotted bleak, *Alburnus caeruleus* was originally described from Aleppo-Syria and was listed as Least Concern (LC) in the IUCN Red List because of its widespread occurrence (Turkey-in-Asia, Iraq, Iran, and Syria) and tolerance to many threats (Freyhof, 2014). For Turkish inland waters, Turan, Kaya, Ekmekçi & Doğan (2014) described a new species, *Alburnoides recepi* from the Merzimen Stream, a tributary of the Euphrates River. However, Birecikligil, Eagderi, Jouladeh-Roudbar & Çiçek (2017) examined the morphometric, meristic and molecular characters of the fish samples from the same locality and found a large overlap with those of *A. caeruleus,* hence they reported this species to be treated as synonym of *A. caeruleus*.

In Turkey, *A. caeruleus* is found in the Euphrates and Tigris River basins and the information on the distribution of the species has only been presented in a few studies. It was reported in the Tigris River basin along with the Eğil Dam Lake (Diyarbakır), Dicle University Pond (Diyarbakır), Stream Bitlis (Siirt), Ambar Stream (Diyarbakır) and Başur, Zarova and Bağlıca streams (Siirt) (Kaya, Turan, & Ünlü, 2016; Freyhof & Turan, 2019). In addition, the distribution areas of the species in the Euphrates River basin were listed as Balıklıgöl Lake (Şanlıurfa), Erikliyayla Spring (Kilis), Stream Çakal (Adıyaman) and Merzimen, Karasu and Nizip streams (Gaziantep) (Bekleyen & İpek, 2010; Birecikligil & Çiçek; 2010; Freyhof & Turan, 2019). The species was also reported in the Sinnep Stream (Kilis), which is one of the small headwater streams of the Qweik River that flows to northern Syria (Birecikligil et al., 2017). In Iran, *A. caeruleus* has a narrow distribution range (Mohammadian-kalat et al., 2015) and is known from a few localities in the Tigris River basin; Gamasiab and Doab rivers (Esmaeili, Gholamhosseini, Mohammadian-Kalat, & Aliabadian, 2018), Maroon River (Zareian, Esmaeili, Zamanian Nejad, & Vatandoust, 2015), and Chardaval River (Zareian, Esmaeili, Zamanian Nejad, & Vatandoust, 2015; Mousavi-Sabet et al., 2015).

Both freshwater habitats and freshwater fishes are now notably sensitive to several major threats such as habitat modification, fragmentation, destruction, overfishing, invasive species, pollution, and climate change (Reid, Contreras MacBeath, & Csatádi, 2013). Recently, there has been increasing concern for the conservation status of native fish, therefore, it is quite important to update and increase the knowledge of the geographic distribution of freshwater fishes (Baigun & Ferriz, 2003). The goals of this study were i) to report additional localities to the geographic distribution of *A. caueruleus* in the Euphrates and Tigris River basins, ii) to estimate the length-weight relationship of the species living in these two river basins.

Studies for the conservation of fish cannot be separated from a detailed examination of their bio-ecological characteristics and an estimation of the length-weight relationship of fish is one such useful biological parameter (Hossain, Rahman, Ahamed, Ahmed, & Ohtomi, 2013; Giannetto et al., 2015). The length-weight relationship data is needed by fishery biologists, managers, or conservationists to compare the life histories of fishes among different geographic locations (Hossein et al., 2011; Akhtar & Khan, 2018). Also, the length-weight relationship is helpful for converting fish lengths into biomass in field surveys. The measurement of a fish length is more easy and rapid compared with its weight; therefore it is considered to be more practical to estimate weight where only the length is known (Harrison, 2001; Froese, Tsikliras, & Stergiou, 2011). The information on the length-weight relationship of *A. caeruleus* was available only in three previous studies. In Turkey, Birecikligil & Çiçek, (2011) reported the length-weight relationship of this species, inhabiting a river in the Euphrates River basin. In the other two studies, Mousavi-Sabet, Khataminejad, & Vatandoust (2014) and Valikhani et al. (2020) presented the findings of this equation obtained from the different populations inhabiting the inland waters of Iran.

MATERIALS AND METHODS

Fish specimens were inspected from the samples preserved in the Istanbul University Science Faculty Hydrobiology Museum (IUSHM). Fish were collected by electro-fishing in June 2008 and September 2009. The fish samples were measured to the nearest 0.1 cm for total length (TL) using a digital calliper and weighed to the nearest 0.01 g for body weight (W) on an electronic balance. The length–weight relationship was calculated using the equation: $W=aL^b$, where W is the total weight (g), L is the total length (cm), *a* and *b* are regression parameters (Le Cren, 1951; Froese, 2006). The 95% confidence interval (CI) of parameter *b* was calculated to exhibit significant deviation from the isometric condition (*b*=3) (King, 2007). The map (Figure 1) was created using the QGIS v. 3.4 software available from http://qgis.org.

RESULTS AND DISCUSSION

An examination of the fish samples in the IUSHM collection, revealed *A. caeruleus* samples collected from ten different sites, both in the Euphrates and Tigris River basins (Figure 1 and Table 1). The fish was a new record for one site (Eğri Stream) in the Euphrates and two sites (Savur Stream and River Tigris (5 km west of Hasankeyf)) in the Tigris drainages (Figure 1, Table 1). With these new contributions, the distribution area of *A. caeruleus* in the Euphrates River basin has extended to the Eğri Stream, which is one of the tributaries of Atatürk Reservoir in the northeast. It is

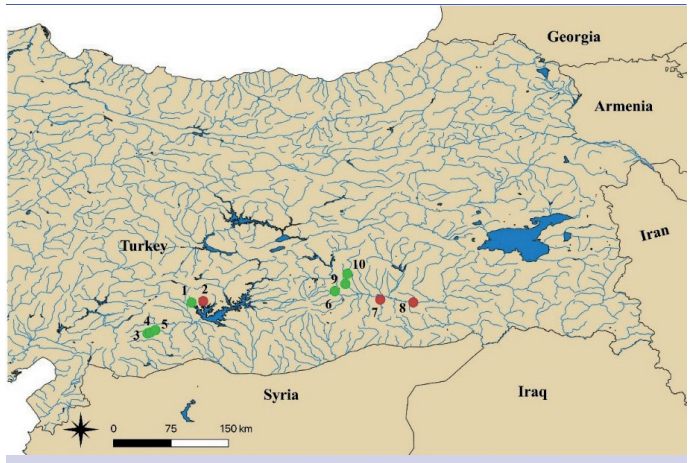

Figure 1. Distribution of *A. caeruleus* in the Euphrates and Tigris River basins in Turkey. Numbers refer to site numbers and red points mean new localities.

Table 1. The data of the sites, sampling dates, individual numbers (n), total lengths, body weights (W) and IUSHM collection codes of *A. caeruleus* in the Euphrates and Tigris River basins in Turkey.

Site No	Site	Coordinate	Province	Basin	Sampling date	n	TL (cm, min-max)	W (g, min-max)	IUSHM collection code
1	Stream Çakal (13 km west of Adıyaman, tributary to Atatürk Dam Lake)	37°43'19.9"N, 38°09'55.0"E	Adıyaman	Euphrates	20 June 2008	56	3.4-6.2	0.31-2.22	IUSHM 37800-351
2*	Stream Eğri (6 km southeast of Adıyaman, tributary to Atatürk Dam Lake)	37°44'30.0"N, 38°20'06.0"E	Adıyaman	Euphrates	20 June 2008	5	5.2-6.4	1.20-2.59	IUSHM 37800-349
3	Merziman Stream-1 (a tributary of the stream south of Yavuzeli)	37°16'36.0"N, 37°31'55.9"E	Gaziantep	Euphrates	27 Sept. 2009	2	5.2-5.7	1.00-1.10	IUSHM 2017-1290
4	Merziman Stream-2 (south of Yavuzeli)	37°17'31.9"N, 37°34'19.9E"	Gaziantep	Euphrates	27 Sept. 2009	10	5.1-11.2	0.90-12.71	IUSHM 2017-1291
5	Merziman Stream-3 (at Bağtepe)	37°19'28.9"N, 37°38'39.9"E	Gaziantep	Euphrates	28 Sept. 2009	5	4.1-13.0	0.42-15.57	IUSHM 2017-1292
6	River Tigris-1 (south of Diyarbakır at ten-eye-bridge)	37°53'12.9"N, 40°13'46.9"E	Diyarbakır	Tigris	19 June 2008	13	4.9-6.5	0.83-2.75	IUSHM 37800-341
7*	Savur Stream (between Bayındır and Ahmetli east of Tepe)	37°45'49.0"N, 40°53'02.0"E	Diyarbakır	Tigris	26 Sept. 2009	4	3.5-6.0	0.26-1.15	IUSHM 2017-1293
8	River Tigris-2 (5 km west of Hasankeyf)	37°43'25.0"N, 41°21'37.0"E	Batman	Tigris	25 Sept. 2009	17	4.3-6.8	0.52-2.03	IUSHM 2017-1294
9	Ambar Stream (at road to Silvan, 25 km east of Diyarbakır)	37°59'24.0"N, 40°22'55.9"E	Diyarbakır	Tigris	26 Sept. 2009	4	5.7-8.8	1.14-4.22	IUSHM 2017-1295
10	Ambar Stream (west of road from Diyarbakır to Bingöl about north of junction 15 km)	38°08'13.9"N, 40°24'46.0"E	Diyarbakır	Tigris	26 Sept. 2009	5	6.4-8.2	2.09-4.65	IUSHM 2017-1296

* indicates new distribution sites for the species.

assumed that *A. caeruleus* may have a wider distribution area, hence it is suggested that the Euphrates and Tigris River basins should be extensively studied in detail to determine the recent status of this species and other fishes inhabiting the region.

The total length and body weight of *A. caeruleus* in the present study varied between 3.4-13.0 cm and 0.31-15.57 g for the Euphrates population and 3.5-8.8 cm and 0.26-4.65 g for the Tigris population. Birecikligil and Çiçek (2011) reported that the total length and body weight distribution of this species inhabiting the Euphrates River basin were 3.8-7.1 cm and 0.25-2.85 g. Mousavi-Sabet et al. (2015) found the total length distribution of the species as 6.7-8.2 cm for the Chardaval River and 5.0-9.2 cm for the Gamasiab River in the Tigris River basin. Although the same fishing method (electro-fishing) was used in these three studies, the total length range in the present study was wider. However, the total length distribution presented in this study is similar to the data reported from the Tigris River drainages by Mousavi-Sabet et al (2015). The different habitat characteristics of the sampling sites (e.g. water depth, temperature, and flow rate) are considered to affect the size distribution of the fish. In addition, for *A. caeruleus*, a new maximum total length was found in the present study; the specimen of 13.0 cm examined from the Merziman Stream in the Euphrates River basin was longer than the previously reported as 9.2 cm TL in FishBase (Froese & Pauly, 2019).

The length-weight relationship of *A. caeruleus* living in two different river basins was calculated: i) Stream Çakal (Site-1) in the Euphrates River basin, ii) River Tigris (Site-6 and Site-8 in the main branch of the river). The sample size (n), length and weight distribution, parameters of the length-weight relationship (*a* and *b*) with 95% confidence intervals and correlation coefficients (r) values are summarized in Table 2. The values of *b* (3.243 and 3.340) estimated for two different *A. caeruleus* populations were within the expected range of 2.5 and 3.5 (Froese, 2006), therefore the results can be used as valid. In addition, the slopes of the

Table 2. Descriptive statistics and estimated parameters of length–weight relationships of *A. caeruleus* collected in Stream Çakal and River Tigris.

Species	n	a	b	95% CI of b	r	TL (cm, min–max)	W (g, min–max)
A. caeruleus (from Stream Çakal)	56	0.005	3.243	3.104-3.383	0.988	3.4-6.2	0.31-2.22
A. caeruleus (from River Tigris)	30	0.004	3.340	3.041-3.699	0.968	4.3-6.8	0.52-2.28

length-weight relationship of *A. caeruleus* indicated that two populations living in both the Euphrates and Tigris River basins had positive allometric growth (3.243±0.139 for Stream Çakal and 3.340±0.329 for River Tigris). The values of the correlation coefficient (r>0.95) for both populations indicated a strong positive relationship between length and weight (Table 2).

For the Euphrates population, Birecikligil & Çiçek (2011) calculated the length-weight relationship from 16 specimens with a narrow size range (3.8-7.1 cm) and reported positive allometric growth in the species (*b*=3.515; ±95% CI of *b*=3.099–3.930). This *b* value is in agreement with the one obtained from the present study. Whereas, for the Tigris population, Mousavi-Sabet et al. (2014) calculated this equation from 13 specimens with a very narrow size range (6.6-8.2 cm) and found an isometric growth (*b*=3.072; ±95% CI of *b*=2.417-3.908). Although the growth of a fish is species-specific, it can vary depending on factors such as size range (more small or large specimens) and environment in which they live (Bagenal & Tesch, 1978; Froese, 2006; Kachari, Abujam, & Das, 2017).

Valikhani et al. (2020) studied the length-weight relationship of *A. caeruleus* living in the shallow littoral waters of the Shadegan Wetland (Iran) and reported a negative allometric growth (*b*=2.84±0.15). Their fishing method is different from the studies above. They used a seine-net with 5 mm mesh size. In addition, they calculated the equation from 55 specimens with a narrow size range (3.2-8.5 cm). Compared to the present study in which positive allometric growth was calculated, this difference between the two populations is expected. Apart from size distribution, both the sampling method (electro-fishing vs. seine-net) and habitat differences (river vs. wetland) are considered as factors influencing the length-weight relationship in these two populations. Furthermore, for all populations compared, fish growth is affected by a number of other factors including sex, gonad maturity, season, degree of stomach fullness, and health (Bagenal & Tesch, 1978).

CONCLUSION

The results of the present study will contribute to the distribution range of *A. caeruleus* in the two river basins (Euphrates and Tigris) with new data. This study also provided the first data on the length-weight relationship of *A. caeruleus* population living in the Tigris River basin. The data presented in this study might constitute important background information for establishing further studies on this species.

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Original Article

Bio-designing of Culture Conditions for *Chlorella vulgaris* Using Response Surface Methodology

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ABSTRACT

Microalgae are microscopic organisms and show a geographical distribution depending on the physical, dynamic, and chemical factors of the environment. These factors are mostly important for attachment and development of microalgae. Substrate, temperature, light, agitation, and turbidity can be given as examples of physical factors, whereas salinity, pH value, and vitamins can be categorized as chemical factors. In this study, the optimization of *Chlorella vulgaris* production was carried out by response surface methodology (RSM) using two factors of agitation rate (100-250 rpm) and nitrogen source concentration (1-4 g/L) in the cultivation of BG11 medium. Moreover, the usage of urea instead of $NaNO_3$ was investigated and discussed.

Keywords: *Chlorella vulgaris*, Response surface methodology, Optimization, Urea

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INTRODUCTION

Microalgae are distributed across the tree of life with the most genetic diversity on the planet and they are members of a group of aquatic organisms of the kingdom Protista predominantly (Barkia et al., 2019). Thus, the capability of microalgae and their products have been studied for centuries.

Industrial microalgae cultivation will provide to the development of a sustainable large-scale production for biomass as well as its products. The industrial microalgae production potential was shown for various species of microalgae (Støttrup and McEvoy, 2008). However, there are several challenges to run commercial trials. The most affecting factors for those challenges are less biomass concentrations and insufficient information on growth conditions (Khan et al., 2018). Microalgae can be cultured under different conditions depending on the physical, dynamic, and chemical factors of the environment. Substrate, temperature, light, and turbidity can be given as examples of physical factors. Salinity, pH value, and vitamins can be categorized as chemical factors whereas, agitation and pressure are dynamic factors. Those factors are mostly important for the growth of industrial-scale biomass production.

Photosynthesis occurs in almost all microalgae owing to the chlorophyll-a and much of what is known about photosynthesis was discovered firstly by studying green alga. *Chlorella* sp. *Chlorella* sp. has a high amount of lipids and fatty acids, carbohydrates, peptides and proteins, inorganic minerals, phenolic compounds, and vitamins in its structure (Becker, 2007; Hariskos and Posten, 2014; Yeh et al., 2010). *C. vulgaris* has high photosynthetic capacity with regard to vascular plants due to the high concentration of chlorophyll-a. Moreover *C. vulgaris* is rich in B-group vitamins, especially B12, which are vital for the formation and development of blood cells. Owing to these rich contents, *C. vulgaris* can be used in cosmetics, wastewater treatment, pharmaceuticals, fruit and vegetable preservatives, tablets, powders, nectar, and noodles (Chisti, 2007; Priyadarshani and Rath, 2012;

Stolz and Obermayer, 2005). *Chlorella* sp., therefore, is considered a promising feedstock for several sustainable and value-added bioproducts in various cultivation modes for renewable energy, food, biopharmaceutical, and nutraceutical manufacturing.

Nitrogen source concentration and agitation rates play major roles in *C. vulgaris* cultivation. Different works that have aimed to observe the effect of nitrogen source concentration show that there is an inverse proportion between *C. vulgaris* production and the present nitrogen source concentration in a growth medium. As reported earlier, the *C. vulgaris* growth rate increased up until saturation levels, while the nitrogen source concentration in a growth medium decreased (Tam and Wong, 1996). Moreover, it was observed that the maximum level of lipid contents of *C. vulgaris* depended on when microalgal cultivation was achieved, and when the nitrogen source concentration was at a minimum level (Converti et al., 2009). In addition, microalgae can be damaged at high agitation rates because of the leakage of important chemicals from within the cell (Sacasa Castellanos, 2013). This study was aimed at determining the optimization of *C. vulgaris* production. The optimization of *C. vulgaris* production was provided by Response Surface Methodology (RSM) using two factors of agitation rate (100-250 rpm) and nitrogen source concentration (1-4 g/L) in the cultivation of BG11 medium. Moreover, the usage of urea instead of $NaNO_3$ was investigated and discussed.

MATERIALS AND METHODS

Maintenance and growth conditions of *C. vulgaris*

C. vulgaris was obtained from EGE MACC, Izmir-Turkey. The sample was incubated for three days in a refrigerated shaker incubator at 22 ± 2 °C with a stirring speed of 100 rpm under continuous illumination that measured as 320 lux. At the end of the third day, the stock culture was transferred into two 250 mL Erlenmeyers which contained 100 ml of sterile BG11 medium prepared under laboratory conditions and used for cultivation of *C. vulgaris* as equal amounts to prepare the inoculum culture aseptically. Both Erlenmeyers were allowed to incubate at 22±2°C, under a yellow light in the incubator, at a stirring rate of 100 rpm for ten days. The ten-day-old cultures were used as inoculum at 10% volume for all experiments.

The *C. vulgaris* strains were cultured in the 250 mL Erlenmeyer containing 90 mL growth medium in the refrigerated shaker incubator under a temperature of 22 ± 2 ° C at different concentrations of nitrogen and different agitation rates. The *C. vulgaris* strains were incubated either for 8 days when $NaNO_3$ was used as a nitrogen source type or for 10 days when urea was used as a nitrogen source type. Illumination was provided by refrigerated shaker incubator (Mikrotest MCS-55). Irradiance was measured with a Luxmeter (Benetech Gm1010 Digital Light Meter).

RSM and optimization studies

C. vulgaris production optimization was provided using 22 full factorial experiment designs with five replicates at a central point (175 rpm and 2.5 g/L) according to Central Composite Design (CCD) by the Response Surface Methodology (RSM) using Design Experiment Pro 7.0.0. $NaNO_3$ and urea were used as nitrogen source types. The range of nitrogen source concentration and agitation rates selected were 1-4 g/L and 100-250 rpm respectively. Determined factors' codes, ranges, and their levels can be seen in Table 1. There were five different agitation rates; *A*-rpm (69, 100, 175, 250, 281) and five different nitrogen source concentrations; and *B*-g/L (0.37, 1, 2.5, 4, 4.62) was studied for *C. vulgaris* production optimization. It was considered that these levels have potential effects on response function; and biomass concentration *(Y*, mg/L). The CCD can be seen in Table 2. In total, 13 experimental sets were used for determination of optimum level selected factors. All experiments were performed in duplicate and the average values of experimental sets were recorded.

Table 1. Experimental range and levels of the independent variables.

Independent Variables	Symbol Coded	Coded Levels				
		-α	-1	0	+1	+α
Agitation rate (rpm)	*A*	69	100	175	250	281
Nitrogen source concentration (g/L)	*B*	0.37	1	2.5	4	4.62

Table 2. CCD for *C. vulgaris*.

Number of Experimental Sets	Factor 1 Agitation Rate (rpm) (*A*)	Factor 2 Nitrogen Source Concentrations (g/L) (*B*)	
		$NaNO_3$	Urea
1	281	2.5	2.5
2	69	2.5	2.5
3	175	2.5	2.5
4	175	0.37	0.37
5	175	2.5	2.5
6	175	2.5	2.5
7	250	4	4
8	175	2.5	2.5
9	100	1	1
10	100	4	4
11	175	4.6	4.6
12	175	2.5	2.5
13	250	1	1

In accordance with these experimental sets, the growth medium where *C. vulgaris* was cultivated prepared as 100 mL into the 250 mL Erlenmeyer without any pH value. The difference between the growth medium and the original BG11 growth medium was the nitrogen source type and the nitrogen concentration. Then, 10 ml of each growth medium was pipetted into two different schott bottles according to the type of nitrogen source. Ten mL of *C. vulgaris* was inoculated into the 250 mL Er-

lenmeyer which contained 90 mL growth medium. In addition, a new inoculum culture was prepared by adding 90 mL of BG11 growth medium and 10 mL of *C. vulgaris* to an Erlenmeyer for use in subsequent sowing. Inoculated Erlenmeyer and newly prepared inoculum culture were put into the shaking incubator and cultivated in different periods which changed according to the type of nitrogen source at 22 ± 2 °C according to the experimental set-up. The Erlenmeyer which contained $NaNO_3$ as a nitrogen source was cultivated for eight days, The Erlenmeyer which contained urea as a nitrogen source and inoculum culture were cultivated for ten days.

The mathematical relationship of these independent variables on response can be approximated by a quadratic polynomial equation as can be seen in Equation 1:

$$Y=\beta_0+\beta_1A+\beta_2 B+\beta_{12}AB+\beta_{11}A2+\beta_{22}B2 \quad (1)$$

Where *Y* represents the response variable, β_0 is model constant, β_1 and β_2 are linear coefficients, β_{12} is interaction effect coefficient, β_{11} and β_{22} are quadratic coefficients, *A* and *B* are the coded levels of independent variables. The terms *AB, A*^{2} and *B*^{2} represents the interaction term between factors and quadratic terms of factors respectively. The equation (1) expresses the relationship between predicted response value and the independent variables in coded values. The quality of the developed model was determined by value of correlation value (R^2). Analysis of variance (ANOVA) was used for evaluation of the statistical significance of the model with values of regression and mean square of residual error (Deniz et al., 2015).

Dry-weight analysis

After two replicated productions of *C. vulgaris* for each experimental set up, the dry weight of these was measured by using filter paper. Firstly, the filter paper which was dried at 60 °C for one night in vacuum oven (Daihan Wov-70) and cooled in desiccators for 45 minutes was tarred by using precision scales (Shimadzu Atx224). Then it was moistened with 5 mL of distilled water. Secondly, a 50 mL sample was taken from the Erlenmeyer which was measured and dropped onto the filter paper slowly. Lastly, 5 mL of distilled water was dropped onto the filter paper again. These wetting and dropping procedures were performed by using a vacuum pump (Diaphragm Lh-185Lh). Then, the filter paper was dried at 60 °C for one night to reach a constant weight and cooled in desiccators for 45 minutes the next day. After these procedures, the filter paper was weighed again, and dry weight calculations were made. The results were recorded to an experimental design table.

RESULTS AND DISCUSSION

This set of experiments were designed by CCD using RSM and evaluated the effects of factors (agitation rateand nitrogen source concentration) on the production of *C. vulgaris*. As seen in Table 3, the range of factors selected were 100-250 rpm and 1-4 g/L at the end of the literature review, biomass concentration which changed depending on selected factors which ranged from 0.013 to 0.55 mg/L and 0.025 to 0.132 mg/L for $NaNO_3$ and urea respectively. The *C. vulgaris* production was performed five times at the central point (175 rpm and 2.5 g/L) of factors for optimization. According to the results of these five replications, the average values of biomass concentration were calculated as 0.32 mg/L for production which contained $NaNO_3$ as a nitrogen source type in growth media and 0.054 mg/L for urea. In addition, the maximum and minimum values of biomass concentration were reported as 0.013-0.55 mg/L and 0.025-0.132 mg/L for $NaNO_3$ and urea respectively.

Table 3. Experimental design matrix and experimental results.

Runs	*A (rpm)*	*B (g/L)*	Biomass concentration (mg/L)	
			$NaNO_3$	Urea
1	281	2.5	0.05±0.01	0.036±0.00
2	69	2.5	0.022±0.00	0.06±0.02
3	175	2.5	0.4±0.02	0.032± 0.00
4	175	0.37	0.3±0.03	0.132±0.04
5	175	2.5	0.3±0.01	0.04±0.01
6	175	2.5	0.3±0.01	0.03±0.00
7	250	4	0.014±0.01	0.01±0.00
8	175	2.5	0.4±0.01	0.1±0.03
9	100	1	0.072±0.01	0.094±0.04
10	100	4	0.084±0.00	0.01±0.01
11	175	4.6	0.55±0.01	0.025±0.00
12	175	2.5	0.2±0.21	0.02±0.03
13	250	1	0.013±0.01	0.1±0.03

Response Surface Methodology For Biomass Concentration For $NaNO_3$

The biological, chemical, and physical parameters play important roles in biomass production. In this study, the agitation rate and nitrogenous source concentration were physical parameters which played a dynamic role in the stimulation of biomass production and the factor ranges selected were 100-250 rpm and 1-4 g/L respectively.

The statistical testing of the model of *C. vulgaris* biomass production in a growth medium containing $NaNO_3$ was done by *Fisher's F* test for ANOVA as shown in Table 4. The *F* value was shown as 4.72 and where the *p* value was less than 0.05 with 0.0298 value, there was only a 2.98 chance that a "Model F Value" this large could occur due to noise. The values of *F* and *p* implies that the quadratic model was significant for production optimization of *C. vulgaris*. "Lack of fit *F* value" of 3.09 implied that the "Lack of fit" was not significant relative to pure error. There was a 14.99% chance that a "Lack of Fit F Value" this large could occur due to noise. The insignificance of "Lack of fit" value was a desired circumstance for convergence of the model as close to reality as possible. Statistically, the significance of the model and the insignificance of "Lack of fit" value indicated that the model was appropriate.

As seen in Table 4, the regression coefficient R^2 of 0.7025 value indicated that the regression model represented 70.25% of the experimental results and expressed a good fit response. The quality of fit explained by the model given by the multiple coefficient of determined R^2 value and if R^2> 0.7 insured, the model

Table 4. Analysis of variance (ANOVA) of the model for biomass concentration for $NaNO_3$.

Source	Sum of Squares	Degree of Freedom	Mean Square	*F*-Value	*p*>*F*
Model	0.27	4	0.068	4.72	0.0298
Factor A Agitation Rate	9.991E-004	1	9.991E-004	0.070	0.7984
Factor B Nitrogen Source Concentration	0.017	1	0.017	1.17	0.3104
AB	3.025E-005	1	3.025E-005	2.112E-003	0.9645
A^2	0.25	1	0.25	17.65	0.0030
Residual	0.11	8	0.014		
Lack of Fit	0.087	4	0.022	3.09	0.1499
Pure Error	0.028	4	7.000E-003		
Correlation Total	0.39	12			
Standard Deviation	0.12	R^2	0.7025		
Average	0.21	Adjusted R^2	0.5538		
C.V.%	57.52	Predicted R^2	0.2293		
Press	0.47	Adequate precision	6.179		

was suitable and adequate in biological production (Hanrahan et al., 2007). By adding factors to the model, the R^2 value increased regardless of factors significant or non-significant (Montgomery, 2001; Myers et al., 2016). Generally, although incensement of R^2, the adjusted R^2 (adj. R^2) value did not increase by the addition of factors to the model. Large differences between R^2 and adj. R^2 indicated that the model included non-significant terms. The adj. R^2 coefficient showed that significance of the model was high (Myers et al., 2016). In addition, the adj. R^2 value increased by deleting unnecessary factors of the model (Fermoso et al., 2010; Mazaheri et al., 2010). In this study, the adj.R^2 value was 0.5538 which eliminated non-significant terms from the model.

Adequate precision value (adeq. precision) measures the noise level of signals. There are circumstances in which an adequate precision value of more than 4 is desirable and this study determined that a model can be used to navigate the design space with an adeq. precision of 6,179 value. If the predicted R^2 is less than 0 (in this study with 0.2293 value), then the overall mean is a better predictor of response than the model.

The effect of factors on *C. vulgaris* production are expressed mathematically in a quadratic polynomial equation, Equation 2, for a growth medium including $NaNO_3$. In Equation 2, *Y* was the expected response; biomass concentration (mg/L), *A* and *B* were the coded values of factors; agitation rate (rpm) and nitrogenous source concentration (g/L) respectively.

$$Y = 0.32 - 0.011xA + 0.046xB - 2.750E\text{-}003xAxB - 0.19xA^2 \quad (2)$$

It is clear that the most affecting factor is A^2, in other words the square of the agitation rate, on biomass concentration and the square of the agitation rate is followed by nitrogenous source concentration, agitation rate, and interaction of factors respectively with regard to circumstances of terms in Equation 2 and Table 4.

The relationship between obtained biomass concentration response values from optimization studies which were performed in accordance with experimental sets and calculated biomass concentration results by using the Equation 2 can be seen in Figure 1. The optimum conditions described by the model as the point in which the biomass concentration values which were obtained by optimization studies close to the calculated biomass concentration results by using the Equation 2. In Figure 2, the effect of the interaction of factors selected which were the agitation rate and nitrogenous source concentration and change in range of 100- 250 rpm and 1-4 g/L respectively, on biomass concentration can be seen. The shape of the response surface showed an interaction between these two factors. The weakest effect on the response was observed for the nitrogenous source concentration with 1 g/L value, regardless of the maximum and minimum levels of agitation rate. In response surface 3D plot, the effect of the agitation rate could be seen clearly. The obtained biomass concentration response values from optimization studies were related closely with the agitation rate in which *C. vulgaris* productions were performed.

In this study, the level of physical parameters of agitation rate and nitrogenous source concentration were fixed as low and high, in the range of -1 to +1 and the maximum value of the response was aimed. All relevant factors were limited as seen in Table 5 for production optimization studies of *C. vulgaris*. The *C. vulgaris* production optimization solutions corresponded to 172 rpm and 4 g/L for agitation rate and nitrogenous source concentration respectively in regards to response at maximum desirability and predictability. Furthermore, the amount of biomass concentration obtained at the end of the production of *C. vulgaris* at optimum conditions were 0.370 mg/L with an appropriate predicted value with the desirability of 0.666. According to the model seen in Equation 2, optimum conditions of biomass production of *C. vulgaris* were determined as 172 rpm agitation rate and 4 g/L nitrogenous source concentration as $NaNO_3$.

3.2. Response surface methodology for biomass concentration for urea

The physical parameters effective on the production of *C. vulgaris* were selected as agitation rate and nitrogenous source concentrations varying from 100-250 rpm and 1-4 g/L respectively. Each experimental set seen in Table 2 were studied twice for determining the optimum production conditions of *C. vulgaris*. As seen in Table 6, the variance analysis (ANOVA) used for response analysis at the end of the different combinations of factors which was effective on *C. vulgaris* production. According to this, the biomass concentration model *F* value of 4.60 implies the model is significant and that there is only a 3.52% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob> f" less than 0.05 indicate model terms are significant. The *p* value associated with the F value is used to determine whether the F value was large enough to show statistical significance (Jaliliannosrati et al., 2013). The "Lack of Fit F-value" of 0.15 implies the Lack of Fit is not significant relative to pure error. There is a 92.53% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good for convergence to reality of the model. According to this study, the statistical significance of the model and the insignificance of lack of fit implies that the model is significant.

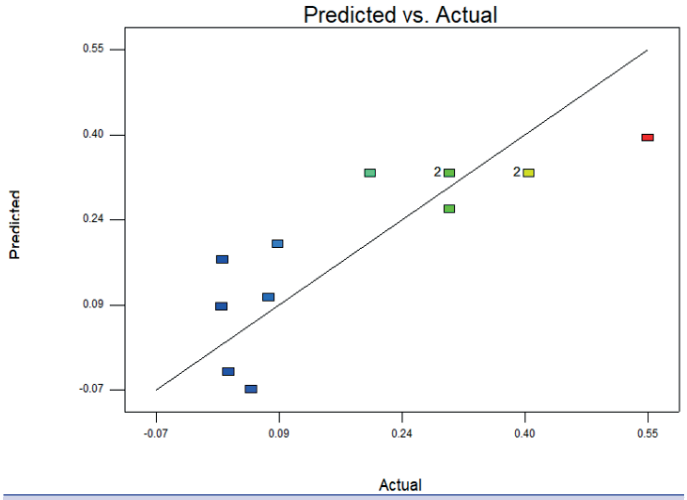

Figure 1. The relationship between performed optimization studies values and calculated values for *C. vulgaris* biomass concentration.

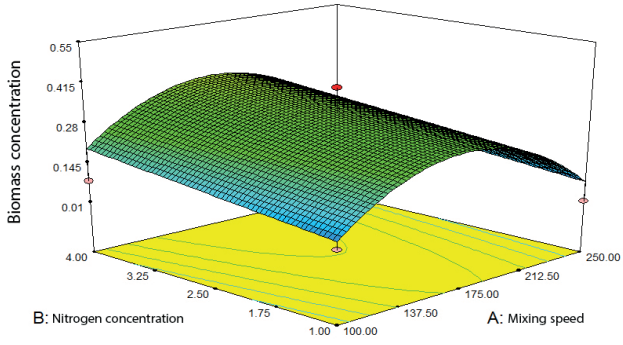

Figure 2. The response surface 3D plot of agitation rate and nitrogenous source concentration effects on *C. vulgaris* biomass concentration.

In this study, R^2 value was 0.7668 and it implies that a regression model did not correspond to the experimental results in ratio of 23.32%. The adjusted R^2 value was high with 0.6003 and the model was highly significant.

If there are *p* values lower than 0.0001 level in an analysis of variance, then a quadratic polynomial model has high significance and it is enough for the interrelated independent factors and responses (Guo et al., 2012). The *p* value of the model was 0.0352. According to the model, the *p* value of factors which affected biomass and coded as *A* and *B* were 0.0027 and 0.9093 respectively. In this situation, although factor *A* is assumed as significant for the model because the *p* value was lower than 0.05, factor *B* was insignificant because the *p* value was greater than 0.1. In addition to that, the interaction coefficient term of *AB* was insignificant because the *p* value was higher than 0.05. In this situation, individual effects of factors were greater than the effect of factor interaction on *C. vulgaris* biomass concentration and the most effect was caused by factor *B*.

When insignificant terms are decreased in the model, an improvement was on the carpet. The coefficient variation was high with a 47.95 value and low values of coefficient variation were needed of a high precision degree in providing the experimental data's reliability. Noise ratio of signals were measured by using an adequate precision value with a desired value not greater than 4. In this study, the adequate precision value was 6.859. For this reason, the model can be used for 3D design. The predicted R^2 value was 0.5058.

The growth medium which included urea as a nitrogenous source type, the effects of selected factors of agitation rate

Table 5. Optimum conditions for maximum biomass concentrations of *C. vulgaris* for $NaNO_3$.

Factors-Responses	Goal	Lower Limit	Upper Limit	Optimum conditions for *C. vulgaris*	Desirability
Agitation rate, *A*, (rpm)	Is in range	100	250	172.27	
Nitrogenous source concentrations, *B*, (g/L)	Is in range	1	4	4	
Biomass concentration of *C. vulgaris* (mg/L)	Maximize	0.013	0.55	0.370	0.666

Table 6. Analysis of variance (ANOVA) of the model for biomass concentration for urea.

Source	Sum of Squares	Degree of Freedom	Mean Square	*F*-Value	*p>F*
Model	0.015	5	2.973*E*-003	4.60	0.0352
Factor A Agitation Rate	9.759E-005	1	9.759*E*-005	0.15	0.7090
Factor B Nitrogen Source Concentration	0.013	1	0.013	20.48	0.0027
AB	9.000*E*-006	1	9.000*E*-006	0.014	0.9093
A^2	2.827*E*-006	1	2.827*E*-006	4.377E-003	0.9491
B^2	1.485*E*-003	1	1.485*E*-003	2.30	0.1732
Residual	4.521*E*-003	7	6.459*E*-004		
Lack of Fit	4.538*E*-004	3	1.513*E*-004	0.15	0.9253
Pure Error	4.067*E*-003	4	1.017*E*-003		
Standard Deviation	0.025	R^2	0.7668		
Average	0.053	Adjusted R^2	0.6003		
C.V.%	47.95	Predicted R^2	0.5058		
Press	9.582*E*-003	Adequate precision	6.8589		

and nitrogenous source concentration and coded as *A* and *B* on *C. vulgaris* production was indicated in a second order polynomial equation which was obtained by using multiple regression analysis, Equation 3, which can be used for calculations of predicted response value with any combination of relevant factors in experimental ranges. In Equation 3, *Y* is the predicted response; biomass concentration (mg/L), *A* and *B* are coded factors; agitation rate (rpm) and nitrogenous source concentration (g/L) respectively.

$$Y= 0.44 – 3.493E\text{-}003xA – 0.041xB - 1.500E\text{-}003xAxB – 6.375E\text{-}004xA^2+0.015xB^2 \quad (3)$$

According to the coefficients of terms in Equation 3, the *p* value of *B* was small and this showed that the dominant factor on biomass concentration was nitrogenous source concentration followed by the square of nitrogenous source concentrations, agitation rate, and interactions of factors which coded as *A* and *B* and lastly, the square of agitation rate.

Biomass concentration values obtained at the end of calculations by using Equation 3 and biomass concentration value obtained at the end of the optimization studies performed according to the experimental sets interaction given in Figure 3. Biomass concentration values obtained from performed optimization studies and predicted biomass concentration values calculated by using Equation 3 were close to each other.

The individual and interaction effects of independent factors which were selected as agitation rate and nitrogenous source concentration and affect to the biomass concentrations of *C. vulgaris* can be seen as a 3D response surface in Figure 4 by using the Design Expert in range of 100-250 rpm and 1-4 g/L respectively. In 3D design, the inconvenience of a factors range which affected *C. vulgaris* production can be seen. There was an inverse proportion between biomass concentration obtained at the end of the performed optimization stud-

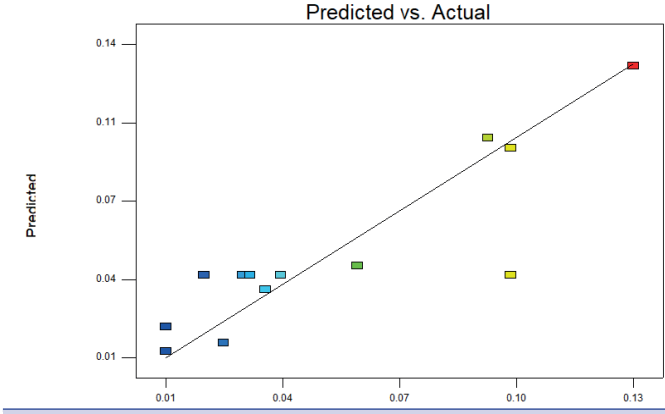

Figure 3. Interaction between biomass concentration values of performed studies and predicted and calculated.

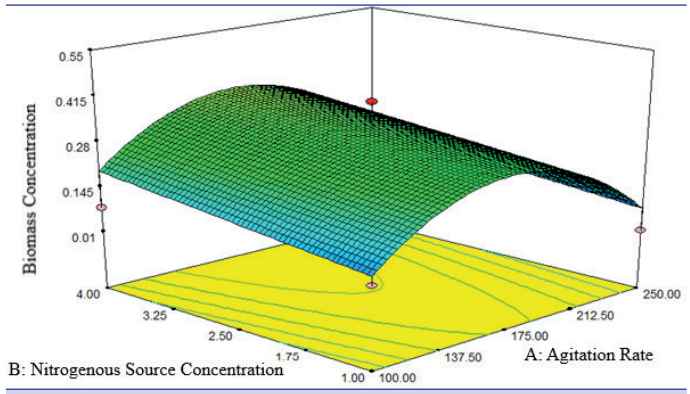

Figure 4. 3D design of agitation rate and nitrogenous source concentration effects on biomass concentration of *C. vulgaris*.

ies and present nitrogenous source (urea) concentration regardless of agitation rate values, such that the maximum level of response obtained at minimum level of nitrogenous source concentration likewise minimum level of response reported at maximum level of nitrogenous source concentration. Despite this situation, it was understood that when urea was used as a nitrogenous source in growth medium, the agitation rate did not affect the biomass.

In this study, the maximum response was aimed and the selected physical variables of agitation rate and nitrogenous source (urea) concentration values were fixed in a range -1 (low) to +1 (high). The *C. vulgaris* production optimization solutions were determined as 100 rpm and 1 g/L for agitation rate and nitrogenous source concentration respectively because of the maximum desirability and predictability value of response as seen in Table 7. Furthermore, the amount of biomass concentration of *C. vulgaris* at the end of the production of optimum conditions was predicted as 0.101033 mg/L and it was in agreement with the predicted value, with the relative desirability of 0.746, in which the model showed high desirability. According to the model, optimum conditions were 100 rpm agitation rate and 1 g/L nitrogenous source concentration for *C. vulgaris* biomass production by using urea as a nitrogenous source type.

In this study, *C. vulgaris* production optimization was provided by a Response Surface Methodology (RSM) which depended on agitation rate as a nitrogenous source concentration. When $NaNO_3$ was used as a nitrogenous source in growth medium, optimum conditions were determined as 172 rpm and 4 g/L in order to obtain maximum *C. vulgaris* biomass concentration. These optimum conditions were determined as 100 rpm and 1g/L for urea. It is understood from these results, that the agitation rate was mostly effective on biomass concentration described as a response function for growth medium which contained $NaNO_3$ as a nitrogenous source type. If urea was used as a nitrogenous source type in growth medium, the nitrogenous source concentration played a major role in *C. vulgaris* production in regards to obtained results and the decrease of a present nitrogen source concentration had a positive effect on biomass concentration. In a previous study, maximum lipid productivity of *C. vulgaris* of 247.16 mg l^{-1} d^{-1} was achieved when the concentration of $NaNO_3$ was 2.06 g l^{-1} (Xie et al., 2012). However, (Kong et al., 2012) reported the maximum biomass of *C. vulgaris* yield of 4.28 g/L when the concentrations of KNO_3 was 1.30 g/L.

After determination of optimum conditions for maximum level of *C. vulgaris* biomass concentration, it is understood that the physical parameter which was mostly effective on biomass concentration changes according to the nitrogenous source type such that the mostly effective physical parameter was agitation rate for $NaNO_3$, and nitrogenous source concentration was the most effective physical parameter for urea. Optimum conditions for *C. vulgaris* production were found to be as 100 rpm in BG11 medium supplemented with 1 g/L urea instead of $NaNO_3$. The utilization of urea is important because of its accessibility, being non-explosive, having low cost compared to $NaNO_3$.

At the end of this study and literature research our study is in accordance with (Tam and Wong, 1996) and (Converti et al., 2009) and the determined optimum agitation rate level was found to be close to the study of (Imamoglu et al., 2014) where the maximum level of protein contents of *C. vulgaris* obtained 168 rpm. In another study, the optimum agitation rate obtained was 150 rpm for maximum level of biomass of *C. vulgaris* (Razack et al., 2015). Differences between our study and other studies are caused by differences of growth medium used for *C. vulgaris* cultivation, *C. vulgaris* cultivation temperature, or the period of the incubation. After the optimum conditions were determined, a new *C. vulgaris* production was performed according to optimum conditions, predicted and obtained values of the results were controlled and validated. In Table 8, potential *C. vulgaris* biomass concentration values can be obtained at different confidence intervals when predicted result validation was performed at optimum conditions which were determined by using Design Expert. Biomass productions of 0.35 and 0.11 mg/L were obtained for $NANO_3$ and urea respectively at optimum conditions. According to the model, the predicted and performed responses were close together and appropriate to ranges thus it showed that the model was validated.

Determined mathematical models should be compatible with the experimental results. In this study, the aim was to show the maximum effects of selected parameters on *C. vulgaris* biomass concentrations depending on the nitrogenous source type used in *C. vulgaris* growth medium. This study presented an experimental approach for new research about the optimization of physical process parameters which are effective on *C. vulgaris* biomass concentrations.

CONCLUSION

In this study, the optimization of *C. vulgaris* production was performed and the factors which affect the *C. vulgaris* production were selected as nitrogen source type, nitrogen source concentration, and agitation rate. The optimum conditions of biomass production of *C. vulgaris* were determined as 172 rpm and 4 g/L

Table 7. Optimum conditions for optimum *C. vulgaris* biomass production.

Factors-Responses	Goal	Lower Limit	Upper Limit	Optimum conditions for *C. vulgaris*	Desirability
Agitation rate, *A*, (rpm)	Is in range	100	250	100	
Nitrogenous source concentrations, *B*, (g/L)	Is in range	1	4	1	
Biomass concentration of *C. vulgaris* (mg/L)	Maximize	0.01	0.132	0.101033	0.746

Table 8. Potential *C. vulgaris* biomass concentrations which can be obtained at the end of the productions at optimum conditions.

Predicted Values							
$NaNO_3$							
Response	Prediction	Standart Error Mean	95% Confidence Interval Low	95% Confidence Interval High	Standart Error Prediction	95% Prediction Interval Low	95% Prediction Interval High
C. vulgaris Biomass Concentration (mg/L)	0.370467	0.061	0.23	0.51	0.13	0.61	0.68
Urea							
Response	Prediction	Standart Error Mean	95% Confidence Interval Low	95% Confidence Interval High	Standart Error Prediction	95% Prediction Interval Low	95% Prediction Interval High
C. vulgaris Biomass Concentration (mg/L)	0.101033	0.020	0.054	0.15	0.032	0.024	0.18

nitrogenous source concentration as $NaNO_3$. Also, the optimum conditions were 100 rpm and 1 g/L nitrogenous source concentration for *C. vulgaris* biomass production by using urea as nitrogenous source type. The study aimed to provide a new nitrogen source for *C. vulgaris* production and also to utilize urea as an alternative substrate in biotechnology owing to the low cost and high accessibility in regard to $NaNO_3$. Because $NaNO_3$ can also be used in agriculture, construction, and the petroleum chemical industry along with the active substance in production of explosive devices. For this reason, availability and conservation of it is very difficult.

Conflict of interests: -

Ethics committee approval: -

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Disclosure: -

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Original Article

Effects of Salt/Sugar Brine Storage Solutions on Shelf Life of the Salted Atlantic Bonito

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ABSTRACT

In this study, Atlantic bonito was prepared as a salted traditional fish product known as Lakerda using the combined techniques of dry salting and brine salting. The product was preserved in brine containing 15% salt according to the traditional method and was analysed by comparison with 6.5% salt (Group A) and 6.5% salt-5% sugar (Group B) containing brine in cold storage. According to sensory analysis findings, Group A could be safely consumed from cold storage for 7 weeks and Group B for 9 weeks. The international acceptable limit values for analysis findings were not exceeded in both groups. However, the chemical and microbiological analysis results of the samples stored in the salt-sugar containing brine were found to be statistically lower than the salt-containing brine. It is concluded that the application of salt-sugar brine with acceptable results in terms of product safety and sensory properties can be recommended in the preservation of lakerda.

Keywords: Lakerda, brine, sugar, salt, shelf life, Atlantic Bonito

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INTRODUCTION

Salting is a traditional fish processing method and one of the preservatives most used in many countries of the world since ancient times, it is widely used with positive technological effects on taste and low cost, all around the world (Giuffrida et al., 2017). Microbial and enzyme activity in seafood can be controlled with reducing water activity by salting, resulting in the extend shelf life of the fisheries products. Salt also increases the texture and taste of the product. Depending on the size and composition of the fish, different salting methods such as dry and brine salting can be used (Horner, 1997; Sen, 2005). Salting of fish may be performed by dry-salting, brining, brine injection, or a combination of these techniques. In the traditional salting process, the water content is usually reduced from approximately 82% to about 54% (Oliveira et al., 2012). Dry salting is a method where salt crystals are applied to the flesh and fish are stacked in dry salt. As the salt penetrates the extracted moisture is allowed to drain away. This method is more preferred for lean fish. In salting, if the water separated from the tissue is allowed to drain it can be identified as "kench (dry) salting" and if this liquid is not allowed to be removed, the term "pickle" can be used (Sen, 2005). The brine water formed by water separated from the tissue should be drained away continuously in kench type dry salting. Especially in semi-dry salting and pickle type salting, there is an essential effect on product maturation by autolysis and proteolytic enzymes and microorganism activity (Horner, 1997). The ripening time required for the formation of the salty fish's characteristic flavour, appearance and texture will vary according to fish species, fish flesh thickness, fat content, and desired degree of curing. Brine salting is the method of preserving fish in a concentrated saturated salt solution. Pickling and brining are advantageous for fatty fish since being immersed in brine, fat is protected from atmospheric oxygen (Sen, 2005). Various factors such

as fish flesh thickness, composition and concentration of brine, ratio of brine to product and salting time are effective factors for the intramuscular diffusion of salt in this type of salting (Oliveira et al., 2012). It is an advantage to allow the fish to be soaked with brine to give sufficient time for the muscle to absorb a significant amount of salt. This reduces time and increases the weight efficiency of the salting process (Thorarinsdottir et al., 2004; Oliveira et al., 2012).

Salted products are generally defined as traditional fish products in the world and our country. Northern and Southern Europe countries such as Norway, Iceland, Spain and Portugal mostly consume salted cod. Several different species like *Gadus morhua, Gadus macrocephalus*, and *Gadus ogac* are used in the production of salted cod (Oliveira et al., 2012). In Sudan, consumers tend to prefer little fat in salted products and mostly use certain lean fish types such as *Hydrocynus spp* "Kass" (Ahmed et al., 2010). Whereas in South-eastern Europe, mostly in Greece, salted herring, anchovy, and marinade (herring) are widely consumed. Waxed caviar made from mullet roe is also consumed widely in Turkey. There are also some products made with combined processing techniques, such as lakerda which is a traditional salted fish product very popular in both Turkey and Greece, prepared from both dry salting and brining. One of the salted products prepared by applying combined techniques is salted cod. In salted cod, unlike lakerda, brine salting is applied first and then dry salting is applied. The salting process is followed by packaging and storage.

Nowadays, this type of traditional product should be developed in accordance with consumer taste and aroma demands. With the changes in dietary habits, consumers tend to prefer appropriate, quality and safe foods such as salt and sugar-processed seafood. These types of products are considered as a different, attractive and promising alternative to processed traditional seafood products (Lyu et al., 2017).

Like the effect of salt, sugar use extends the storage time of products by reducing water activity in foods (Zhang et al., 2015). However, there is little information in the literature on how using salt and sugar together affects changes in the quality or shelf life of fishes.

In this study, the effect of salt and salt-sugar containing brine on the quality and shelf life of seafood made from Atlantic bonito (*Sarda sarda*, Bloch, 1793) preserved in brine was examined.

MATERIALS AND METHODS

Materials

Salted fish was prepared from 14 pieces of Atlantic bonito (*Sarda sarda*) obtained from İstanbul Gürpınar Wholesale Fish Market (Turkey). The average length and weight of the fish was 39.69±2.04 cm and 995.60±133.51 g, respectively.

Production of salted Atlantic bonito

Atlantic bonito were brought to the laboratories in two hours with polystyrene boxes in ice. The fish were headed, gutted, their dorsal, caudal, and lateral fins were removed then they were cut into pieces, with a maximum width of 8 cm (Figure 1). Then their blood clots and bone marrow were completely removed. After this, the fish were dipped in an iced solution containing 2% salt. The fish were dry salted [fish weight: salt weight (1:1)] for 7 days at 4±2°C, brined in a 15% salt solution, fish to salt solution ratio was 1:1, for 13 days at 4±2°C. At the end of the ripening process, the fish were divided into two groups: Group A containing 6.5% salt and Group B containing 6.5% salt and 5% sugar. The production process of salted fish is given in Figure 1. The salted fish products were stored in these different storage solutions while being in cold storage at 4±2°C. All analytical determinations were done every two weeks to determine the shelf life.

The proximate composition and pH of the samples were determined throughout the processing steps from raw material until storage. The chemical analysis was determined both in the salted fish flesh and the brine. The microbiological and sensory attributes were analysed in fish flesh throughout the storage.

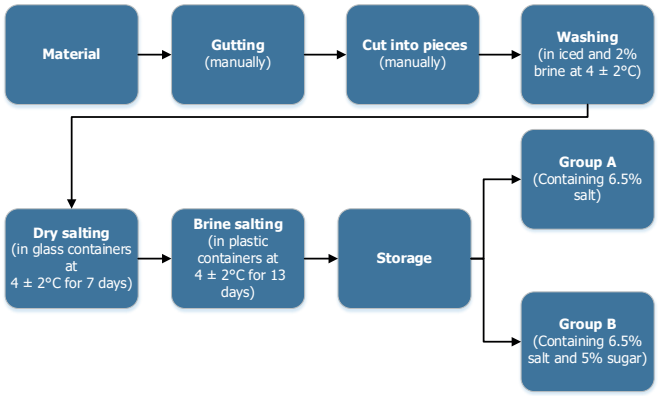

Figure 1. The production process of salted fish prepared from *Sarda sarda*.

Proximate composition

The lipid content (Weilmeier & Regenstein, 2004), moisture content (Cunniff, 1998), ash content (Cunniff, 1990a) and total crude protein content (Cunniff, 1990b) of the samples were analysed until the ripening in brine process.

pH

The pH of the samples was evaluated in raw material through the processing steps until storage solutions part. The pH was measured at room temperature in homogenates of fish flesh in distilled water (1/10 w/w) (Vyncke, 1981). The monitoring of the pH was performed using a calibrated WTW-pH-Meter.

Determination of total volatile basic nitrogen (TVB-N)

Total volatile basic nitrogen (TVB-N) analysis was performed according to Antonacopulous & Vyncke (1989). For TVB-N in fish flesh, a 10g sample was homogenized in an Ultra Turrax (IKA T 25 Basic, Staufen, Germany) with 6% perchloric acid (90 mL) for 1 minute. The same procedures were applied to the brine for TVB-N, 10 mL of the brine solutions were stirred with 6% perchloric acid (90 mL) for 1 minute in an Ultra Turrax. The homogenates were filtered through a filter paper (Whatman No. 1) and alka-

lized with NaOH (20%) before distillation. After the distillation (with the VELP UDK 140, Milan, Italy) the filtrates were titrated with 0.01 N HCl and calculated with the following formula.

$$TVB-N(mg/100g) = \frac{(V1-V0)\times 0.14\times 2\times 100}{M}$$

V1: Volume of 0.01 N HCl solution (in mL) for the sample

V0: Volume of 0.01 N HCl solution (in mL) for the blank

M = Weight of the sample in g

Determination of trimethylamine nitrogen (TMA-N)

TMA-N was determined by the modified method of Erkan & Özden (2008). Ten grams of homogenized samples were blended with 90 mL of 7.5% trichloroacetic acid solution in an Ultra Turrax and filtrated. 4 mL of the filtered solution was transferred into test tubes and 1 mL formaldehyde solution (20%), 10 mL anhydrous toluene, and 3 mL KOH solution were added. The tubes were shaken for 3 minutes and 5 mL of the top layer was pipetted and transferred into test tubes. 5 mL picric acid working solution (0.02%) was added to the pipetted top layers. The mixed contents were transferred to a spectrophotometric cell and measured in a UV-VIS spectrophotometer at 410 nm absorbance against the blank. Samples were calculated as mg/100 g in fish flesh according to the equation of the curve obtained from the standards.

Determination of thiobarbituric acid (TBA)

The Erkan & Özden (2008) modified method was used to determine the thiobarbituric acid (TBA) reactive substances. The fish flesh samples were placed in a 50 mL centrifuge tube and homogenized at high speed for 2 min in Ultra-Turrax with addition of 16 mL of 5% (w/v) TCA solution and 100 µL of butylated hydroxytoluene. The mixture was then filtered through a Whatman No. 1 filter paper. The 5 mL filtrate was mixed with 1 mL of a 0.01 M aqueous 2-TBA solution. The mixture was heated in a boiling water bath (70-80°C) for 30 minutes until the pink color was completely formed and then cooled to room temperature. TBA level was measured at 532 nm in UV-VIS spectrophotometer (Shimadzu Model 1610, Riverwood Drive Columbia, MD).

TBA values were expressed as milligrams of malondialdehyde (MDA)/kilogram of fish flesh. The concentration of MDA was calculated from a standard curve using solutions of the MDA precursor (same molecular weight) 1,1,3,3-tetraethoxy-propane (TEP) into distilled water after the addition of a quantity of TBA solution. The TBA value (mg of MDA/kg of fish meat) was obtained by the formula: The concentration of MDA calculated from standard curve x dilution factor/samples weight.

Microbiological analysis

Samples (25 g) were transferred aseptically to a Stomacher bag containing 225 mL of 0.1% peptone water (Merck, 107228) and homogenized for 60 s using a Lab Blender 400, Stomacher at high speed (Stomacher, IUL Instrument, Spain). For microbial count, 0.1 mL samples of serial dilutions (1:10, diluents, 0.1% peptone water (Merck, 107228, Darmstadt, Germany) of fish homogenates were spread on the surface of agar plates. Additionally 3.5% salt was added to agar and peptone. Plate count agar (PCA; Merck 1.05463) was used for psychrotrophic and mesophilic bacteria and incubated at 7°C for 10 days. Dichloran Rose Bengal Chloramphenicol (DRBC; Merck 1.00466) agar was used for mold-yeast and incubated at 25°C for 5 days. Results are expressed as a logarithm of colony forming units (log cfu) per gram of sample (Baumgart, 1986).

Sensory analysis

Salt brine and salt-sugar brine fish samples were assessed by 5 experienced panellists on the basis of appearance, taste, odour and texture characteristics using a ten-point descriptive scale, performed under controlled light, temperature and humidity conditions in individual booths. A score of 10.0-9.0 indicated "excellent" quality, a score of 8.9-8.0 "very good" quality, a score of 7.9–6.0 "good" quality, a score of 5.9-4.0 "acceptable" quality and a score below 4.0 indicated "unacceptable" quality (Karl et al., 2001).

Statistical analysis

Significant differences between the samples were calculated by IBM SPSS Statistics 21 (IBM Corp., USA) using a significance level of $p<0.05$ by the independent samples *t*-test. Possible differences between mean values of proximate composition results were analysed using ANOVA and when needed, post-hoc comparisons were done using Tukey's test.

RESULTS AND DISCUSSION

Proximate composition

The lipid, moisture, ash and total crude protein contents of the fresh, salted and ripenned samples in brine are given in Table 1. The lipid content of the fresh bonito was 32.99% (Table 1) which is similar to a study by Koral (2006) who reported 38.99%. Çağlak (2009) reported the lipid content of the fresh bonito samples as 23.4%, which is relatively lower. After salting, the amount of nutrient components changed - the amount of protein and ash increased by 6% and 8%, respectively (Table 1). Ormancı (2013) found similar results to our study in terms of decrease in lipid content after the salting process. At the beginning of the salting process, the meat swells with the entry of salt into the fish meat and the fish meat retains the water in its body. In the main salting process, protein coagulation occurs due to the intensive salt inflow into the fish meat and water leaves the fish muscle through diffusion (Ormancı, 2013). After the ripening period in brine, the moisture content of lakerda was 51.77% - similar to Ormancı who reported the value as 51.28%. The ratio of nutrient components such as lipid, protein moisture and ash in fish meat can vary de-

Table 1. The lipid, moisture, ash and protein content of the bonito samples (%).

Proximate composition	Fresh bonito	After salting	After ripening brine
Lipid	32.99±0.51[a]	25.68±0.52[b]	25.11±0.45[a]
Moisture	49.97±0.22[a]	42.78±0.28[b]	51.77±0.07[c]
Ash	1.05±0.10[a]	9.52±0.11[b]	7.76±0.04[c]
Protein	15.94±0.76[a]	21.93±0.70[b]	15.24±0.49[b]

All values are the mean±standard deviation (n=3). Different letters (a,b,c) in the same line indicate significant differences between groups ($p<0.05$).

pending on the type of fish, nutrition, reproduction, age and environment (Tülsner, 1994).

pH

Immediately after capture, the pH values of the fish were reported between 6.0 and 6.5. Quality of the fish were acceptable up to a pH of 6.8 but were considered to be spoiled above a pH of 7.0 as stated in Koral & Köse (2018). According to the literature data, the pH value of the raw material is within the freshness limits. As seen in Table 2, it ranged from 5.82 to 6.38 in Group A and from 5.86 to 5.62 in Group B during the 11-week storage period. Ormanci and Colakoglu (2017) reported a decrease in pH values from 6.38 to 5.94 in lakerda from Atlantic bonito. The pH decrease in the storage process of our samples is also related to this. These decreases in pH are explained by the increase in the ionic strength of the solution in the cells (Goulas & Kontaminas, 2005).

Total volatile basic nitrogen (TVB-N)

TVB-N production is generally used as an index to assess the preservation of the quality and shelf life of seafood products, and its production is closely related to the activity of spoilage bacteria (Erkan et al., 2009). The European Union set the limits of TVB-N for unprocessed fishery products at 25-35 mg/100 g (EC, 2008). The legal TVB-N limit determined for oily fish (sardines, herring and mackerel) is 20 mg/100 g (Erkan et al., 2009).

Indexes for TVB-N in the fish samples from both Group A, B and their brine solutions are shown in Table 3. During 11 weeks of storage Group A and B did not exceed the limits. However, the TVB-N levels of the Group A brine solution exceeded the limits at the 9th and 11th weeks. TVB-N values of the brine solution of the Group A at 9th and 11th weeks were 23.84 mg/100 g and 27.47 mg/100 g, respectively.

Erkan et al. (2009), found a low TVB-N value in brine stored Atlantic bonito compared to those stored in vacuum packs and oil packed during storage, as a result of which the total volatile basic nitrogenous compounds in flesh are dissolved in the brine. Although small fluctuations were observed, 8.20 mg/100 g was measured in the lakerda packed in brine at the end of the 4 month storage period (Erkan et al., 2009). In this study, as found in Erkan's study (2009), volatile basic compounds formed in the lakerda were transferred to brine solution during storage.

As in the study of Koral and Köse (2018), of the Atlantic bonito samples to which different concentrations (1:3, 1:4, 1:6 fish: salt ratio) were applied, those from dry salting were found to have an increased TVB-N value during storage, thus it was possible to comment on the quality of the product according to TVB-N freshness criteria.

In the later stages of storage, the absence of an increase in TVB-N can be attributed to the CO_2 solution in the fish muscle metabolised by lactic acid bacteria. After CO_2 absorption, the acidic solution neutralized basic metabolites (ammonia and amine compounds) from fish spoilage and caused the TVB-N to fall slightly. Significantly lower TVB-N values may be associated

Table 2. The changes of pH values of the salted Atlantic bonito stored in different brine solutions at 4±2°C.

Analysis	Raw Material	Group	1st week	3rd week	5th week	7th week	9th week	11th week
pH	5.84±0.01	*A*	5.82±0.01^{a}	5.85±0.01^{a}	5.73±0.01^{a}	5.34±0.02^{a}	5.97±0.01^{a}	6.38±0.05^{a}
		B	5.86±0.01^{b}	5.71±0.02^{b}	5.56±0.00^{b}	5.16±0.01^{b}	5.59±0.01^{b}	5.62±0.02^{b}

All values are the mean±standard deviation (n=3). Different letters (a,b) in the same column indicate significant differences ($p<0.05$).

Table 3. The changes in the values of TVB-N, TMA-N and TBA of salted Atlantic bonito stored in different brine solutions (4±2°C).

Analysis	Raw Material	Groups	1st week	3rd week	5th week	7th week	9th week	11th week
TVB-N (mg/100 g)	6.31±0.11	*A*	1.38±0.06^{a}	1.41±0.12^{a}	2.79±0.11^{a}	4.90±0.10^{a}	8.87±0.16^{a}	16.49±0.10^{a}
		A (Brine)	1.12±0.06^{a}	2.17±0.04^{a}	4.77±0.03^{a}	10.36±0.18^{a}	23.84±0.03^{a}	27.47±0.05^{a}
		B	0.88±0.04^{b}	1.45±0.09^{a}	1.61±0.07^{b}	1.89±0.04^{b}	2.25±0.02^{b}	3.87±0.10^{b}
		B (Brine)	0.84±0.05^{b}	1.34±0.13^{b}	1.93±0.04^{b}	2.86±0.06^{b}	3.55±0.02^{b}	5.39±0.03^{b}
TMA-N (mg/100 g)	1.70±0.18	*A*	0.63±0.07^{a}	0.53±0.18^{a}	0.33±0.04^{a}	0.31±0.03^{a}	0.63±0.02^{a}	0.40±0.01^{a}
		A (Brine)	0.72±0.14^{a}	0.69±0.07^{a}	0.53±0.05^{a}	0.52±0.01^{a}	0.94±0.05^{a}	0.77±0.05^{a}
		B	0.40±0.02^{a}	0.35±0.02^{a}	0.26±0.04^{a}	0.25±0.01^{b}	0.50±0.03^{b}	0.32±0.01^{b}
		B (Brine)	0.84±0.01^{a}	0.64±0.02^{a}	0.35±0.03^{b}	0.33±0.02^{b}	0.58±0.01^{b}	0.43±0.02^{b}
TBA (µg MDA/g)	0.77±0.01	*A*	6.15±0.30^{a}	4.29±0.04^{a}	2.45±0.02^{a}	3.39±0.02^{a}	4.29±0.06^{a}	5.82±0.08^{a}
		A (Brine)	0.20±0.03^{a}	0.98±0.02^{a}	1.82±0.01^{a}	2.05±0.03^{a}	2.29±0.05^{a}	2.39±0.01^{a}
		B	6.27±0.03^{a}	5.17±0.15^{b}	3.74±0.02^{b}	2.88±0.02^{b}	2.83±0.07^{b}	2.73±0.02^{b}
		B (Brine)	0.79±0.01^{b}	1.23±0.01^{b}	2.61±0.07^{b}	2.35±0.01^{b}	2.62±0.03^{b}	2.85±0.02^{b}

All values are the mean±standard deviation (n=3). Different letters (a,b) in the same column indicate significant differences ($p<0.05$). The groups were evaluated within themselves [A,B and A(Brine), B(Brine)].

with this. As Wang et al. (2016) mentioned in their study, this reduction can be due to the addition of sugar as an energy source to microorganisms. There are also other studies about significant effects of salt and sugar treatment which results in decreasing TVB-N and pH levels (Zhang et al., 2015; Wang et al., 2016; Qin et al., 2017; Li et al., 2018).

Trimethylamine nitrogen (TMA-N)

The TMA-N values of the salted Atlantic bonito stored in different brine solutions are shown in Table 3. In marine fish, trimethylamine oxide (TMAO), which is reduced by bacterial and enzymatic action to, TMA which is a spoilage product and is one of the main substances that causes stale fishy odor (Koral and Köse, 2018). Sikorski et al. (1990), proposed an acceptable level for TMA of 5 mg/100 g.

Similar to the results of the TVB-N analysis, the TMA values, which are volatile nitrogenous compounds, were found to be quite low in this study. The TMA values of the fresh Atlantic bonito was 1,70±0,18 mg/100 g which indicates the raw material was very good quality. At the beginning of storage, the TMA values were 0.63±0.07 mg/100 g (Group A) and 0.40±0.02 mg/100 g (Group B). The TMA levels of the brine solutions of groups A and B were 0.72±0.14 mg/100 g and 0.84±0.01 mg/100 g, respectively. After 11 weeks of storage the TMA levels of both groups and their brine did not show any significant increase, nevertheless they were measured at a fairly low levels. It is thought this was caused by the preservative effects of sugar and salt.

Koral and Köse (2018), reported the TMA levels of the group with 1:3 salt: fish ratio between 2.28- 3.86 mg/100 g during 12 weeks of storage. Similarly, Erkan et al. (2009) reported levels of TMA of the brine packed lakerda between 3.81-5.67 mg/100 g during 16 weeks of storage (Erkan et al., 2009).

The notable loss of nitrogenous components during brining is related to increased protein solubility in the tissues resulting from increased salt content, proteins and the destruction of these components may result in leaching into the brine (Martinez et al., 2012). However, in the present study, our data shows very low levels of TMA both in flesh and brine which can be explained by solubility of these nitrogenous components.

Thiobarbituric acid (TBA)

In traditional salted fish products, depending on preference, fatty fish such as mackerel, sardine, anchovies, bonito and lean fish such as cod, meagre can be preferred, but fat oxidation is a major problem in the quality of these products.

Fish are rich in polyunsaturated fatty acids that are very valuable in muscle tissue, food and nutritional physiology, but these fatty acids are easily oxidized in aerobic environments. The first oxidative oxidation products in fish oil tissue are unstable peroxide compounds. Since peroxides are unstable compounds that deteriorate rapidly to other degradation products, measuring the peroxide value is not a good parameter for making accurate determinations about product quality. Peroxides are divided into more stable components called malonaldehydes during oxidation. The TBA value generally shows a steady increase in fish and fish products, as in our study, and a decrease in correlation with changes in acceptable flavor and taste (Selmi et al., 2010).

There are several advantages to brining, such as protection against oxidative degradation (Oliveira et al., 2012). The changes in TBA values, which are indicative of the oxidative degradation rate, are shown in Table 3. The TBA value of the fresh Atlantic bonito was 0.770 mg MDA/kg. The TBA values of Group A and B were determined as 6.15 mg MDA/kg and 6.27 mg MDA/kg, respectively. At the end of the storage period, the TBA values of groups A and B were determined as 5.82 mg MDA/kg and 2.73 mg MDA/kg, respectively.

Erkan et al. (2009) reported that brine packed lakerda (1.52 mg MDA/kg) had the lowest TBA values compared to oil packed (3.03 mg MDA/kg fish) and vacuum packed (2.54 mg MDA/kg) groups. This occurs due to the fatty substance of brine-packaged salted fish separates from the product and pass into the brine during the storage. The fat content of the raw material also affects TBA values.

According to Faraji and Lindsay (2005), some sugars exhibit antioxidant activity and suppress oxidation (and therefore oxidative degradation) to some degree. Martinez et al. (2012), investigated the quality of salmon with different salting methods (dry and brine salting) with sugar additions. The dry salted and sugar added group appeared to provide the best results in shelf life analysis at the end of the 45-day storage. In their study, they concluded that sugar can protect the fish meat from oxidative deterioration, which is also consistent with our results.

Microbiological analysis

The total aerobic plate counts of mesophilic and psychrophilic bacteria are shown in Table 4. The total viable count limit for marine fish species is 7 log cfu/g (ICMSF, 1986). Initial mesophilic aerobic bacteria counts of the Group A and B were 4.07±0.35 log cfu/g and 3.61±0.11 log cfu/g, respectively. Psychrophilic aerobic bacteria counts of both groups' results were similar to the mesophilic bacteria. At the end of the storage mesophilic and psychrophilic bacteria counts of groups A and B were 4.59, 4.15 log cfu/g and 5.14, 5.05 log cfu/g, respectively. However, yeast and mould were not found in the products. Similarly Ahmed et al. (2010), reported that the total viable count load in salted Kass was below the limits and no yeast-mold was found. Throughout the 11-weeks of storage the bacterial counts of the products did not show a significant change. Both groups were not deteriorated in term of bacterial limit, which could be attributed to the inhibitory effect of salt on spoilage bacteria.

Erkan et al. (2009) reported that the fresh Atlantic bonito and bonito lakerda's initial psychrotrophic bacteria counts were 3.29 log cfu/g and 3.76 log cfu/g, respectively and brine-packaged samples after 11 weeks of storage has reached to 5.65 log cfu/g. The differences in the bacterial counts can be explained by the effect of salt and raw material.

Li et al. (2018) studied the quality of the salted and sugar-salted bream and concluded that bream fillets brined with a 1.8% salt or 1.8% salt and 0.9% sugar can delay deterioration and microbial growth. Similarly, Zhang et al. (2015), who studied the quality of carp fillets treated with different concentrations of salt and sugar, indicated that 6.0% salt intensely restrained the growth of total viable count compared to the other group (treated with 2.0% salt and 1.0% sugar). This shows that the ratio of salt and sugar used in

Table 4. The changes of total mesophilic aerobic and psychrophilic aerobic bacteria counts of salted Atlantic bonito stored in different brine solutions (4±2°C).

	Group	1st week	3rd week	5th week	7th week	9th week	11th week
Total Mesophilic Aerobic Bacteria (log cfu/g)	*A*	4.07±0.35^{a}	5.46±0.1^{a}	5.73±0.25^{a}	4.81±0.11^{a}	4.60±0.35^{a}	4.59±0.09^{a}
	B	3.61±0.11^{b}	3.75±0.12^{b}	4.16±0.02^{b}	4.27±0.2^{b}	4.54±0.07^{a}	4.15±0.13^{b}
Total Psychrophilic Aerobic Bacteria (log cfu/g)	*A*	3.96±0.14^{a}	5.12±0.1^{a}	5.24±0.1^{a}	5.52±0.5^{a}	5.56±0.35^{a}	5.15±0.46^{a}
	B	3.71±0.06^{b}	3.98±0.12^{b}	4.03±0.1^{b}	4.58±0.19^{b}	4.66±0.16^{b}	5.05±0.1^{a}
Mold-Yeast (log cfu/g)	*A*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
	B	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*

All values are the mean±standard deviation (n=4). Different letters (a,b) in the same column indicate significant differences ($p<0.05$). n.d: not detected

the product is effective in the formation and suppression of microbial activity, which directly affects the overall quality.

Sensory analysis

The results of sensory analysis are a very important parameter in determining the quality of all processed fish products, including salted seafood products. Sugar treatment promoted the generation of objectionable odours. This phenomenon may be attributed to acidic metabolites produced by certain anaerobic microorganisms; these metabolites were regarded as off-flavour compounds by sensory panellists (Wang et al., 2016).

Ormanci and Colakoglu who studied the nutritional and sensory properties of the lakerda, characterize this traditional salted Atlantic bonito product as whitish-milky coloured, cohesive and soft rubbery textured, salty and sweat-smell odored (Ormanci and Colakoglu, 2015).

Evaluation of different sensory attributes such as appearance, odour, texture, and flavour of salted Atlantic bonito products by the panellists are given in Table 5. Group A, in the 9th week of storage and Group B in the 11th week of storage in terms of odour and flavour was identified as non-consumable by the panellists. The results for sensory components of the samples showed that the products stored in salt-sugar brine had a better overall sensory acceptance value compared to the salt brined group. Sugar treatment suppressed the production of unwanted odours, resulting in a significant effect ($p>0.05$) on the overall sensory quality. At the end of the storage it was found that the group with sugar addition had higher mean odour-flavour scores than the group with only salt addition. According to Wang et al. (2016), the combination of salt (1.3%) and sugar (1.0%) may delay physical, chemical and microbial changes, while preserving other characteristics of flavour and quality. In this study, the changes in total bacteria counts of both groups were not consistent with sensory evaluation results and the products had a longer shelf life according to the microbiological acceptability limit compared with sensory analysis at 4±2°C. This is in correlation with Wang et al. (2016). The panellists compared both groups and stated in the comments that Group B (6.5% salt + 5% sugar) had a better overall quality in terms of texture, appearance, and odour. In our study, sensory scores were a more effective quality index, compared with chemical and microbial results, in determining the shelf life of salted and salt-sugared bonito product. In relation to this, other chemical parameters support our overall sensory quality. The low levels of TBA values developed during storage of the salted Atlantic bonito products (Table 3) support

Table 5. The changes in sensorial attributes of the salted Atlantic bonito stored in different brine solutions (4±2°C).

		1st week	3rd week	5th week	7th week	9th week	11th week
Odour + Flavor	*A*	8.82± 0.83^{a}	8.37±0.67^{a}	7.63±1.07^{a}	5.78±1.44^{a}	3.12±1.46^{a}	1.49± 1.36^{a}
	B	9,33±0.39^{a}	8.38±0.42^{a}	8.11±0.78^{a}	7.10±0.89^{b}	5.28± 1.49^{b}	3.10±1.78^{b}
Mean	*A*	9.03± 0.80^{a}	8.68±0.84^{a}	7.52±0.97^{a}	6.01±1.26^{a}	3.93±1.68^{a}	2.70±1.80^{a}
	B	9,33±0.38^{a}	8.38±0.47^{a}	8.01±0.77^{a}	7.01±1.19^{b}	5.80±1.46^{b}	4.12±1.70^{b}
Appearence	*A*	9.30±0.88^{a}	9.16±0.81^{a}	7.25±1.17^{a}	6.48±1.01^{a}	5.33±1.51^{a}	3.90±1.43^{a}
	B	8.70±0.45^{a}	7.92±1.07^{a}	7.10±1.52^{a}	6.57±1.25^{a}	5.28±0.85^{a}	3.48±0.98^{a}
Texture	*A*	9.17± 0.68^{a}	8.36±0.92^{a}	7.58± 0.74^{a}	6.00±1.17^{a}	4.15±1.44^{a}	3.90±1.43^{a}
	B	8.06±0.44^{a}	7.92±0.49^{a}	6.74±1.60^{a}	6.07±1.40^{a}	5.00±0.79^{b}	3.23±0.93^{a}
Odour	*A*	9.50± 0.55^{a}	8.68±0.43^{a}	7.67±1.03^{a}	5.60±1.67^{a}	3.48±1.10^{a}	1.70±0.97^{a}
	B	8.50±0.50^{a}	8.40±0.51^{a}	7.22±0.91^{a}	5.48±1.29^{a}	3.70±0.45^{b}	3.60±0.73^{b}
Flavor	*A*	8.13± 0.29^{a}	8.06±0.75^{a}	7.58±1.11^{a}	5.96±1.34^{a}	2.75±1.78^{a}	1.28±1.76^{a}
	B	8.26±0.34^{b}	7.82±0.93^{a}	6.98±0.96^{a}	5.08±1.77^{a}	2.50±2.45^{b}	1.23±1.84^{a}

All values are the mean±standard deviation (n=3). Different letters (a,b) in the same column indicate significant differences ($p<0.05$).

our results in the case that no major rancidity flavour was detected by the panellists. The best strategies to extend shelf life of fish and fish products include the use of natural preservatives. As the effect of enzymes, which break down proteins and fats, the salting process of the compounds in the tissue (proteins, water-soluble vitamins, minerals, and oil) end up breaking up/dissolving into brine (Oliveira et al., 2012).

CONCLUSION

The results of the analysis showed that there was no difference between the applications in terms of the shelf life of the Atlantic bonito preserved in salt and salt-sugar containing brine. However, chemical and microbiological analysis results were found lower; sensory analysis results were higher in the bonito stored in sugar-containing brine, with this study it was shown that sugar containing brine can be recommended for product safety and sensory properties. Preservation in brine containing salt and sugar gave the lakerda 30% more shelf life.

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