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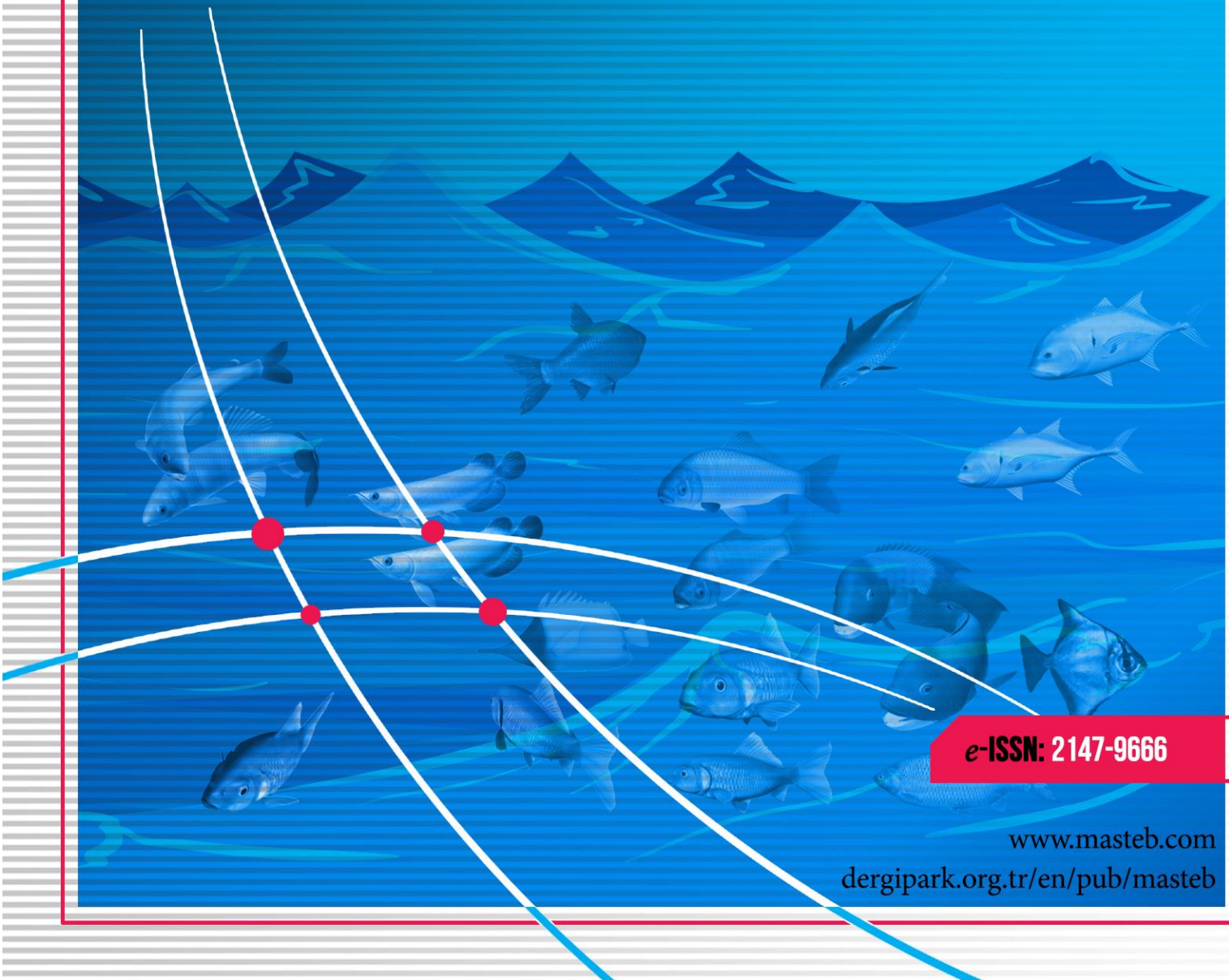

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RESEARCH ARTICLE

Age, growth and reproduction of *Neogobius melanostomus* (Pallas 1814) (Perciformes: Gobiidae) in the southern Black Sea

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ABSTRACT

Round goby (*Neogobius melanostomus*, Pallas 1814) which belongs to Gobiidae, inhabits in the Black Sea, the Marmara Sea, the Caspian Sea and the rivers that flows into fore stated seas. The main population parameters, such as age, length, sexual composition, length-weight relationship, growth, condition factor, gonadosomatic index and reproduction of *N. melanostomus* were investigated from coasts of the southern Black Sea in this study. A total of 2408 individuals were sampled between July 2017 and June 2018. Results showed that average length and weight were found 14.97 cm (10.50-26.20) and 43.90 g (15.28-212.2), respectively. Sex ratio between female and male was found 1:1.77 (P>0.05). Age of samples varied between one and eight years old. The von Bertalanffy growth parameters were calculated as L_{∞} = 26.5 cm, k = 0.1980 year^{-1} and t_0 = -1.3487 year for all individuals. The length-weight relationship was found as W = 0.0069 TL$^{3.1972}$ for all individuals. Total mortality, natural mortality, fishing mortality, growth performance index and condition factor were calculated as 0.625, 0.479, 0.145, 2.143, and 1.66, respectively. Spawning occurs between March and May and the maximum value of gonadosomatic index reached in April. Average relative fecundity was 318.88 number/g (124.8-958.9), while average diameter of eggs was found 2025.58 µm ± 262.86 (1112.0-2703.4). This study will contribute for information gap on the species as well as provide information source for future studies.

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Introduction

Round goby, *Neogobius melanostomus* Pallas 1814, distributes widely in coastal shallow waters of tropical and subtropical region. There are 1578 identified species of goby in the world. There are 74 goby species in the Black Sea and Mediterranean Sea, 35 of them were reported from coasts of Turkey (Miller, 1986; Nelson, 1994; Froese and Pauly, 2009;

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Engin and Bektaş, 2010; Sezgin et al. 2017). The Black Sea, the Marmara Sea and the Caspian Sea are natural habitats for species of *N. melanostomus* (Berg, 1949). It is also reported that the species distribute in various seas (Baltic, Gulf of Gdańsk, North America etc.) as an invasive species carried by ballast water (Skora and Storlarski, 1993).

The species is invasive and opportunistic because it has well adaptive skills, high fecundity, short life-span and rapid growth properties (Lodge, 1993). The species prefers habitats of sandy, rocky and pebble in shallow waters down to 20 m in the Black Sea and Sea of Azov (Miller, 1986), especially vegetative (*Zostera spp.*) habitats down to 70 m in the Caspian Sea (Moskal'kova, 1996). While Jude and Deboe (1996) reported that the species distribute from creep flow rivers, lagoon to brackish waters down to 20 m in spring and autumn, Miller (1986) reported their migration to deeper water down to 60 m.

N. melanostomus is one of the benthic feeding habit species. Crustaceans and mollusks contribute to an important portion of their diet. Additionally, it is reported that their diet includes species of polychaetas, small fish and benthic eggs (Berg, 1949; Miller, 1986).

It can be classified as discard fish in coastal fisheries of the Black Sea. Öztürk (1998) reported it has low abundance for Turkish coastal waters of the Black Sea. Recently, it became more abundant species in fishing operations, and it has started to sold commercially in fish markets (approx. 3 USD) (Dr. Mehmet AYDIN personal observations). The species is not commonly known and not preferred by local people.

There are various studies in the regions for this species (Slastenenko, 1956; Kovtun, 1979; Abdoli et al., 2009; Yankova et al., 2011, Hôrková and Kováč, 2014; Macun, 2017) however studies from the Black Sea, especially the southern part are scarce (Engin, 2008; Gözler et al., 2003; Kurt, 2005; Kasapoğlu, 2016).

In scope of this study, it is aimed to determine the main population parameters (age, length, sexual composition, length and weight relationships, growth, condition factor, gonadosomatic index and breeding properties) of *N. melanostomus* from coasts of the central-southern Black Sea. This research will be one of the most detailed study for the Turkish coast of the Black Sea focus on the marine environment and it will provide a significant value to the literature for the future of population.

Material and Methods

The study was conducted between July 2017 and June 2018 by using trammel net (1000 m length, 2 m depth) with mesh size of 17-24 mm which is used commonly by fishers from southern coasts of the Black Sea. A total of 2408 individuals were sampled in 48 operations during the period of the study.

The study was performed in central coasts (41°08'41.93"N-37°17'41.29"E and 40°57'55.68"N- 38°07'24.97"E) of the southern Black Sea, off Ordu province (Figure 1).

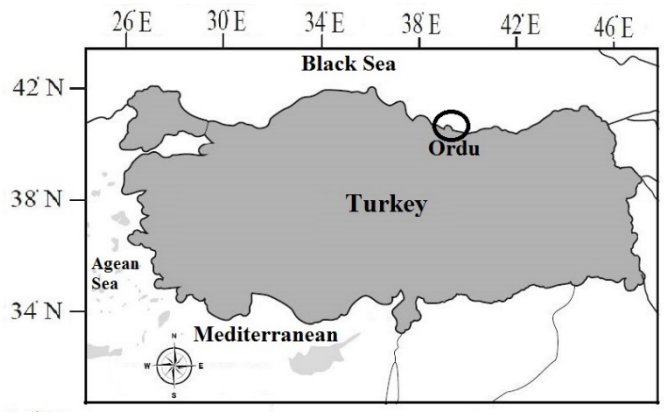

Figure 1. Study area

Captured fish were preserved in iceboxes till examination in the laboratory. Total length (TL, in nearest 0.1 cm), total weight (W) and gonad weight (GW) (at 0.01 g precision) of all specimens were recorded. The sex of each specimen was determined by gonads macroscopically.

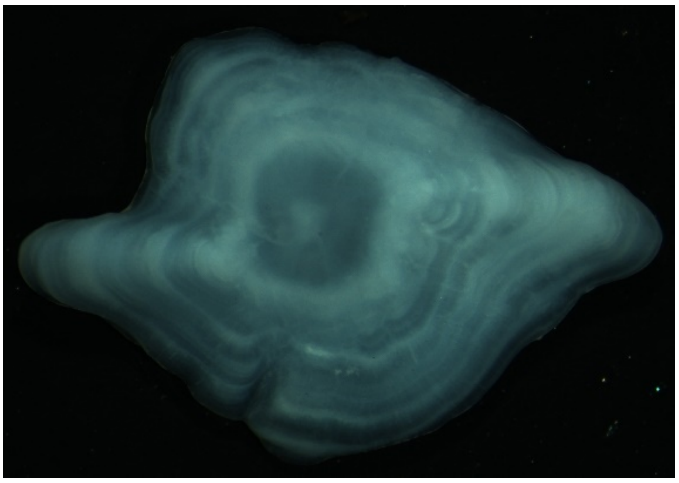

Figure 2. Otolith of 5 years old specimens of *Neogobius melanostomus*

Age Determination

Otolith of each specimen was used for age determination in this study. Otoliths were prepared for age determination and placed into black plate. Steromicroscope (up to X10 zoom) was used by illumination from top and side.

Length and Weight Relationships

The total length-weight relationship (LWR) of the species was estimated by the exponential regression model, $W = aTL^b$, where *a* and *b* are constants (Ricker, 1975). LWR relationships

were performed by using all samples as well as female and male, separately. The regression co-efficient for isometric growth is "3", while values greater or lesser than this value indicate an allometric growth. The Pauly's *t*-test was used to compare the "*b*" values of male and female groups (Pauly, 1984) whether significant difference or not.

Growth Parameters

The von Bertalanffy growth equation (VBGE) was used to describe the growth of *N. melanostomus* for the whole individuals sampled (Sparre and Venema, 1992; King, 1995).

$$L_t = L_\infty\left(1 - e^{-k(t-t_0)}\right)$$

where L_t is the total length at age t, L_∞ is the asymptotic length, k is the growth coefficient, and t_0 is the theoretical age when the fish was at zero total length. The same function was used for growth in weight;

$$W_t = W_\infty\left(1 - e^{-k(t-t_0)}\right)^b$$

where W_t is the total weight, W_∞ is the asymptotic weight, and b is the power constant of the length-weight relationship. Values of L_∞, t_0 and K, which are parameters of the von Bertalanffy growth equation (VBGE), were estimated by using method of Ford-Walford (Pauly, 1984; Gulland, 1988). Growth parameters by using these values in following formulas were found (Sparre and Venema, 1992; King, 1995):

$$L_\infty = \frac{a}{1-b}$$

$$k = \ln b$$

$$t_0 = t + \left(\frac{1}{k}\right) \times \ln\left[1 - \left(\frac{L_t}{L_\infty}\right)\right]$$

Munro's phi-prime growth performance (Φ') was calculated the formula of Pauly and Munro (1984):

$$\varphi' = \log(k) + 2\log(L_\infty)$$

Condition Factor

Fulton's coefficient of condition factor (C) of *N. melanostomus* was calculated monthly for the sampling by

$$C = \left(\frac{W}{TL^3}\right) \times 100 \text{ (Ricker, 1975)}$$

Gonadosomatic Index

Monthly values of the gonadosomatic index (GSI) were calculated for each sex.

$$GSI = \left(\frac{GW}{W}\right) \times 100 \text{ (Devlaming et al., 1982)}$$

Fecundity

Eggs of female individuals were collected and counted in April and May, when GSI reached the maximum in spawning season. A total of 206 individuals were examined for this purpose. The gonads were collected and eggs were counted in ovaries, immediately. Subsamples were counted using the gravimetric method and then calculated according to the following formula (Holden and Raitt, 1974):

$$F = \frac{G}{g} \times n$$

where; F is the total number of eggs in the ovary, G is ovary weight (g), g is the weight of the subsample taken from the ovary (g), and "n" is the total number of eggs (including previtellogenic oocytes) in the ovary. It should be noted that only mature oocytes were taken into account while measuring egg diameters.

Mortality Rates

The natural mortality (M) and fishing mortality (F) rates were calculated by means of the following relationships. Total mortality rate (Z) is calculated by using the survival rate (S). (Gulland, 1969; Ricker, 1975).

$$S_{(t)} = \frac{N_{(t+1)}}{N_{(t)}}$$

where $N_{(t)}$ is the number of fish in the related age group and $N_{(t+1)}$ is the number of fish in the related age group at the end of one year. In this equation, the relation between the survival rate and the total mortality rate is as follows:

$$S_{(t)} = e^{-Z(t)}$$

Total mortality rate (Z) is calculated as follows:

$$Z = -ln(S)$$

Natural mortality (M) is calculated according to Pauly (1980).

$$LogM = -0.0066 - 0.279 logL_\infty + 0.6543 logk + 0.463 logT$$

Here, T is the CTD derived average annual water temperature of the environment in which the *N. melanostomus* lives (°C). Fishing mortality (F) was estimated as

$F = Z - M$ (Beverton and Holt, 1957).

Statistical Analysis

T-test and χ^2 square test were used to compare statistically the parameters obtained in this study. Statistical applications were performed by using software's of Microsoft Office Excel and SPSS 18.

Table 1. Sex ratio data according to months

Months	N		(F:M)	Chi-square (χ^2)
	Female	Male		
July 2017	17	37	1 : 2.17	7.407*
August 2017	9	28	1 : 3.11	9.757*
September 2017	11	21	1 : 1.90	3.125
October 2017	10	121	1 : 12.10	94.053*
November 2017	52	285	1 : 5.48	161.095*
December 2017	37	235	1 : 6.35	144.132*
January 2018	73	228	1 : 3.12	79.817*
February 2018	108	155	1 : 1.43	8.399*
March 2018	178	145	1 : 0.81	3.372
April 2018	138	144	1 : 1.04	0.128
May 2018	233	132	1 : 0.56	27.948*
June 2018	4	7	1 : 1.75	0.818
Total	**870**	**1538**	**1:1.77**	**185.309***
	2408			

Note: * (1df, 5%)

Table 2. Total length and weight data of *N. melanostomus* for each sex and the pooled data

	L (cm)			W (g)		
	Mean ± SD	**Min.**	**Max.**	**Mean ± SD**	**Min.**	**Max.**
All	14.97±2.43	10.50	26.20	43.90±27.63	15.28	212.20
Female	13.55±1.40	10.50	20.90	31.06±13.24	15.28	133.91
Male	15.78±2.51	11.90	26.20	51.17±30.82	15.86	212.20

Results

Length-Frequency Distribution

A total of 2408 individuals were obtained between 1-120 m depth during the study period. Especially, the big size males were caught around 120 m depth. A large amount of samples (1652 individuals) were ranged between 12-15 cm length (68.60%). The highest frequency was found 13 cm length group with 599 individuals (24.87%). While minimum length was 10.5 cm, the maximum length was 26.2 cm. Frequency distribution of each length group and monthly length frequency distribution were presented in Figure 3 and 4, respectively. Weight distribution of sampling ranged between 15.28 g and 212.2 g.

Sex Composition

Results showed that percentages of female and male individuals were calculated as 36.13 % (870) and 63.87 % (1538) during this study, respectively. Sex ratio of female and male was found 1:1.77 (Table 1). It should also be noted that difference between sex was statistically significant (χ^2= 185.309, df= 1, P>0.05).

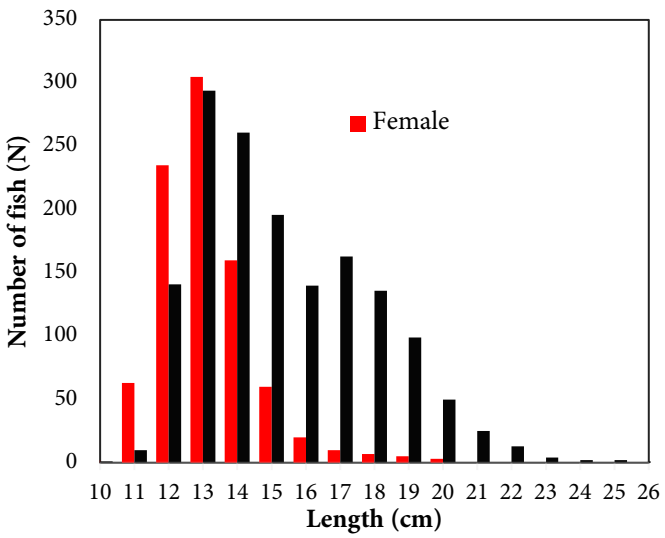

Figure 3. Frequency distribution of total length for male and female

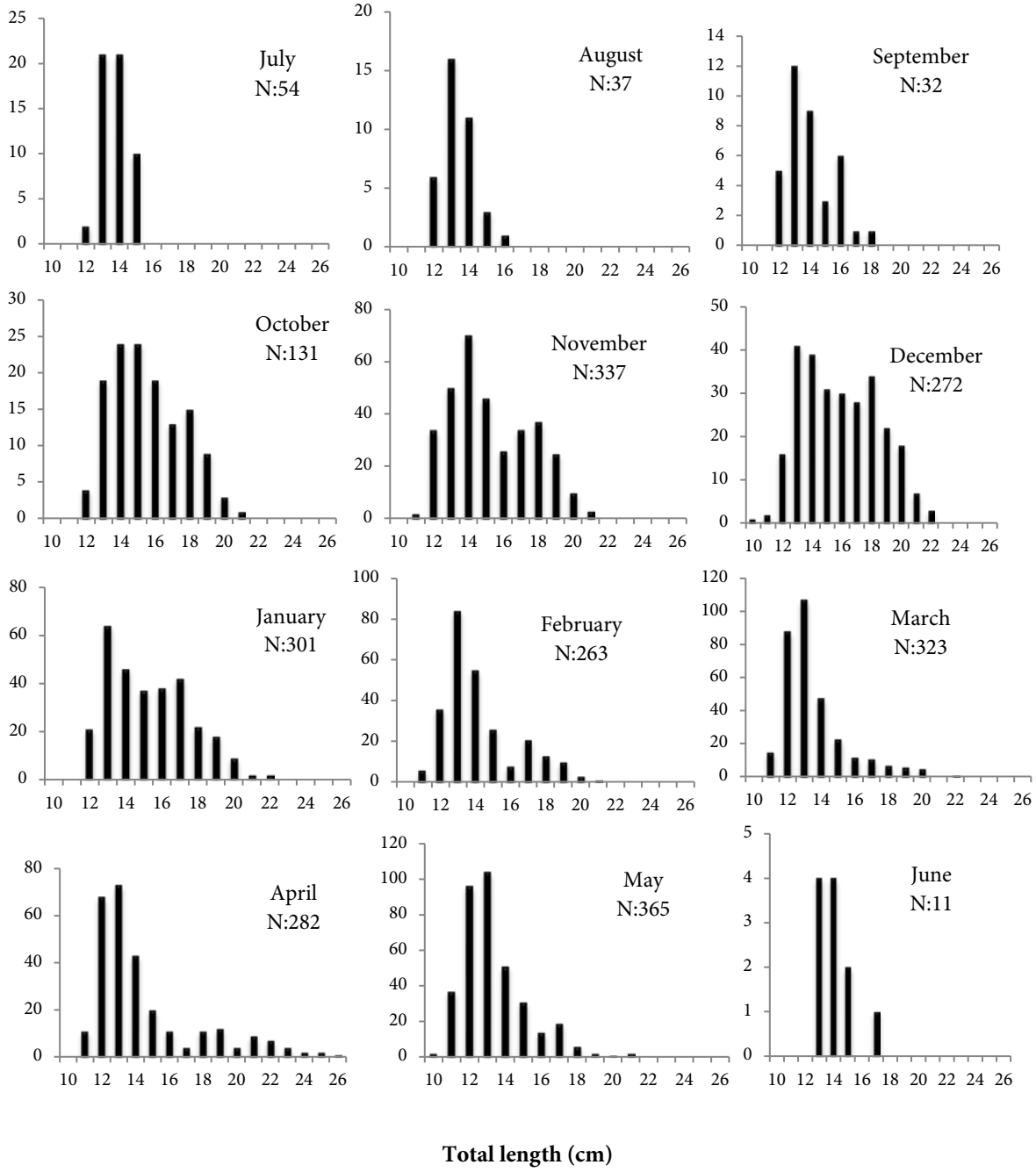

Figure 4. Monthly total length- frequency distributions

Table 3. Total length and weight data of the *N. melanostomus* by age

Age	(N)	%	L (cm) (Mean ± SD)	W (g) (Mean ± SD)
1	3	0.1	10.7±0.20	16.3±1.52
2	1387	57.6	13.40±0.86	28.1±5.73
3	473	19.6	15.59±1.31	44.9±12.07
4	282	11.7	18.15±1.61	77.6±23.91
5	172	7.1	18.72±1.44	85.6±26.28
6	74	3.1	19.98±1.50	110.3±36.01
7	13	0.5	22.54±2.67	155.0±46.54

8	4	0.2	22.63±3.64	150.1±57.80
Total	**2408**	**100**	**Mean: 14.97±2.43**	**Mean: 43.90±27.63**

Table 4. Von Bertalanffy growth parameters and growth equations in *N. melanostomus*

Growth Parameters					**Length-growth functions**	**Weight-growth functions**
L_∞	**W_∞**	**K**	**t_0**	**b**	**$L(t)= L_\infty (1-e^{-k(t-to)})$**	**$W(t)= W_\infty (1-e^{-k(t-to)})^b$**
26.5	245.05	0.1980	-1.3487	3.1972	$L(t)= 26.5 (1-e^{-0.1980(t+1.3487)})$	$W(t)=245.05(1-e^{-0.1980(t+1.3487)})^{3.1972}$

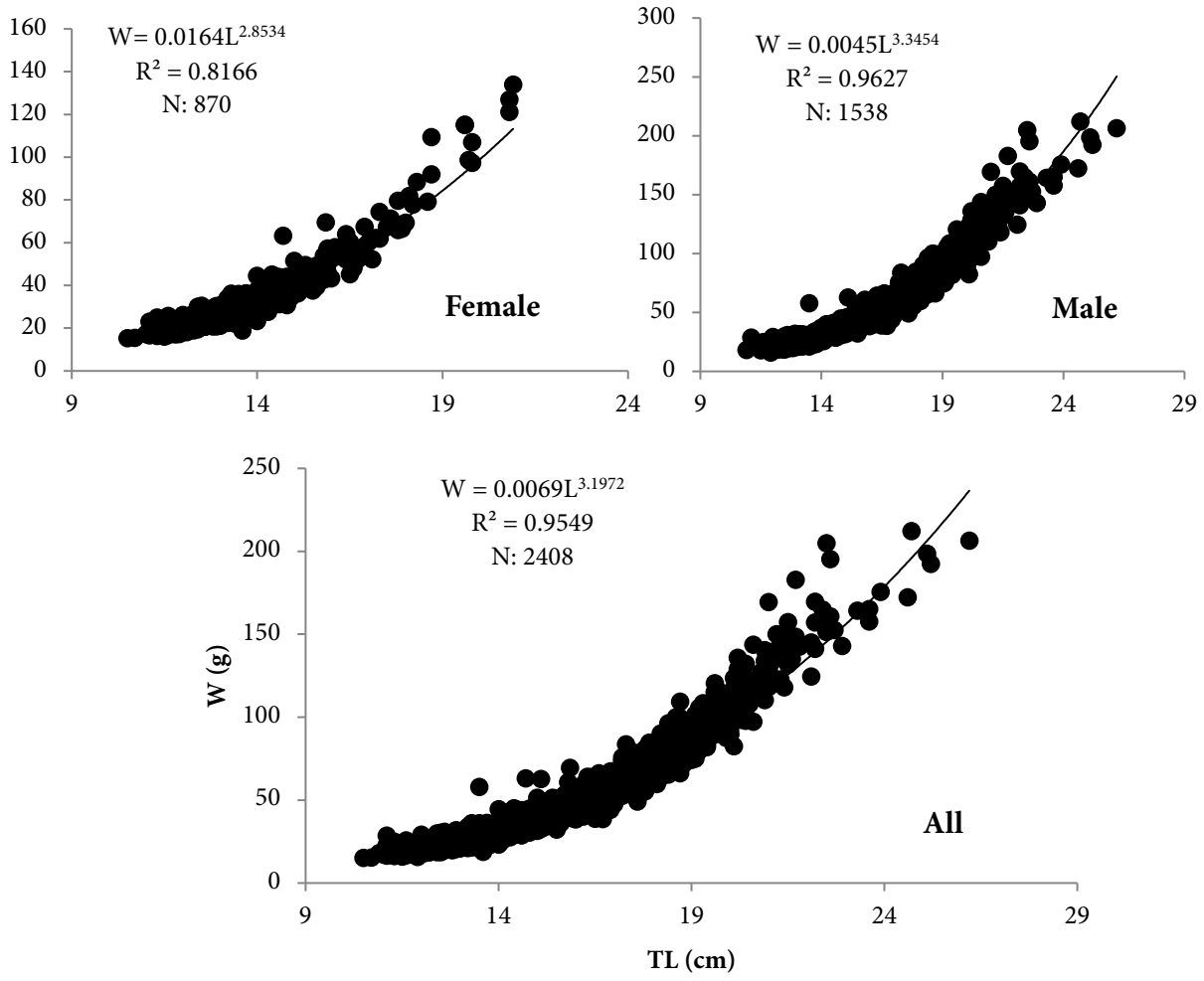

Figure 5. Total length-weight relationship of *N. melanostomus*

Length and Weight Relationship

LWR was determined by using a total of 2408 individuals in this study. The relationship was found for all individuals and each sex, separately (Figure 5). The average length and weight were presented in Table 2. Results indicated that as female (Pauly's t test: t = 1.326, P>0.05) cluster showed negative allometry (b<3), clusters of all individuals (Pauly's t test: t = 13.96, P<0.05) and male (Pauly's t test: t = 20.28, P<0.05) showed positive allometry (b>3) (Figure 5).

Age Composition

Age composition was ranged between one and eight years old. Most of samples were found two years age class (57.59) (Table 3). Even though it is seems that male individuals are relatively larger than females, there was no statistically significant difference between them (P<0.05).

It must be noted that age zero was not determined in this study. Although, minimum mesh size (17 mm) used in this study, no individual was found smaller than 10 cm.

Von Bertalanffy Growth Parameters

Growth parameters of von Bertalanffy and equations were calculated without any clustering such as sexes and presented in Table 4. Growth performance value (Φ') was also calculated and found 2.143. Estimated and observed values of relation between length and age were presented in Figure 6.

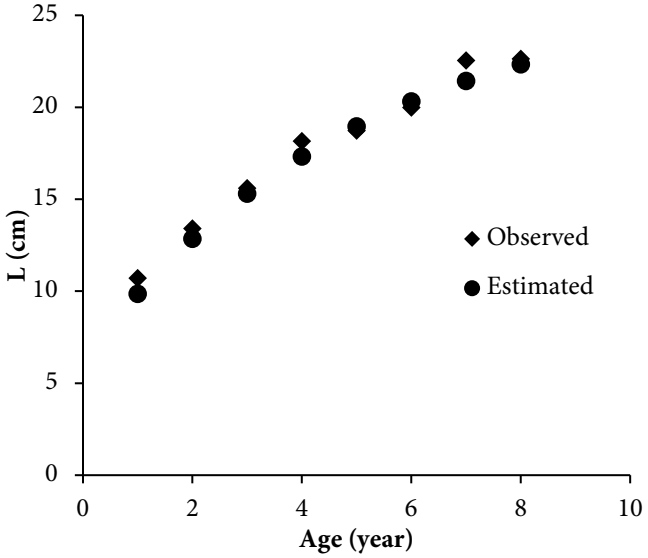

Figure 6. Age-total length relationship

Spawning Properties

Value of GSI of each month was calculated separately. Value of GSI has started to increase after December and it reached peak in April for females. This result indicated that spawning season occurred between March to May, and spawning reached maximum in April.

The maximum value of GSI in female was estimated as 3.898 (0.56-24.4) in April, while minimum value of GSI was found as 0.131 (0.04-0.25) in August. Average value of GSI was found 1.190±1.22 for female individuals. The value of GSI for male cluster was systematically lower than female cluster (Figure 7).

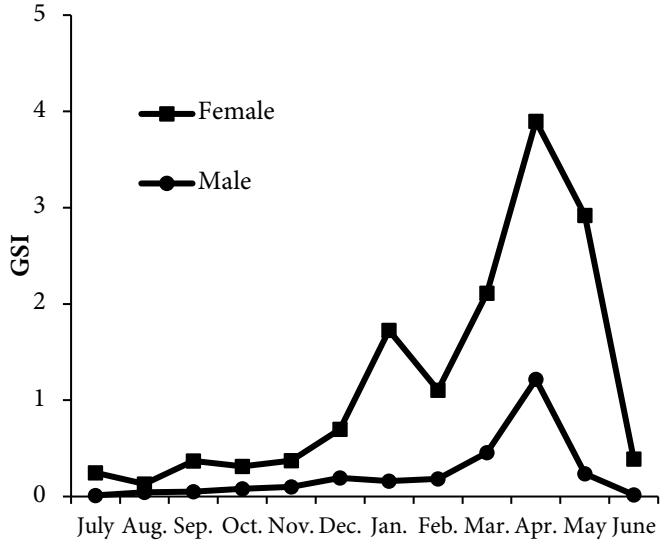

Figure 7. Monthly distribution of gonadosomatic index (GSI) values

Fecundity

A total of 206 female individual were examined between April and May for fecundity. Individuals were dispersed between 11.0 cm and 20.8 cm lengths, and 17.3 g and 127.01 g weights, respectively. Minimum egg number was found as 355.9, the maximum number was found 3953.7. Average fecundity was calculated as 1005.7 ± 534.1. Relative fecundity was calculated as 318.88 number /1g (124.8-958.9). Average diameter of egg was 2025.58 µm ± 262.86 (1112.0 - 2703.4).

Condition Factor

Condition factor was determined seasonal or annual changes on feeding of organisms. In this study, average condition factor of *N. melanostomus* was calculated as 1.66 ± 0.04 (1.05-1.21) (Figure 8).

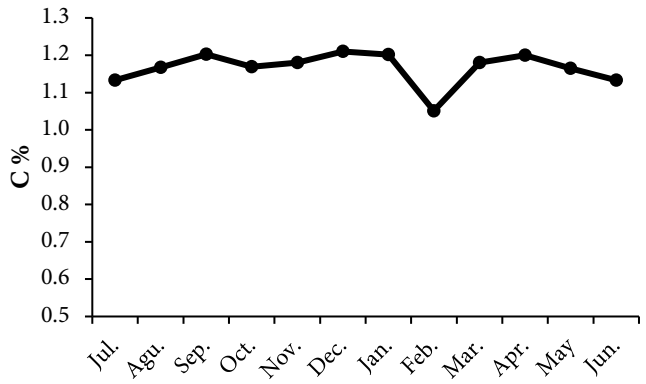

Figure 8. Monthly distribution of the Condition factor (C) for *N. melanostomus*

Mortality

Mortality parameters are the most important indicators for showing decreases in stocks. Total mortality rate (Z) was found 0.625, while survival rate (S) was calculated as 0.535. Natural mortality rate (M) was calculated by using growth parameters of the species and average temperature at depth that species lived. It was determined that average depth was 20±15 m, and average temperature was 15°C for the species. Based on these parameters and Pauly's approach, M and F were calculated 0.479 and 0.145, respectively.

Discussion

A total of 2408 individuals were obtained during this study. This study reached the maximum sampling size among previous studies conducted in the Black Sea (Table 5).

The sex composition is expected close 1:1 due to various reasons such as differences of natural and fishing mortality on sexes, spawning migration and, size and habitat differences between sexes (Nikolskii, 1980). Previous studies on population of *N. melanostomus* reported that male individuals were more abundant than female ones (Skora and Stolarski, 1996; Gözler et al., 2003; Gümüş and Kurt, 2009) as parallel to this study, that was found 1:1.77.

Length-weight datasets for *Neogobius melanostomus* from the Black Sea coasts given by the previous studies were tabulated in Table 5. These results state a length variation of this species between 3.6 cm (Abdoli et al., 2009) and 35 cm (Ak et al., 2009), however length distribution ranged from 10.5 cm to 26.2 cm in this study. Slastenenko (1956) and Sapota (2012) reported that this species reaches a maximum total length of 25 cm which is similar to the 24.6 cm maximum record given by Skora et al. (1999). Velkov et al. (2014) focused on the growth characteristics of this species in different habitats and concluded that populations in marine habitats have larger individual sizes than the fresh and brackish habitat populations. Results of this study clearly shows that the maximum total length is larger than many of the previous literature reports (Slastenenko, 1956; Skora et al., 1999; Sapota, 2012), but still stays well below the value reported by Ak et al. (2009).

Most frequent length cluster was reported as 13.4 cm by Gözler et al. (2003); 15.8 cm for male and 13.4 cm for female by Kurt (2005). Engin (2008) reported most frequent length ranges for each sex, which was 14.1-16 cm for males and 16.1-18 cm for females. In this study, frequent length clusters were estimated as 13 and 14 cm, which correspond to 42.36 % of the total sampling. It can be concluded that similar frequent distributions are observed with a reasonable uncertainty.

Most of the studies, including this one, reported positive allometry for this species except for Samsun (1995) who reported a negative allometry instead.

Kurt (2005), a study from the Black sea, off Samsun, reported that; even though morphological anomalies can be observed in otoliths, these rigid tissues are the most reliable part for the age determination for this species. Otoliths were used for the purpose of age determination in this study due to its proven reliability.

Table 5. Growth parameters of previous studies from the region

References	N	Mean	Lmin-Lmax	Wmin-Wmax	a	b	r^2	Region
Samsun (1995)	1425		8.0-20.5	6.25-98.74	0.0243	2.85		Black Sea
Gözler et al. (2003)	263		9.00-23.30	9.00-186.65	0.1145	3.08	0.93	Black Sea
Demirhan and Can (2007)	99	---	8.6-19.1		0.0047	3.39	0.95	Black Sea
Engin (2008)	300		5.1-28.4	25.9-370	0.0095 0.0055	♀3.15 ♂3.32	0.95 0.95	Black Sea
Abdoli et al. (2009)	758	---	3.6-13.3		0.0112	3.08	0.97	Caspian Sea
Ak et al. (2009)	73	--	9.1-35	8.58-381.4	0.010	3.033	0.89	Black Sea
Gümüş and Kurt (2009)	♀ 397 ♂ 471	13 14	7.5-19.7 7.4-25		0.0076 0.0110	3.23 3.07	0.94 0.96	Black Sea

Yankova et al. (2011)	3910	15.45	13.6-19.2	37.5-113	0.006	3.346	0.98	Bulgaria
Kasapoğlu (2016)	172	12.86	6.5-26.4		0.0114	3.088 ♀2.79 ♂3.06	0.96	Black Sea
Macun (2017)	266		9.1-19	12-119				Black Sea Freshwater
Çalık and Erdoğan Sağlam (2017)	58	--	26	8-265	0.0059	3.3062	0.99	Black Sea
This study	2408	14.97	10.50-26.20	15.28-212.20	0.0069	3.1972	0.96	Black Sea

Gözler et al. (2003) and Kurt (2005) concluded that the most frequent age cluster of this species is two. Engin (2008) reported the most frequent age as two and three for male and females respectively. These findings are in parallel with our results which indicates a most frequent age cluster of two with 57.59 % of the total sampling with an age range between one to eight years old.

According to the results, male individuals were statistically larger than females in this study. While average length of female was 13.55 cm, it was 15.78 cm for males. Previous studies also concluded the similarly (Gümüş and Kurt, 2009, Skora and Stolarski, 1996). Skora and Stolarski (1996) reported that males reached 21.2 cm when they are four years old. Kurt (2005) reported the length at age for both sexes as 18.2 cm and 19 cm at five years old for female and male, respectively. More detailed study by Engin (2008) reported that males can reach 28.4 cm and 93.4 g when they are seven years old, as females can reach 18 cm and 93.4 g when they are five years old. Comparison of these studies indicated remarkable length difference for the same age. It is thought that reason for this difference might be sampling in different habitats or methods.

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An extensive review for values of growth parameters of Von Bertalanffy and growth performance index were presented in Table 6.

Table 6. A list for values of growth parameters of von Bertalanffy (L_∞, k, t_0) and growth performance index (Φ') from previous studies

Sex	L_∞	k	t_0	Φ'	References
♂♀	26.0	0.20	-2.24	4.12	Gözler et al. (2003)
♂	21.3	0.38	9.70	4.22	Kurt (2005)
♀	25.2	0.18	5.75	4.05	Kurt (2005)
♀	19.7	0.43	-0.04	3.84	Engin (2008)
♂	24.1	0.25	-0.06	4.10	Engin (2008)
	36.1	0.14	-1.57		Kasapoğlu (2016)
	26.5	0.19	-1.34	2.14	**This study**

The instantaneous total mortality rate (Z), natural mortality (M) and fishing mortality (F) were estimated as 0.625, 0.479 and 0.145, respectively. Similarly, Kasapoğlu (2016) found Z=0.54, M=0.29 and F=0.145 in similar study region.

Table 7. Fecundity and egg size of the species from previous studies.

Areas	Fecundity (egg/ind.) Min-Max (Avg.)	Egg diameter (mm) Min-Max (Avg.)	References
Azov Sea	325-3323		Slastenenko (1956)
Azov Sea	200–9771		Kovtun (1979)
European waters	200-9771		Charlebois et al. (1997)

Detroit River	310-5210		Macinnis and Corkum (2000)
Danube	103.5–1938.2 (557.1)	0.12–2.35 (0.81)	Lavrincikova and Kovac (2007)
Eastern Black Sea	423–2396 (1325)	1.3–2.5 (2.02)	Engin (2008)
Bulgaria	419 - 7865 (3512)	1.72 -2.75 (2.25)	Hôrková and Kováč (2014)
Slovenia	1578 - 10605 (4413)	1.50-2.73 (2.03)	Hôrková and Kováč (2014)
Black Sea	1420-2477		Macun (2017)
Black Sea	355.9-3953.7 1005.7 ± 534.1	1.11-2.70 2.02±0.26	**This Study**

Nikolskii (1954) reported that the species reaches sexual maturity at two years old in females and three years old in males. Engin (2008) reported it as length 9.09 cm and 6.36 cm for males and females. Studies from various regions resulted that the species has spawning period between April and November (Kazancheev, 1981; Skora and Stolarski, 1996; Lavrincikova and Kovac, 2007). Engin (2008) reported the period between April and June. Parallel to this study, Hôrková and Kováč (2014) reported from various regions that the species reach its peak in April for spawning and it continues in May. All previous studies point the same period for spawning.

Comparison among studies concluded that similar egg diameter ranges were obtained in studies from the Black Sea (Table 7). However, there are some variations in number of eggs. On the other hand, comparison among fish species showed that *N. melanostomus* has a less eggs than other species. As mentioned by Macinnis and Corkum (2000), survival rate of the species is higher due to some certain reasons. One is having larger egg diameter. Larger larval size is another reason. It is also important to choose secure place for spawning and protection of eggs by males for these strategies.

Skora et al. (1999) reported vertical distribution limit of the species is 30 m. In this study, individuals of the species, especially males, were caught from 120 m. Another information from Fishbase pointed out that the species maximum length reaches to 24.6 cm and six-year-old as maximum age (Froese and Pauly, 2009). These values are updated as 26.4 cm and seven years old by contribution of this study.

Conclusion

Due to the decreasing stocks of the traditionally consumed fish species of the Black Sea with an economical value in Turkey, species like round goby started to become commercially valuable in recent years in the Black Sea coast of the Turkey. Being less important as a commercial product in the past, round goby did not take enough attention and there are limited numbers of studies in the current literature where the population characteristics are hardly known yet. However, as mentioned above, the commercially growing demand should not be ignored and should be carefully managed for round goby which has a significant importance for the health of the coastal ecosystem. This can only be succeeded by understanding the population characteristics of this species by continuous monitoring studies, therefore this research is definitely important despite its regional scope.

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Compliance with Ethical Standards

Conflict of Interest

The author declares that there is no conflict of interest.

Ethical Approval

This study was conducted in accordance with ethics committee procedures of animal experiments.

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RESEARCH ARTICLE

Emission analysis of LNG fuelled molten carbonate fuel cell system for a chemical tanker ship: A case study

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ABSTRACT

Since sea transportation is one of the sources of air pollution and greenhouse gas emissions, so restrictive regulations are entering into force by the International Maritime Organisation to cope with the ship sourced emissions. Alternative energy generating systems are one of the key concepts and fuel cells can be one of the solutions for the future of the shipping industry by their fewer hazardous emissions compared to diesel engines. In this perspective, a Liquefied Natural Gas using molten carbonate fuel cell is evaluated instead of a conventional marine diesel engine for a chemical tanker ship. As a case study, the real navigation data for a tanker is gathered from the shipping company for the 27 voyages in 2018. Emissions are calculated respecting fuel types (marine diesel oil and heavy fuel oil) and designated Emission Control Areas for both diesel engine and fuel cell systems. The results show that more than 99% reduction in SO_x, PM, and NO_x emissions and a 33% reduction in CO_2 emissions can be reached by the fuel cell system. At last, fuel cells seem very promising technologies especially for limited powered vessels under 5 MW for propulsion to use as main engines by complying with current and new coming emission limitations on the way of emission free shipping.

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Introduction

Shipping transportation is more energy-efficient among different transportation modes (Alföldy et al., 2013; Zhu et al., 2018), and around 90% share of global trade is carried out by shipping transportation (Dere and Deniz, 2019; Harrould-Kolieb, 2008). Since 1990, more than a 150% increase occurred for the transportation of goods by sea and continuing to increase depending on the economic growth (Baldi et al., 2020). Despite its advantages in terms of cost, efficiency, operation ability, and reliability, shipping exhaust gases are substantially harmful emission sources for the environment. Besides the

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major pollutants; nitrogen oxides (NO_x), sulphur oxides (SO_x), particulate matter (PM), carbon dioxide (CO_2), and volatile organic compounds (VOCs) emissions are also taking an important place for the maritime industry (Ammar and Seddiek, 2020). According to the Third Greenhouse Gas Study which is carried out by the International Maritime Organization (IMO), for the year 2012, the total and international shipping CO_2 emissions were estimated to approximately 938 and 796 million tons which contribute 3.1% and 2.6% of global CO_2 emissions, respectively (IMO, 2015). In 2018, by aiming to minimize the total amount of greenhouse gases with environmental negative impacts by at least 50% by 2050 compared to levels in 2008, IMO put into force more stringent regulations (IMO, 2018). From this perspective, IMO has introduced the Energy Efficiency Design Index (EEDI), the Ship Energy Efficiency Management Plan (SEEMP), and Energy Efficiency Operational Indicator (EEOI) in MARPOL Annex VI in 2013 (IMO, 2011). To achieve emission reduction from ships, and to stay under limitations, various options must be well analysed and introduced such as increasing engine efficiency with a more efficient energy management plan (Uyanık et al., 2020), load, road, and speed optimization (Psaraftis and Kontovas, 2014), slow steaming (Dere and Deniz, 2020), alternative marine fuels (Deniz and Zincir, 2016; Hansson et al., 2019), auxiliary solar PV systems (Karatuğ and Durmuşoğlu, 2020), hybrid and electric propulsion systems (Bennabi et al., 2016) or exhaust gas cleaning systems (Lee et al., 2020; Zhu et al., 2018). However, according to exhaust gas contents, treatment technology varies, and therewithal to reduce NO_x emission, there are Exhaust Gas Recirculation (EGR) and Selective Catalytic Reduction after treatment (SCR) (Raptotasios et al., 2015). Besides, scrubbers play a key role to reduce SO_x emission from power generation and propulsion on board (Brynolf et al., 2014).

On the other hand, possible changes in ship designs such as modification of hull form or propelling systems, are also taking interest of researchers, however still do not address the final solution of this emission problem (Baldi et al., 2020). Another important option for shipping is Diesel-electric propulsion systems which help to reduce emissions by fuel-saving because engines can operate with high efficiency at high and constant load, however maritime stakeholders still need to comply with the environmental regulations (Ghenai et al., 2019) The battery-powered ships also seem another option for the future of shipping mostly used in hybrid systems with expected savings up to 20% but there are only infrequent cases and because of the limited battery capacity and high cost, it is not feasible for long-range intercontinental shipping (Moe, 2016).

In this regard, fuel cells are very promising and they can play a key role in their environmentally friendly power generation capacity (Inal and Deniz, 2018). Fuel cells can generate power without any air pollutants except CO_2 even using carbon included fuel. Furthermore, they are highly modular and this is a very important factor specifically for limited space applications in transportation like submarines or commercial ships. Among the five commercial types of fuel cells; the proton exchange membrane fuel cells (PEMFC) are one of the most popular types (Sohani et al., 2020) with its high efficiency and technological maturity. However, the need for pure hydrogen as a fuel is very hard to handle for ships. Also, limited power output is a disadvantage for being the propulsion power generator for cargo ships (Inal and Deniz, 2020). For this reason, molten carbonate fuel cells (MCFC) and solid oxide fuel cells (SOFC) gain importance with their fuel flexibility and higher power capacity (van Biert, et al., 2016). Both are classified as high-temperature fuel cells and total system efficiency can be raised by heat recovery systems using high-quality exhaust gases (Wee, 2011; Martinić et al., 2018). The heat recovery capacity of the SOFC is higher than the MCFC thanks to its higher operating temperature which is approximately 200°C above (Buonomano et al., 2015). However, SOFC has some difficulties such as excessive thermal expansions due to its very high operating temperatures, less maturity than MCFC, and mechanical disadvantages (van Biert et al., 2016; Ahn et al., 2018). On the other hand, MCFC is demonstrated and commercially wider than SOFC, and on board ship applications were already practiced (McConnell, 2010; Tronstad et al., 2017). From this perspective, in the maritime industry, some fuel cell applications have been put into practice for on board electricity production instead of the diesel generators (De-Troya et al., 2016). For instance, in some of the projects; FellowSHIP project, a 330 kW MCFC is installed to offshore supply vessel "Viking Lady" and the system is operated for 18 hours (Tronstad et al., 2017); METHAPU project, methanol fed 20 kW SOFC is applied to a RoRo ship (Strazza et al., 2010); FELICITAS project, a 250 kW SOFC is tested in a mega yacht (Tse et al., 2011); SchIBZ project, a 500 kW diesel internal reforming SOFC powered the propulsion (van Biert et al., 2016), and methanol fed 500 kW MCFC is installed into an offshore vessel by hybridization with diesel engine (Díaz-de-Baldasano et al., 2014). The fuel cells seem promising for the future of the shipping industry by their fewer hazardous emissions and depending on the technological developments they can be replaced marine diesel engines not only electric generation but also propelling.

The purpose of this paper is to investigate the emission variance between a commercial LNG fuelled molten carbonate

fuel cell and marine diesel oil and fuel oil using diesel engines for a chemical tanker at the same power output. In this study, ten months of voyage data is collected from a shipping company of a chemical tanker ship in 2018. The tanker ship has a 4-stroke diesel engine with 2880 kW output power. Instead of the main engine, LNG using molten carbonate fuel cell with 2800 kW output would be installed for propelling. A new propulsion system is designed according to ship machinery room and out of use fuel tank conversion is discussed. The rest of this paper is organized as follows. In section 2, MCFC's working principles and the designed system is described. In section 3, the case study is carried out by giving case ship and routes properties. In section 4, emissions of diesel and fuel cell versions are calculated and advantages and disadvantages were investigated. Finally, section 5 concludes the paper.

Molten Carbonate Fuel Cell (MCFC) and System Description

In this study, molten carbonate fuel cell is chosen to apply the tanker ship as a case study. The same generated propulsion power and being commercial type are the main motivation sources for selecting this fuel cell. Furthermore, fuel flexibility is another major effect of shipping. Also, the assessment of fuel cell types for commercial shipping was investigated in a previous study (Inal and Deniz, 2020).

Generally, in literature, fuel cells are categorized according to their working temperatures as low and high, and the molten carbonate fuel cell is one of the high temperature working fuel cells. Among high temperatures; MCFC works around 650°C and this high operation temperatures raise the total system efficiency (Marefati and Mehrpooya, 2019). The electrolyte is carbonates (Li_2CO_3 and K_2CO_3) and the electrodes in the MCFC are made of nickel materials (Mehmeti et al., 2016). As mentioned before, MCFC operating temperature is around 650°C and inside ion, conductivity occurs thanks to melted carbonate at 500°C (Ahn et al., 2018).

The proposed propulsion power generation system of the ship is illustrated in Figure 1. As seen in the figure, fuel is transferred to the mixer through a compressor and the mixture of natural gas and water is fed into the fuel cell stack. The delivered mixture pass to an internal reformer where water and natural gas react and produced hydrogen is given to the anode side of the fuel cell system. Before the internal reformer, reactants have to be heated to be prepared for an effective steam methane reforming (SMR) (2.1) and water gas shift reactions (WGS) (2.2) (Ahn et al., 2018) which are given below:

SMR: $$CH_4 + H_2O \leftrightarrow CO + 3H_2 \quad (2.1)$$

WGS: $$CO + H_2O \leftrightarrow CO_2 + H_2 \quad (2.2)$$

These two important reactions which occur to produce hydrogen and carbon monoxide inside of the fuel cell stack (Muñoz de Escalona et al., 2011). Since the reforming reaction is a deeply serious endothermic cycle, it ousts the heat delivered by the hydrogen oxidation (Kim and Lee, 2017). The electrochemical reactions in the MCFC are the followings (Mench, 2008; Ovrum and Dimopoulos, 2012):

Anode: $$H_2 + CO_3^{2-} \rightarrow CO_2 + H_2O + 2e^- \quad (2.3)$$

$$CO + CO_3^{-2} \rightarrow 2CO_2 + 2e^- \quad (2.4)$$

Cathode: $$\frac{1}{2}O_2 + CO_2 + 2e^- \rightarrow CO_3^{2-} \quad (2.5)$$

Overall: $$H_2 + \frac{1}{2}O_2 + CO_2 \rightarrow H_2O + CO_2 \quad (2.6)$$

In this study, a commercial MCFC SureSource 3000 by Fuel Cell Energy Company is chosen for the case study. The fuel cell is comprised of two 1400 kW modules with total capacity of 2800 kW. The emission data of the fuel cell is collected from the manual of the product which is given in Table 1.

Table 1. Fuel cell specifications (Fuel Cell Energy, 2017)

Specification	SureSource 3000 MCFC
Power Output	2800 kW
Standard Frequency (Optional)	60 Hz (50 Hz)
Exhaust Temperature	370 – 400 °C
NO_x Emission	0.0045 g/kWh
SO_x Emission	0.000045 g/kWh
CO_2 Emission	444.5 g/kWh
PM Emission	9.07×10^{-6} g/kWh
Efficiency	47 +/- 2%
Fuel Consumption (NG)	615.12 m^3/h
Sound Level	72 dB at 3m
Maximum Height	6.6 m
Cell Unit Length	6.5 m
Cell Unit Width	13.1 m

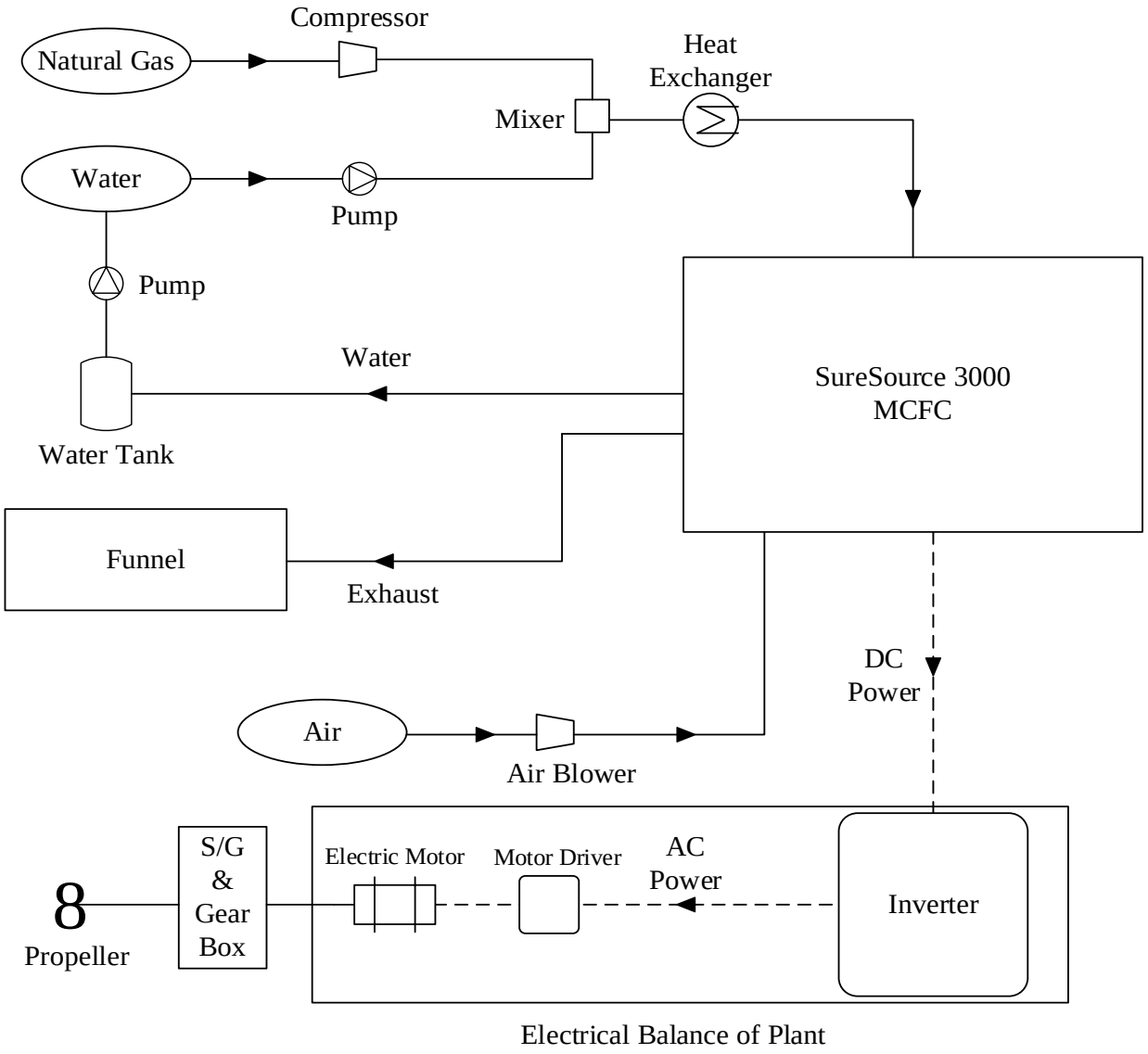

Figure 1. Schematic representation of the propulsion system

In Figure 1, the proposed propulsion system for the case ship is represented. As seen in Figure 1, an air blower supplies the oxygen for the cathode reaction in the system. As well, the produced water by the fuel cell is gathered in a water tank to use again by the system for mixing with natural gas. After several electrochemical reactions in the fuel cell which are given above, the produced DC power is transformed into usable AC power by an inverter system which is included in the total fuel cell system under the title of the electrical balance of plant. The electrical power is converted to mechanical power after passing through the motor driver and electric motor. Then, the RPM of the electric motor is decreased to designed propeller RPM by a gearbox. At this point as being at the original version of the ship, a shaft generator can be used for the electrical need of the ship to use for navigation, HVAC, or accommodation space. Furthermore, the high-temperature exhaust gases can be used in a combined gas or steam turbine system as exhaust gas recovery to increase to total system efficiency. By the way, the ship is not equipped with a waste heat recovery system at the original version. Therefore, the system has not been analysed and is beyond the scope of this article.

Case Study

Case Ship Description

An oil/chemical tanker ship that has MAN STX 6L 32/40 with 2880 kW main engine power output is chosen as the case study reference ship for the molten carbonate fuel cell system. The case ship is equipped with a shaft generator, therefore during the courses, the electric need of the ship is provided by this system and diesel generators are in service during manoeuvring, emergencies, and when the vessel is berthed. Moreover, the ship is equipped with a controllable pitch propeller (CPP), the speed of the vessel is set by changing the angle of the blades of the propeller. Therefore, the main engine can work at fixed RPM to be more efficient.

The reference ship is using heavy fuel oil or marine diesel oil according to the sailing area. If the vessel enters to emission control area (ECA), fuel change over procedures entry into force due to emission limitations in 2018. Otherwise, because of the economic advantage, the company prefers to use fuel oil. In this paper, emissions are calculated for both marine diesel oil and fuel oil. The reference ship properties are listed in Table 2.

Table 2. Reference ship specifications

Specifications	
Ship Type	Oil / Chemical Tanker
Gross Tonnage	4829
Deadweight	6970 tonnage
Length	119.1 m
Breadth	16.9 m
Year Built	2009
Main Engine Power	2880 kW, 750 RPM
Main Engine Sizes	10m × 5.5m × 2.5m
Shaft Generator	1500 kW
Diesel Generator	3 set, 500 kW (each)

The ship has different tanks such as the fuel oil (F/O), marine diesel oil (MDO), and lubricating oil (L/O) which can be transformed into LNG tanks. The tanks capacities according to ship plans are listed in Table 3. Other tanks will be needed during ship operations, such as; freshwater tanks, oily water tanks, bilge tanks, black and grey water tanks. After removing the main engine F/O, MDO and LO tanks won't be needed anymore. To sum up, the total capacity of the tank, which can be switched to LNG tanks, is approximately 530 m^3.

Table 3. Ship tank capacities

Tank	Volume (m^3)
F/O	421.64
MDO	66.79
L/O	41.57
TOTAL	530 m^3

After removing the main diesel engine some of the auxiliary equipment will be out of use. The list of the equipment with their approximate weights is given in Table 4. Some of the equipment names are given as a system due to their auxiliary equipment such as pumps, valves, and lines. This is why the approximate weights are increased.

The vessel engine room consists of 3 floors. The main engine, shaft, several tanks, and pumps are located at the bottom floor, the plan is in Figure 2. Three diesel generators, fuel oil, diesel oil and lubricating oil separators (located in separator room), air compressors and start air tanks, freshwater generator system, some other tanks, and steering room are located in the second floor, as seen in Figure 3. The third floor consists engine control room, workshop, incinerator room, and boiler room, therefore any kind of displacement due to the fuel cell system will not occur on this floor. Also, some of the tanks like freshwater or fuel oil are longitudinal, so, they have parts on both floors. The total engine room volume is fairly enough for the proposed modular fuel cell system and its auxiliary units.

Table 4. Out of use auxiliary equipment list

Equipment	Quantity	Approx. Weight (kg)
Fuel Oil Separator	2 set	2 × 250
Fuel Conditioning System	1 set	1 × 150
Start Air Tubes	2 set	3 × 100
Start Air Compressors	2 set	2 × 75
Fresh Water Generator	1 set	1 × 200
Lubricating Oil Separator	1 set	1 × 250
Sea Water Cooler System	1 set	1 × 300
Fresh Water Cooler System	1 set	1 × 400
TOTAL		2250 kg

Route Description

The total ship voyage data in 2018 is collected from the company logs. According to collected data, the ship had 27 voyages generally in the Mediterranean Sea. The departure and arrival ports, distances (nautical mile), times spent at sea (hours) and average speeds (knots) of the vessel are given in Table 5. During its course for ten months, the case ship has sailed for approximately 3228 hours and 31469 nautical miles, generally in the Mediterranean Sea.

Several ports at the ship routes are in the Emission Control Areas (ECA) such as; Antwerp (Belgium) and Rotterdam (Netherlands). To enter to ECA zones, the ship must switch its fuel to low sulphur diesel oil from heavy fuel oil. This changeover causes to changes in emissions. Therefore, in this paper, to have more accurate calculations, a software program Netpas (Figure 4) is used to determine the correct distance by regarding the correct fuel type. Indeed, emission calculations are done by separating ECA.

For instance, in a case of total distance includes an ECA, the starting point of the area, and the destination port is calculated with low sulphur diesel oil besides the rest of the voyage is calculated with fuel oil.

Some of the distances between the same ports are different due to the change in the ship course as given in table 6. The average speed of the ship is calculated as 9.7 knots with a maximum speed of 10.91 and a minimum speed of 7.67 knots. The average speed variance graph is given at the Figure 5.

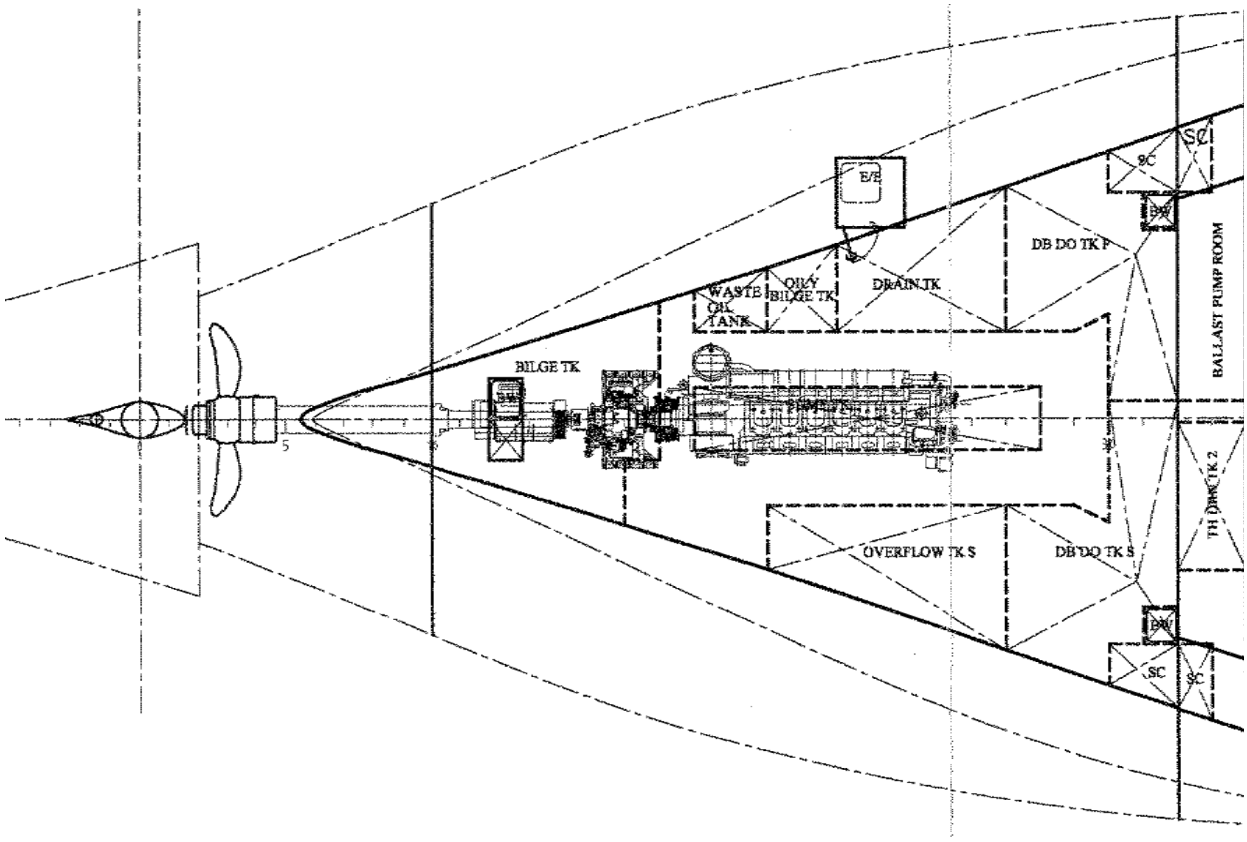

Figure 2. Bottom floor of the engine room

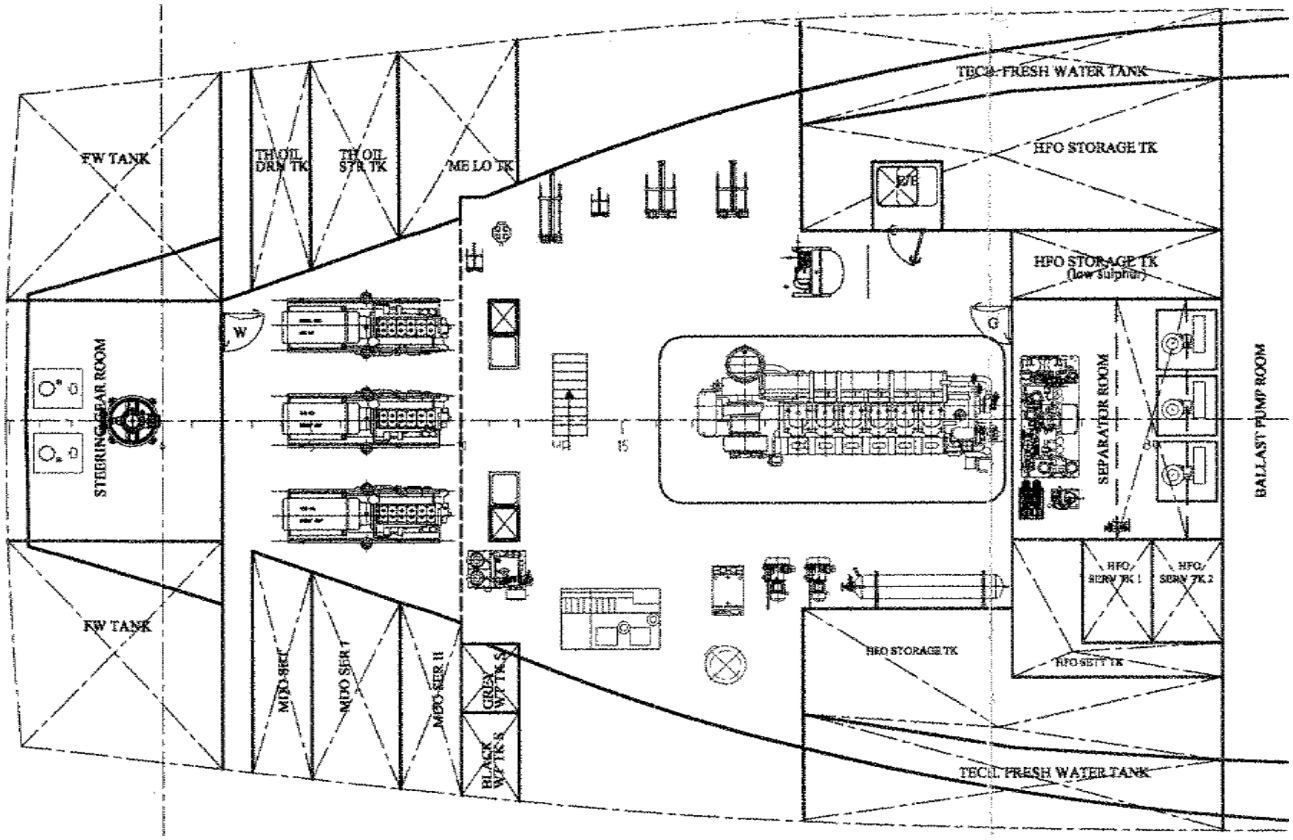

Figure 3. Second floor of the engine room

Table 5. Reference ship routes

No	Arrival	Departure	Distance (nm)	Time Spent at Sea (h)	Average Speed (kts)
1	Ravenna	Antwerp	3119.6	322	9.69
2	Koper	Ravenna	136.5	13.83	9.87
3	Kulevi	Koper	1815.85	176.17	10.31
4	Constantza	Kulevi	618.9	61.03	10.14
5	Elevsis	Constantza	616.7	69.5	8.87
6	Ravenna	Elevsis	953.7	87.42	10.91
7	Runcorn	Ravenna	3085.9	302.58	10.20
8	Aughinish	Runcorn	576	57.33	10.05
9	Port Said	Aughinish	3177.5	330	9.63
10	Haifa	Port Said	223	25.25	8.83
11	Fos	Haifa	1698.5	166	10.23
12	Aliaga	Fos	1488.9	136.5	10.91
13	Gemlik	Aliaga	324	31.05	10.43
14	Izmit Bay	Gemlik	79	8	9.88
15	Augusta	Izmit Bay	859.2	93.4	9.20
16	Berre	Augusta	736.7	71,4	10.32
17	Augusta	Berre	723	77.23	9.36
18	Izmit Bay	Augusta	904.6	83.55	10.83
19	Aliaga	Izmit Bay	306	35	8.74
20	Algeciras	Aliaga	1673.3	159.4	10.50
21	Leixoes	Algeciras	531.5	63.55	8.36
22	Safi	Leixoes	851.5	111	7.67
23	Lavera	Safi	1576.8	198	7.96
24	Livorno	Lavera	299	29	10.31
25	Genoa	Livorno	102	11.33	9.00
26	Haifa	Genoa	1534.5	157	9.77
27	Rotterdam	Haifa	3457	352	9.82

Table 6. Total ECA distance of case ship

Voyage No	Departure	Arrival	Total Distance (nm)	ECA Distance (nm)	ECA Time (h)
1	Ravenna	Antwerp	3119.6	417	43
27	Rotterdam	Haifa	3457	406	41.3
TOTAL			6576.6	823	84.3

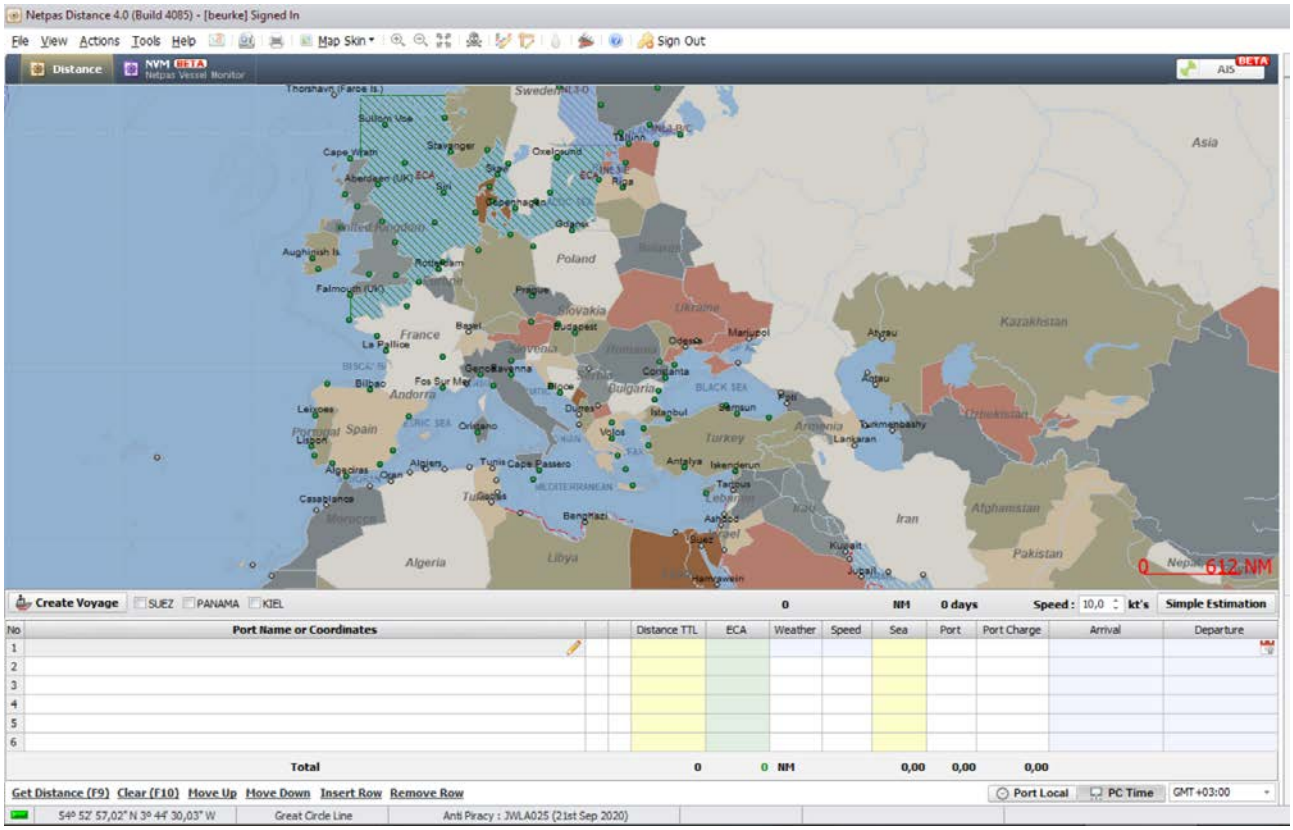

Figure 4. User interface of Netpas software

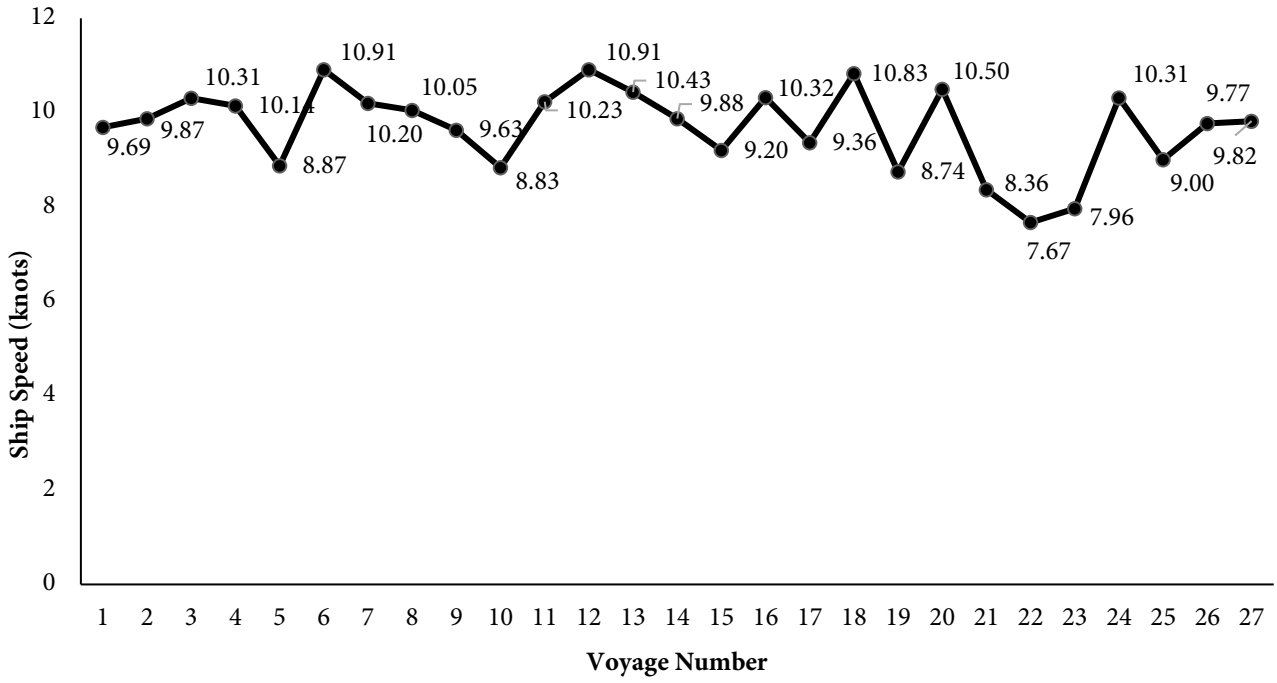

Figure 5. Average ships speed for each voyage

Also, the average speed of the ship changes regarding to ship load, sea and weather conditions. In addition, the average speed of the ship is accepted as the actual speed per each voyage for load ratio calculations in formula (4.1) where the actual and design power ratio is crucial for approximate emission calculations.

Results and Discussion

In this study, emissions are calculated according to main engine fuel consumption. Despite the specific fuel oil consumption (SFOC) and ship emissions depend on the engine load, generally, the total system load is smaller than the main

engine power capacity because usually ships sail between 60-80% engine load (Lee et al., 2020; Berstad, et al., 2013). Therefore, the specific fuel consumption is directly related to engine load. In this perspective, previously it should be calculated the load of the engine to find the SFOC. The ratio of the actual power (P_{actual}) and design power (P_{design}) is used to calculate the load ratio of the main engine. Also, load ratio (L_R) can be expressed as the ratio of actual speed (v_{actual}) and design speed (v_{design}) of the ship, prime the speed coefficient (α) (Dere and Deniz, 2019; Moreno-Gutiérrez et al., 2015). The speed coefficient can vary between 2.5 and 3 (Moreno-Gutiérrez et al., 2015) and it is taken as 2.5 in this study. In addition, the design speed of the ship is 12 nautical miles and it is used for calculating the load ratio.

$$Load\ Ratio\ (L_R) = P_{actual} / P_{design} = (v_{actual} / v_{design})^{a} \quad (4.1)$$

Where P_{actual} becomes;

$$Pactual = (v_{actual} / v_{design})^{a}\ x\ P_{design} \quad (4.2)$$

The SFOC actual can be calculated as following:

$$SFOCactual\ (g/kWh) = SFOCbase\ x\ SFOCratio \quad (4.3)$$

The $SFOC_{base}$ is calculated according to the data at the manual of the main engine respecting each load per each voyage which is given in Table 5. On the other hand, the load ratio in formula (4.1) is used to calculate $SFOC_{ratio,}$ and the relationship between them is given as follows (Dere and Deniz, 2019; Moreno-Gutiérrez et al., 2015):

$$SFOCratio\ (g/kWh) = 0.455L_R^2 - 0.71L_R + 1.28 \quad (4.4)$$

Finally, the total estimated fuel consumption (EFC) according to main engine load of the ship is calculated in kilogram as following where the P_{actual} is calculated from the formula (4.2):

$$EFC\ (kg) = Pactual\ (kW) \times SFOCactual\ \left(\frac{g}{kWh}\right) \times Time\ (h) \times 10^{-3} \quad (4.5)$$

In case study fuel oil and diesel oil tanks would be transformed to LNG tanks. The total fuel oil tank capacity is 421 m^3 and total diesel oil tank capacity is 66 m^3 as mentioned in table 3. The maximum LNG consumption of the fuel cell for its longest route is 360 m^3 for all voyages. The membrane type tank is selected for application in order to easier transformation and to be an already self-proving technology. The Moss type tank is another important alternative, however due to its spherical shape, during the transformation and adaptation of the system it would cause a severe volume loss inside the tanks. So, combined membrane system technology for LNG storage shows a great coherence during the transformation of the conventional oil tanks, related to its lower weight and membrane thickness. The volume reduction because of the insulation is calculated 96 m^3 according to isolation layer of the combined system for the total of the both diesel oil and fuel oil tanks which is 487 m^3, and this equals to 20% of volume loss from the total. Therefore, the maximum LNG transportation capacity reduces to 391 m^3. The longest voyage of the chosen ship is determined and it is clearly seen that the new tank volume is satisfying for the vessel's routes, so vessel wouldn't need any LNG bunkering operation during its longest voyage.

Approximate total consumption of 1400 tons of marine diesel oil and heavy fuel oil are saved in 27 voyages via transformation, and according to 2020 ship bunker price, 95.000$ saving is expected when compared to LNG price. Furthermore, as a result of the system changing, lesser operational and periodic maintenance expenditure and so labour force add more financial gain and diminish the risk due to human factor in the system as well. The major equipment for maintenance is the mechanical equipment of plant and its components. These types of auxiliary maintenance are almost same with the conventional diesel engines and their equipment such as pumps, compressors, valves and filters, so neither disadvantages nor advantages cannot be designated. The major fuel cell maintenance need occurs at the end of its lifecycle by changing of the electrolyte and electrodes. However, the lifetime and carbon footprint of the fuel cells should be investigated and therefore the difference with diesel engines and costs for the renewing the electrolyte should be identified.

CO_2 Emissions

The emissions of main diesel engines have been calculated according to estimated fuel consumption. The case ship can use both fuel oil and marine diesel oil in diesel engine, so CO_2 emissions are calculated according to fuel type including LNG for fuel cell, as seen in Figure 6. The fuel changeover has been taken into account regarding ship routes in ECA and emissions are calculated respecting fuel types.

The CO_2 emission coefficients are taken 3.206 and 3.114 for marine diesel oil and heavy fuel oil, respectively (MEPC, 2018). The table according to conversion factor between fuel consumption and CO_2 emission is shown at Table 7. On the other hand, for both LNG fuel cell system, it is calculated by using manual data at given in Table 1.

The calculations are done according to following formulas:

$$CO2\ Emission\ (kg) for\ MCFC\ =\ 444.5\left(\frac{g}{kWh}\right)\ x\ Pactual(kW)\ x\ Time(h)\ x\ 10^{-3} \quad (4.6)$$

$$CO2\ Emission\ (kg)\ for\ MDO\ Diesel\ Engine\ =\ 3.206\ x\ EFC\ (kg) \quad (4.7)$$

$$CO2\ Emission\ (kg)\ for\ HFO\ Diesel\ Engine\ =\ 3.114\ x\ EFC\ (kg) \quad (4.8)$$

Table 7. Conversion factor between fuel consumption and CO2 emission (MEPC, 2018)

Fuel Type	Reference	Carbon Content	Conversion Factor
Diesel / Gas Oil	ISO 8217 Grades DMX through DMB	0.8744	3.206
Light Fuel Oil (LFO)	ISO 8217 Grades RMA through RMD	0.8594	3.151
Heavy Fuel Oil (HFO)	ISO 8217 Grades RME through RMK	0.8493	3.114
Liquefied Natural Gas (LNG)	-	0.75	2.750

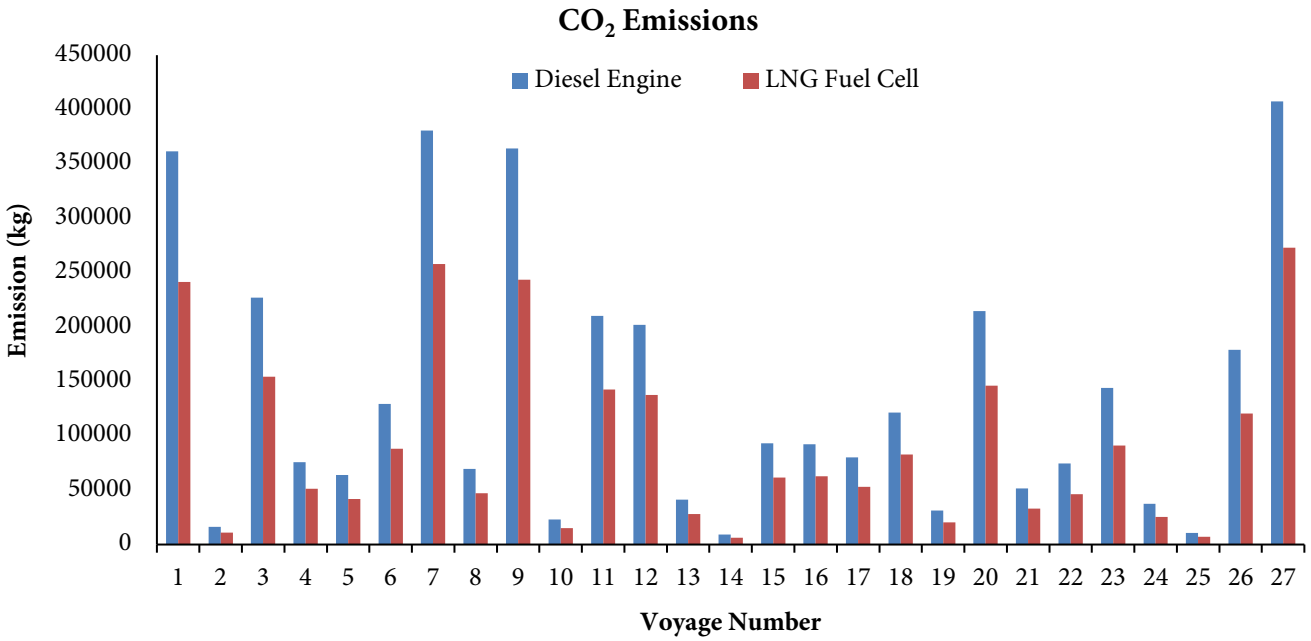

Figure 6. CO_2 emissions according to the fuel type and voyage number

The results show that more than 1200 tons of CO_2 emissions decrease approximately 33% with the LNG fuel cell system for the same power output during the same route and sailing hours compared to the diesel engine. The case ship is already equipped with a shaft generator. Therefore, the diesel generators are not in use for energy needs of the ship during sailing. However, during tank washing, as a classical need for a chemical tanker, the total electrical need is maximizing. Since the selected fuel cell type has a high temperature exhaust, gas a waste heat recovery system can be applied. As a result, this additional power need can be supplied by a waste heat recovery system by increasing the total system efficiency and also by decreasing greenhouse gas emission.

As a result of the proposed system, the new EEDI is calculated according to new CO_2 emissions to see the ship is in compliance with the requirements. The calculation has several steps to reach the attained EEDI so firstly, baseline (4.9) and required EEDI (4.10) must be found for benchmarking.

$$Baseline = a\ \times\ b^{-c} \quad (4.9)$$

In equation (4.9), a is 1218.80, b is deadweight of the ship and lastly, c is 0.488 for a tanker ship (IMO, 2015).

$$Required\ EEDI = \left(1 - \frac{x}{100}\right) \times\ Baseline \quad (4.10)$$

In equation (4.10), x is defined as reduction factor and varies according to ship's deadweight and EEDI phase. In this paper, phase 1 (01 January 2015 – 31 December 2019) and phase 2 (01 January 2020 – 31 December 2024) are taken into account for calculations because of the case ship's voyage period which was during phase 1 but new reduction factor during preparations of this paper which is in phase 2.

$$\frac{(\prod_{j=1}^{M} f_j)\ \mathrm{x}\ (\sum_{i=1}^{nME} P_{MEi} \times C_{FME} \times SFC_{ME}) + (P_{AE} \times C_{FAE} \times SFC_{AE}) + ((\prod_{j=1}^{M} f_j \times \sum_{i=1}^{nPTI} P_{PTI(i)} - \sum_{i=1}^{neff} f_{eff(i)} \times P_{AEeff(i)}) C_{FAE} \times SFC_{AE}) - \sum_{i=1}^{neff} f_{eff(i)} \times P_{eff(i)} \times C_{FME} \times SFC_{ME})}{f_i \times f_c \times f_l \times Capacity \times f_w \times V_{ref}} \quad (4.11)$$

Table 8. Parameters used in equation (4.11)

Parameter	Explanation
P_{MEi}	Main engine power
C_{FME}	Carbon content of fuel used in main engine
SFC_{ME}	Specific fuel consumption of main engine
P_{AE}	Auxiliary engine power
CF_{AE}	Carbon content of fuel used in auxiliary engine
SFC_{AE}	Specific fuel consumption of auxiliary engine
P_{PTI}	Power consumption of shaft motor
P_{AEeff}	Power of innovative technology
P_{eff}	Efficiency of the innovative technology
Capacity	Deadweight tonnage of the ship
V_{ref}	Ship speed
f_j	Correction factor to account for ship specific design elements
f_{eff}	Availability factor of innovative energy efficiency technology
f_i	Capacity factor for any technical limitation on capacity
f_c	Cubic capacity correction factor
f_w	Coefficient indicating the decrease of speed caused by sea condition

Table 9. EEDI results

Baseline	16.23
Required EEDI phase 1	15.92
Required EEDI phase 2	15.62
Diesel Engine EEDI	15.34
Fuel Cell EEDI	10.38

NO_x Emissions

The case ship has an engine that satisfies IMO Tier II NO_x emission limits. Since 2011, the Tier II global emission limitation depends on the engine speed, and for the engines working between 130 and 2000 rpm can be calculated with (4.12) where the n is the engine speed (IMO, 2016).

Therefore, since the main engine maximum operating speed is 750 rpm for our case ship, according to the formula (4.12), the maximum allowable NO_x emission can be found 9.59 g/kWh. Therefore, NO_x emission per voyage can be found using the following equations (4.13) and (4.14):

The results of emissions are shown in Figure 7 where the fuel cell emissions are at the left column and the diesel engine emissions are at the right according to ship voyage.

As a result, more than 99% emission, more than 53 tons of NO_x decrease is calculated, totally. As expected, the main reason for NO_x formation in diesel engines is the air for internal combustion. However, in fuel cell systems, the electrical power is produced directly by converting the chemical energy of the fuel. So, the differentiating factor and the source of nitrogen at this point is the need for air for conventional engines. In other words, fuel cell NO_x emission is negligible compared to diesel engines and this shows great potential for the future of shipping and in the meaning of strict emission regulations.

SO_x Emissions

In the maritime industry, the dominant fuel types are sulphur blended fuels such as marine diesel oil and heavy fuel oil which is the main reason for SO_x formation by marine diesel engines. In this paper, the reference ship routes are collected for the year 2018. However, at the beginning of 2020 outside ECA SO_x emission limit is decreased to 0.5% from 3.5%. On the other hand, inside ECA sulphur content in fuel was reduced to 0.1% since 2015. Therefore, in this research both possible options are investigated in the meaning of sulphur oxide emission.

In this context, 2 different fuel emissions for diesel engine are calculated under acceptable limits for both the 2018 and 2020 years. The voyages in ECA for our case ship are already summarized in table 6. Thus, fuel with a sulphur content of 0.1% for ECA and 3.5% and 0.5% for outside ECA, for the years 2018 and 2020, respectively, were accepted in the calculations according to ISO 8217. SO_x emissions are calculated according to formulas (4.15) and (4.16) where, 0.97553 is the fraction of fuel sulphur converted to SO_x and 2 is the ratio of molecular weight of SO_x and sulphur (IMO, 2015):

$$The\ maximum\ allowable\ NO_x\ emission\ (kg) = 44 \times n^{-0.23} \quad (4.12)$$

$$NO_x\ emission\ for\ diesel\ (kg) = 9.59\ \left(\frac{g}{kWh}\right) \times P_{actual}\ (kW) \times Time\ (h) \times 10^{-3} \quad (4.13)$$

$$NO_x\ emission\ for\ MCFC\ (kg) = 0.0045\ \left(\frac{g}{kWh}\right) \times P_{actual}\ (kW) \times Time\ (h) \times 10^{-3} \quad (4.14)$$

$$SO_x\ \left(\frac{g}{kWh}\right) = SFOC_{actual}\ \left(\frac{g}{kWh}\right) \times 2 \times 0.97753 \times \%fuel\ sulphur\ fraction \quad (4.15)$$

$$SO_x\ (kg) = SO_x\left(\frac{g}{kWh}\right) \times P_{actual} \times Time\ x\ 10^{-3} \quad (4.16)$$

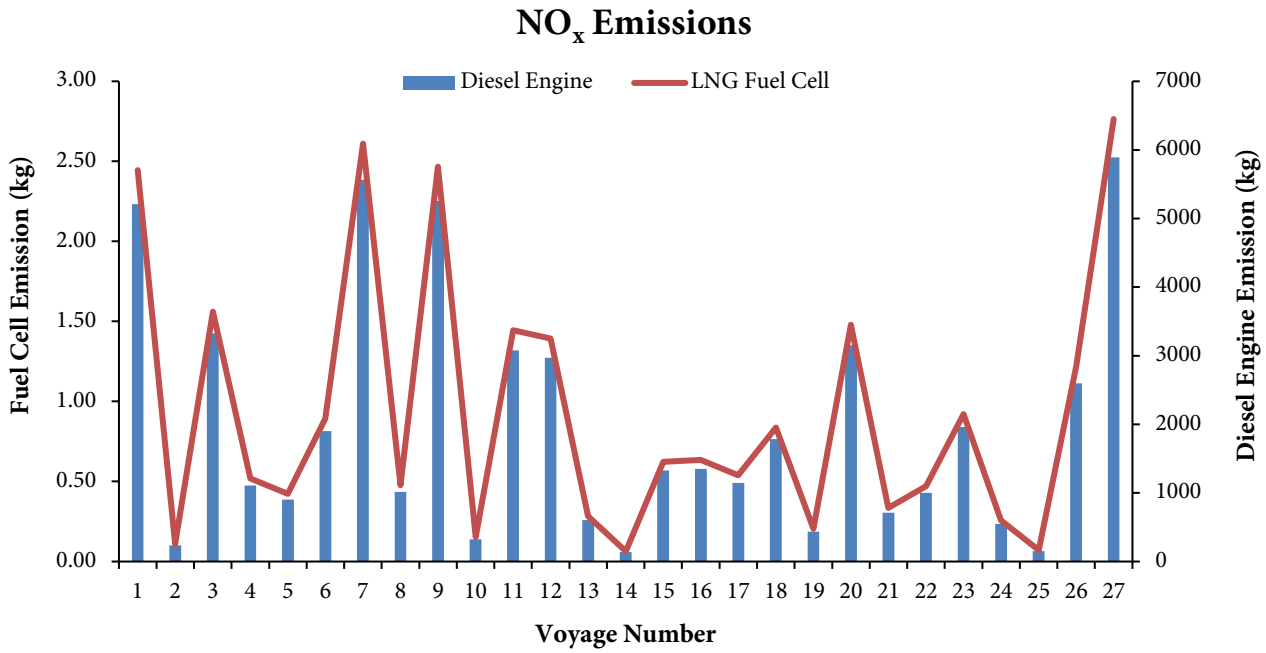

Figure 7. NO_x emissions according to the fuel type and voyage number

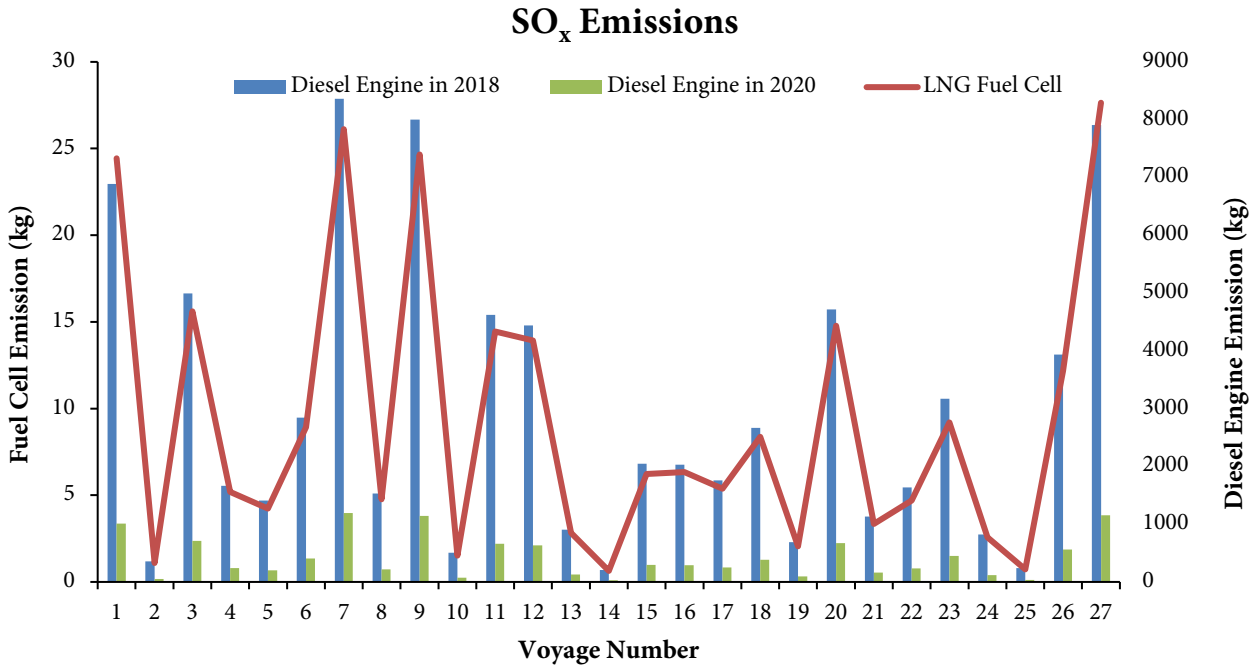

Figure 8. Total SO_x emissions according to the fuel type and voyage number

In Figure 8, SO_x emissions are shown with two different emission regulations for diesel engines for each voyage. The emission results for diesel engine in 2020 are according to today's rules. Since the case ship navigated in ECA, total sum of diesel oil and fuel oil is calculated for voyage number 1 and 27.

The total emissions for 27 voyages in 2018 are calculated and more than 99% and 98% emission reduction can be reached against 2018 and today's fuel types. Regarding to 2020 emission regulations, more than 11 tons of SO_x emission reduction is reached with fuel cell systems. Since the natural gas is a sulphur free fuel, it is a good option with the aim of staying under limits for the maritime industry also with dual-fuel marine engines.

Particulate Matter (PM) Emissions

Particulate matter is one of the polluting emissions from ships. PM emissions are directly dependent on the sulphur content of the consumed fuel like SO_x. In the case of reducing sulphur blended fuel usage, PM emissions are also showing a decreasing trend. According to MARPOL Annex VI emission regulations, PM emissions are one of the major topics. PM emission for the diesel engine is calculated using formula (4.17) where SF is the sulphur fraction of the fuel (IMO, 2015):

$$PM(kg) = [1.35 + SFOC\ x\ 7\ x\ 0.02247\ x\ (SF - 0.0246)]\ x\ P_{actual}\ x\ Time\ (h)\ x\ 10^{-3} \quad (4.17)$$

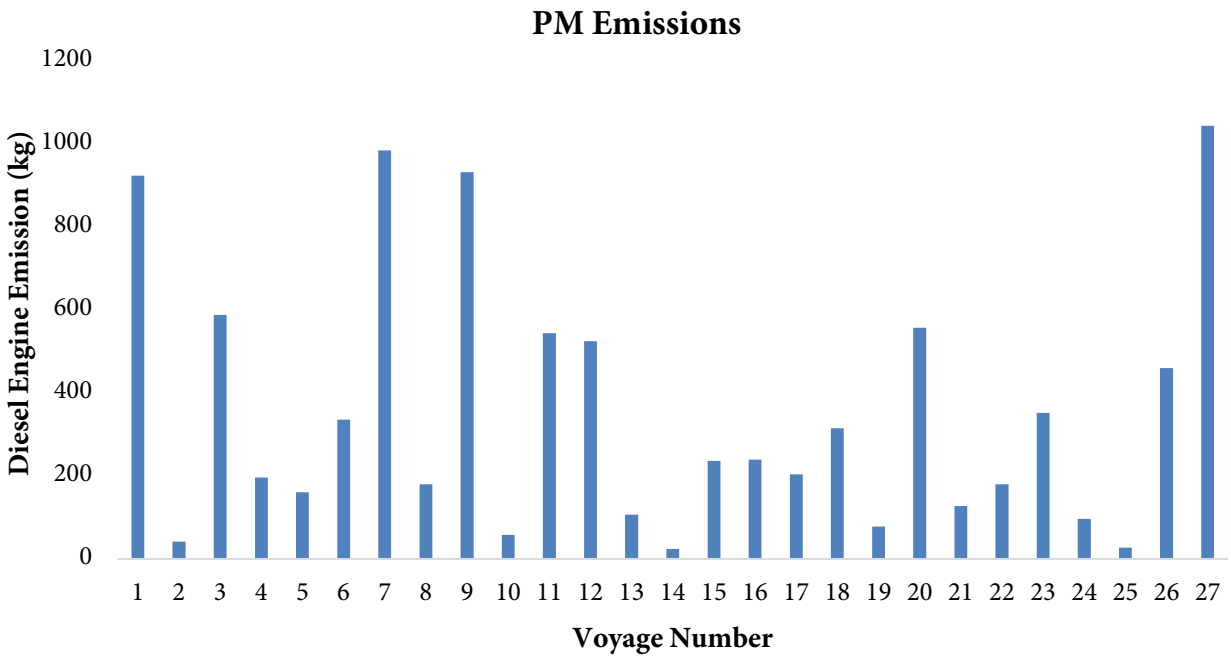

Figure 9. Total PM emissions according to the fuel type and voyage number

The total PM emission for the diesel engine is 9506 kg for all 27 voyages. However, fuel cell's PM emissions are negligible and almost totally eliminated compared to diesel engines. Therefore, it is not shown in Figure 9.

Conclusion

There are numerous methods to reduce the ship sourced emissions in shipping industry; however, their high operational and installation costs and diminishing of fossil fuel reserve are forcing ship owners to invest in new environmental friendly power sources. Due to strict international regulations on GHG and air polluting emissions in shipping, as a major exhaust gas producers, main engines of vessels must be switched from diesel engines to zero emission power producing technologies. At this point hydrogen fuel cells are important alternatives with their water emissions but high cost of hydrogen production and difficulties on storage of hydrogen make their usage difficult in shipping. Therefore, another fuel cell type, molten carbonate, a high temperature working fuel cell, is investigated in this research thanks by considering to its capability of using LNG as a fuel.

In this paper, a chemical tanker was dealt with case study using real routes in 2018 that were received from the company logs. LNG fuelled MCFC, which has same power output with ship's main diesel engine, is studied for the ship main propulsion system. The case routes are investigated respecting ECA using Netpas software and fuel switching procedures. The fuel type of the main engine is taken into account while calculating the emissions. Approximately, more than 99% of SO_x, PM, and NO_x and 33% of CO_2 emission reductions are calculated. Furthermore, 11402 kg of SO_x, 9506 kg of PM, 53668 kg NO_x and 1223 tons of CO_2 emissions is reduced just by one ship for 27 voyages in the Mediterranean Sea. The reduction at SO_x was expected due to LNG characteristics but in contrast, the reduction at CO_2 emissions is important in comparison to LNG fuelled diesel engines. However, the lifetime and carbon footprint of the fuel cells should be investigated and therefore the difference with diesel engines and costs for the renewing the electrolyte should be identified.

The new system is designed with respect to auxiliary system components like compressors or additional pumps. Hence, previous equipment such as fuel separators, fresh water generator, lubricating oil tanks and related pumps and heat exchangers will be out of use. The transformation of the fuel tanks to LNG may be another challenge for the new system, at least LNG use in shipping is a mature technology than hydrogen usage and there is enough experience to handle it. The electrical components are very reliable systems for years, so, their failure and maintenance need is neglected. In addition, suitability for co-generation systems for the MCFC by waste heat recovery system was another motivating reason for ship case study. However, in the original version, case ship is not equipped by a waste heat system. So, comparison with cogeneration system would not be useful to see the results clearly.

For further studies, similar cases can be investigated for different ship types and different routes under economical perspective with considering supply chain and bunkering operations of hydrogen or LNG. Moreover, noise and vibration effects and response for instantaneous load changes of the system can be investigated for the fuel cell powered ships. Operational procedures, system risk assessments and maintenance cost and frequency can also be an interesting area to study.

Compliance with Ethical Standards

Conflict of Interest

The authors declare that they have no competing interests.

Ethical Approval

For this type of study, formal consent is not required.

Availability of Data and Materials

The data that support the findings of this study are available from Chemfleet Ship Management but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of Chemfleet Ship Management.

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RESEARCH ARTICLE

A binary logistic regression model for prediction of feed conversion ratio of *Clarias gariepinus* from feed composition data

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ABSTRACT

Aquaculture in developing countries faces a lot of challenges that are barely being addressed. With feed taking nearly 70% of the total production cost, it becomes imperative to develop means of optimizing how research is conducted into feed development. Feed conversion ratio as a measure of feed quality can be used to quantify in retrospect the appropriateness of feed fed to livestock, particularly, *Clarias gariepinus*. From the study, binary logistic regression can in simple terms, determine if prospective feed will perform below or above the acceptable level of 1.5, based on its composition and proximate analysis values. Data from similar experiments are normalized and split into train and testing data to fit a logistic regression model, three numerical optimizers were used including liblinear, Newton-CG, SAG and accuracy of the models were compared using the confusion matrix, and Jaccard similarity score. An accuracy value of 0.8 was observed in the model regardless of the numerical optimizer, this indicates the appropriateness of the model in predicting either high or low FCR for feed types. The probability of prediction showed disparity among liblinear and SAG/Newton-CG solvers. Liblinear solver showed close probabilities in predicting if values will be 1 or 0. While a similar prediction was made by all solvers, this indicates a possible affinity for error when the solver is used. This is also indicated with a logloss of 0.65 as compared to 0.51 in both SAG and Newton-CG solvers.

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Introduction

The variety of factors that could be responsible for an observed outcome in any biological system requires the need to conduct several experiments and trials to get the desired result. In aquaculture, factors including the composition of a single feedstuff contribute alongside all other factors in the performance of the entire nutrient performance of the

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compounded feed. This accounts for the use of a large amount of human and capital resources.

Aquaculture plays a very important role in food systems, especially in middle-income regions where the industry employs labor and serves as a major source of animal protein. As stated by FAO (2016), global fish production reached a peak of 171 million tonnes, 47% of which was produced by aquaculture. However, if aquaculture is to remain an alternative to dwindling capture fishery stocks, there's the need to reduce the cost of production which is mostly accounted for by the cost of feeding, taking between 60% and 80% of the total cost of production (Ng et al., 2013).

Fishmeal is an indispensable component of fish feed due to its amino-acid profile, fatty acids, flavor, and other essential nutrients. The ecological cost of fishmeal and high demand from other livestock species necessitates the need for a similar substitute. (Farahiyah et al., 2016). Hence optimization of the protein content of feed relies heavily on successfully substituting fishmeal with other more affordable feedstuff for optimal growth performance (Degani et al., 1989). The FCR is simply the amount of feed it takes to grow a kilogram of fish. For example, if it requires two kilograms of feed to grow one kilogram of fish, the FCR would be two, this means that when a feed has a low FCR, it takes less feed to produce one kilogram of fish than it would if the FCR were higher. A low FCR is a good indication of a high-quality feed. FCR is a valuable and powerful tool for the fish farmer. It allows for an estimate of the feed that will be required in the growing cycle. Knowing how much feed will be needed then allows a farmer to determine the profitability of an aquaculture enterprise. This means that FCR allows the farmer to make wise choices in selecting and using the feed to maximize profitability. (USAID-HARVEST, 2011)

Several factors can influence the way fish respond to feed. Stage of culture, size, water quality, genetics, pond management, and the composition of other feedstuff.

Binary logistic regression studies the association between a category of the dependent variable and a set of independent variables. Logistic regression is used when the outcome has only two possible values (0 and 1), and is opposed to multinomial regression where the outcome could be three or more possible outcomes or prediction. Logistic regression as opposed to linear regression is used for the prediction of categorical response variables. It is assumed to be more suited for modeling because it does not assume a normal distribution for the independent variables (NCSS, 2020).

Materials and Methods

Data Generation and Preprocessing

FCR reported, based on specific feed composition as reported by Chor et al. (2013), Oyekanmi et al. (2013), Dudusola and Akinlade (2014), Falaye et al. (2015), and Aniebo et al. (2009) were used as historical data. Experimental results from feeding trials on *Clarias gariepinus* comprising of feed components used in each of the trials. Some components are present in nearly all the trials, i.e. fishmeal and lipids. Other components include maggot-meal, feather meal, blood-meal, etc. Feed proximate analysis data, with similar experimental design and analytical procedure as outlined by AOAC (1990) were collected as relevant to the feed composition data. The initial entry was done on excel spreadsheets, feed component and proximate data are loaded into rows, columns are based on feed trial indicator, and source.

Feed component data included in the model comprises the most utilized feedstuff for the formulation of feed for African catfish (Table 1). This is expected to facilitate the ease of using the model by a third party in the prediction of Feed conversion ratio.

Five code indicators are used in the columns to indicate the source of the data, they include FTM, MGT, CMGT, MAIZE, and FSHML representing Feather-meal, Maggot-meal, Maggot-meal, and Fishmeal respectively, each referring to the theme of feeding trial from which the corresponding data was obtained.

Binary Classification

A feed conversion ratio of 1.8 to 1 was observed by Li et al. (2014) to be typical in experimental set-up and that was used to categorize the FCR values in the historical data. FCR values between 0 and 1.5 were categorized as 1 while FCR values greater than 1.5 were classified as 0 as shown in Table 2.

Regression

Logistic regression uses the independent variable from historical data (feed composition and proximate analysis as data shown above) to produce a formula that predicts the probability of the class label (FCR churn). Logistic regression fits a special s-shaped curve by transforming the numeric estimate into a probability using the sigmoid function. Hence the model predicts the particular class for which a hypothetical feed composition belongs (1 meaning good FCR and 0 meaning bad FCR), and also gives the probability of having that class.

Table 1. Feed components included in the model

Feed	FSHML	PRN	FTML	BRWWST	SBML	BLDML	Lipid	MGTML	WTBRN	YMZ	GNC	CMC	Vit	Chromic	Minerals	Calcium	Cellulose	Tapioca
FTM0	56.5	0	0	0	0	0	6.43	0	0	0	0	2.36	3	0.5	4	1	5.34	28.25
FTM20	45.32	0	9.86	0	0	0	7.54	0	0	0	0	2.36	3	0.5	4	1	6.1	28.39
FTM40	33.99	0	19.7	0	0	0	8.63	0	0	0	0	2.36	3	0.5	4	1	6.88	28.53
FTM60	22.66	0	29.6	0	0	0	9.73	0	0	0	0	2.36	3	0.5	4	1	7.66	28.67
FTM80	11.33	0	39.4	0	0	0	10.8	0	0	0	0	2.36	3	0.5	4	1	8.43	28.81
FTM100	0	0	49.3	0	0	0	11.9	0	0	0	0	2.36	3	0.5	4	1	9.21	28.94
MGT0	31.73	0	0	0	0	0	0.25	0	11.18	22.4	31.7	0	0.5	0	1.5	1	0	0
MGT50	22.77	0	0	0	0	0	0.25	22.77	9.57	19.1	22.8	0	0.5	0	1.5	1	0	0
MGT33	31.89	0	0	0	0	0	0.25	15.95	11.07	22.2	16	0	0.5	0	1.5	1	0	0
MGT66	17.76	0	0	0	0	0	0.25	35.57	8.66	17.3	17.8	0	0.5	0	1.5	1	0	0
MGT75	14.55	0	0	0	0	0	0.25	43.65	8.8	16.7	14.5	0	0.5	0	1.5	1	0	0
CMGT0	25	0	0	0	34	10.3	5.2	0	11	11	0	0	0.2	0.2	0.15	3	0	0
CMGT12	12.5	0	0	0	39	10	3.5	12.5	8	11	0	0	0.2	0.2	0.15	3	0	0
CMGT25	0	0	0	0	43.5	10	1	25	6	11	0	0	0.2	0.2	0.15	3	0	0
MAIZE0	13.63	0	0	0	27.26	0	1	0	0	28.9	27.3	0	0.5	0	0.5	2	0	0
MAIZE25	13.3	0	0	0	26.63	0	1	0	0	21.7	26.6	0	0.5	0	0.5	2	0	0
MAIZE50	13.3	0	0	0	26.63	0	1	0	0	14.7	26.6	0	0.5	0	0.5	2	0	0
MAIZE75	13	0	0	0	26	0	1	0	0	7.65	26	0	0.5	0	0.5	2	0	0
MAIZE100	13	0	0	0	26	0	1	0	0	0	26	0	0.5	0	0.5	2	0	0
FSHML100	62	0	0	4.25	0	9	2.5	0	0	15	3	0	0.3	0	0.3	2.9	0	0.5
FSHML75	47	15	0	4.25	0	9	2.5	0	0	15	3	0	0.3	0	0.3	2.9	0	0.5
FSHML50	31	31	0	4.25	0	9	2.5	0	0	15	3	0	0.3	0	0.3	2.9	0	0.5
FSHML25	15	47	0	4.25	0	9	2.5	0	0	15	3	0	0.3	0	0.3	2.9	0	0.5
FSHML0	0	62	0	4.25	0	9	2.5	0	0	15	3	0	0.3	0	0.3	2.9	0	0.5

Note: Abbreviations in header row and meanings: FSHML- Fishmeal; SBML- Soyabean Meal; PRN- Rocky-prawn; BRWWST- Brewers Waste; BLDML- Blood-meal; MGTML- Maggot-Meal; WTBRN- Wheat-bran; YMZ- Yellow maize; GNC- Groundnut Cake; CMC- Carboxymethyl Cellulose; Vit – Vitamin premix

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Table 2. Proximate analytical data for compounded feed

Feed	Protein	Fat	Ash	Crude fiber	NFE	Moisture	Culture period	Fish weight	FCR	FCR churn
FTM0	39.82	11.45	86.55	4.811	25.85	4.78	4	2.85	1.24	**1**
FTM20	39.51	11.23	87.43	4.811	25.85	5.12	4	2.85	1.34	**1**
FTM40	41.25	11.87	89.1	4.811	25.85	5.03	4	2.85	2.9	**0**
FTM60	38.95	12.14	89.7	4.811	25.85	5.89	4	2.85	3.08	**0**
FTM80	40.34	11.5	90.7	4.811	25.85	4.43	4	2.85	2.85	**0**
FTM100	40.67	11.26	91.27	4.811	25.85	5.37	4	2.85	1.91	**0**
MGT0	44.2	4.76	13.37	3.74	23.98	9.96	10	21.73	5.07	**0**
MGT50	43.5	4.6	12.7	3.76	25.47	9.9	10	21.8	3.96	**0**
MGT33	44.23	4.5	13.11	3.74	24.52	9.8	10	21.67	3.57	**0**
MGT66	43.48	4.76	13.16	3.85	24.8	9.94	10	21.8	4.16	**0**
MGT75	44.03	4.85	13.6	4.1	28.54	4.9	10	21.77	3.13	**0**
CMGT0	40.76	9.2	10.59	4.1	25.85	9.65	10	10	1.15	**1**
CMGT12	40.59	8.98	9.1	4.87	25.85	9.22	10	10.02	1.17	**1**
CMGT25	40.74	8.51	8	5.22	25.85	9.87	10	10	1.16	**1**
MAIZE0	37.42	4.36	15.78	4.2	28.45	9.96	12	4.03	0.65	**1**
MAIZE25	35.96	4.98	15.88	5.53	28.2	9.78	12	4.06	0.68	**1**
MAIZE50	36.42	5.24	16.02	5.51	27.3	9.89	12	4.06	0.7	**1**
MAIZE75	38.25	5.46	15.95	5.6	24.92	9.93	12	4.05	0.68	**1**
MAIZE100	40.2	6.66	16.22	5.58	22.63	9.88	12	4.05	0.62	**1**
FSHML100	38.3	6.81	8.61	3.44	39.96	8.88	13	10.73	2.33	**0**
FSHML75	37.6	6.45	8.78	3.56	34.2	9.41	13	10.57	2.33	**0**
FSHML50	38.9	7.63	9.44	3.73	30.47	9.83	13	10.56	2.16	**0**
FSHML25	37.8	7.49	9.53	3.63	32.09	9.46	13	10.58	2.3	**0**
FSHML0	36.6	7.33	9.59	3.69	33.3	9.49	13	10.61	2.62	**0**

Note: Abbreviations used in index column and meanings (values attached in the table indicates level of inclusion in the compounded feed): FTM-Feather meal; MGT-Maggot meal; CMGT-Maggot meal as reported by Aniebo et al. (2009); MAIZE-Maize inclusion meal; FSHML- Fish Meal.

Mathematically, the sigmoid function is represented as:

$$\sigma: h(x)=\sigma(\theta TX)= \frac{e(\theta 0+\theta 1x1+\theta 2x2+\ldots)}{1+e(\theta 0+\theta 1x1+\theta 2x2+\cdots)} \quad (1)$$

Where θTX= regression results (sum of variables weighted by the coefficients), (θTX) =sigmoid or logistic function. Probability of a class calculated by Equation (2).

$$P_i(Y=1|X)=\sigma(\theta TX_i)= \frac{e\theta TX}{1+e\theta TX} \quad (2)$$

Where P_I = Probability of having an acceptably high value for FCR, X_I is a vector of explanatory variables, θTX is unknown parameters to be estimated.

Normalization, Train, and Test Splitting

Normalization was done using Standard scaler from preprocessing Sci-kit learn library to have an equal representation of each feature within the groups. Using train_test_split library, data was split into the train and testing set. Test size is set at 20%, while 80% is used for training the model.

Modelling and Fitting

The inverse of the regularization strength also known as the 'C' parameter is set at 0.01, the numerical optimizer is set as liblinear, SAG, and Newton-CG solvers are also applied to know the optimal solver. A test set comprising of 5 data points was used to test the model.

Measurement of Accuracy

Jaccard index/ Jaccard similarity score: is estimated using metrics from sklearn. The index is a measure of size of the intersection divided by size of the union of two label sets (0 and 1), i.e. if all predicted labels for a particular set matches with the true labels, subset accuracy is 1.0, if none match, and it is 0.0.

Confusion matrix shows the number of correctly predicted points versus wrong predictions side by side. The first row is for FCR whose actual churn value in the test set is 1, the second row is for FCR with an actual churn value of 0. The first column holds total number of correct predictions and second column holds the number of wrong predictions. These values can then be interpreted as true positives, false positives, true negatives, and false negatives. Classification report comprises precision, recall, and F1 score. Precision measures accuracy provided class label has been predicted by Equation (3).

$$\text{Precision}= \frac{TP}{(TP+FP)} \quad (3)$$

Recall is the rate of true positives. Recall calculated by Equation (4).

$$\text{Recall} = \frac{TP}{(TP+FN)} \quad (4)$$

F1 score is the harmonic average of both precision and recall. Best value = 1 while worst = 0. Log loss measures the performance of the classifier where output to be predicted is binary.

Results and Discussion

Results

Accuracy metrics results for test set data are given in following tables (Table 3, Table 4, Table 5). Matrix for logistic regression using different solver are presented in following figures (Figure 1, Figure 2, Figure 3). Classification reports for different solvers are tabulated in Table 6, Table 7, and Table 8.

Table 3. Accuracy metrics using liblinear solver

0	1	Prediction	Jaccard Similarity Score	Log Loss
0.520076	0.479924	0	0.8	0.65
0.518691	0.481309	0		
0.539945	0.460055	0		
0.544009	0.455991	0		
0.438961	0.561039	1		

Table 4. Accuracy metrics using Newton-CG solver

0	1	Prediction	Jaccard Similarity Score	Log Loss
0.72107	0.27893	0	0.8	0.51
0.704345	0.295655	0		
0.652111	0.347889	0		
0.68603	0.31397	0		
0.341602	0.658398	1		

Table 5. Accuracy metrics using SAG solver

0	1	Prediction	Jaccard Similarity Score	Log Loss
0.72107	0.27893	0	0.8	0.51
0.704345	0.295655	0		
0.652111	0.347889	0		
0.68603	0.31397	0		
0.341602	0.658398	1		

Table 6. Classification report for liblinear solver

	Precision	Recall	F1-Score	Support
0	0.75	1	0.86	3
1	1	0.5	0.67	2
Accuracy			0.8	5
Macro avg	0.88	0.75	0.76	5
Weighted avg	0.85	0.8	0.78	5

Table 7. Classification report for Newton-CG solver

	Precision	Recall	F1-Score	Support
0	0.75	1	0.86	3
1	1	0.5	0.67	2
Accuracy			0.8	5
Macro avg	0.88	0.75	0.76	5
Weighted avg	0.85	0.8	0.78	5

Table 8. Classification report for SAG solver

	Precision	Recall	F1-Score	Support
0	0.75	1	0.86	3
1	1	0.5	0.67	2
Accuracy			0.8	5
Macro avg	0.88	0.75	0.76	5
Weighted avg	0.85	0.8	0.78	5

Discussion

Similar Jaccard similarity score form tables 3.1.1, 3.1.2 and 3.1.3 shows all solvers predicted the output of the 5 test data with an 80% accuracy, this also corresponds to the similar values in the prediction columns. However, probability of prediction observed using a liblinear solver has very low margins. The Probability of the first prediction made using a liblinear solver, are 57% for 0 and 42% for 1 were recorded. This may make the solver more prone to error. Logarithmic loss is also highest under the liblinear solver when compared to the other solvers.

Newton-CG and SAG solvers show very high probabilities in the accurate predictions made. But when compared to liblinear solver, SAG and Newton-CG solver places very high probability on predicting the last data point which was wrong. An accurate value for point 5 was supposed to be 0 but was predicted as 1, while liblinear solver apportioned a probability

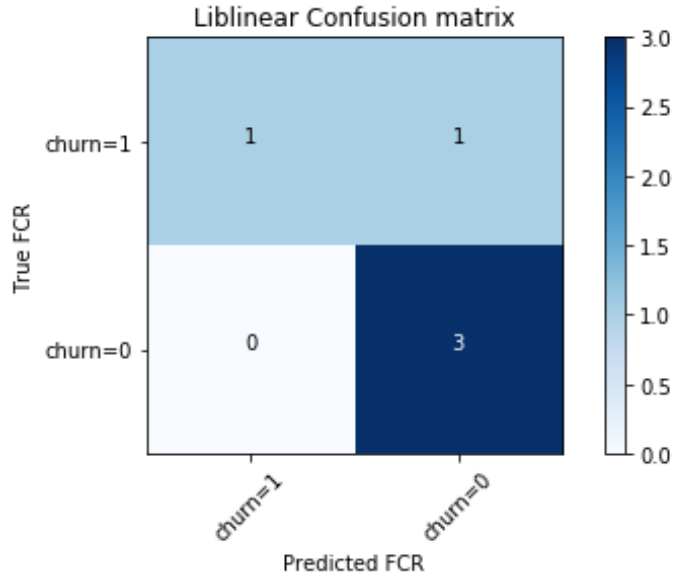

Figure 1. Matrix for logistic regression using liblinear solver

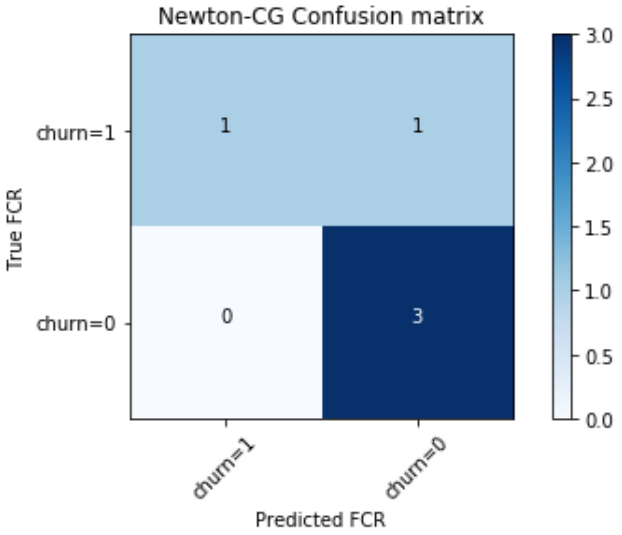

Figure 2. Matrix for logistic regression using Newton-CG solver

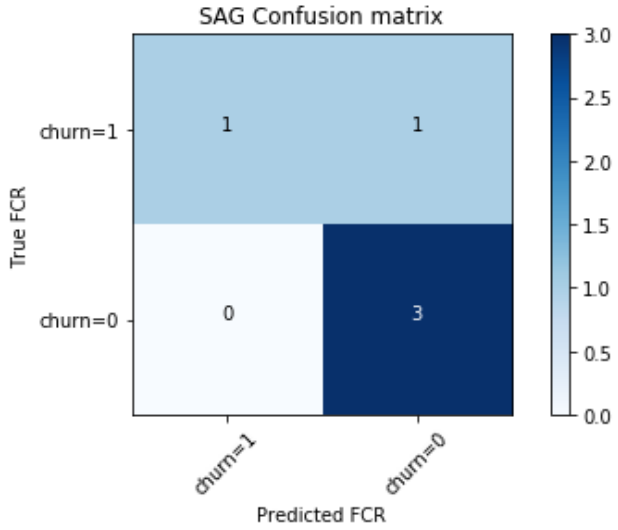

Figure 3. Matrix for logistic regression using SAG solver

of 43% on 0, both Newton-CG and SAG apportioned a probability of 56%.

Confusion matrix indicates similar numbers of true positive (1), false positive (1) and true negatives (3), no false negatives were observed. This translates to having 4 right and one wrong prediction regardless of the solver used.

Conclusion and Recommendation

Binary logistic regression simply underlies the use of data to answer questions with two possible outcomes. This can be used in simply predicting either result will be high or low. This machine learning method can also be used to predict multiple outcomes (multinomial regression). Application to aquaculture especially when extensive laboratory experiments are unavailable will help rural farmers, feed manufacturers, and researchers have an idea of the expected Feed conversion ratio of feed being compounded.

From the probability results obtained, different solvers provide closely similar results but may differ in the probabilities of prediction made. While Newton-CG and SAG solvers perform better than liblinear solver, the results indicate the need to run the same prediction with multiple solvers and compare the resulting probabilities.

The study indicates logarithmic regression can be used to successfully predict the FCR of feed compounded for *Clarias gariepinus* as either high (1) or low (0). As long as feed composition contains any of the following set of feedstuff: Fish-meal, rocky-prawn, Feather-meal, brewers-waste, soybean-meal, blood-meal, Lipid, maggot-meal, wheat-bran, yellow-maize, groundnut-cake, Carboxymethylcellulose (CMC), Vitamin, Chromic, Minerals, Calcium, Cellulose and Tapioca.

Also, proximate analysis data needed for the model include: Feed Protein content, fat content, ash content, crude-fiber, Nitrogen Free Extract, moisture, culture period, and fish weight at the onset of the experiment.

The study utilized historical data in making predictive analysis, the quantity and quality of data used in training models determined the accuracy and robustness of such models. This can be made easier with the use of cloud relational databases that hold experimental data and make them easily accessible. This would enhance aquaculture development especially in areas where experimental funding is quite a challenge.

Compliance with Ethical Standards

Conflict of Interest

The author declares that there is no conflict of interest. Historical data utilized in the research is appropriately cited.

Ethical Approval

For this type of study, formal consent is not required.

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RESEARCH ARTICLE

Determination of some biological characteristics and population parameters of the blotched picarel (*Spicara flexuosa* Rafinesque, 1810) distributed in the Eastern Black Sea (Rize - Hopa)

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ABSTRACT

This study was carried out in order to determine some biological characteristics of the fish species of *Spicara flexuosa*, which is distributed on the Eastern Black Sea coast, and to contribute to the management of the picarel in all the seas of our country. Between October 2015 and September 2016, 599 fish samples were obtained and examined in the laboratory. It was determined that the examined individuals were distributed between the ages of I-VII which 31.22% of the population was male and 68.78% was female. The minimum-maximum total length values of the samples were between 8.7 and 21.8 cm; and the weight values ranged from 7.1 to 129.94 g. It has been determined that the mean total length of males is statistically different from that of females ($P<0.05$). Von Bertalanffy growth equilibrium was calculated as "L_∞=22.71 cm TL, K=0.243 year^{-1}, t_0=-2.306 year^{-1}; W_∞=118.27 g" for females, "L_∞=38.34 cm TL, K=0.063 year^{-1}, t_0 = -6.381 year^{-1}; W_∞=755.37 g" for males, and "L_∞=33.42 cm TL, K=0.080 year^{-1}, t_0=-5.381 year^{-1}; W_∞=401.24 g" for all individuals. The length-weight relation of all individuals was found as $W= 0.0118*L^{2.9727}$ (R^2=0.9487). When the ratio of gonadosomatic index (GSI) data and gonadal maturity stages were examined over the year, it was determined that the reproduction of the picarel in the Black Sea occurred between June and September.

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Introduction

Species that belong to the genus *Spicara* occur in shallow rocky and muddy bottoms (in the coastal shelf up to 130 m depth) throughout the Mediterranean and the Black Sea, in the Atlantic from Portugal to Morocco and around the Canary Islands (Fischer et al., 1987; Froese & Pauly, 2013). The genus *Spicara* has posed numerous identification problems and consequently many different species have been described, leading to a variety of synonyms. For many years *Spicara flexuosa* Rafinesque, 1810 considered as the synonym of *Spicara maena* (Linnaeus, 1758) but cariological and genetic studies conducted in the recent years prove that those both are two different species (Vasiliev, 1980; Chiba et al., 2009; Imsiridou et al., 2011; Turan, 2011). It is possible to distinguish *S. maena* and *S. flexuosa* using meristic and morphometric features (Tortonese, 1986; Fischer et al., 1987; Rizkalla, 1996). *Spicara flexuosa* is distributed widely along the Turkish coastline (Bilecenoğlu et al., 2014) and various studies (Vasiliev, 1980; Vasilieva & Salekhova, 1983; Ilkyaz et al., 2007, Imsiridou et al., 2011, Turan, 2011; Minos et al., 2013, Bektaş et al., 2018) on the genetics and morphology of the species are available. There are studies conducted by Sever (2019) and Lipskaya & Salekhova (1980) on the nutrition of the species in the Mediterranean. However, studies on the biology of the species are very limited (Hattour et al., 1985; Mytilineou, 1987; Mytilineou & Papaconstantinou, 1991; Şahin & Genç, 1999; Mater, 2001; Malkav, 2002; Özvarol, 2014). The aim of this study is to reveal the age, growth, reproduction, mortality and population structure of *S. flexuosa* distributed in the South East Black Sea.

MATERIALS AND METHODS

Study Area and Sampling

The samples of *S. flexuosa* were obtained monthly between October 2015 and September 2016 (except August 2016) from the fishermen engaged in commercial fishing by using gillnets with mesh sizes from 16 mm to 20 mm from the Southeast Black Sea coast (Rize-Hopa). A total of 599 (412 females, 187 male) fish sample were taken to the laboratory and total length (TL) and weight measurements were made according to gender. The TL of each picarel was measured with 1 mm interval and the specimens were weighed (wet weight) on a balance with a sensitivity of 0.001 g. Each pair of sagittal otoliths was also removed and stored in plastic eppendorf tubes for further process (aging).

Age Determination

The otoliths were kept in 4% NaOH solution for 15-20 minutes and after the skin residues were cleaned, they were made transparent by passing through 40% and 70% alcohol series. Afterward, ages were determined by reflecting light from the top with a magnification of 0.8-8.0 under the Nikon SMZ1000 brand stereomicroscope. The photographs of each otolith were taken using a Nikon DSFI1 digital camera and two readers were recorded the ages independently. The age of 494 fish were successfully recorded from a total of 599 sagittal otoliths, while 105 of them could not be measured.

Growth

Size Frequency

Monthly size–frequency distributions for both sexes were calculated as 2 cm total length-class intervals. Size–frequency distribution analysis for females and males was conducted using the Kolmogorov–Smirnov two-sample test. Comparison of the mean total length between female and male was performed using t-test. Statistical analyses were considered significantly different at the level of α= 0.05. Statistical analysis carried out using a computer program PAST v2.14 (Hammer et al., 2001).

Length-Weight Relationship

Length-weight relationships were estimated by fitting an exponential curve to the data (Equation 1) (Ricker, 1973, 1975). Parameters a and b of the exponential curve were estimated by linear regression analysis over log-transformed data (Equation 2).

$$W = aL^b \quad (1)$$

$$\log W = \log a + b \log L \quad (2)$$

where W is the total weight (g), L is the total length (cm), a is the intercept and b is the slope, using the least-squares method. The association-degree between variables of W and L was calculated by the determination coefficient (r^2). Additionally, 95% confidence limits of the parameter b were estimated. The Student's t test was used for comparison of the slopes (Zar, 1996).

The growth parameters were measured by using von Bertalanffy Growth Model (Equation 3) (Ricker, 1975).

$$L_t = L_\infty \left[1 - e^{-k(t-t_0)}\right] \quad (3)$$

In this formula, L_t: The average length of the fish at age t (cm), L_∞: Maximum length that a fish can reach theoretically at infinity (cm), k: Growth coefficient (year^{-1}), t: Age (year), t_0: Theoretical age of the fish before they are hatched (year). The growth constants calculated in this study and the constants calculated in other studies were compared by using the Munro's Phi index and the t test. When this test was being applied, the growth constants (k) and (L_∞) values, which were obtained from the previous studies on the same species, were used. For each of these values, φ' values were calculated using Equation 4. The hypothesis assuming that there were no differences between the growth constants calculated and the other constants in previous studies was accepted (ts<tt) (Pauly & Munro, 1984; Avşar, 1998).

$$\varphi' = \log(k) + 2 \times \log(L_\infty) \quad (4)$$

Sex Ratio

The sex ratio of the *S. flexuosa* used in the study was examined by months and lengths. Whether the sex ratio (female / male ratio) is different from 1/1 or not was tested by the two-square test (χ^2).

Mortality

The total mortality rate (Z) was calculated by taking advantage of using average length according to Beverton & Holt (1957) and the Equation 5.

$$Z = \frac{K \times (L_\infty - \bar{L})}{\bar{L} - L'} \quad (5)$$

In this equation, K is the growth constant, $\bar{L}$ is the average length of fish used in the calculation of growth constants and L', for the fish used in the data set, shows the size corresponding to the class range in which the smallest fish are found. In order to calculate the natural mortality rate, the following formula (Equation 6) suggested by Pauly (1980) was used.

$$M = 0.8 \times exp(-0.0152 - 0.279 \times \ln(TL_\infty) + 0.6543 \times \ln(K) + 0.4634 \times \ln(TC^\circ)) \quad (6)$$

In the formula, TC° refers the average of annual surface water temperature and is considered as TC°=15.2°C, which is the long-term average of the Black Sea region (Bat et al., 2007). L_∞ is the asymptotic total length (cm) and K is the growth coefficient (Gayanilo et al., 2005). Fishing mortality rate is calculated with the following formula using the Z value and the mortality rates are calculated according to Pauly (1980) method (Equation 7).

$$F = Z - M \quad (7)$$

So where F *is* fishing mortality rate (year^{-1}), M refers natural mortality rate (year^{-1}) and Z is the instant total mortality rate (year^{-1}). The exploitation rate (E) is calculated by the ratio of the fishing mortality factor (F) to the total mortality factor (Z) (Equation 8).

$$E = \frac{F}{Z} \quad (8)$$

where, Z is the total mortality rate (year^{-1}), M is the natural mortality rate (year^{-1}), F is the fishing mortality rate (year^{-1}) E is the exploitation rate.

Reproduction Biology

Gonad Maturity and Spawning Time

Morphological characters were primarily used in sex determination of *S. flexuosa* fish. In males, the upper part of the lateral line is dark brown and there are distinct blue-purple bands on the side of the body, with distinct blue-purple bands and spots on the dorsal and caudal fins. During the breeding period, this coloration becomes brighter. In females, the body is bright yellowish and the abdomen area is white, only in the breeding period, faint blue-purple stripes are visible on the side of the body. In the breeding period, the same coloration in female individuals is seen only on the dorsal fin, unlike male individuals (Matic-Skoko et al., 2004). In individuals whose gender could not be determined morphologically, the fish was dissected and the sex determination of the individuals who reached sexual maturity was done macroscopically. In individuals who did not reached sexual maturity yet, gonads were examined under a microscope and their genders were determined. The individuals with a granular structure, yellowish orange color, swollen and abundant blood vessels gonads were evaluated as female, others as male. The development of gonads is morphologically divided into 5 categories as follows (Matic-Skoko et al., 2004):

Stage 1: Not mature; gonads are cylindrical, soft translucent, pink and white in females, while in males, they are very small and seem like only translucent filamentous strings.

Stage 2: Precocity; gonad development of females can be easily observed with the naked eye. Pink gonads are located on both sides of the body cavity like soft tulle. In males, the gonads are thicker and longer. Pink or white male gonads can be noticed with the naked eye.

Stage 3: Development; gonads in females are granular, colored, dark yellow-slightly orange. The development of blood vessels can be observed easily. Gonads in males are well

developed, thickened and white in color. In males and females, the ovaries occupy half the body cavity.

Stage 4: Mature; gonads cover the entire body cavity in orange-red color in females. There are prominent blood vessels scattered over the surface. The membrane covering the eggs is very thin and bursts with light pressure. In males, the gonads are creamy white and cover the entire body cavity.

Stage 5: Spent-Relaxation; in females, ovaries are elongated, honey-colored, saggy and loose, while the gonads in males are in skin color, bloody and wrinkled.

Gonadosomatic Index

Gonads were weighed in 0.01 g precision balance to determine the reproductive period and gonadosomatic index value. In determining the breeding season, the gonadosomatic index (GSI) value for both sexes was calculated according to the formula given in the Equation 9 (Erkoyuncu, 1995).

$$GSI = \left(\frac{W_g}{W}\right) \times 100 \quad (9)$$

Here, W_g is the gonad weight, and W is the total fish weight.

Size at Sexual Maturity

In the breeding season, individuals with gonad development stages 1 and 2 were considered immature, and individuals with gonad development stages 3, 4 and 5 were considered as mature (Matic-Skoko et al., 2004). Using the ratio of mature and immature individuals with 2 cm length intervals of female and male *S. flexuosa* obtained during breeding time, 50% maturity length of the gender was calculated according to the formula given in the Equation 10 for both genders (Campbell, 1985; King 1995).

$$P = {}^{1}/_{1 + e^{(a+bTL)}} \quad (10)$$

In this formula, P is the ratio of mature female and male fish, a and b refer equation constants, TL is the total fish length (cm). The 50% maturity length is calculated by using the equation constants (a and b) for female and male individuals by (a/b) formula.

Results

Size Composition

Total 599 *S. flexuosa* (412 females, 187 males) were obtained between October 2015 and September 2016. Total length of females ranged between 10.0 and 20.6 cm (mean 14.10±2.0709 cm) and weights ranged between 11.21 and 109.1 g, (mean 32.96±0.952 g) whereas total length of males varied between 8.7 and 21.8 cm (mean 16.4±2.0638 cm) and their weights varied between 7.1 and 129.94 g (mean 52.13±1.45 g) (Figure 1). The average total length of males is greater than that of females, and the difference was statistically significant (P=7.99-32). In addition, the length-frequency distribution was found to be statistically different between male and female individuals (Kolmogorov-Smirnov test: d=0.47455, P=3.66E-26). The dominant length group was calculated to be between 12 and 16 cm (32.3%) in females and 14 and 18 cm (35%) in males.

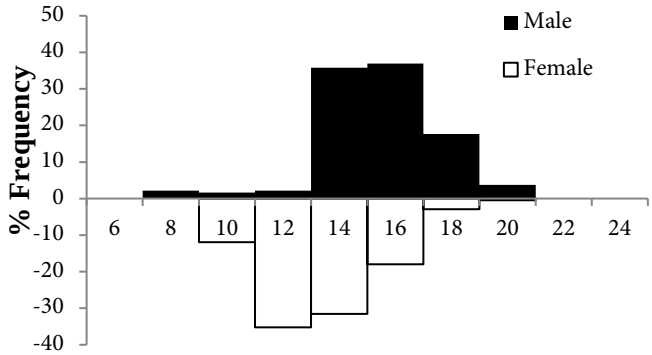

Figure 1. Length frequency composition of *S. flexuosa* individuals

Throughout the year, the smallest individual was sampled in April and the largest individual was sampled in November. The small individuals were very common in the stock in April, and a single 14.1 cm of male individual was sampled in May (Figure 2).

Length - Weight Relationship

The relationship between the length-weight values obtained from *S. flexuosa* individuals was calculated from the $W= aL^b$ growth equation. The length-weight relationship for females and males was calculated as follows: Female; $W=0.0172L^{2.8292}$ (R^2=0.9670), male; $W=0.0059*L^{3.2249}$ (R^2=0.9487), all individuals; $W=0.0118L^{2.9727}$ (R^2 =0.9487). The length-weight equation slope coefficient (b) was found to be different from isometric growth (b=3) in female (Pauly's t test=4.2744) and male (Pauly's t test=4.2613) individuals. While female individuals showed negative allometric growth, positive allometric growth was detected in male individuals. This difference is thought to be related to the nutritional conditions.

Age Composition

Age readings were made on a total of 494 individuals (318 females and 176 males). It was determined that the oldest individual was 7 years old for both males and females. It was determined that the dominant age group was 1 year (39.6%) for females and 3 years age group for males (31.8%). Approximately 81.8% of the population were found to be between 1 and 3 years old (Figure 3).

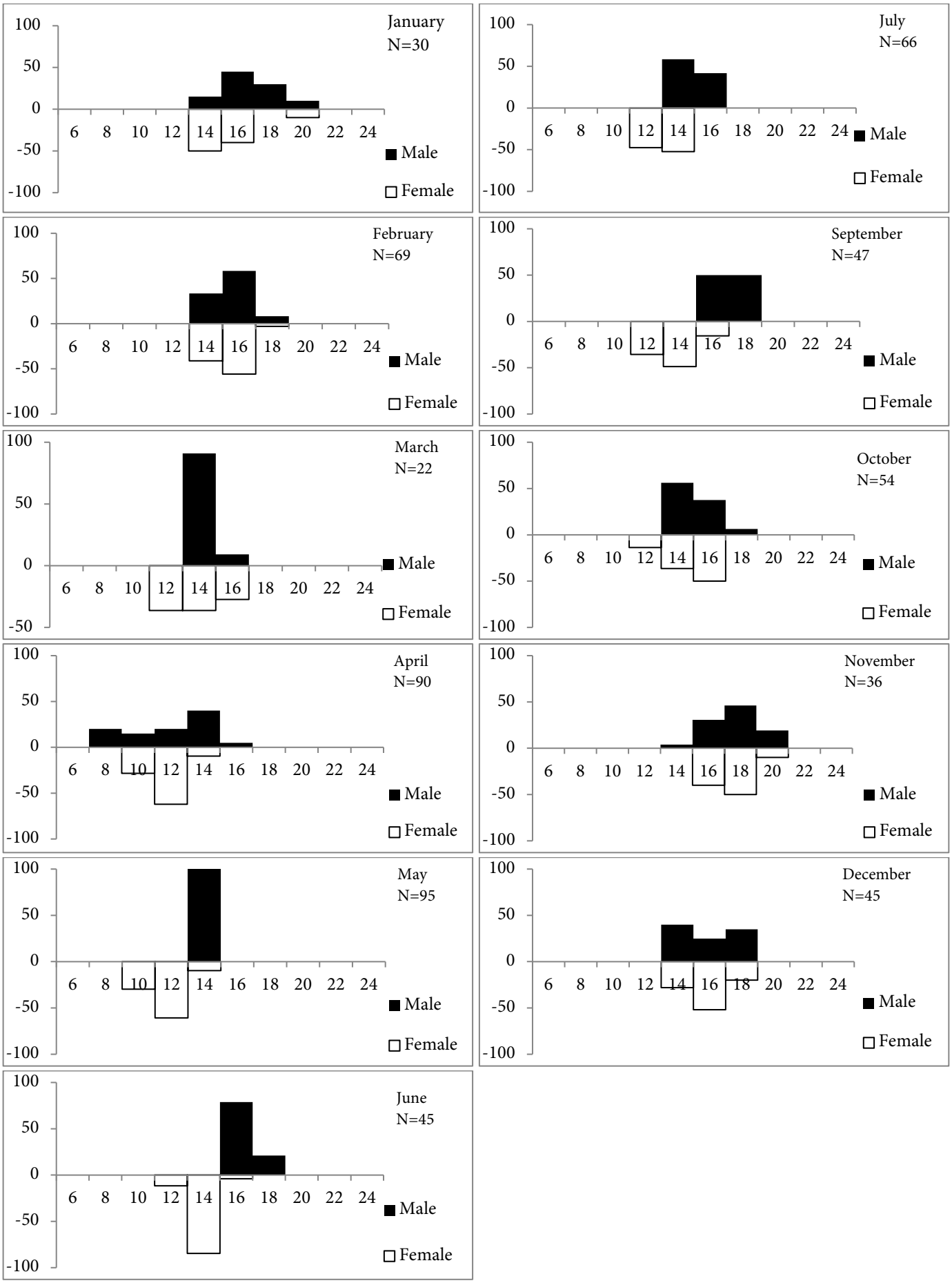

Figure 2. Monthly frequency distribution of *S. flexuosa* individuals

Table 1. Growth model parameters of *S. flexuosa* individuals, L_∞=asymptotic length, K=growth factor, t_o=age when fish length is theoretically zero, W_∞=asymptotic weight, parameter, *Φ'*=growth performance index

Parameters	L_∞	k	t_o	W_∞	(*Φ'*)
Total	33.42	0.080	-5.381	401.24	1.95
Female	22.71	0.243	-2.306	118.27	2.09
Male	38.34	0.063	-6.381	755.37	1.96

Growth

Among the extreme values that affect the calculations in female and male individuals are represented as a single individual in age groups, individuals of 20.6 cm for females and 21.8 cm for males were excluded from the evaluation. L_∞ value was higher in males than females. This value was calculated as 22.71 cm for females and 38.34 for males. The K value was higher in females than males, 0.243 in females and 0.063 in males. The growth performance index (Φ') value calculated using the parameters calculated by growth models (L_∞ and K) was determined to be 2.09 for females and 1.96 for males (Table 1).

Mortality

Fishery mortality rate (F) and exploitation rate (E) were calculated from the data obtained as Z year^{-1} 0.800; F year^{-1} 0.3239; M year^{-1} 0.4761; E year^{-1} 0.404. It has been determined that the stock exploitation ratio of *S. flexuosa* is operated at a value lower than the optimum value (E=0.5), that means, there is no fishing pressure. In the study, it was seen that the fishery mortality rate was caused only by discard.

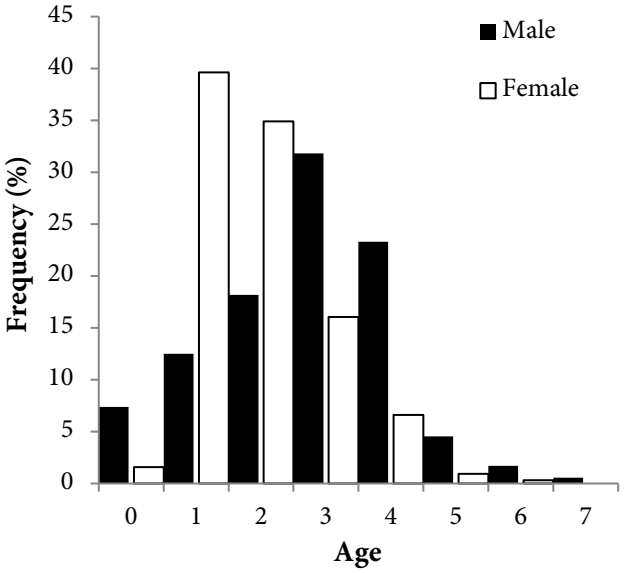

Figure 3. Age composition of male and female fish of *S. flexuosa* individuals

Reproductive Biology

Sex Ratio

In all the *S. flexuosa* samples examined, the female/male ratio (2.20) was in favor of the females, and the difference was statistically significant (χ^2 = 83.488, P < 0.001).

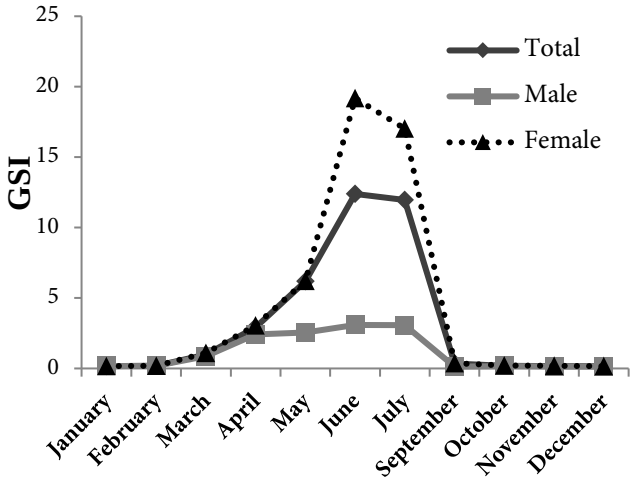

Figure 4. Monthly gonadosomatic index (GSI) values of females, males and all *S. flexuosa*

Spawning Season

According to the monthly gonadosomatic index (GSI) values, a peak occurred in June and the GSI value reached its lowest level in September by decreasing regularly after June. According to the monthly fluctuations of GSI values and the monthly development of gonad maturity stages, it was determined that the spawning time of the *S. flexuosa* in the study area is between June and September (Figure 4). Due to scarce of sample in August the value was left as blank in Figure 4. Male and female individuals in first and second gonad development stages were determined between September and April. Females in the 3rd and 4th stages were generally determined between March and July, while the males in the 3rd and 4th stages were mostly determined between April and July. Individuals in the emptied rest stage (5th stage) were identified in September. (Figure 5 and Figure 6).

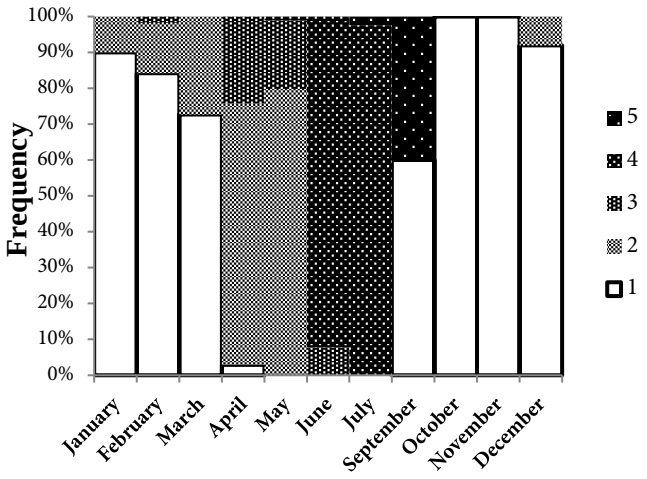

Figure 5. Percentage rates of monthly gonad stage (1; immature, 2; developing, 3; mature, 4; spawing, 5; spent) of female *S. flexuosa*

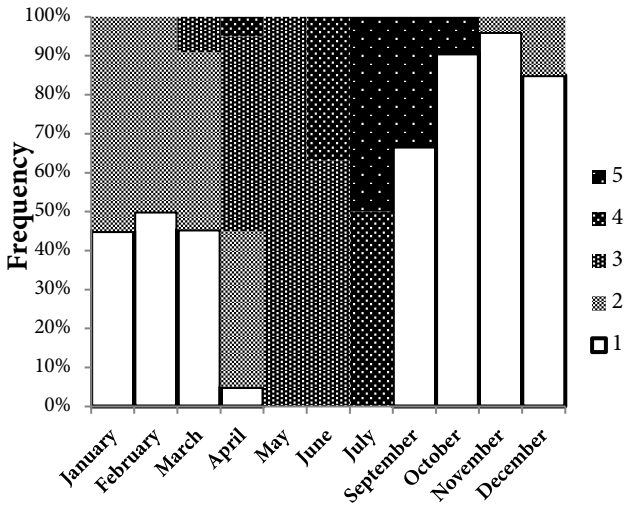

Figure 6. Percentage rates of monthly gonad stage (1; immature, 2; developing, 3; mature, 4; spawing, 5; spent) of male *S. flexuosa*

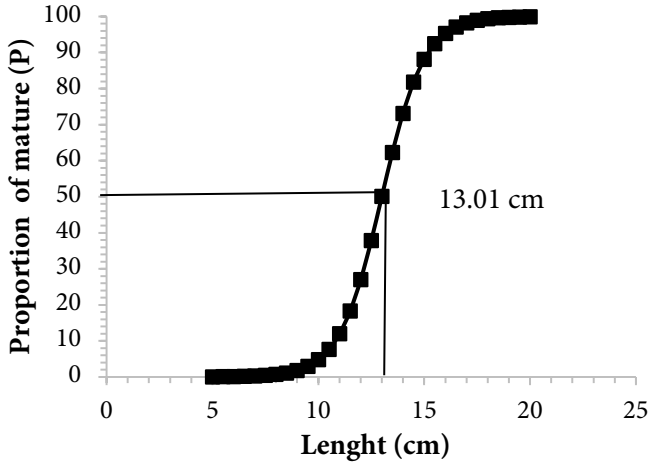

Figure 7. 50% sexual maturity length of male *S. flexuosa*

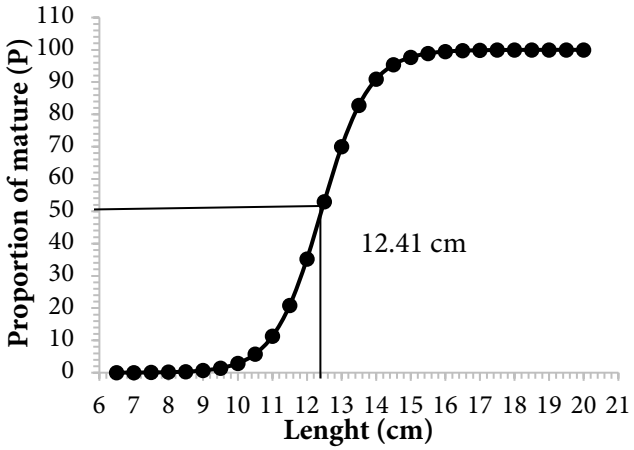

Figure 8. 50% sexual maturity length of female *S. flexuosa*

Size at Maturity

The sexual maturity was calculated using data of 176 female (131 mature) and 41 male (25 mature) individuals sampled in the breeding season in 2 cm length class interval. The maturity length of the breed was calculated as 12.41 cm for females and 13.01 for males. (Figure 7 and Figure 8).

Discussion

Population Structure

Many morphological and genetic studies have been conducted on the *Spicara* genus. From these studies, it has been discussed that *Spicara smaris* species sampled in the waters of our country can be distinguished from *S. flexuosa* and *S. maena* species morphologically and that *S. flexuosa* and *S. maena* species can be the same species. (İlkyaz et al., 2007; Froese & Pauly, 2018). However, in another study conducted recently and in which up-to-date genetic analysis of the species has been carried out, it was stated that all of the picarel living on the Black Sea coast were *S. flexuosa*, and the other two species probably did not enter the Black Sea due to their salinity tolerance (Bektaş et al., 2018). In the light of the mentioned study, it is thought that the fish studied in the research conducted by Şahin & Genç (1999) and İşmen (1995) previously in the Black Sea were *S. flexuosa* instead of *S. smaris*. In the study, the smallest individual was sampled in April, and an intense recruitment to the stock was observed in the same month. The finding of a single male individual in the specimens in May is thought to be due to the species' nesting behavior for spawning. The maturation of the gonads in May supports our interpretation (Figure 4). In another study 440 *S. flexuosa* specimens were examined in Antalya Bay (Mediterranean Sea) and the length range was reported as 9.0-17.3 cm (Özvarol, 2014). Two studies were done in 1989, 1991 and 1992 years in a region (Trabzon coast) very close to the region where our sampling took place.

Among them, Şahin & Genç (1999) reported the length range from 11.1 cm to 22.5 cm for females and 11.3 to 22 cm for males. In another study, it was reported that all individuals varied between 10.0 and 18.5 cm in 1991 and 1992, and no female individuals larger than 17.0 cm were encountered (İşmen, 1995). In the north of the Black Sea, male pickarels can reach 19.4 cm in length and 82 g in weight, and females can reach up to 15.7 cm in length and 45 g in weight (Slastenenko, 1956). The weights of male individuals obtained in the study varied between 7.1 and 129.94 g, while females varied between 11.21 and 109.1 g. Şahin & Genç (1999) reported that male *S. flexuosa* individuals varied between 12.94 and 90.54 g and females varied between 11.88 and 120.03 g. İşmen (1995) reported that *S. flexuosa* fish can be found in the Black Sea between 17.81 and 64.53 g, regardless of gender. Our findings are in agreement with Şahin & Genç (1999) when compared with other studies conducted in the Black Sea. The differences with our study and those two studies would be due to the different sampling methods used.

Length-Weight Relationship

When the relationship between length and weight values obtained in the study was examined, the "b" value was found to be 3.2249 for males, 2.8292 for females and 2.9727 for all individuals. In previous studies in the Black Sea, İşmen (1995) found the "b" value to be 3.26; Şahin & Genç (1999) reported 3.12604 for males and 3.22926 for females, so, they calculated a positive allometric growth in *S. flexuosa*. With the results we have obtained, it is thought that the difference in growth observed in females in previous studies would be due to the nutritional conditions. Erkoyuncu (1995) reported that different populations of the same species or possibly the same population may differ in various years according to feeding conditions.

Age Composition

In the study the dominant age group in females was 1 year (39.6%), while it was 3 years (31.8%) in males. Among the samples examined, the oldest male individual is 7 years old. On the Mediterranean coast of Greece, the dominant age group was 1 year for females and 3 years for males, and the oldest individual was 5 years old (Mytilineou & Papaconstantinou, 1991). Mater et al. (2001) reported that the dominant age group of *S. flexuosa* was 2 years for females and 3 years for males, and the oldest individual was 4 years old in Izmir Bay. In the Black Sea, Şahin & Genç (1999) found the dominant age group to be 2 years for females, 3 years for males, and the oldest individual to be 6 years old, while İşmen (1995) reported that the oldest individual was 5 years old. The data obtained from the study and other studies are in harmony and it has seen that the dominant age group for males in all seas is 3 years. The 7-year-old individual identified in this study is the oldest fish reported from all seas and one can say that the current population is less exploited compared to the other seas. It is also thought that this situation may be related to the physical and chemical parameters of the environment and nutrient abundance.

Growth

Considering to the data obtained in the study, it was calculated as L_∞=33.42 cm, K=0.080 years^{-1}, t_0=-5.381 years for all individuals. The results of studies on stocks of the same species living in different seas are given in table 2. accordingly, the lowest L_∞ value was given as 19.05 cm for female *S. flexuosa* fish in Aegean Sea (Malkav, 2002), while the highest value was reported as 33.52 cm for female *S. flexuosa* fish in Eastern Black Sea (Şahin and Genç, 1999). L_∞ is theoretically defined as the maximum (asymptotic) length of the fish and inversely proportional to the growth coefficient, K. These parameters can vary considerably even within the same species. Generally, the "K" value is higher in warm seas, the "L_∞" value is lower, and in cold seas the "K" value is lower and the "L_∞" value is higher (Erkoyuncu, 1995). As seen in table 2, Şahin and Genç (1995) found the "L_∞" value to be high for females and lower for males, contrary to our study. This result is thought to originate from a single 22.40 cm female individual obtained in the study. Similarly, in our study, the female individual of 20.6 cm obtained from the samples increased the "L_∞" value, therefore single individuals in the age groups for female, male and all individuals were excluded from the calculations. The t_0 value, which corresponds to the age, when the fish length is theoretically zero, is the value that is considered the beginning of the growth curve, which has no biological meaning (Erkoyuncu, 1995). The t_0 value calculated for all individuals in the study is mostly similar when compared to other studies. However, İşmen (1995) gave the t_0 value as -0.01 years. This value differs from many other studies. İşmen (1995) stated in his study that he could not sample the 1-year old group due to the selectivity of the sampling gear. The reason for the change of K value according to years and regions may be due to the different size composition of the studied fish, age composition, biotic (such as prey predator relationship, genetic variation) and abiotic (such as temperature, salinity) environmental factors. The growth performance index (Φ') which was calculated according to Von Bertalanffy growth constants was calculated using the "L_∞" and "K" values and it was found to be Φ'=1.9514 for all individuals, to be Φ'=2.0986 for female individuals and to be Φ'=1.9692 for male individuals.

Table 2. Growth and mortality rates in studies *S. flexuosa*

Reference	L_∞	K	T_0	W_∞	Z	M	F	Φ	Region
İsmen (1995)	20.05	0.44	-0.01	87.20	1.58	0.87	0.71	2.2476	East Black Sea
Şahin (1995)									
Female	33.52	0.125	-2.848	-	-	-	-	2.1602	East Black Sea
Male	27.37	0.192	-2.404					2.1475	
Mater (2001)	19.45	0.205	-0.399	78.31	-	-	-	1.8895	Aegean Sea
Malkav (2002)									
Total	21.86	0.25	-2.36	-	-	-	-	2.077	
Female	19.05	0.23	-2.23	-	-	-	-	1.9215	Aegean Sea
Male	23.97	0.28	-2.28	-	-	-	-	2.2064	
Yeldan et al. (2015)	21.72	0.385	0.135	118.87	-	0.62	-	2.2591	Mediterranean
This study									
Total	33.42	0.080	-5.381	401.24	0.80	0.48	0.32	2.1096	East Black Sea
Female	22.71	0.243	-2.306	118.27	-	-	-	2.0752	
Male	38.34	0.063	-6.381	755.37	-	-	-	1.9675	

In other studies, conducted in the Eastern Black Sea region, İşmen (1995) reported Φ'=2.2476 for all individuals, while Şahin and Genç (1999) reported Φ'=2.1602 (Table 2). The results obtained in the study gave similar results with the previous studies in the Black Sea, and the difference between the values reported in previous studies was not found to be statistically significant ($P > 0.05$, $t_{0,05} = -1.239 \times 10^{-4}$). In population dynamics studies, the growth performance index was proposed to determine the average growth of the species by evaluating the L_∞ and K value together, and it was reported that it could be used to compare the growth under the effect of different environmental factors (Pauly, 1991).

Mortality

In the study, natural and fisheries-related mortality rates of *S. flexuosa* in the Black Sea were found to be 0.4761 $year^{-1}$ and 0.3239 $year^{-1}$, respectively. And the total mortality rate was determined as Z=0.800 $year^{-1}$. When the results were examined it was determined that the stock belonging to the *S. flexuosa* species was operated at a value lower than the optimum value (E=0.5). that means there was no fishing pressure. In the study, it was seen that the fishing mortality rate was caused only because bycatch of small fish. In an other study conducted in the eastern Black sea "Z" value as 1.58 $years^{-1}$ (İsmen. 1995). As given in table 2 when comparing our study with the previous study it is thought that the difference in the mortality rate may have been caused by the fishing pressure by years, the type of fishing gear used and the biotic and abiotic factors that keep growth characteristics under control with the participation of new individuals. In the study area National Statistical Institue is not announce *S. flexuosa* fishing and according to our findings only 12.1 tons fish was captured as discard species from other fishing operations such as purse seining, gill nets.

Sex Ratio

The ratio of male *S. flexuosa* fish to females in this study was 2.19 in favor of females. This rate has been reported as 1.25 in *S. flexuosa* species which have been studied in the Mediterranean for long years (1994-2002) (Ragonese et al., 2004). Mater et al. (2001) reported the ratio of males to females for the species in Izmir Bay as 0.71. In the Black Sea Şahin and Genç (1999) reported the female-to-male ratio as 1.59. The differences between our study and other studies are thought to be due to the variation in sampling gears used and according to the number of individuals sampled by months. Changes in sex ratio may be due to differences in mortality by sex and migration.

Spawning Season

When the monthly GSI values and gonad development stages are examined, *S. flexuosa* shows reproductive activity between May and September during the study period. Şahin & Genç (1999) stated that the GSI value of *S. flexuosa* in the Black Sea reached the highest in May. Fisher et al. (1987) reported that the breeding activities of the *S. flexuosa* fish in the Black Sea were carried out between May and September. Our results are in agreement with other studies. Determining the breeding season and the first breeding length of the fish species living in the ecosystem, accordingly, determining the minimum fishing length in the product to be landed and the season in which the fishing will take place is extremely important for a sustainable fishing model (İlkyaz et al., 2018). In the Communique Regulating Fisheries for Commercial Purposes published in 2016 by the Ministry of Food Agriculture and Livestock (Republic of Turkey) there is no regulation for *S. flexuosa* fishing in our country. There is a lack of information regarding the first breeding length and minimum landing size in the studies of the *S. flexuosa* species. Soykan et al. (2010) reported the first breeding length for *S. maena* as 11.51 cm for females and 13.12 cm for males in their study in the Aegean Sea and İlkyaz et al. (2018) recommended the minimum fishing length for the species as 13 cm according to the results obtained.

Conclusion

Regular studies and monitoring of natural stocks are essential for both fisheries management and fisheries biology to ensure the sustainability of the ecosystem. Especially in the Black Sea, conducting researches on the biology and population characteristics of fish that are not subject to fishing prohibition and monitoring the effects of these species on the ecosystem are important in terms of the balance of fish stocks. The results would be useful for optimum management and future positive fisheries management strategies.

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Compliance with Ethical Standards

Authors' Contributions

Author GD designed the study, IOE and HO wrote the first draft of the manuscript, YC performed and managed statistical analyses. All authors read and approved the final manuscript.

Conflict of Interest

The authors confirm that no conflicts of interest exist and the funders had no role in study design data collection, analysis, and decisions.

Ethical Approval

For this type of study, formal consent is not required.

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RESEARCH ARTICLE

Effects of two different macroalgae (*Ulva lactuca* and *Jania rubens*) species on growth and survival of juvenile red swamp crayfish (*Procambarus clarkii*) as feed additive

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Survival

ABSTRACT

The effects of dietary supplementation of two different macroalgae *Ulva lactuca* and *Jania rubens* on the growth performance, survival and feed conversion ratio of juvenile red swamp crayfish juvenile (*Procambarus clarkii*) were investigated. Red swamp crayfish with an average total length of 56.2±6.67 mm and an average weight of 3.77±0.2 g were placed at tanks (10 crayfish at each tank) and offered diets 8 weeks. Different levels of macroalgae were added to commercial sea bass feed, and no seaweed was used as a control group. It was observed that crayfish fed with 10% feed had higher growth performance (in terms of length and weight) than those fed with 15% diet and control group ($P<0.05$). The lowest feed conversion rate was observed in juvenile crayfish fed with 15% feed ($P<0.05$). The highest survival rate was 50.0% at group fed with 15% feed, followed by 46.66% (control group) and 43.33% (10% diet groups), respectively. This study showed that there was no statistical difference in survival rate among treatment groups ($P>0.05$). However, the frequency of molting was mostly observed in the group fed with 10% diet. Therefore, the results showed that seaweed (*Ulva lactuca* and *Jania rubens*) could be used as a supplement for red swamp crayfish diet (*Procambarus clarkii*) at 10% to improve growth performance with no adverse effects on feed efficiency or survival rate.

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Introduction

The red swamp crayfish *Procambarus clarkii* is an important commercial aquaculture freshwater crayfish with an annual globally production of almost 700 tons (FAO, 2017). *P. clarkii* is successfully grown in most tropical regions of the world due to its rapid growth at low oxygen levels and high temperatures (23-31°C), high egg productivity and the ability to grow and reproduce rapidly (Mazlum & Eversole, 2004, 2005). Crayfish are invertebrates and have polytrophic feeding habits. Crayfish are classified as herbivorous, detritivorous, omnivorous and sometimes mandatory carnivores (Correia, 2002; Nystrom, 2002; Mazlum & Yilmaz, 2012; Mazlum & Şirin, 2020). They are capable of living in many habitats in terms of physiological, morphological and behavioral characteristics. Crayfish are abundant and predominantly found among all invertebrates. This organism play an important role in the freshwater food chain by feeding on the residues and detritus of thousands of animals, from living and rotten plants, cereals, algae and vertebrates to smaller vertebrates such as small fish species (Lodge et al., 2012; Twardochleb et al., 2013).

Recently, seaweed (known as macroalgae) have been drawing attention as alternative protein sources for aquaculture as nutritional content of aquatic organisms due to their relatively high protein values (Sinurat & Fadjriah, 2019) , essential amino acid content, vitamins and trace metals (Morais et al., 2020; Peñalver et al., 2020) and also low cost availability in tropical countries (Nakagawa & Montgomery, 2007). Annual seaweed production was reported to be 31.2 million tons in 2016 (Buschmann, 2017; Ferdouse, 2018), and the seaweed industry worldwide was over 6 million USD (FAO, 2018). However, it is still difficult to use the applicable and sustainable methodology in seaweed cultivation and to be economical and productive in various parts of the world. (Golberg et al., 2020; García-Poza et al., 2020).

Ulva lactuca (Chlorophyta) and *Jania rubens* (Rhodophyta) are the dominant seaweed plants in Iskenderun Bay. The biochemical composition, nutritional ingredient and mineral composition of these two species were previously evaluated by Turan et al. (2015). *U. lactuca* is also known as "sea lettuce", which has high nutritional value due to its high polysaccharides, proteins, vitamins and trace minerals (Ashour et al., 2020). *Jania rubens* has significant nutrient compounds such as protein, lipids and carbohydrates (Morais et al., 2020). The nutritional value of dietary supplements of seaweed in aquaculture is mostly estimated in terms of growth performance, feed intake and survival rate. It has been revealed by the researchers seaweed have a good vitamin and mineral profile and are particularly rich in ascorbic acid (Ortiz et al., 2006; García-Casal et al., 2007).

The use of seaweed as a feed supplement has shown favorable positive effects on human and animal health and nutrition. These findings by scientific researchers have revealed that the application of seaweed or extract as a feed additive has many benefits. Adding seaweed to diets has been shown to significantly alter the gut flora, improve immunity, and improve growth performance, feed utilization and disease resistance for many aquatic animals and reduced nitrogen release to the environment (Ashour et al., 2020). Therefore, studies conducted with the addition of macroalgae to feed in recent years have focused on both shellfish and fish species. These studies mainly on pacific shrimp (Rodríguez-González et al., 2014; Cárdenas et al., 2015), trout (Soler-Vila et al., 2009; Güroy et al., 2013), European seabass (Valente et al., 2006), African catfish (Abdel-Warith et al., 2016; Al-Asgah et al., 2016), sea bream (Emre et al., 2013), red tilapia *Oreochromis sp.* (El-Tawil, 2010), Nile tilapia (Güroy et al., 2007; Marinho et al., 2013; Valente et al., 2016), Senegalese sole (Moutinho et al., 2018) and Atlantic salmon (Kamunde et al., 2019). They reported that adding macroalgae to feed at low rates did not adversely affect growth. Although some studies have investigated the use of seaweed as a partial feed substitute for the shrimp diet, there is no study on (*Ulva* + *Jania*) being used as a feed additive in the crayfish diet. Therefore, this study is considered as a preliminary study as the determination of alternative feed sources for crayfish is one of the key factors for successful crayfish culture. Thus, the specific purpose of this study was to determine the effect of two different seaweed (*Ulva* + *Jania*) dietary supplements on growth performance, survival and feed conversion rate of juvenile red swamp crayfish.

Material and Methods

The study was conducted at Aquaculture Research and Application Center (ISTE-DUM) breeding laboratory of Iskenderun Technical University, Hatay, Turkey during a period of 60 days.

Experimental Crayfish

Brood crayfish, *Procambarus clarkii,* were obtained from an aquarium pet shop in İskenderun, Turkey. Once the adult crayfish transported to the Aquaculture Research and Application Center (ISTE-DUM), they were kept in troughs and fed with a commercial feed until hatching time. Adult crayfish were fed with a commercial trout diet (50% crude protein and 12% cure lipid) during the acclimation period. A total of 90 juvenile crayfish with an initial mean total length (56.2±6.67 mm) and weight of 3.77±0.2 g were used in this

study. Ten juvenile crayfish were randomly stocked in 9 tanks (100 L water capacity) in triplicate and supported by continuous aeration. Juvenile crayfish were fed twice a day, five days a week. Uneaten food was siphoned out from the aquariums 1 h after feeding. PVC pipe pieces with a diameter of 2-3 cm were placed into all aquariums as shelters. Experimental crayfish were weighed at the beginning of the experiment and then at 4-week intervals for 8 weeks. Water temperature was maintained at 25.2±1.23°C, dissolved oxygen at 4.05±0.48 mg/L (YSI oxygen meter) and pH at 7.49±0.14 (YSI). During the experiment, a 12-hour light and 12-hour dark photoperiod was applied. Crayfish were fed twice a day, on the morning and evening. Growth performance was monitored monthly. Morphometric characteristics of crayfish were measured as total length (mm) and weighed (g) with an electronic balance. Total length measurements were performed at from the rostrum tip to the telson by using a vernier caliper.

Experimental Diets

The macroalgae *Ulva lactuca* and *Jania rubens* were collected from June to July 2019 along the Konacık coastline in Iskenderun Bay. The macroalgae species were collected freshly and the samples were brought to Iskenderun Technical University, Faculty of Marine Sciences and Technology, Algal Biotechnology Laboratory in cooling containers. Macroalgae were identified according to their species, then they were cleaned from pollutants such as sand, epiphyte, and stored at –20°C until analysis after washing and drying processes were performed (Ye et al., 2009). The ash, lipid and protein amounts of macroalgae were determined by following the methods of Vollenweider (1974) and Bligh & Dyer (1959). Nutrient components (Nitrogen (N), Carbon (C), Hydrogen (H)) of algae samples were determined according to method described by Dumas (1831). Methods suggested by Santoso et al. (2006) were followed in the determination of macro (Na, Ca and Mg) and micro mineral contents of *J. rubens* (Rhodophyta) and *U. lactuca* (Chlorophyta). The macroalgae required for the experiment dried in an oven at 50°C for 48 hours, and seaweed powder was prepared. A 55% CP was used in the present study (crude protein for control diet was formulated with two different seaweed).

Diets Formulation and Preparation

The percentage of the biochemical (protein, lipid, ash), nutrient components (Nitrogen (N), Carbon (C), Hydrogen (H) and mineral contents of two different macroalgae species (*J. rubens, U. lactuca*) (g/100g dry weight) are given in Table 1.

Table 1. The percentage of the biochemical (protein, lipid, ash), nutrient components (Nitrogen (N), Carbon (C), Hydrogen (H)) and mineral contents of two different macroalgae species (*J. rubens, U. lactuca*) (g/100g dry weight).

Species	*Ulva lactuca*	*Jania rubens*
Chemical Composition		
Crude protein	16.89±0.12	5.991±0.773
Lipid	1.08±0.33	0.39±0.103
Ash	26.47±0.20	78.740±0.066
Nutritional Ingredients (%)		
Nitrogen	1.004 ± 1.061	1.716 ± 1.030
Carbon	16.382 ± 0.541	17.415 ± 0.022
Hydrogen	1.702 ± 0.129	1.339 ± 0.007
Micro mineral ingredients		
Fe	97.12 ± 0.71	51.09 ± 0.02
Zn	1.08 ± 0.03	0.09 ± 0.26
Cu	0.53 ± 0.01	0.69 ± 0.12
Macro Mineral Ingredients		
Na	1098±0.07	806±0.22
Ca	16345±0.19	359±0.01
Mg	1934±3.01	973±1.09

The experimental commercial diets were pelleted and placed into the mixer chamber of Alphie (Hexagon Product Development Pvt. Ltd. India) with two different seaweed 3-D mixing feature and 25 min (1000 μ), 20 min (1200μ), 15 min. (1500 μ) at 80 rpm with stirring. Feed sizes were adjusted according to crayfish measurements in 30-day periods. Prepared feeds were stored at +4°C until used in plastic containers. A new diet was formed by adding 10% and 15% seaweed (*U. lactuca and J. rubens*) to commercial sea bass feed containing 55% crude protein and 9.8% cure lipid (Sirin and Mazlum, 2016; Yazıcı et al., 2020).

Growth Performance

Crayfish in the different experimental groups were weighted at the end of the 8th week feeding trail to estimate the growth parameter. Growth parameter was analyzed in terms of final individual crayfish body weight (BW g), weight gain (WG g), specific growth rate (SGR, %day^{-1}), feed conversion ratio (FCR). The formulas were used as follows:

$$\text{WG} = \text{Final weight (g)} - \text{Initial weight (g)}$$

$$\text{SGR} = \frac{\ln \text{final body weight} - \ln \text{initial body weight}}{\text{Rearing duration (60 days)}} \times 100$$

$$\text{FCR} = \frac{\text{Dry feed intake (g)}}{\text{wet weight gain (g)}}$$

$$\text{Survival rate} = \frac{\text{Total number of crayfish harvested}}{\text{Total number of crayfish stocked}} \times 100$$

$$\text{Total Molting Frequency} = \frac{\text{Number of molt crayfish}}{\text{Total number of crayfish}} \times 100$$

Statistical Analysis

The data were analyzed by using the Statistical Package for the Social Sciences software (SPSS, 2012, Version 17.0, SPSS, Chicago, IL, USA). The results were subjected to Levene's test to determine homogeneity of variance and no transformation was required. One-way ANOVA was used to determine the effects of the diets on the various responses including final wet weight, molting frequency, survival, SGR, and FCR for all treatments. Multiple range test (Duncan's) was used to compare means of treatments. Results were considered to be significant at the (P<0.05) level. Mean values were presented with ± standard deviation (SD) in tables.

Results

The initial total length (ITL) and initial body weight (IBW) of the treatment group were the same at the beginning of the experiment (P>0.05). The results of this study indicated that final total length (FTL), final body weight (FBW), weight gain (WG), specific growth rate (SGR) and feed conversion ratio (FCR) of red swamp crayfish (*Procambarus clarkii*) were affected (P<0.05) by using different levels of seaweeds (*U. lactuca*) in the diet (Table 2).

Juvenile crayfish fed on control and experimental groups (10% and 15%) attained 62.57, 66.15 and 60.06 mm final length. Final total length was found significantly different among the treatment groups (Table 2). The highest FBW, WG and SGR%/day values were obtained with the crayfish kept at 10% level, were found to be 7.72 g, 3.95 g (Table 2) and 1.19 g% day (Figure 1), respectively. Followed by crayfish maintained control and %15 level did not significant differences (P<0.05) (Table 2). However, the least values of FBW, WG and SGR%/day were recorded with crayfish maintained at 15% level with the value of 6.25 g, 2.46 g and 0.83 g%/day, respectively (Table 2).

FCR was found to be 2.25, 1.98 and 3.07, respectively. FCR did not enhanced with increasing the level of addition rate of seaweed in the diet. The substantial improvements (P<0.05) in feed conversion ratio were achieved when seaweed level in the diet 10%. The best SGR was detected with the crayfish maintained at 10% seaweed level (1.19). It was considerably different from SGR of the crayfish maintained at 15% seaweed level and control group. Lastly, the lowest (P<0.05) SGR value (0.83) was detected with crayfish maintained at 15% seaweed level in the diet. At the end of the experiment, the survival rate was the same among treatments, and there was no significant difference (P>0.05). The highest survival rate (50.0%) was obtained using 15% diet, followed by control diet (46.66%), and then by 10% diet (43.33%). However, molting frequency was mostly observed in the group fed with 10% diet.

Table 2. Supplementation of macroalgae on growth performance of red swamp crayfish (*Procambarus clarkii*) fed at different dietary levels.

	Treatments		
Parameter	**Control**	**10%**	**15%**
ITL(mm)	56.2±6.67[a]	56.2±6.67[a]	56.2±6.67[a]
FTL (mm)	62.57±5.21[a]	66.15±6.02[b]	60.06±7.43[a]
IBW (g)	3.76±0.09[a]	3.77±0.01[a]	3.79±0.15[a]
FBW (g)	7.13±1.86[a]	7.72±2.92[b]	6.25±2.26[a]
WG (g)	3.37±0.08[a]	3.95±0.06[b]	2.46±0.02[c]
SGR (%/day)	1.06±0.02[a]	1.19±0.010[b]	0.83±0.006[c]
FCR	2.25±0.02[a]	1.98±0.01[b]	3.07±0.09[c]
SR (%)	46.66[a]	43.33[a]	50.0[a]
MF (%)	53.33[a]	66.66[a]	56.66[a]

Note: Means values expressed as mean± standard deviation (SD). Means in each row followed by different letters are significantly different (P<0.05). Initial total length (ITL mm), Final total length (FTL mm), Initial body weight (IBW), Final body weight (FBW gr), Weight gain (WG gr), specific growth rate (SGR %/day), feed conversion ratio (FCR), Survival rate (SR %) and Molting frequency (MF %)

Discussion

Seaweed is alternative protein sources for aquaculture as nutritional content of aquatic organism due to their relatively high protein values, essential amino acid content, vitamins and trace metal and production rates in tropical countries (Nakagawa & Montgomery, 2007). It has been reported that seaweed chemical structure can be affected several parameters such as by temperature, light, salinity or food source during cultivation (Sinurat & Kusumawati, 2017). The results of this study showed that different level of seaweed supplementation improved the growth performance and feed efficiency of red swamp crayfish with no adverse effects in the diet (Table 2). Similar results were reported by Qiu (2017) could addition up to 2% fish meal in shrimp feed without causing negative effects on the growth of shrimp. He noted that adding up to 2% to shrimp feed did not have a negative effect on shrimp growth.

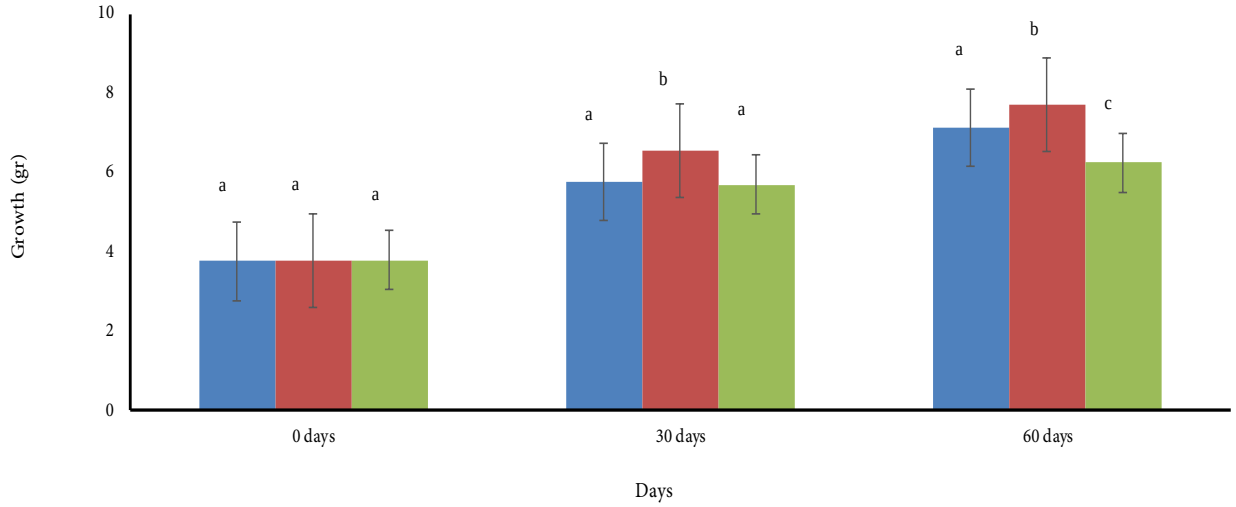

Figure 1. Mean and standard deviation of red swamp crayfish growth for macroalgae added to feed in an 8-week study.

Also, in a number of studies, involvement levels of less than 5% of seaweed meals include Pacific white shrimp (Rodríguez-González et al. 2014; Cárdenas et al., 2015), rainbow trout (Soler-Vila et al., 2009; Güroy et al., 2013), European perch (Valente et al., 2006), African catfish *Clarias gariepinus* (Abdel-Warith et al., 2016; Al-Asgah et al., 2016), gilthead seabream *Sparus aurata* (Emre et al., 2013) red tilapia *Oreochromis sp.* (El-Tawil, 2010) and Nile tilapia (Güroy et al., 2007; Marinho et al., 2013; Valente et al., 2016) were reported not to adversely affect growth.

However, in many studies, the results of low, medium, and high inclusion have shown a discrepancy between the levels of seaweed meals. Qiu (2017) reported that high level of *Ulva* meal as an additional in meal resulted in depressions in shrimp growth performance. In other studies, *Ulva rigida* species added to Nile tilapia feed were reported to have the best performance at the level of 5% algae inclusion and decreased by 10-20% (Diler et al., 2007; Güroy et al., 2007). Rodríguez-González et al. (2014) reported that adding *U. lactuca* meal to the diet meaningfully reduced the weight of *Litopenaeus vannamei* by 10% and 15%, whereas shrimps fed diets containing similar levels of seaweed (*Gracilaria parvispora*) showed no change in weight gain. In a study by Felix and Brindo (2014), they showed that 10, 20, and 30% inclusion of raw *U. lactuca* flour in the diet causes a decrease in growth performance in the giant freshwater shrimp *Macrobrachium rosenbergii*. Ergun et al. (2009) stated that the inclusion of 5% Ulva flour (*U. rigida*) in diets improved *Oreochromis niloticus* growth performance, feed efficiency, and nutrient and body composition. They concluded that Ulva meal can be added to feeds as a dietary component. In addition, Nakagawa et al. (1993) showed that adding 2.5-5.0% of the Ulva meal to the diet gives good results. Therefore, in our study, crayfish fed a diet supplemented with 10% seaweed (*Ulva* + *Jania*) showed a slight increase in growth performance, whereas these parameters decreased for crayfish fed with a 15% diet. At the end of this study, it shows that the growth performance of red swamp crayfish was affected by the addition of various levels of seaweed in the diet. The highest values of final live weight, weight gain and SGR %/day were obtained by keeping crayfish at 10% level. Increasing the level of Ulva added to the food above 15% did not have a significant effect on growth compared to the control group. These results are consistent with the results of previous studies in terms of growth performance.

Azaza et al. (2008) concluded in their study that green algae (*U. rigida*) can be included in *Oreochromis niloticus* diets up to 20% no any adverse effects on growth parameters. Güroy et al. (2007) observed that two seaweed meals effects (*U. rigida* and *Cystoseira barbata*) on feed intake, growth, and food use at three levels (5%, 10% and 15%) of young Nile tilapia (*Oreochromis niloticus*). They concluded that the adding of 5% ulva meal to the fish diet did not have a adverse effect on growth performance, feed use and body composition; however, they stated that using levels between 10% and 20% may result in negative consequences.

Valente et al. (2006) found that the young sea bass *Dicentrarchus labrax* had maximum fish growth performance in 10 weeks when 10% seaweed (*Ulva sp.*) was added to their diet. Elmorshedy (2010) showed that the starting body weight of 0.094 g in mullet *Liza ramada* significantly increased with the body weight, weight gain and specific growth rate up to 28% seaweed level (*Ulva sp.*) In addition, Diler et al. (2007) recommended that the addition of 5 to 15% dietary Ulva pulp instead of wheat flour in carp diets better growth and could be

suitable for carp. Also, Guroy et al. (2007) indicated that fish mass gain was high of *Oreochromis niloticus* fed diets added with numerous levels of Ulva flour were achieved for in fish fed 5 to 10% Ulva diets. They think that the differences in the results of certain proportions of seaweed can be variable depending on dietary habits, age, and species of algae and fish.

Our result showed that 10% seaweed significantly increased the growth performance of red swamp crayfish ($P<0.05$). Mustafa & Nakagawa (1995) stated that adding algae as a feed supplement significantly improves growth, digestive efficiency, disease tolerance, chemical composition and quality meat. In addition, Nakagawa & Montgomery (2007) stated that fish diet prepared with the exception of fish fed a diet containing 25% *Ulva* has a positive effect on FCR. However, previous study results such as Güroy et al. (2007) and Diler et al. (2007) showed that adding 20% *Ulva* meal to the diet effect negatively on FCR value.

Conclusion

This study supports previous outcomes displaying that seaweed can be used as possible additive in crayfish feed. Inclusion of seaweed up to 10% has shown that the red swamp crayfish allows for an increase in growth performance and feed utilization. However, the extent to which higher feed additions and continued long-term testing affect growth and feed use should be studied in more detail. The results of this study concluded that the use of seaweeds can provide dietary alternatives supplement of juvenile crayfish as they increase growth performance and feed utilization. With the results obtained, it was concluded that seaweed can be supplemented with an optimum 10% diet of red swamp crayfish (*Procambarus clarkii*) no adverse effects to increase growth parameter.

Compliance with Ethical Standards

Authors' Contributions

YG: experimental design, feed preparation, data collection, writing and analysis, original draft review and editing. MY: data collection, feed preparation, original draft review and editing. SS: supply macroalgae, analysis of macroalgae, species identification, review and editing. OH: analysis of algae SU: analysis of algae.

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors. According to scientific research procedures in Turkey, if you are investigating arthropods such as shrimp and crayfish you do not have to get ethical permission.

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RESEARCH ARTICLE

Investigations on endohelmint fauna of teleost fishes of Aras and Murat Rivers in Turkey

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ABSTRACT

In this study which has been done between April 2008 and June 2009, fishes captured from Ağrı and Erzurum were examined by endoparasite fauna. Examined fishes are as follows: *Acanthobrama marmid* Heckel, 1843, *Alburnus akili* Battalgil, 1942, *Barbus plebejus* Bonaparte, 1839, *Barbus mursa* Güldenstädt, 1773, *Capoeta barroisi* Lortet in Barrois, 1894, *Capoeta capoeta* Güldenstädt, 1773, *Cyprinus carpio* Linnaeus, 1758, *Leuciscus cephalus* Linnaeus, 1758. Consequently, a total of 908 individual parasites were detected from six parasite species; *Rhabdochona denudata* Dujardin, 1845 (Nematoda), *Neoechinorhynchus* sp. and *Pomphorhynchus* sp. (Acanthocephala), *Bothriocephalus acheilognathi* Yamaguti, 1934, and *Caryophyllaeus laticeps* Pallas, 1781 (Cestoda), *Allocreadium isoporum* Looss, 1894 (Digenea). The distribution of the infection prevalence, mean intensity, and abundance values of parasite species were determined. As a result of our study, 93 of 233 (39.91%) fish were reported with parasites.

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Introduction

Fishes are one of the important basic nutritional elements in animal food sources and provide high-quality protein and a large variety of vitamins and minerals (Öztürk, 2005; Balami et al., 2019). Fishes are constantly together with parasites in the natural environment, parasitic diseases constitute one of the most important problems of fisheries (Taşçi & Topçu, 1990; Öztürk, 2005; Aydoğdu & Selver, 2006). Parasites exogenously live in the gills, skin, fins, and eyes, endogenously live in various internal organs of fish species (Dörtbudak et al., 2019). It is reported that almost 50% of the fish larvae die from parasitic infections in crowded pools (Dörücü & Mutlu, 2008). Parasites are reducing the nutritional value of the fish and also prevent them from growing, reproducing, and feeding (Özan & Kır, 2005).

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Helminth parasites can cause disease in the tissues and organs of the fish (Aydoğdu & Selver, 2006). During their development, helminths cause effects such as poor appetite, discoloration, swimming disorder, blindness, weakness, hemorrhage in tissues, and gill and skin deformities. Especially inflammation and degeneration in the intestines lead to fish deaths (Dörtbudak et al., 2019; Aktürk et al., 2020). The subject of fish diseases increasing aquaculture sector in Turkey has gained great importance day by day. So, it is necessary to know the parasite fauna of fish in inland waters (Karatoy & Soylu, 2006).

This study aimed to investigate the endohelmint fauna of teleost fishes of Aras and Murat Rivers in Turkey.

Material and Methods

This study was carried out between April 2008 and June 2009. Fish samples were caught using fishing nets and fishing rods from between Pasinler and Köprüköy in Erzurum (39°57' N-41° 51' E) and from Tutak (39° 29' N-42° 40' E), Hamur (39° 36' N-42° 57' E), and Taşlıçay (39° 35' N-43° 35' E) regions of the Ağrı as shown in Figure 1.

A total of 233 fish specimens were evaluated as part of this study, of which 98 were from the Murat River (Ağrı) and 135 were from the Aras River (Erzurum). The fish specimens are, *Acanthobrama marmid* Heckel, 1843, *Alburnus akili* Battalgil, 1942, *Barbus mursa* Güldenstädt, 1773, *Barbus plebejus* Bonaparte, 1839, *Capoeta barroisi* Lortet in Barrois, 1894, *Capoeta capoeta* Güldenstädt, 1773, *Cyprinus carpio* Linnaeus, 1758, *Leuciscus cephalus* Linnaeus, 1758 species.

The fish specimens were transported as alive within aquariums to the Atatürk University Parasitology Laboratory of the Biology Department of Faculty of Science. The method follows by Kuru (1975), Balık & Ustaoğlu (1992), Geldiay & Balık (1996) were used to identification of the fish species. In this study, the body cavities, livers, stomachs, and intestines of fishes were examined in terms of helminth fauna. Only helminths were found in the body cavity, stomach, and intestines. After the examinations were completed with aid of a binocular stereomicroscope (Olympus BH-2, Japan), the nematodes were taken into plastic tubes containing 70% alcohol. These tubes were labelled with the date, the fish species, parasite type, and count, and were stored. Preparations out of these were made by covering them with glycerin jelly. Other parasites were taken between the glasses and cover slides and were fixed using A.F.A solution. These were stained as suggested by Pritchard & Kruse (1982).

Identification of the parasites was performed using the guidelines specified by the researcher (Markevich, 1951; Bykhovskaya-Pavlovskaya, 1962; Yamaguti, 1963a-c). The prevalence, mean intensity and mean abundance percentage values were calculated as suggested by Bush et al. (1997).

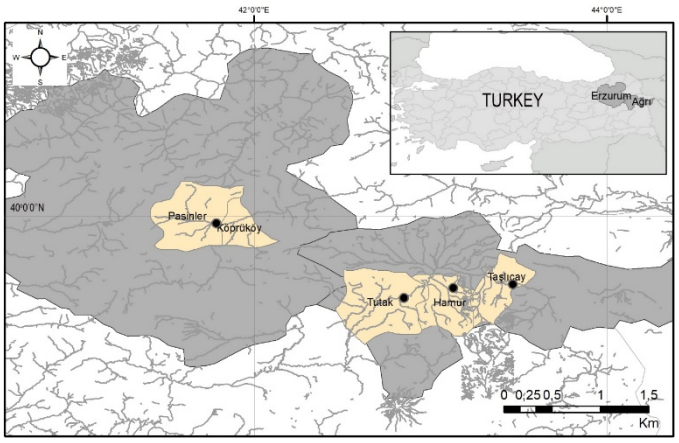

Figure 1. Map showing the Aras and Murat Rivers with black circles where the fish caught

Results

A total of 233 fish were caught as part of this study, 98 of which were from the Murat River and 135 from the Aras River. These 233 fish consist of 17 *Acanthobrama marmid,* 11 *Alburnus akili*, 4 *Barbus mursa*, 22 *Barbus plebejus*, 156 *Capoeta capoeta*, 15 *Capoeta barroisi*, 3 *Cyprinus carpio,* and 5 *Leuciscus cephalus* of which 93 (39.91%) were found to be infected with parasites. The inspections have revealed two species of Cestoda (*Bothriocephalus acheilognathi, Caryophyllaeus laticeps*), two species of Acanthocephala (*Neoechinorhynchus* sp., *Pomphorhynchus* sp.), one species of Nematoda (*Rhabdochona denudata*), and one species of Digena (*Allocreadium isoporum*) to be present amongst the fish helminths (Figure 2).

Of those, *Bothriocephalus acheilognathi* was found in *Cyprinus carpio* caught from the Aras River, *Caryophyllaeus laticeps* was found in *Acanthobrama marmid* and in *C. capoeta* caught from the Aras River, *Pomphorhynchus* sp. was found in the *Leuciscus cephalus, C. capoeta* and *Barbus plebejus* caught from the Aras River, while no *Pomphorhynchus* sp. was determined in fish caught from the Murat River. *Allocreadium isoporum,* on the other hand, was determined only in *Barbus plebejus* caught from the Murat River. *Neoechinorhynchus* sp. was determined in *Capoeta capoeta* and *Barbus plebejus* caught from the Aras River and in *Capoeta barroisi* caught from the Murat River. *Rhabdochona denudata* was present in *Capoeta capoeta* and *Barbus plebejus* of both Murat and Aras River fish, in addition to the *Barbus mursa* of the Aras River. No parasites were determined in *Leuciscus cephalus* and *Alburnus akili* fish caught from the Murat River (Table 1).

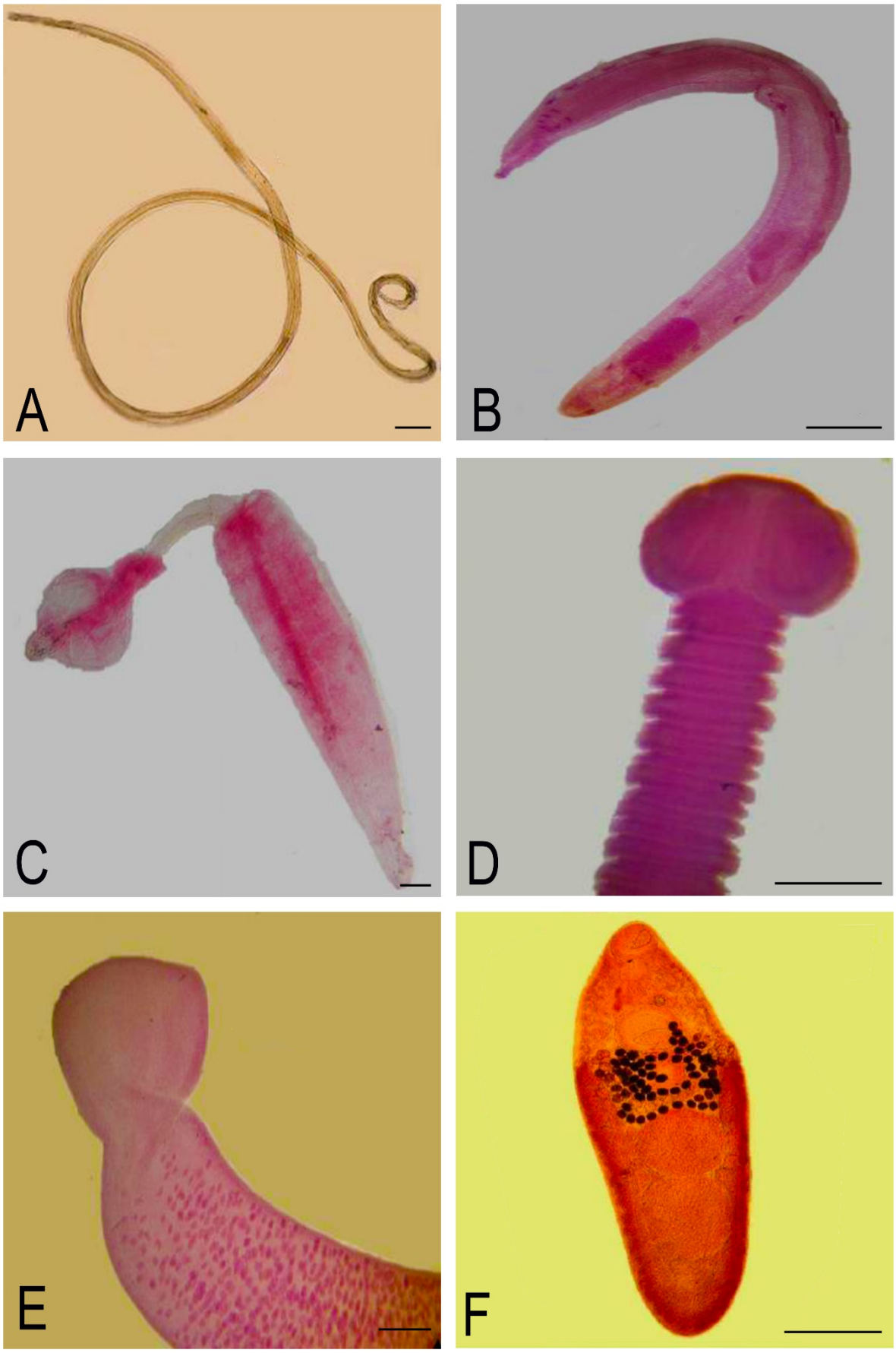

Figure 2. Some types of helminths A) *Rhabdochona denudata* (Dujardin, 1845) Railliet, 1916 (♂), B) *Neoechinorhynchus* sp. Stiles and Hassall, 1905 (♂), C) *Pomporhynchus* sp. Monticelli, 1905 (♀), D)*Bothriocephalus acheilognathi* Yamaguti, 1934 scolex parts, E) *Caryophyllaeus laticeps* (Pallas, 1781) Lühe, 1910 anterior part, F) *Allocreadium isoporum* (Looss, 1894) Looss, 1902, Scale bars: 0.5 mm.

Discussion

Parasitic nematodes are very commonly found in fish. While there are numerous studies performed on nematodes in Turkey (Türkmen & Tüzer, 1992; Aydoğdu & Altunel, 2002; Öztürk et al., 2002; Kır & Özan, 2005; Özan & Kır, 2005), studies involving *Rhabdochona denudata* which we were able to identify as part of the present study are quite limited studied in Turkey. The presence of *Rhabdochona* species in freshwater fishes in Turkey was first determined in Balıklı Kaplıca and Topardıç streams (Sivas), in *C. macrostomus* and *G. rufa* (Saygı & Bardakçı, 1990).

In the present study, only one nematode species *Rhabdochona denudate* was determined. The prevalence of *R. denudata* in *C. capoeta* fish caught from the Aras River was determined as 12%, while mean intensity was determined as 2.82 and mean abundance was determined as 0.33. In the *C. capoeta* fish caught from the Murat River, its prevalence was 5%, the mean intensity was 2.33, and the mean abundance was 0.11. For the *B. plebejus* caught from the Aras River, its prevalence was determined as 15.4%, while its mean intensity was 1.5 and its mean abundance was 0.23, but for the same species of fish caught from the Murat River, these values are 11%, 1, and 0.11, respectively. The highest prevalence for *R. denudata* is determined in the *B. mursa* fish caught from the Aras River with 25%.

In studies conducted in Turkey related to Acanthocephalan, it is reported that *Neoechinorhynchus* is quite common. In a study performed in the İznik Lake, seven out of 145 common carps (*Cyprinus carpio*) were found to have *Neoechinorhyncus rutile* (Aydoğdu et al., 1997). The same parasite was reported to be present in (with a total count of 14) in 37 *Capoeta trutta* fish caught from the Kocakale region of the Keban Dam Lake of the Elazığ province into which the city sewers are being discharged (Sağlam & Sarıeyyüpoğlu, 2002). It is reported that 165 out of the 423 (39.01%) sailton pupfish (*Aphanius chantrei*) caught from the Sarıkum Lagoon (Sinop province) were infected with *Neoechinorhyncus rutile* (Öztürk, 2005).

Another acanthocephalan species, *Pomphorhynchus laevis*, was reported in a total of seven fish distributed amongst the *Leuciscus cephalus*, *Carassius carassius*, *Carassius auratus*, *Nemacheilus* sp., and *Alburnus alburnus* species caught from the Enne Dam Lake (Kütahya) (Koyun, 2001). Buhurcu (2006) reports that 21 *Alburnus nasreddini* out of 34 (61.8%) caught from the Akşehir Lake were infected with *Pompmhorhynchus laevis*.

In the present study, the prevalence of *Neoechinorhynchus* sp. in *Capoeta capoeta* fish caught from the Aras River was determined as 58%, while the median intensity was 2.2 and the median abundance was 1.3. No acanthocephalan species were encountered in *Capoeta capoeta* fish caught from the Murat River, however. While the *Barbus plebejus* fish caught from the Aras River were found to have a *Neoechinorhynchus* sp. prevalence of 23.1%, the median intensity of 5, and median abundance of 1.1, no *Neoechinorhynchus* sp. was encountered in *Barbus plebejus* from the Murat River. That being said, all (100%) of the *Capoeta barroisi* fish caught from the Murat River were found to be infected with *Neoechinorhynchus* sp., for which the median intensity and median abundance was determined as 43.80. The parasite was not encountered in any other fish species caught from either of the Aras and Murat Rivers. In the present study, *Pomphorhynchus* sp. prevalence for the Aras River alone was determined as 6% for *Capoeta capoeta*, 15.4% for *Barbus plebejus*, and 50% for *Leuciscus cephalus*.

In the studies performed related to *Bothriocephalus acheilognathi* in Turkey; 4 out of 72 (5%) common carps (*Cyprinus carpio*) caught from the İznik Lake were found to be infected with *Bothriocephalus acheilognathi,* and in 13 out of 72 (18%) zander fish (*R. frisii*) (Türkmen & Tüzer, 1992). Oğuz et al. (1996) report that of the 46 common carps (*Cyprinus carpio*) caught from the Apolyont Lake, a total of 9 *Bothriocephalus* sp. were identified. The same parasite was encountered in 54 out of the 337 *Alburnus alburnus* caught from the Enne Dam Lake (Koyun, 2001). *Caryophyllaeus laticeps* is also commonly encountered in Turkey. A study has reported 25 out of 72 (35%) carps (*Cyprinus carpio*) in İznik Lake contained the parasite (Türkmen & Tüzer, 1992), while in Kovada Lake the rate of infection is 58 out of 147 common carps (*Cyprinus carpio*) (Becer & Kara, 1998). *C. laticeps* was also encountered in the carps living in the Dalyan Lagoon (Karacabey) (Aydogdu et al., 2001), while mirror carps of the Seyhan River were found to contain *Caryophyllaeus* sp. (Cengizler et al., 2001) and *C. laticeps* was determined in the freshwater trouts (*Abramis brama*) of the Terkos Lake (Karatoy & Soylu, 2006). Furthermore, Soylu (2006) reported the presence of *C. laticeps* (Cestoda) in the white seabream fish inspected in their study.

In the present study, the prevalence of *Bothriocephalus acheilognathi* in a single *C. carpio* of 11.5 cm length caught from the Aras River was recorded as 33%, with a mean intensity of 2 and mean abundance of 0.67. *Caryophyllaeus laticeps*, on the other hand, were found in two specimens of *Capoeta capoeta* amongst the 94 total caught from the Aras River with 2% prevalence, mean intensity of 1, and mean abundance of 0.02.

Table 1. Distribution of the identified parasites based on fish species

		Aras River					Murat River			
	Fish species	*A.marmid*	*B.mursa*	*B.plebejus*	*C.capoeta*	*C.carpio*	*L.cephalus*	*C.barroisi*	*C.capoeta*	*B.plebejus*
Number of fish samples		17	4	13	94	3	4	15	62	9
Parasitic Fish Count	*A. isoporum*	0	0	0	0	0	0	0	0	1
	B. acheilognathi	0	0	0	0	1	0	0	0	0
	C. laticeps	1	0	0	2	0	0	0	0	0
	Neoechinorhynchus sp.	0	0	3	55	0	0	15	0	0
	Pomphorhynchus sp.	0	0	2	6	0	2	0	0	0
	R. denudata	0	1	2	11	0	0	0	3	1
Infection Rate (%)	*A. isoporum*	0	0	0	0	0	0	0	0	11.1
	B. acheilognathi	0	0	0	0	33.3	0	0	0	0
	C. laticeps	5.9	0	0	2.1	0	0	0	0	0
	Neoechinorhynchus sp.	0	0	23.1	58.5	0	0	100	0	0
	Pomphorhynchus sp.	0	0	15.4	6.4	0	50	0	0	0
	R. denudata	0	25	15.4	11.7	0	0	0	4.8	11.1
Total Parasite Count	*A. isoporum*	0	0	0	0	0	0	0	0	2
	B. acheilognathi	0	0	0	0	2	0	0	0	0
	C. laticeps	1	0	0	2	0	0	0	0	0
	Neoechinorhynchus sp.	0	0	15	123	0	0	657	0	0
	Pomphorhynchus sp.	0	0	7	14	0	3	0	0	0
	R. denudata	0	40	3	31	0	0	0	7	1

Only one out of the 17 *A. marmid* fish caught from the Aras River contained the parasite, resulting in 6% prevalence, mean intensity of 1, and mean abundance of 0.06.

Many researchers have performed studies on Digeneans. One such study reports that, amongst the 26 common rudds (*Scardinius erythropthalmus*) obtained from the Apolyont Lake, seven were infected with *Asymphylodora markewitschi* (Oğuz & Öztürk, 1993). Zander fish (*Stizostedion lucioperca*) of the Eğridir Lake were found to contain *Bucephalus polymorphus* (Yıldırım et al., 1996). According to a study performed on the *Barbus* fish of the Doğancı Dam Lake reports that a total of 35 *A. isoporum* were observed in the 47 fish included in the study (with a 19.1% prevalence) (Aydoğdu & Altunel, 2002).

In this study, only *Allocreadium isoporum* (Digenea) was found in 16 cm length one *B. plebejus* caught from the Murat River. The average prevalence of *A. isoporum* was found to be 11%.

Among the reasons for the differences observed in the study, the location where the fish are caught, pollution rate of water, host and intermediate host population, seasonal variations, and methods used can be included.

Conclusion

A large portion of the parasites determined as part of the study consists of *Neoechinorhynchus* sp. of Acanthocephala phylum. It is followed by the *Rhabdochona denudata* which is a nematode, and *Pomphorhynchus* sp., which is an acanthocephalan. While *Bothriocephalus acheilognathi*, *Caryophyllaeus laticeps,* and *Allocreadium isoporum* were also identified, there were in small numbers. This study was an attempt to determine the endohelminth species in the fish fauna of Aras and Murat Rivers, and the prevalence, intensity, and abundance values of encountered parasite species were evaluated. We believe that our findings will provide useful information for future studies.

Acknowledgements

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"*Endohelminths Encountered in Certain Cyprinides of Murat and Aras Rivers*", and in the 17th National Parasitology Congress-the Caucasus and the Middle East Parasitic Diseases Symposium held in Kars between 04-10 September 2011 with the title "*The Relationships of Endohelminths Encountered in Cyprinids Caught from Aras River with the Fish Length*".

Compliance with Ethical Standards

Authors' Contributions

BAÇ and MCO designed the research plan and organized the study. BAÇ performed the fieldwork, collected the samples, carried out the laboratory work, analyzed the data, and wrote the manuscript. MCO read and approved the final manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

For this type of study, formal consent is not required.

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RESEARCH ARTICLE

Extraction and characterization of polyhydroxybutyrate (PHB) from *Bacillus flexus* MHO57386.1 isolated from marine sponge *Oceanopia arenosa* (Rao, 1941)

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ABSTRACT

Polyhydroxybutyrate (PHB) is the most widely studied biodegradable plastic that does not release any toxins or residues in the environment like petroleum based plastics. This work has been undertaken to screen PHB accumulating microorganisms from marine sponges and a total of sixteen isolates were collected and purified. Screening of isolated strains was done by Nile blue staining and observed under Leica LSCM to confirm the production of PHB. Yellow pigmented AB8a isolate from *Oceanopia arenosa* scored positive for PHB accumulation and subjected to morphological, biochemical and phylogenetic characterization. The biopolymer was extracted by dispersion of sodium hypochlorite and chloroform solution and characterized by FT-IR and 1H NMR for the confirmation as PHB. The highest PHB production (70.25% /100 ml) was achieved at pH 7.0 by applying dextrose as medium at incubation temperature 30°C and 150 rpm agitation speed. The FTIR spectrum of the PHB sample showed major peaks at 3457, 1692, 1550, 1454, 1420, 1190 and 1050 cm^{-1}, whereas the remaining peaks are closely laid between 3450 cm^{-1} and 600 cm^{-1}. ^{1}H NMR spectrum of PHA isolated from dextrose media indicated characteristic signals of PHB. The spectrum also revealed the presence of three groups of signals characteristic of PHB by the doublet at 1.3 ppm attributed to the methyl group coupled to one proton; and the spectrum of the quadruplet at 2.57 ppm, the methylene group adjacent to an asymmetric carbon atom bearing a single proton and the multiplet at 5.28 ppm indicated signals of PHB. The PHB accumulated bacterium identified as *Bacillus flexus* strain based on characterization studies and 16S rRNA sequence analysis and confirmed the presence of intracellular accumulated polymer substantiated as PHB.

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Introduction

Bacterial polyhydroxyalkanoates (PHAs) are alternates for petroleum based polymers due to their eco-friendliness, which are produced and stored by prokaryotes as cytoplasmic inclusion bodies in response to environmental stress (Castilho et al., 2009; Chen, 2009; Rehm, 2010). PHAs are polyesteric biological macromolecules and reported tobe outstanding because of their biodegradability and biocompatibility (Koller, 2018). In bacteria, PHA synthesis is triggered by stressful conditions and it can be produced industrially when the medium contains excess of carbon source with nitrogen limitation (Saharan et al., 2014). Polyhydroxybutyrate (PHB) is the most widely studied and best characterized derivative of PHA (Bhuwal et al., 2014).

Various bacteria from different environmental niches have been sourced for PHBs production and marine bacteria are rarely discovered for PHBs synthesis (Numata & Morisaki, 2015). The majority of PHB producing bacteria was isolated from soil and activated sludge (Getachew et al., 2016). Recently, new bioresources such as marine environments were also explored regarding their potential to harbour new PHB producers. In general, marine ecosystems are unique habitat of microbes which are exposed to a wide variety of environmental conditions including extremes in temperature, salinity, nutrient limitation and pressure (Poli et al., 2017).

To date majority of chemicals have been identified from marine invertebrates of which sponges predominate (Lie and Zhou, 2002). Considering its pharmaceutical and drug development prospective, they are known to produce excellent resource of novel bioactive secondary metabolites (Koopmans et al., 2011). As marine sponges are usually having symbiotic relationship with different microorganisms and the marine sponge-associated symbionts have been accepted as prosperous resource of biological macromolecules, research on sponge-associated bacteria will provide remarkable new avenues for biopolymer research in future (Lie & Zhou, 2002).

The first documented PHA producing bacterial genera was *Vibrio* isolated from different marine arenas (Baumann et al., 1971; Oliver & Colwell, 1973). In marine haloarchaea, the first PHB accumulation was reported in *Halobacterium* sp. from Dead Sea and it was authenticated through free-fracture technique (Kirk & Ginzburg, 1972) followed by the genera like *Halococcus, Halorubrum, Haloarcula, Haloquadratum, Haloterrigena, Haloferax, Natronococcus, Natrialba* and *Natronobacterium* which were also established as active producers of PHB (Poli et al., 2011). The notable problem in PHB production is the process of optimization to reduce the production cost. Hence, researchers are focusing on the selection of cheap raw materials for the production of PHB from marine bacteria.

The present study describes isolation and identification of bacteria having PHB productivity and special emphasis has been given to optimize the most significant variables such as temperature, pH and substrates in order to optimize the production of PHB. The purified biopolymer was characterized by FTIR and NMR analysis by comparing with the standard PHB. Hence, the present research work has been aimed to screen marine sponge symbiotic bacteria as biological tools for production of PHB.

Material and Methods

Collection and Identification of Marine Sponges

Marine sponges were collected from the rocky shores of Kovalam (Lat. 8°22'0.01"N; Long. 76°59'48.01"E), Southern West coast of India at depth ranging from 6 to 7 m during November 2018 (Figure 1). Details of form, color, surface ornamentation, resiliency and biological associates were also recorded at the time of collection (Rachana et al., 2014). Sponges were identified by studying the spicule's nature using pertinent literatures and keys: Demospongiae of the Gulf of Mannar and Palk Bay (Thomas, 1986); Systema Porifera: A guide to classification of sponges (Hooper & Van Soet, 2002); Sponguide: Guide to sponge collection and identification (Hooper, 2003) and compared it with the original description of the species in World Porifera Database (http://www.marinespecies.org/porifera).

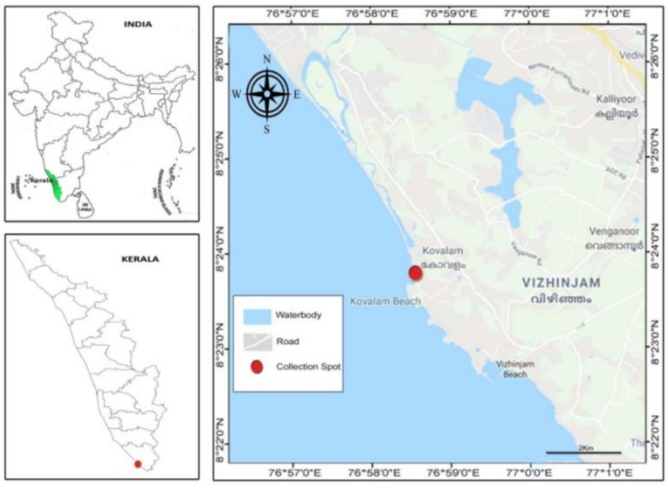

Figure 1. Map showing the collection site

Isolation and Screening of Sponge Associated Bacteria for the Production of PHB

The bacteria from sponges were isolated according to the method of Kim et al. (2006). Nearly 1 cm3 of sponge tissue was excised from the internal mesohyl area using sterile scissors in

aseptic conditions on a sterile ceramic tile. Sterile 0.85% saline (1 ml) was added to squeeze the specimen and homogenization continued till the sponge exudates were obtained (Selvin et al., 2009). The diluted sample was pour plated on modified culture medium of Zobell Marine Agar 2216 medium and incubated for 24 hrs at 32°C (Gandhimathi et al., 2008). The morphologically distinct colonies were re-isolated and maintained on ZMA (HiMedia) at 4°C. The pure cultures were maintained by sub-culturing.

Bacterial isolates were maintained in Minimal Davis Media and cultured for 2-3 days at 37°C supplemented with dextrose (10 ml of 10% in of Minimal Davis Media) as carbon source and screened by Nile Blue staining (Nile blue sulphate 90% dye content for microscopy, 1µg/ml) (Lillie, 1977) and observed under Leica LSCM - Laser Spectral Scanning Confocal Microscope, Model TCSSP 8 (Microscope Model Leica DM 18, software used-LASX). Those bacterial isolates showed bright yellowish-orange color were selected for further study (Ostle & Holt, 1982).

Characterization of Selected Bacterial Strain

Identification of bacteria were made by morphological observations of colony, shape of bacteria, catalase test, test oxidase, test the mannitol motility and the production of compound indole, test O/F (Oxidative/Fermentative), TSIA (Triple Sugar Iron Agar), Citrate test, Lysine, H2S, Urease, Lactose, Glucose and Bile esculin reactions.

The isolate showed significant activity was characterized using 16S rRNA gene sequencing. The methodology and the primers for sequencing were adapted from Kamke et al. (2010). Genomic DNA was isolated using NucleoSpin® Tissue Kit (Macherey-Nagel, Düren, Nordrhein-Westfalen). A small portion of 16S rRNA gene were amplified with the primers 16S-UP-F (5'-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG-3') and 16S-UP-R(5'-CCCGGGATCCAAGCTTACGGCTACCTTGTTACGACTT-3') (Enkicknap et al., 2006). The DNA sequence was edited and aligned using BioEdit sequence alignment editor V.7.0.9.0. (lbisBiosciences, Carlsbad, USA., Hall, 1999). Sequence similarity of specimen was done by using database GenBank (http://www.ncbi.nlm.nih.gov/genbank/). Divergence in the sequence was analyzed using the Kimura 2-Parameter distance model of MEGA (Version 6.0) (Tamura et al., 2013). Maximum likelihood tree was selected for phylogeny analysis.

Disc diffusion method (Bauer et al., 1966) was carried out on Muller Hinton agar plates (Hi-media) to determine the in vitro antibacterial susceptibility test. A 100 µl of test strain (106 CFU/ml bacteria) was spread on the Muller Hinton agar media and 25 different types of antibiotic discs were placed on the medium and were incubated for 24 hrs. After incubation at 37°C in incubator (ROTEK), the area of inhibition zone (mm) was determined using a Hi Antibiotic Zone Scale-C (PW-297, Hi-media).

Optimization of Cultural Parameters for PHB Production

PHB production were optimized by growing the culture with different pH ranging from 6, 7, 8 and 9 and the inoculated flasks were incubated at 30°C at 150 rpm for 48 hrs and PHB was quantified. Effects of different temperatures upon production were optimized by incubating the cultures on a rotary shaker at 20, 30 and 40°C and 150 rpm for 48 hrs and PHB was quantified. Effects of different media also optimized by dextrose, glucose and mannitol media and were sterilized at 121°C for 20 minutes. The inoculated flasks were incubated at 30°C at 150 rpm for 48 hrs and PHB was quantified.

Extraction and characterization of PHB from potent isolates for the extraction of PHB, the fluorescence displayed bacterial isolates were cultured in Minimal Davis Media supplemented with Dextrose as carbon source. The culture were kept in a rotary shaker at 37°C and 150 rpm for 3 days. Extraction of PHB was performed by sodium hypochlorite-chloroform method. After the centrifugation process the solution were appeared as three phases. The upper phase contains hypochlorite solution and the middle phase contains chloroform with cell debris. The bottom phase containing PHB with chloroform were collected and followed by extraction with hot chloroform and precipitated with ethanol and acetone (1:1). The precipitate was allowed to evaporate for dryness at 30°C to obtain PHB crystals (Singh & Parmar, 2011).

The extracted crystals were analysed qualitatively by using Thermo Fisher Scientific Nicolet iS50 FT-IR Spectrophotometer to know the presence of different functional groups at a range of 4000-400 cm^{-1}. IR spectra were recorded at 4 cm^{-1} resolution (Kansiz et al., 2000). The ^{1}H NMR spectra of extracted PHB sample was also obtained at 400 MHz using a model Brucker Avance III HD NMR spectrometer and methanol is used as solvent. Chemical shifts (ppm) and coupling constants (Hz) were recorded.

Results

Ten species of marine sponges were collected and based on morphological features of spicules and other specialized characters the sponges were identified as *Callyspongia (Cladochalina) fibrosa* (Ridley and Dendy, 1886), *Callyspongia (Cladochalina) diffusa* (Ridley, 1884), *Tedania (Tedania) anhelans* (Vio in Olivi, 1792), *Myxilla (Ectyomyxilla) arenaria* (Dendy, 1905), *Sigmadocia carnosa* (Dendy, 1889), *Dysidea fragilis* (Montagu, 1814), *Ecionemia acervus* (Bowerbank, 1864), *Oceanopia arenosa* (Rao, 1941), *Mycale (Carmia) mytilorum* (Annandale, 1914) and *Mycale (Aegogropila) crassissima* (Dendy, 1905) (Figure 2).

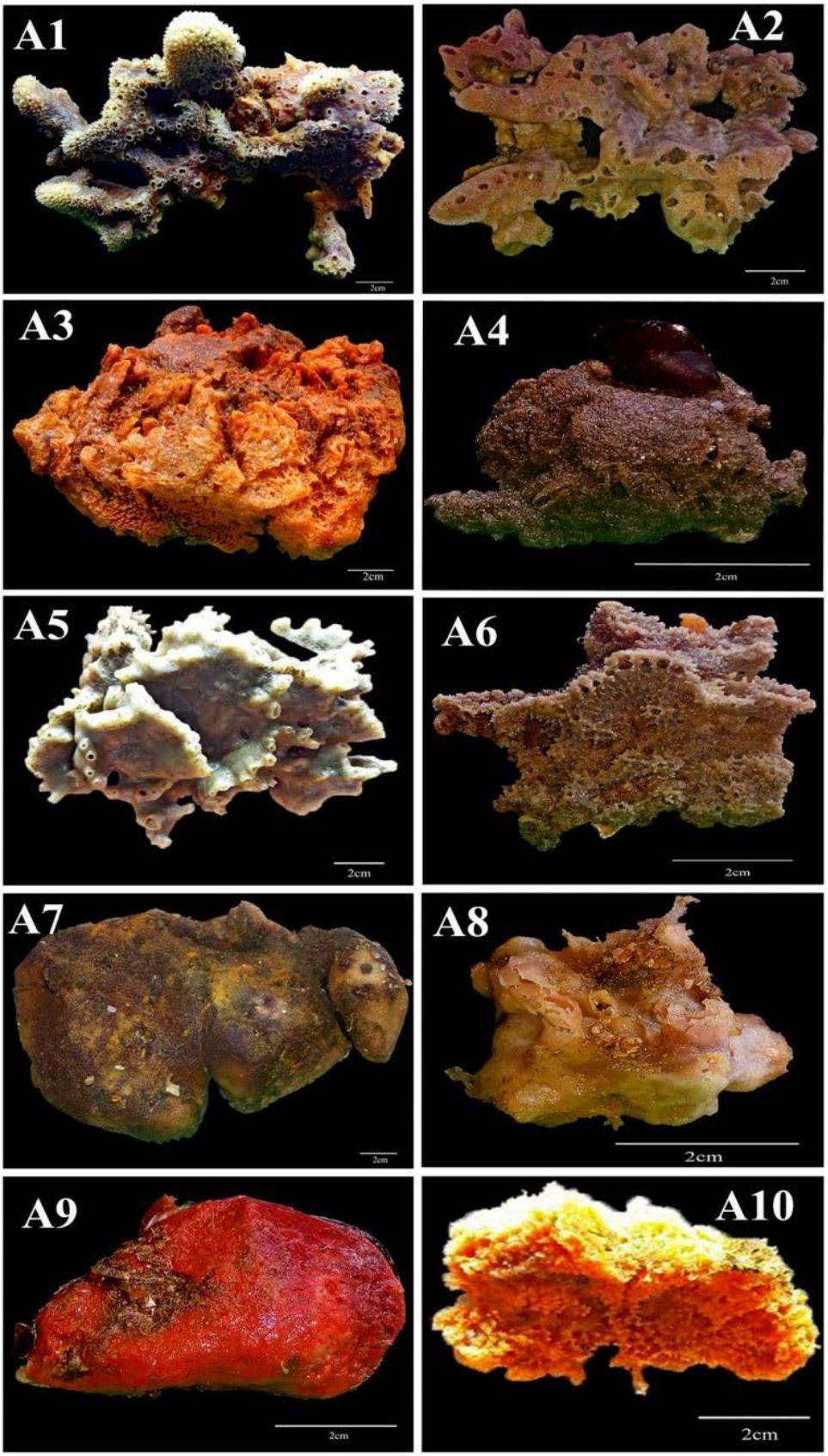

Figure 2. Collected marine sponges; **A1**- *Callyspongia (Cladochalina) fibrosa* , **A2**- *Callyspongia (Cladochalina)*, **A3**- *Tedania (Tedania) anhelans*, **A4**- *Myxilla (Ectyomyxilla) arenaria,* **A5**- *Sigmadocia carnosa*, **A6**- *Dysidea fragilis*, **A7**- *Ecionemia acervus*, **A8**- *Oceanopia arenosa,* **A9**- *Mycale (Carmia) mytilorum,* **A10**- *Mycale (Aegogropila) crassissima*

Screening of isolates for PHB production

The isolates obtained from marine sponges were screened for PHB production using Nile blue staining method and cultured in Minimal Davis Media (Figures 3 & 4). Ten isolates showed growth in Minimal Davis Media were further stained with Nile blue staining and observed under Leica LSCM to confirm the production of PHB. Five isolates such as two strains each from *Callyspongia diffusa* (AB2a, AB2b) (Figure 5), *Mycale mytillorum* (AB9a, AB9b) (Figure 6) and one isolate from *Oceanopia arenosa* (AB8a) (Figure 7) flourish bright yellowish orange color were assumed as the PHB producing colonies. In this study, the AB8a PHB granules were fluoresced as bright orange, and it was concluded that AB8a was the best PHB producer with bright orange fluorescence. So the yellow pigmented AB8a isolate from *Oceanopia arenosa* showed high intensity was selected for further observations and study.

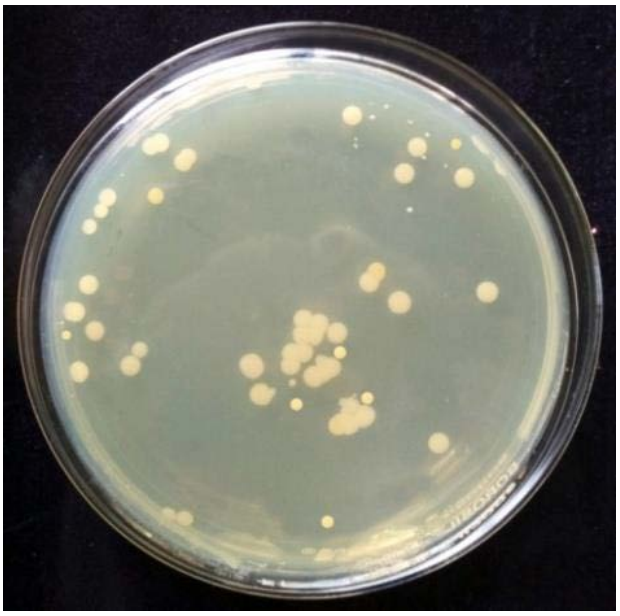

Figure 3. Bacterial isolates - pour plate method

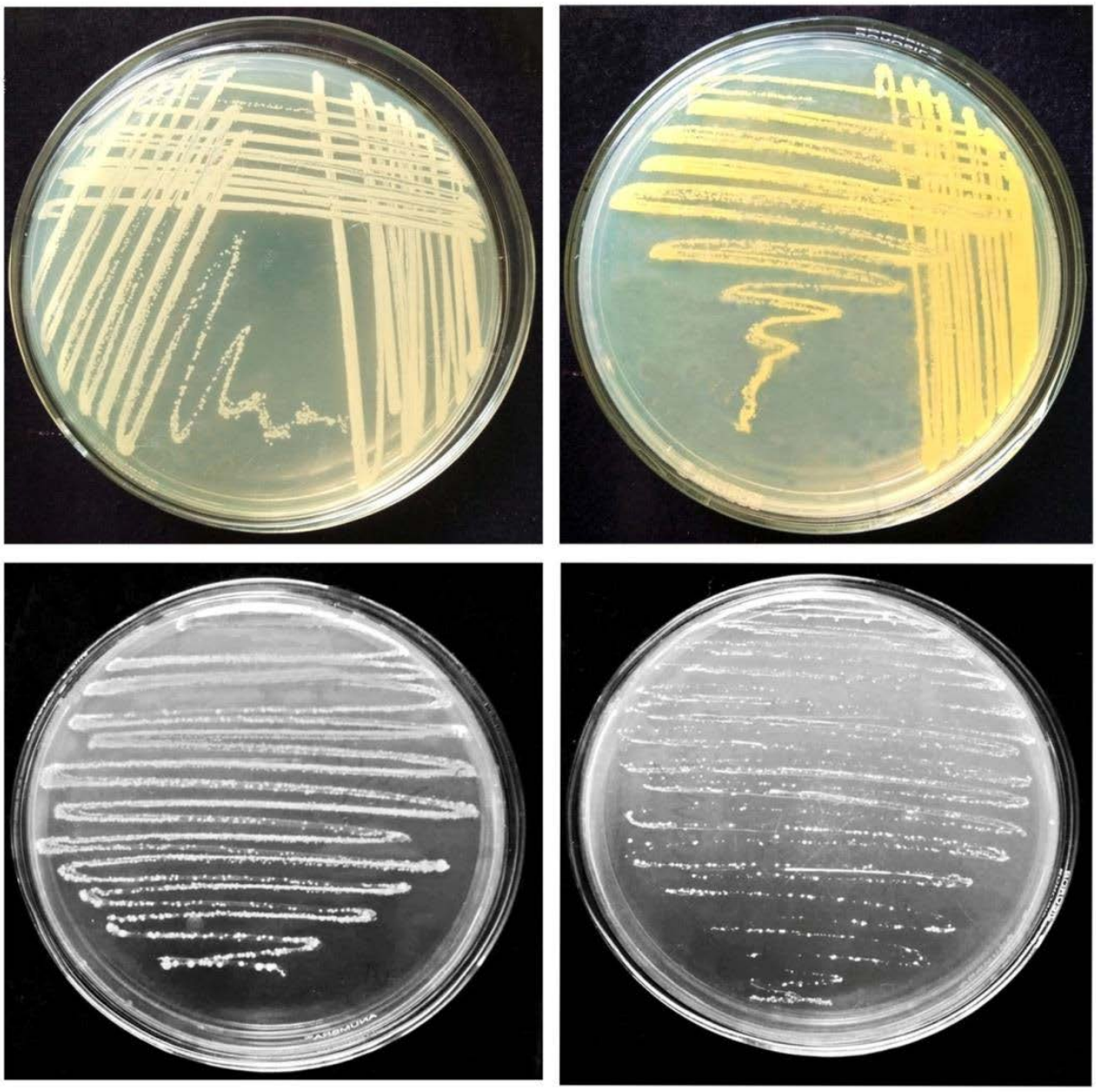

Figure 4. Pure culture of isolates from marine sponge

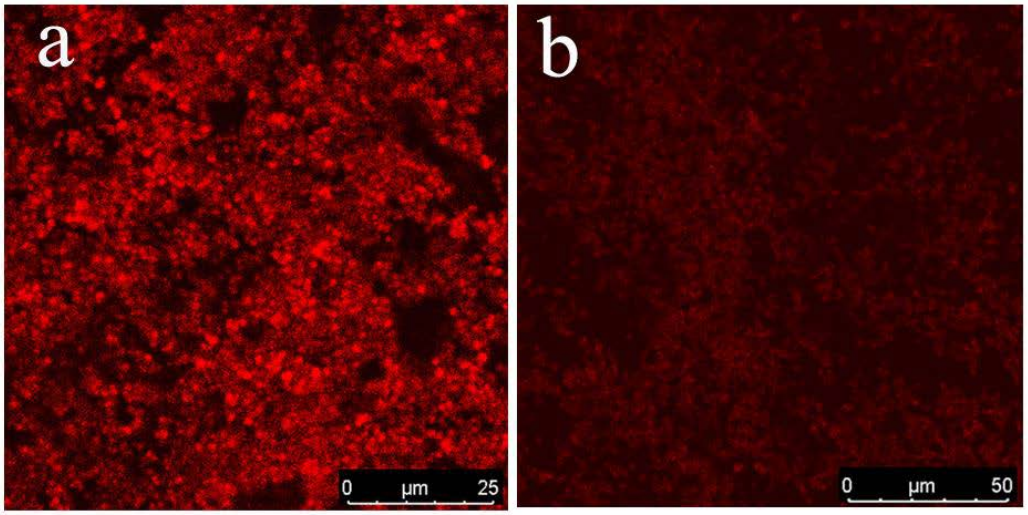

Figure 5. AB2a and AB2b isolates from *Callyspongia diffusa* showing positive result in confocal microscopy

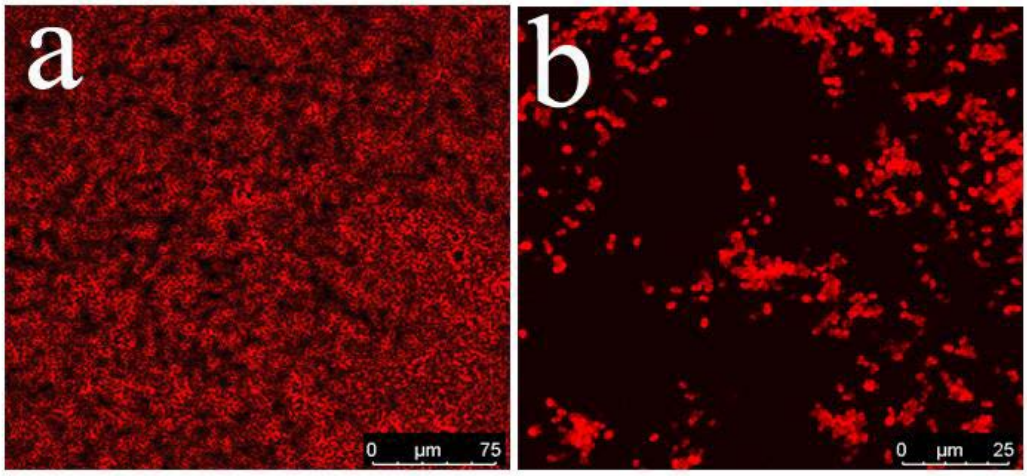

Figure 6. AB9a and AB9b isolates from *Mycale mytillorum* showing positive result in confocal microscopy

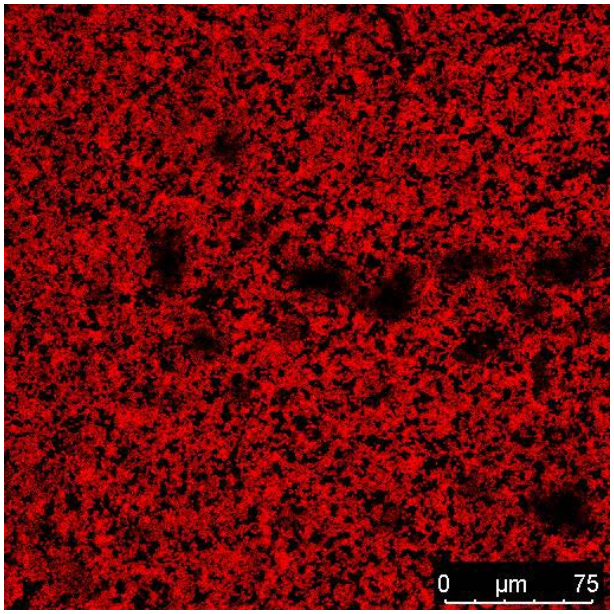

Figure 7. AB8a isolates from *Oceanopia arenosa* showing positive result in confocal microscopy

Characterization of Potent PHB Isolate

To characterize the potent isolate, Gram staining (Figure 8) and biochemical tests (Figure 9, Table 1) were performed. From that result the AB8a isolate was identified as a Gram-positive motile, sugar fermented rod-shaped *Bacillus sp*.

Antibiotic Sensitivity Test

Among twenty five different types of antibiotics used, AB8a was found to be resistant for Ampicillin, Cloxacillin, Cefuroxime, Bacitracin, Amoxyclav and Cephalothinand. The zone diameters of sensitivity of the organism to the antibiotics obtained were recorded (Figure 10, Table 2).

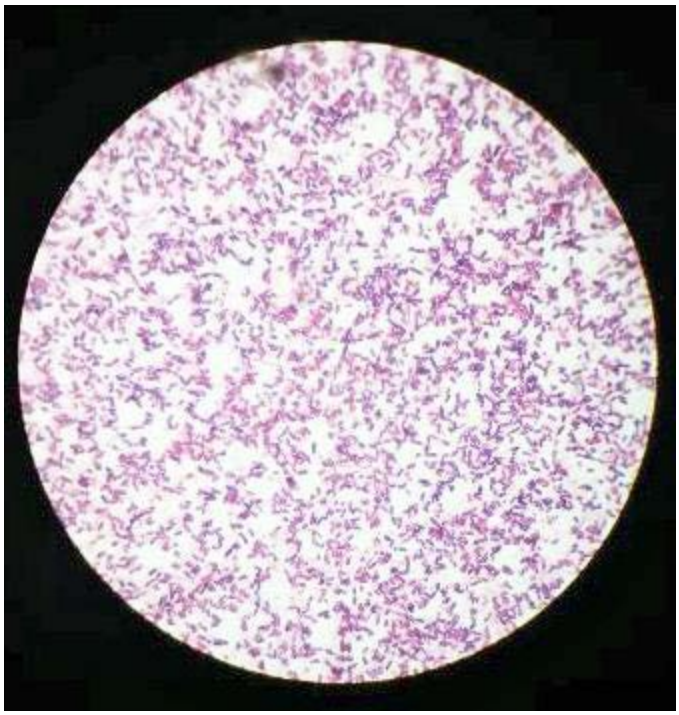

Figure 8. Gram staining of isolated bacteria

Figure 9. Biochemical tests

Table 1. Biochemical characteristics of AB8a

Test	Observation
Motility	Motile, fermented
Gram stain	Gram positive
Catalase	+
Oxidase	+
Mannitol motility	Fermented
Indole	+
Oxidative fermentative	-
TSIA	Sugar fermented A/A
Citrate	-
Lysine	-
H2s	-
Urease	-
Shape of bacteria	Rod
Lactose	-
Glusose	+
Bile esculin	+

Table 2. List of antibiotics used along with zone diameter in mm

Antibiotics	Quantity	Code	Zone (mm)
Ampicillin	10 mcg	AMP	-
Cefotaxime	30	CTX	18
Carbenicillin	100	CB	15
Cloxacillin	1	COX	-
Azithromycin	15	AZM	25
Norfloxacin	10	NX	30
Tobramycin	10	TOB	16
Nalidixic acid	30	NA	22
Cefuroxime	30	CXM	-
Bacitracin	SD 105-1 CT	BT	-
Ceftazidime	30	CAZ	24
Amoxyclav	30	AMC	-
Piperacillin	100	PI	25
Tigecycline	15	TGC	20
Cephalothin	30	CZ	-
Erythromycin	15	E	25
Meropenem	10	MRP	33
Amikacin	30	AK	20
Vancomycin	30	VA	17
Gentamycin	10	GEN	19
Ciprofloxacin	5	CIP	14
Cotrimoxazole	25	COT	20
Nitrofurantoin	300	NIT	17
Pip-Tazobactam	100/10	PIT	19
Cefaperazonesulbactam	75/30	CFS	22

Molecular Analysis for Amplification of the Genes and Phylogenetic Analysis

Amplification and gel electrophoresis of 16S rRNA (Figure 11) showed that AB8a bacteria isolates had approximately 1500bp and it was belonged to bacteria groups. The phylogenic relationship of bacterial isolate (AB8a) was studied using Maximum Likelihood Method. The 16S rRNA strain sequence following pair-wise alignment exhibited 100% similarity at the DNA gene level with the members of the genus Bacillus. The ML tree was prepared (Figure 12) and clade stability was estimated using 1000 non-parametric bootstrap replications. Phylogenetic tree revealed that Bacillus flexus of the present study has got clustered with the identical reference sequence of the *Bacillus flexus* (MHO57386.1) from GenBank with highest boot strap value (100). The isolate of our present investigations was thus identified as *B. flexus*.

Optimization of Cultural Parameters for PHB Production

Effect of different pH on PHB production indicated that, out of different pH of media tested, pH 7.0 was found to be optimum for maximum PHB production by *B. flexus*. Effect of

initial pH studies also showed that as the pH in the medium increases, PHB production increased up to pH 7.0. *Bacillus flexus* showed maximum PHB production (2.85±0.08g/100 ml) at pH 7.0, and at alkaline pH sharp decrease in the production of PHB were found. NoPHB production was observed at pH 6.0 by the isolate. At pH 8.0, all the isolates were found to produce lower yields showing that pH 6.0, pH 8.0 and pH 9.0 were not suitable for PHB accumulation.

Effect of different incubation temperature on PHB accumulation was studied over a range of 20°C to 40°C. The result indicated that the range of 30–35°C was suitable for the PHB production (2.82±0.09g/100 ml). Even though 30-35°C range was found to be suitable for PHB production, 30°C was selected as optimum temperature for further studies. Yield and PHB production was low at 20°C and 40°C.

Among the different carbon sources tested to evaluate their effects on PHB yield, dextrose was found to be the best carbon source. It yielded a mean PHB of 2.83±0.08g/100 ml. This was followed by glucose with a mean PHB of 1.55±0.10g/100 ml.

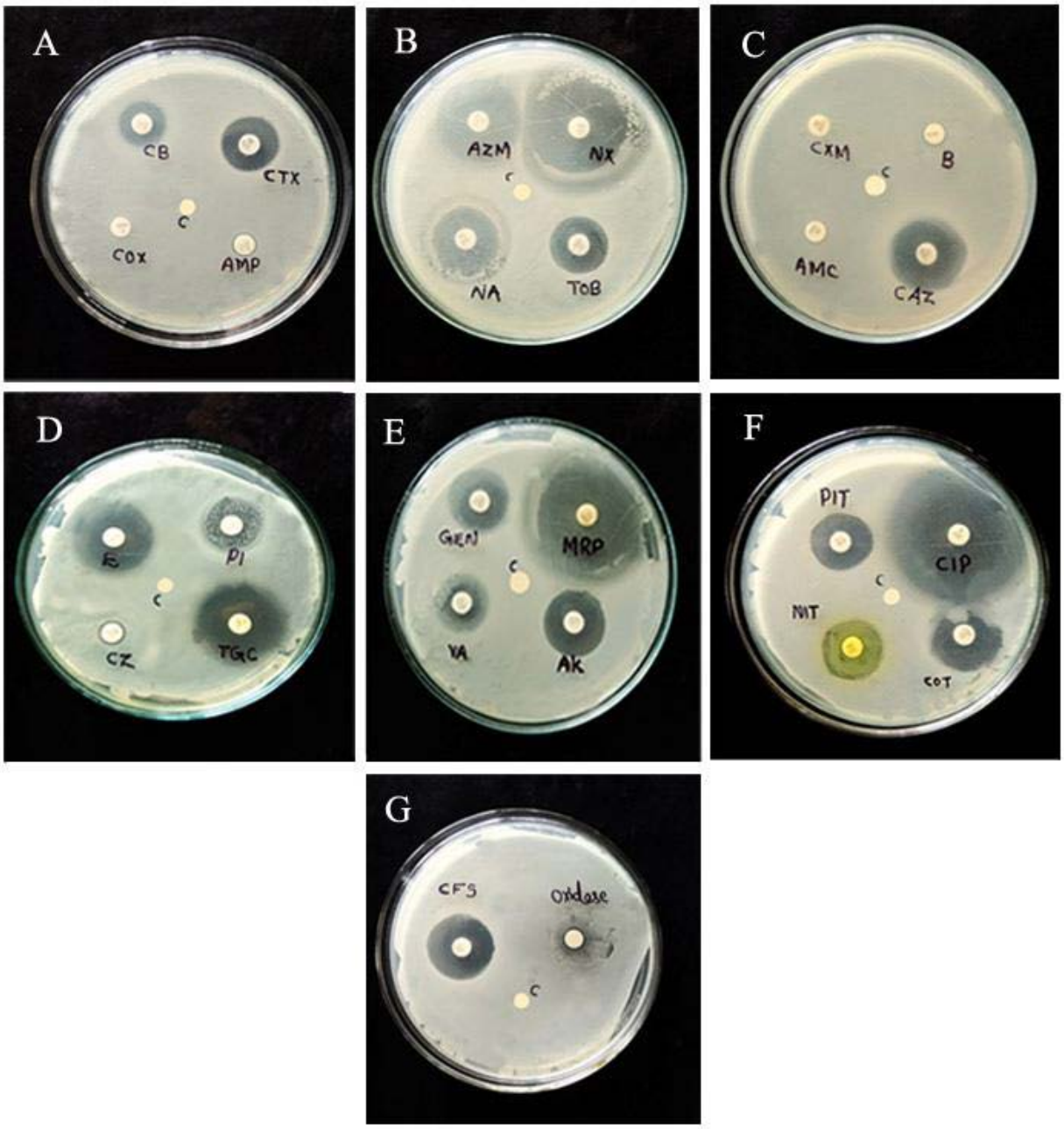

Figure 10. Antibiotic sensitivity test of the isolate; A: CB- carbenicillin; CTX-Cefotaxime; COX-Cloxacillin; AMP- Ampicillin; B: AZM-Azithromycin; NX-Norfloxacin; NA-Nalidixic acid; TOB-Tobramycin; C: CXM- Cefuroxime; BT-Bacitracin; AMC-Amoxyclav; CAZ Ceftazidime; D: E- Erythromycin; CZ-Cephalothin; PI-Piperacillin; TGC-Tigecycline; E: GEN- Gentamicin; MRP-Meropenem; VA-Vancomycin; AK-Amikacin; F: PIT- Pip-Tazobactam; NIT-Nitrofurantoin; COT-Cotrimoxazole; CIP-Ciprofloxacin; G: CFS-Cefaperazone sulbactam

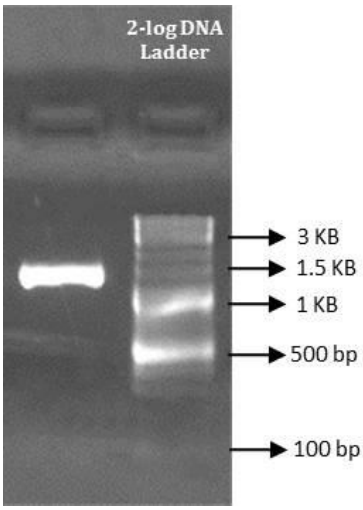

Figure 11. Details of partial 16S ribosomal RNA of AB8a bacteria isolates

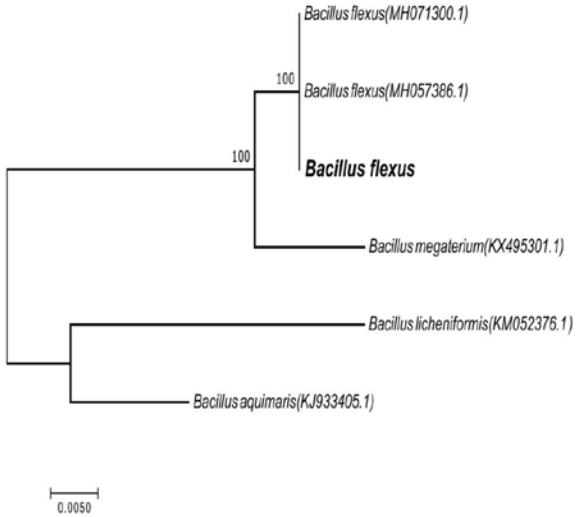

Figure 12. Phylogenetic tree of isolate *Bacillus flexus* and their closest NCBI (BLASTn) strains based on the 16S rRNA gene sequences

Extraction and Characterization of PHB

The sodium hypochlorite method was selected for the extraction of PHB. The precipitate was allowed to evaporate to obtain PHB crystals (Figure 13).

Fourier Transform Infrared Spectroscopy (FTIR)

The IR spectrum of each sample represents its total chemical composition, because every chemical compound in the sample makes its own specific contribution to the absorbance spectrum. The FTIR Spectra (Figure 17) were recorded at 4000 cm^{-1} to 400 cm^{-1} range. From the spectrum obtained it was inferred that the band at 3457 cm^{-1} corresponds to OH (Hydroxyl) group, whereas band at 1692 cm^{-1} represents C=O (Carbonyl) and COO (ester) groups. The band at 1454 cm^{-1} corresponds to CH showing asymmetrical stretching and the band at 1550 cm^{-1} indicating bending vibration in CH3 group, whereas band at 1420 cm^{-1} representing CH2 bond. Stretch of bands ranging from 1050-1190 cm^{-1} showed C-O bonding.

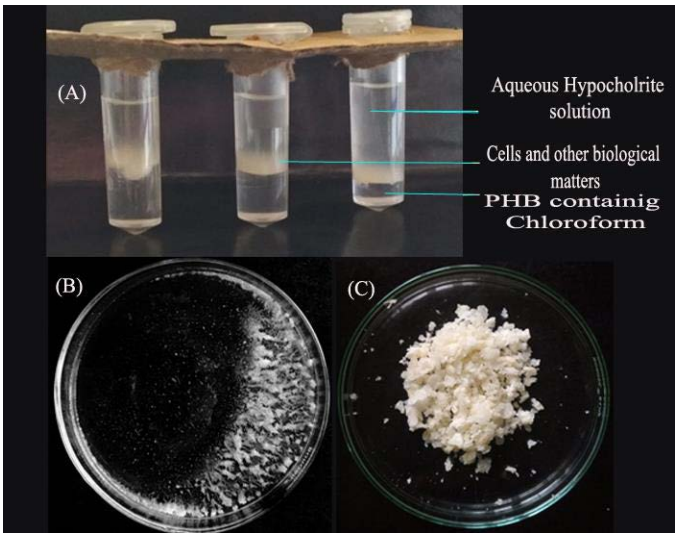

Figure 13. (A) Three different phase appeared after centrifugation, (B) PHB crystals obtained after the evaporation of chloroform by Sodium – Chloroform method, (C) Extracted PHB crystals

Nuclear Magnetic Resonance (NMR) Spectroscopy

The NMR spectrum (Figure 18) showed a triplet at 1.3 ppm which is attributed to the methyl group ($-CH_3$) coupled to one proton. Doublet peak ranging between 2.06-2.6862 is attributed to the methylene group ($-CH_2$) adjacent to an asymmetric carbon atom bearing a single atom. The multiple peak at 4.88 ppm is characteristic of methine group (-CH). Two other signals are observed, a broad one at 3.88 ppm which is due to water and another at 7.93 and 8.599 ppm is may attributed to the solvent used i.e. methanol.

Discussion

The marine environment provides a real untapped resource for novel bacteria and possibly the biopolymers they produce and this study was aimed to isolate a diverse range of PHB accumulating bacteria from marine sponges and the parameters for maximum PHB production were also optimized (Arun et al., 2009; Madison & Huisman, 1999). Based on morphological features and nature of spicules the collected marine sponges specimens were identified as Callyspongia (Cladochalina) fibrosa (Ridley & Dendy, 1886), Callyspongia (Cladochalina) diffusa (Ridley, 1884), Tedania (Tedania) anhelans (Vio in Olivi, 1792), Myxilla (Ectyomyxilla) arenaria (Dendy, 1905),

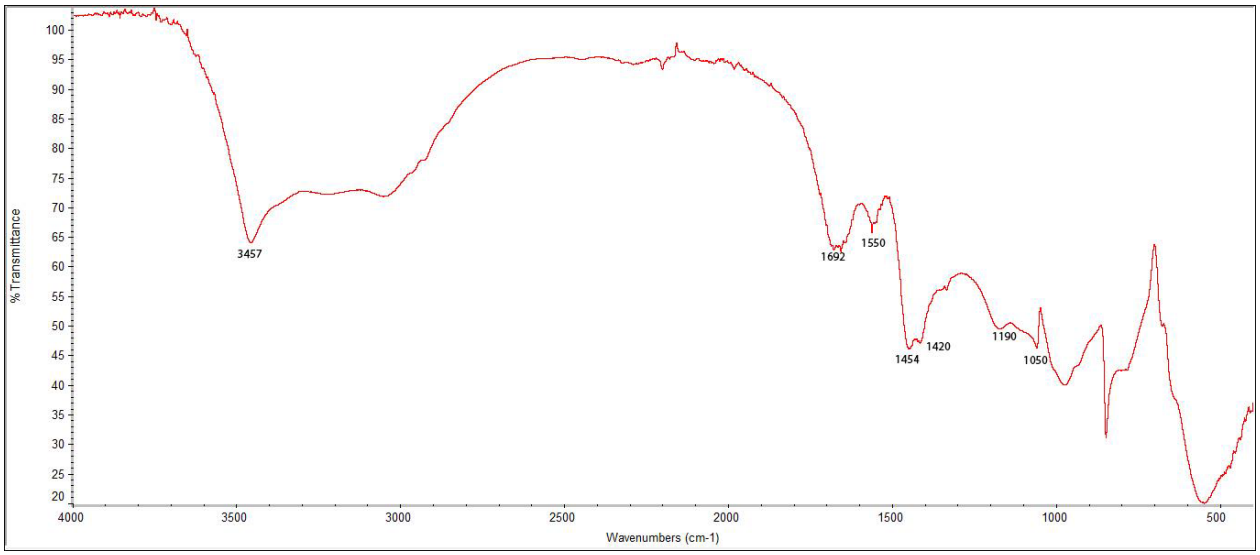

Figure 14. FT-IR spectrum of extracted PHB

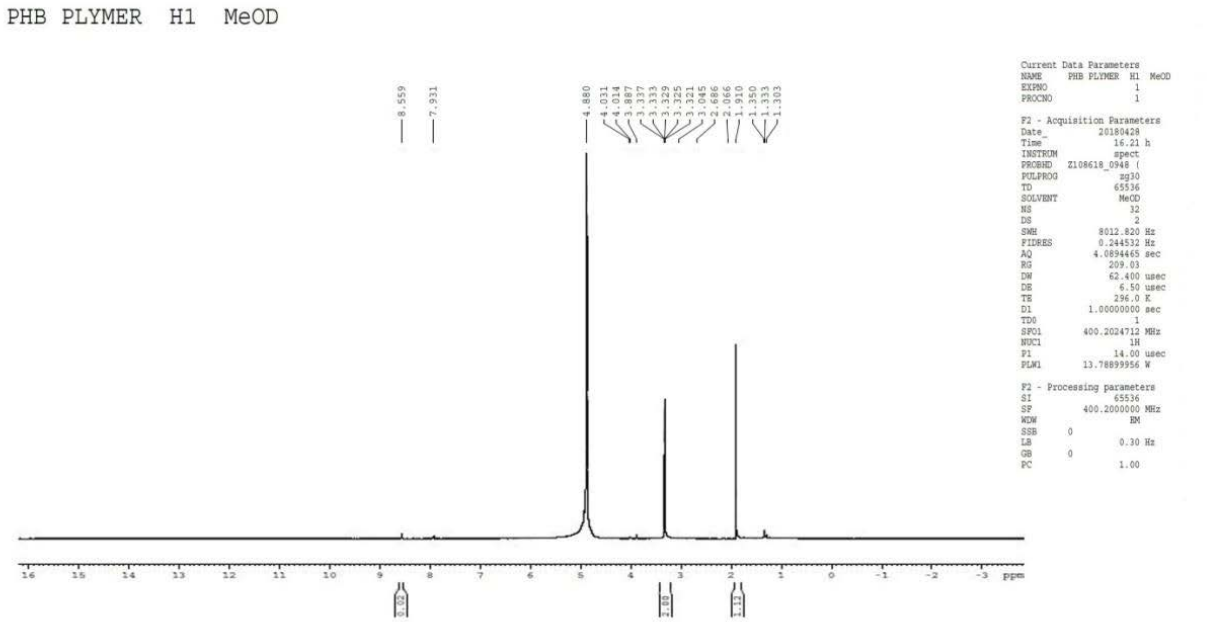

Figure 15. ^{1}H NMR spectrum of extracted PHB

Sigmadocia carnosa (Dendy, 1889), Dysidea fragilis (Montagu, 1814), Ecionemia acervus (Bowerbank, 1864), Oceanopia arenosa (Rao, 1941), Mycale (Carmia) mytilorum (Annandale, 1914) and Mycale (Aegogropila) crassissima (Dendy, 1905). Many reports reveals that these types of marine sponges are very common in Indian coastal areas including Tuticorin coast (Singla et al., 2013), Lakshadweep archipelago, Kavaratti Island (Gopi et al., 2012) and Gulf of Mannar and Mandapam bay (Velho-Pereira & Furtado, 2012). Selvakumar and Dhevendaran (2016) reported the occurrence of Callyspongia diffusa, Mycale mytilorum, Tedania anhelans and Dysidea fragilis from South West coast of India.

The result of the work indicated that five bacterial strains afforded fluorescence signals with Nile-blue test. Moreover, one of them (AB8a) gave a very strong fluorescence signal, whereas the other strains showed faint signals. In addition, the high fluoresced strain was predicted as one of the effective producer of PHB based on the growth rate, the high intensity of fluorescence in viable Nile blue A staining and the presence of lipophilic inclusions. Spiekermann et al. (1999) reported that the Nile-blue stain emitted strongly positive fluorescence signals only with a hydrophobic compound like PHAs and lipids and could be detected by fluorescence spectroscopy or flow cytometry.

Microbiological properties were investigated according to the methods described in Bergey's Manual of Systematic Bacteriology (Kreig & Holt, 1984) and the organism was identified as a member of the genus, Bacillus. The Gram-positive bacteria, such as Bacillus sp. could be considered as ideal candidates for the industrial PHB production due to the

lack of LPS layer. Bacillus sp. has pronounced importance in industry due to their advantages of low nutritional requirements, rapid growth, having machinery enzymes and for utilization of several sugars (El-Sheekh, 2015). Members of this genus are known to grow rapidly, possess various hydrolytic enzymes and produce copolymers from structurally unrelated carbon sources (Halami, 2007; Valappil et al., 2007) and accordingly these characteristics of Bacillus sp. can be considered for the production of PHB with desirable material properties from various low-cost agricultural feed stocks.

Further characterization was confirmed with 16S rRNA sequence. The phylogenic relationship of bacterial isolate (AB8a) was studied using Maximum Likelihood Method. The 16S rRNA strain sequence following pair-wise alignment exhibited 100 % similarity at the DNA gene level with the members of the genus Bacillus. Phylogenetic tree revealed that Bacillus flexus of the present study has got clustered with the identical reference sequence of the Bacillus flexus (MHO57386.1) from GenBank with highest boot strap value (100). The isolate of our present investigations was thus identified as B. flexus. In prokaryotes PHB accumulation property is broadly distributed among the Gram-negative organisms such as Cupriavidus, Pseudomonas, etc., and Gram-positive organisms such as Bacillus, Clostridium, Corynebacterium, Nocardia, Rhodococcus, Streptomyces, Staphylococcus etc. and certain archaeal strains of Halobacterium, Haloarcula, Haloquadratum and Haloferax. Bacillus spp. are well known for their ability to accumulate poly-3-hydroxybutyrate (PHB) (Balakrishna Pillai et al., 2017). According to Beveridge (2001) marine environment have reported that around 36% of the symbiotic isolates are Gram-negative rods. In our study, a Gram positive Bacillus was isolated from Oceanopia arenosa. Generally, bacteria belonging to the genera Bacillus accumulate short chain length polyhydroxyalkanoates such as PHB (Valappil et al., 2007).

Effect of incubation temperature on yield and PHB accumulation was studied over a range of temperature 20°C to 40°C. The result indicated the range of 30-35°C was suitable for the biomass and PHB production (73.40% yield). The incubation temperature ranging from 27 to 30°C favored PHB production and the maximal yield was attained at 30°C. The most adverse effect of incubation temperature on both PHB was recorded above 30°C. This result accorded to a great extent with those obtained by Divyashree et al. (2009a, b), who grew B. flexus strain at 30°C for PHA production. Higher or lower temperatures showed inferior results. This result also coincides with that represented by Aslim et al. (2002), who reported that optimum incubation temperature for PHB production by Bacillus thuringiensis, Bacillus subtilis, and Bacillus pumilis was at 35°C. According to Tamodgan & Sidal (2011) higher and lower temperatures than 30°C lead to decrease in PHB synthesis by Bacillus subtilis ATCC 6633, as well as cell mass, probably due to the low enzymes activity. In this study, the optimum temperature for PHB production was found to be in 30°C. These results are similar with Grothe et al. (1999) that incubation temperature affects polymer accumulation at a range of 30-35°C and over this range, the effect of temperature is negligible. Interestingly, our isolated strain AB8a could accumulate the highest amount of PHB within 36 hrs that is very short time compared to previously reported Bacillus strains. Maximum PHB production from molasses was obtained after 72 hrs by Bacillus flexus ME-77 (El-Sheekh, 2015) and Bacillus thuringiensis (Desouky et al., 2014). Kalaivani & Sukumaran (2015) also reported maximum production of PHB from molasses observed after 76 hrs by Bacillus sp. KSN5.

Dextrose among all the other carbon sources led to the highest PHB production (70.25%) compared to glucose and mannitol. In addition, the enhancing effect of dextrose is probably due to its additional nutrients, such as trace elements, minerals and vitamins as thiamine and riboflavin (Oliveira et al., 2004). Gouda et al. (2001) studied the effect of different carbon sources on the production of PHB using Bacillus megaterium. In that study maximum PHB production was obtained from glucose and maximum cell dry mass was obtained from maltose. In this study among the carbon compounds used, dextrose was found to be best for PHB accumulation and mannitol was found to be poor carbon source.

It has been reported that pH in the range of 6.0-7.5 was the best for microbial growth and PHB production of Alcaligenes eutrophus was reported at optimum pH of 6.9, and the growth declined at pH below 5.4 (Grothe et al., 1999). Even a slight change in pH will cause malfunctioning of metabolic processes (Wei et al., 2011), and drastic changes in PHB production seems to be due to the effect of initial pH on the bioavailability of trace elements (Ramadas et al., 2009). In the present study, maximum PHB production obtained at pH 7.0 (73.55%). Flora et al. (2010) revealed that the maximum PHB production (25%) by Bacillus sphaerius was at pH range from 6.5-7.5. Earlier reports of Sivaprakasam et al. (2008) concluded that the optimum pH for growth of B. flexus was 8.0, while Priest et al. (1988) revealed that B. flexus can tolerate pH range from 4.5 to 9.5.

The biopolymer was extracted from the bacterial pellet using dispersion of sodium hypochlorite and chloroform solution. The extracted PHB was characterized by FTIR and 1H NMR for the confirmation as PHB. The PHB sample extracted

was analyzed qualitatively by FTIR Spectrophotometer to know the presence of different functional groups. In the present study, the FTIR spectrum of the PHB sample shows major peaks at 3457, 1692, 1550, 1454, 1420, 1190 and 1050 cm^{-1}, whereas the remaining peaks are closely lying between 3450 cm^{-1} and 600 cm^{-1}.The IR spectrum reflects both monomeric units in addition a strong absorption band at 1714 cm^{-1} was detected in G1S1 (Bacillus subtilis), as is expected for the C=O (Shah, 2014). All absorptions due to the PHB moiety appeared in the spectrum, and in addition a strong absorption band at 1639 cm^{-1} was detected a thioester bond (Shah, 2012). As an evidence of this finding, the work done by Rohini et al. (2006) can be equated. They identified the polymer with the spectrum which revealed the presence of three groups of signals characteristic of PHB by the doublet at 1.3 ppm attributed to the methyl group coupled to one proton and the spectrum of the quadruplet at 2.57 ppm the methylene group adjacent to the asymmetric carbon atom having single proton and the multiplet at 5.28 ppm to the methylene group.

The results of this study demonstrates that the bacterium, which is isolated from marine sponge Oceanopia arenosa, identified as Bacillus flexus could be an effective and interesting bacterial sp., for production of PHB from dextrose. However, Use of inexpensive substrates could contribute to reducing the PHB production cost and further studies are needed for large scale production of the PHB.

Conclusion

The present investigation provides basis for assessing a potential for using sponge symbiotic bacteria for PHB (a biodegradable plastic) production, which is an economically and environmentally important product. In this study, sixteen marine bacterial strains associated with ten species of sponges *Callyspongia (Cladochalina) fibrosa, Callyspongia (Cladochalina) diffusa* (Ridley, 1884), *Tedania (Tedania) anhalens* (Vio in Olivi, 1792), *Myxilla arenaria* (Dendy, 1905), *Sigmadocia carnosa* (Dendy, 1889), *Dysidea fragilis* (Montagu, 1814), *Ecionemia acervus* (Bowerbank, 1864), *Oceanopia arenosa* Rao, (1941), *Mycale mytilorum* Annandale and *Mycale crassissima* were isolated. Ten isolates showed growth in Minimal Davis Media were further stained with Nile blue staining and observed under Leica LSCM - Laser Spectral Scanning Confocal Microscope to confirm the production of PHB. Five isolates such as two strains each from Callyspongia diffusa (AB2a, AB2b), Mycale mytillorum (AB9a, AB9b) and one isolate from *Oceanopia arenosa* Rao (AB8a) flourish bright yellowish-orange color were assumed as the PHB producing colonies. Yellow pigmented AB8a isolate from *Oceanopia arenosa* showed high intensity was selected for further observations and study. In addition, AB8a was subjected to morphological, biochemical and phylogenetic characterization. The results of the tests showed AB8a to be a Gram-positive, sporulating, motile, catalase,oxidase positive and rod shaped bacteria. The highest PHB production (70.25% /100 mL) was achieved at pH 7.0 by applying dextrose as medium at incubation temperature 30°C and 150 rpm agitation speed. The biopolymer was extracted from the bacterial pellet using dispersion of sodium hypochlorite and chloroform solution. The extracted PHB was characterized by FT-IR and 1H NMR for the confirmation as PHB. Phylogenetic analysis based on comparative analysis of sequenced 16s rRNA of the active strains indicated a preponderance of bacteria belonging to *Bacillus flexus* with 100% sequence similarities.

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Compliance with Ethical Standards

Authors' Contributions

DA and VSP conceived and designed the study, collect specimens, analyze the data and wrote the manuscript. DA carried out the experimental work, interpret the data, reviewed the results. VSP guided the research work, performed editing, critical revision and supervised the findings of the study. Both authors read and approved the final manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

For this type of study, formal consent is not required.

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SHORT COMMUNICATION

Influence of heat shock protein (HSP-70) enhancing compound from red alga (*Porphyridium cruentum*) for augmenting egg production in copepod culture - A new *in silico* report

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ABSTRACT

The present study reports *in silico* investigation of bioactive compounds from marine microalgae capable of escalating copepod fecundity potential through enhanced heat shock protein (HSP-70) production. The structure of ligand (bioactive compounds from microalgae) and hsp-70 obtained from the databases of PubChem and Protein Data Bank (PDB), respectively. Molecular Docking was performed by GOLD software and ligand interaction pathways using web server MANORAA. Fourteen bioactive compounds showed good binding interaction with specific protein HSP-70 and seven of these compounds showed high hydrogen bond interaction with key amino acids (phenylalanine, tyrosine and tryptophan). The highest binding energy of 50.21is recorded in the bioactive compound, arachidonic acid from the red alga *Porphyridium cruentum* TYR 167 involved in the biosynthesis pathway of phenylalanine, tyrosine and tryptophan also showed specific target site of tryptophan synthase (4.2.1.20). Results suggest with *P. cruentum* feed copepod culture could boost their fecundity leading to high density culture.

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Introduction

Marine food webs depend on zooplankton especially, copepods to process and repackage energy harnessed by photosynthetic primary producers. Copepods constitute important primary consumer in all types of aquatic ecosystems and play vital role in the energy transfer from primary producers to secondary consumers (Altaff, 2020). In aquaculture, copepods have been proven to be the preferred and most adequate food for fish larvae (Anandan et al., 2013;

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Vu et al., 2017; Hansen, 2017). The nutritional quality of copepods is accepted to be highly satisfactory for larvae of prawn and finfish species. Biochemical studies have shown that copepods are rich in proteins, lipids, essential amino acids, and essential fatty acids which can enhance reproduction, augment growth, immune stimulation, and color intensification in prawn and fish larvae (Lavens & Sorgeloos, 1996; Aman & Altaff, 2004). For this reason, copepods are cultivated for use as live feed for newly hatched finfish larvae in marine aquaculture systems. Copepods in cultivation systems may be exposed to environmental conditions such as fluctuations in temperature, pH and pathogens that challenge successful large scale production (Petkeviciute et al., 2015). Therefore, it is essential that copepod species chosen for mass culture should be resistance to such environmental conditions. The egg productions of marine copepod may be under favorable or unfavorable environmental conditions. Under favorable conditions, subitaneous eggs are produced that are characterized by hatching within a few days after spawning (Nilsson et al., 2014). In response to adverse environmental conditions, subitaneous eggs enter a quiescent state (direct inhibition of development due to adverse conditions), where embryonic development is delayed until exposure to more favorable environmental conditions. When a copepod embryo undergoes quiescence, it requires a number of stabilizing factors (Nilsson et al., 2014).

The heat shock protein 70 (HSP-70) production is the response of copepods in shallow waters protects them against the adverse environmental conditions such as temperature and pH which otherwise leads to damage the cellular macromolecules through ROS - reactive oxygen species (Nilsson et al., 2014). Aruda et al. (2011) also reported that the HSP-70 of copepod in shallow waters protects proteins against the higher temperatures experienced under these environmental conditions. However, only few copepod species have so far been subject of such *in vivo* studies among the marine copepods. *Acartia tonsa* response to the heat shock was more pronounced at low salinity model (Nilsson et al., 2014; Petkeviciute et al., 2015) and similar impact of sublethal stress of *A. tonsa* using solar UV radiation was also reported (Tartarotti & Torres, 2009). Likewise, Rhee et al. (2009) reported that the HSP-*70* gene expression is elevated when copepods are exposed to elevated temperatures. Based on the above rational, in the present study an attempt is made to search for HSP-70 enhancement bioactive compounds from microalgae using *in silico* modeling. These molecular interaction studies are not reported to our knowledge in any copepod. Present study aims to enhance and regulate fundamental cellular processes during high density culture of marine copepod especially, through biosynthesis of amino acid (Phenylalanine, tyrosine and tryptophan biosynthesis).

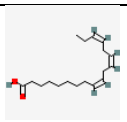
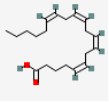
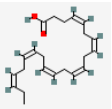
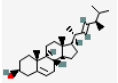
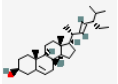
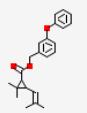
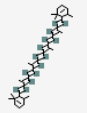
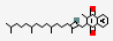
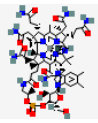
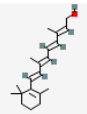
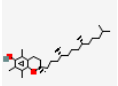
Material and Methods

The molecular docking studies were performed using GOLD software. The structures of bioactive compounds were built using ChemDraw program (Table-1) then were transferred into Discovery Studio 2.5 (Accelrys Inc, San Diego, CA, USA). Compounds were typed with CHARMM force field and partial charges were calculated by Momany-Rone option. The resulting structures were minimized with Smart Minimizer algorithm which performs 1000 steps of steepest descent with a RMS gradient tolerance of 3, followed by Conjugate Gradient minimization. In order to investigate the binding mode of inhibitors, and obtaining nearly bioactive conformations, X-ray crystal structure of HSP-70 complex was taken from PDB (3P9Y) with its resolution 2.10 Å and used for docking studies. The ability of GOLD to produce original ligand binding orientations is greater than 70%. The active site in the HSP-70 protein complex, crystal coordinate was defined as a region with a radius of 10 Å. Preparation for docking process; water molecules were removed; hydrogen atoms were added in the receptor using GOLD. Reproducibility of docking program was checked and compared with original crystal structure. It yields the RMS value of 0.95 Å. This result conform the reproducibility of GOLD program. The early termination option was used to skip the genetic optimization calculation when any five conformations of a particular compound predicted above the rmsd value of 1.5 Å. The best lead molecules were selected based on their binding orientation in the active site and their corresponding GOLD score. Based on molecular docking analysis the highest binding energy compound was selected for further ligand interaction pathway study and investigated with specific biosynthesis of amino acid pathway of phenylalanine, tyrosine and tryptophan using web server MANORAA - Mapping Analogous Nuclei onto Residue and Affinity (http://www.manoraa.org/).

Results and Discussion

Molecular docking is an effective and competent tool for *in silico* screening. Docking is a computational procedure of searching for an appropriate ligand that fits both energetically and geometrically with the protein's binding site (Vijayaraj et al., 2019). During the past decade, for understanding the formation of intermolecular complexes, the application of computational methods has been subjected to intensive research. It is commonly known that molecular binding of

Table 1. List of ligand (Bioactive compounds from microalgae)

Name of the ligands	Molecular formula	Molecular structure	Ligand sources	Reference
α-Linolenic acid	$C_{18}H_{30}O_2$		*Arthrospira* sp.	Cohen & Heimer, 1992
Arachidonic acid	$C_{20}H_{32}O_2$		*P. cruentum*	Ahern et al., 1983
Docosahexaenoic acid	$C_{22}H_{32}O_2$		*I. galbana*	Pulz & Gross, 2004
Brassicasterol	$C_{28}H_{46}O$		*Chaetoceros*	Bandarra et al., 2003
Stigmasterol	$C_{29}H_{48}O$		*I. galbana*	Tsitsa et al., 1993
Pyocyanine	$C_{13}H_{10}N_2O$		*Arthrospira* sp.	Mao et al., 2005
Phenothrin	$C_{23}H_{26}O_3$		*P. cruentum*	Pulz & Gross, 2004
β-Carotene	$C_{40}H_{56}$		*D. salina*	Plaza et al., 2009
Ascorbic acid	$C_6H_8O_6$		*Arthrospira* sp.	Antia et al., 1970
Phylloquinone	$C_{31}H_{46}O_2$		*P. cruentum*	Bandarra et al., 2003
Cyanocobalamin	$C_{63}H_{89}CoN_{14}O_{14}P$		*Pavlova* sp.	De Roeck-Holtzhauer et al., 1991
Retinol	$C_{20}H_{30}O$		*I. galbana*	Li & Xu, 2008
α-Tocopherol	$C_{29}H_{50}O_2$		*Pavlova* sp.	Morse et al., 1979
γ-Aminobutyric acid	$C_4H_9NO_2$		*Porphyridium* sp.	Obrietan et al., 2002

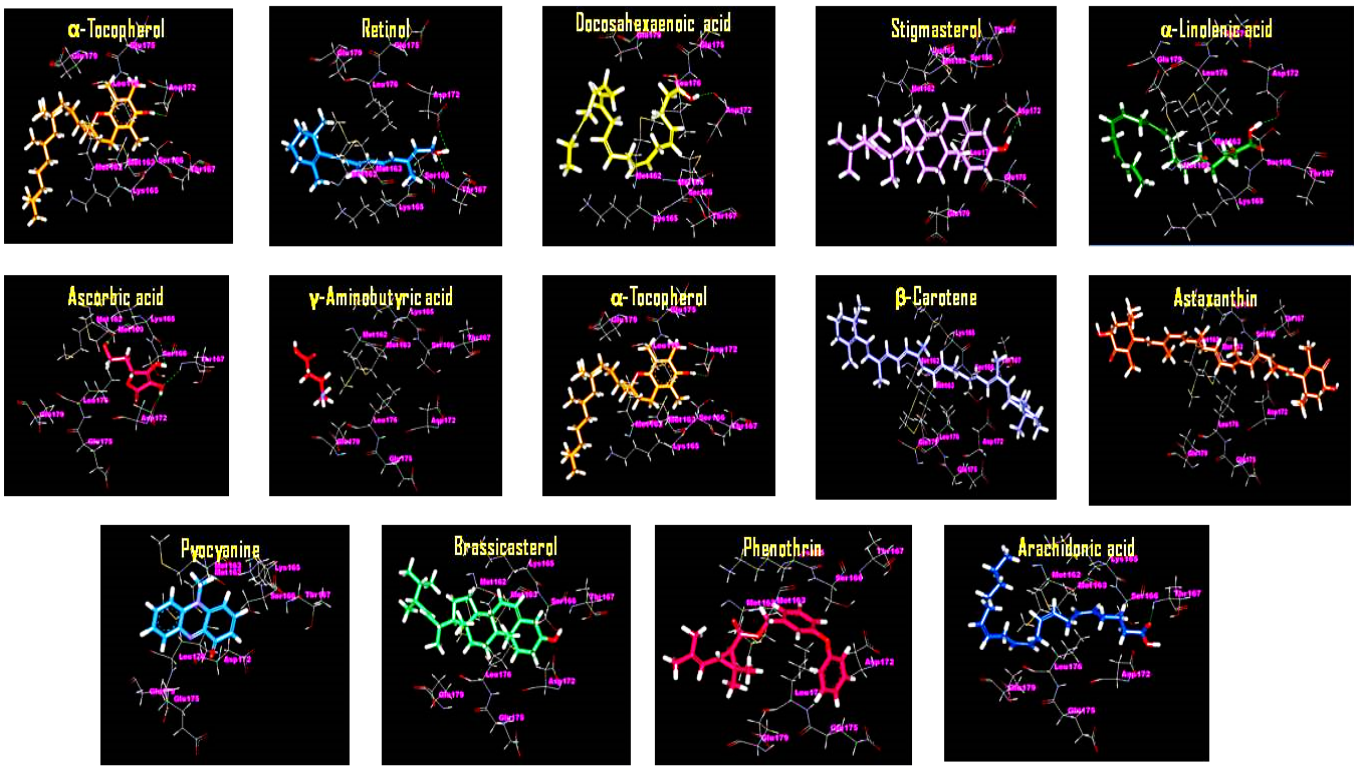


Figure 1. Bioactive compounds binding interaction with specific protein *hsp*-70

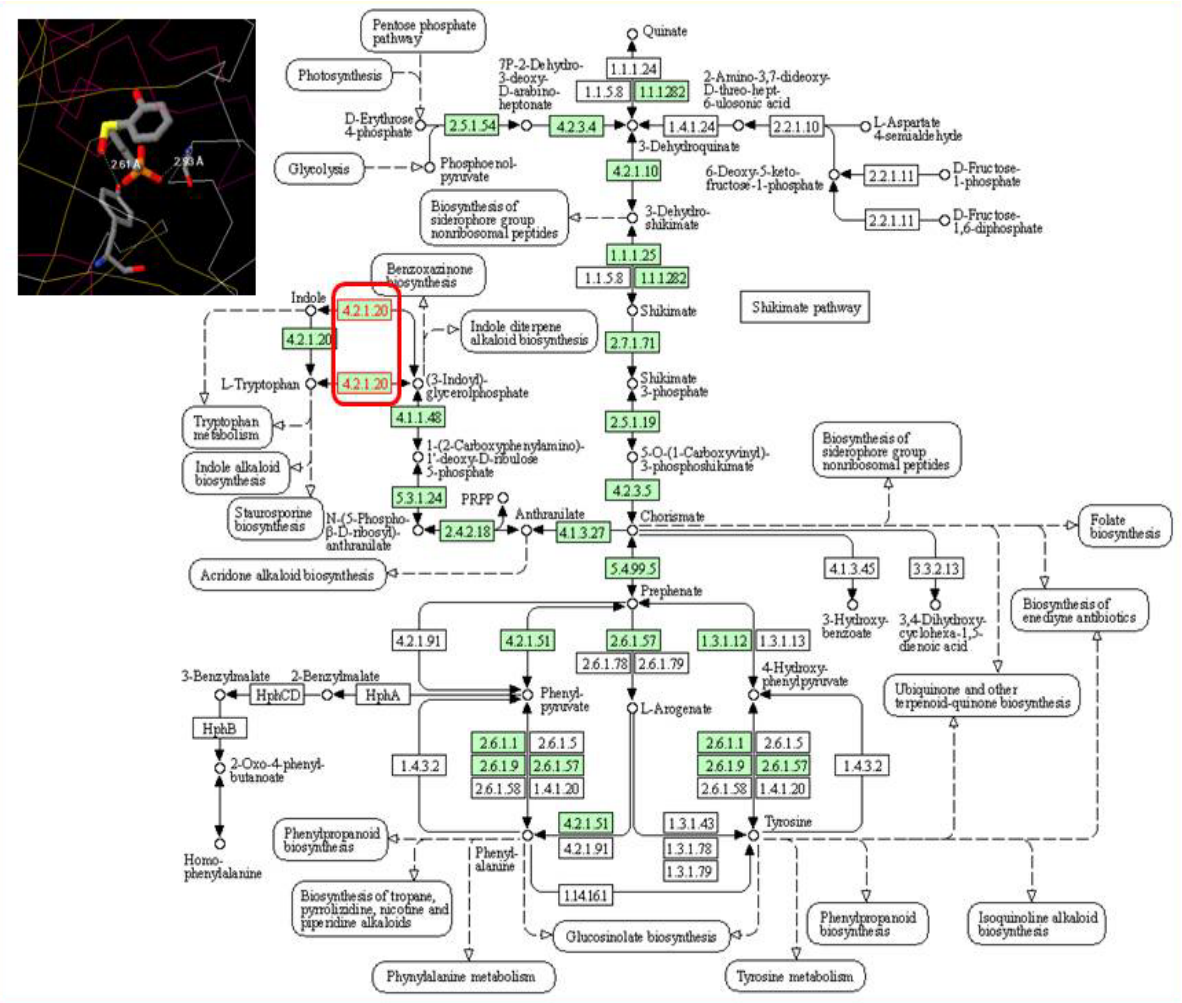


Figure 2. Arachidonic acid influenced phenylalanine, tyrosine and tryptophan biosynthesis pathway

bioactive compounds (ligand) to the pocket of another molecule (protein as a receptor) is responsible for accurate activity of the bioactive compounds. Molecular docking has been proved very efficient tool for novel discovery for targeting protein (Vijayaraj et al., 2020). In the present study, fourteen different HSP-70 enhancements by natural bioactive compounds from various microalgae were investigated. These compounds include α-Linolenic acid from *Arthrospira* sp., Arachidonic acid from *P. cruentum*, Docosahexaenoic acid from *I. galbana*, Brassicasterol from *Chaetoceros* sp., Stigmasterol from *I. galbana*, Pyocyanine from *Arthrospira* sp., Phenothrin from *P. cruentum*, β-Carotene from *D. salina*, Ascorbic acid from *Arthrospira* sp., Phylloquinone from *P. cruentum*, Cyanocobalamin from *Pavlova* sp., Retinol from *I. galbana*, α-Tocopherol from *Pavlova* sp. and γ-Aminobutyric acid from *Porphyridium* sp. (Raposo et al., 2013).

All the fourteen bioactive compounds showed good binding interaction with specific protein *hsp-70* (Figure-1). Among these seven bioactive compounds showed good hydrogen bond interactions with key amino acid (Table 2). The binding energy of the bioactive compound is α-Tocopherol (34.67), Arachidonic acid (50.21), Retinol (34.21), Docosahexaenoic acid (49.18), Phenothrin (41.36), Phylloquinone (42.05), β-Carotene (32.37), Stigmasterol (31.69), α-Linolenic acid (43.81), Astaxanthin (24.83), Brassicasterol (27.90), Ascorbic acid (24.32) and Pyocyanine (32.14). The bioactive compound, Arachidonic acid from microalgae, *Porphyridium cruentum* exhibited highest potential for enhancement of HSP-70 protein compared to other bioactive compounds.

To our knowledge there is no published report in copepods on target enhancement of cellular pathway especially for phenylalanine, tyrosine and tryptophan biosynthesis pathway. Because these three aromatic amino acids are involved in protein synthesis and synthesis of a variety of secondary metabolites a subset of which are involved in numerous anabolic pathways responsible for the synthesis of pigment compounds, hormones and biological polymers which are against reactive oxygen species in the organisms (Parthasarathy et al., 2018). In the present study, the bioactive compound, arachidonic acid is showing highest binding energy 50.21 kcl/mol with specific hydrogen bond interaction with amino acid is TYR 167. The tyrosine - 167 is potential signaling therapeutically importance against ROS (Budiman et al., 2004). The bioactive compound, arachidonic acid shows the potential enhancement of phenylalanine, tyrosine and tryptophan biosynthesis pathway which show the specific target side of 4.2.1.20 (Figure 2).

Table 2. Bioactive compounds binding interaction with specific protein *hsp-70*

Ligand (Bioactive compound)	Binding score (Kcal/mol)	Hydrogen bond interaction with key aminoacid residues
γ-Aminobutyric acid	24.20	-
α-Tocopherol	34.67	ASP 172
Arachidonic acid	50.21	TYR 167
Retinol	34.47	ASP 172 and SER 166.
Docosahexaenoic acid	49.18	ASP 172
Phenothrin	41.36	-
Phylloquinone	42.05	-
β-Carotene	32.37	-
Stigmasterol	31.69	ASP 172
α-Linolenic acid	43.81	ASP172
Astaxanthin	24.83	-
Brassicasterol	27.90	-
Ascorbic acid	24.32	THR 167, LYS 165
Pyocyanine	32.14	-

This is indicating the final step of tryptophan biosynthesis catalyzed by tryptophan synthase (4.2.1.20). This is the first enzymes known to catalyze two different reactions in two separate active sites connected to each other via a tunnel on the interior of the protein. This amino acid is involved in the necessary component of the animal diet. Oba et al. (2009) reported based on the *in vivo* model synthesis of luciferin for various biolumineseces reactions by including phenylalanine, tyrosine and tryptophan to marine calanoid copepod, *Metridia pacifica*. This observation is supported by the earlier report of Zhang et al. (2006) suggesting that an increase in anthranilate phosphoribosyltransferase (a key enzyme in tryptophan biosynthesis) level in response to environmental change such as oxidative and heat shock stress (HSP-70) indicates the presence of a metabolic machinery constantly sustaining life functions. Previous report shows, the enhancement of HSP-70 during the quiescent to subitaneous egg stage has been reported in *A. tonsa* (Nilsson et al., 2013). These studies suggest that, the bioactive compound, arachidonic acid from microalgae, *P. cruentum* can provide enhanced egg production, augmented growth, and immune stimulation in copepod culture combating stress factors.

Conclusion

In the present study, the bioactive compound arachidonic acid from microalgae, *P. cruentum* is showing potential for enhancement of HSP-70 by involving in the main cellular synthesis pathway of phenylalanine, tyrosine and tryptophan biosynthesis with specific target site of tryptophan synthase (4.2.1.20). The inclusion of microalga, *P. cruentum* in the diet of copepod culture could provide enhanced egg production leading to high density culture which in turn promotes marine finfish larval rearing. Further *in vivo* investigations should be carried out on copepods for confirming the egg production with this microalgal diet.

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Compliance with Ethical Standards

Authors' Contributions

Both authors contributed equally for this research works.

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

For this type of study, formal consent is not required.

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RESEARCH ARTICLE

Purification of glutathione reductase from some tissues of *Capoeta umbla* and the inhibitory effects of some metal ions on enzyme activity

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ABSTRACT

The aim of this study was to determine the *in vitro* inhibitory effects of some metal ions (silver ion (Ag^+), cadmium ion (Cd^{2+}), cobalt ion (Co^{2+}), copper ion (Cu^{2+}), nickel ion (Ni^{2+}), lead ion (Pb^{2+}) and zinc ion (Zn^{2+})) on glutathione reductase (GR) enzyme activities that purified from the gill, kidney and liver tissues of *Capoeta umbla*. For this purpose, the enzyme was purified from the gill, kidney and liver of *C. umbla* freshwater fish using ammonium sulfate precipitation and affinity column chromatography methods using 2′,5′-ADP Sepharose 4B. Within this study, the GR enzyme was purified for the first time from the tissues of *C. umbla*. Enzyme purity and molecular weight were determined using the sodium dodecyl sulfate polyacrylamide gel electrophoresis method. In addition, the inhibitory effects of different metal ions (Ag^+, Cd^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+}) on GR enzyme activities of the gill, kidney and liver tissue of *C. umbla* were investigated under *in vitro* conditions. The metal ion concentrations inhibiting 50% of enzyme activity (IC_{50}) were obtained by plotting activity percentage versus [I] figures. Finally, the dissociation constants of the enzyme inhibitor complex (*Ki*), and the inhibition types, were calculated from Lineweaver–Burk plots. *In vitro* inhibition rank order was determined as $Ag^+ > Co^{2+} > Pb^{2+} > Zn^{2+} > Cu^{2+}$ for *C. umbla* gill GR; $Ag^+ > Pb^{2+} > Co^{2+} > Ni^{2+} > Zn^{2+}$ for *C. umbla* liver GR; $Ag^+ > Cu^{2+} > Co^{2+} > Pb^{2+} > Ni^{2+}$ for *C. umbla* kidney GR. From these results, we showed that Ag^+ metal ion is the most potent inhibitor of GR enzyme on gill, liver and kidney tissues. Our results also demonstrate that these metals might be dangerous at low micromolar concentrations for *C. umbla* GR enzyme.

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Introduction

Glutathione reductase (GR) catalyzes the conversion of oxidized glutathione (GSHG) to its reduced form (GSH) and allows the ratio of GSH/GSSG to stay at a certain level (Kuzu et al., 2016). Glutathione (γ-L-glutamyl-L-cysteinylglycine: GSH) is the most important source of nucleophilic thiol equivalents found in cells. Also, the function of some proteins depend on the stability of thiol: disulfide exchange reactions. GSH / GSSG ratios are very important for cell survival, so, it is absolutely necessary to regulate the pentose phosphate pathway system (Akkemik et al., 2011). In the cell, while an increase in GSSG concentration inhibits many important enzyme systems, a decrease in GSH causes oxidative damage and pathological problems like cancer, apoptosis, aging, AIDS, diabetes, Alzheimer's and Parkinson's disease (Thaikovskaya et al., 2005; Fraternale et al., 2009; Simic et al., 2009; Gironi et al., 2011; Raza, 2011; Kirici et al., 2017a).

In this century, our earth is polluted, particularly by human activities like scrap metal disposal and agricultural practices. Therefore, living organisms, especially fish, are intensively exposed to metals in the environment. The toxic effects of metals on aquatic organisms have been investigated by many researchers. In addition, many researchers are attempting to make sense of how metals affect enzymes and the mechanisms at work (Ceyhun et al., 2011; Loro et al., 2012; Qu et al., 2014; Kirici et al., 2016a, b; Kucuk & Gulcin, 2016; Kirici et al., 2017b; Kırıcı et al., 2020). Until now, no studies have been found in published literature on the purification of GR from *Capoeta umbla* gills, liver and kidney tissue, and the effects of Ag^{+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+} metal ions on GR activity of *C. umbla*. The purpose of this study was to contribute to the understanding of any possible effects of metal ions on purified *C. umbla* tissues GR *in vitro*.

Material and Methods

Materials

Chemicals for electrophoresis, 2′,5′-ADP Sepharose-4B, NADPH, protein assay reagents and GSSG were obtained from Sigma-Aldrich. Silver nitrate ($AgNO_3$), copper sulfate pentahydrate ($CuSO_4.5H_2O$), cobalt nitrate hexahydrate ($Co(NO_3)_2.6H_2O$), nickel chloride hexahydrate ($NiCl_2.6H_2O$), lead nitrate ($Pb(NO_3)_2$), zinc chloride ($ZnCl_2$), cadmium sulfate hydrate ($3CdSO_4.8H_2O$) and all other chemicals used were of analytical grade and obtained from Merck.

Preparation of the Homogenates

Fish samples (n = 10; 190 ± 20 g) were caught from the Murat River (Bingöl, Turkey). The fish were dissected in the abdominal region and their kidney and liver tissues were removed. Then the head region was cut off and their gill tissues were removed. Eight grams of gill, kidney and liver tissue samples were washed three times with 0.9% NaCl solution. Then, with the aid of a scalpel, the tissue samples were cut into small pieces. These pieces were homogenized with the aid of liquid nitrogen and suspended in a 50 mM KH_2PO_4 buffer (at a pH of 7.4) that included 1 mM of phenylmethylsulfonyl fluoride (PMSF), 1 mM of ethylenediaminetetraacetic acid (EDTA) and 1 mM of 1,4-dithiothreitol (DTT). The suspension was centrifuged (Hettich Universal 320, Tuttlingen, Germany) at 13500 rpm for 2 hours at 4°C, and then the precipitate was thrown away. The supernatant was stored at 4°C (Le Trang et al., 1983).

GR Activity Assay

Enzyme activity was measured spectrophotometrically with a Beckman Coulter DU730 UV/Vis Spectrophotometer (Beckman Coulter Inc., California, America), at 25°C using the modified method of Carlberg and Mannervik (Carlberg & Mannervik, 1975). The assay system used to measure the enzyme activity comprised 50 mM of Tris–HCl buffer (at a pH of 8.0), containing 0.1 mM of NADPH, 1 mM of EDTA and 1 mM of GSSG.

Ammonium Sulfate Fractionation and Dialysis

The precipitation of ammonium sulfate was carried out according to our previous studies (Kirici et al., 2016a, 2016b, 2017b). The precipitation range of this enzyme was determined to be 30 to 80%, 40 to 80% and 40 to 80% for gill, kidney and liver tissue, respectively, in this study. The precipitate was dissolved in 50 mM of phosphate buffer (pH 7.0). This dissolved precipitate was dialyzed in 1 mM of EDTA and 10 mM of K-phosphate buffer containing 5 mM of β-mercaptoethanol (pH 7.5) for 2 hours with two changes of buffer at 4°C.

2′, 5′-ADP Sepharose 4B Affinity Chromatography

The 2′,5′-ADP Sepharose 4B affinity column (1×10 cm) was prepared according to our previous studies (Kırıcı et al., 2015, 2016b, 2017a). The column material was balanced with 50 mM of K-phosphate buffer including 1 mM of EDTA and 1 mM of DTT (pH 6.0) with a peristaltic pump. The flow rate was adjusted to 50 ml/h. The previously obtained enzyme sample

was loaded onto the affinity column. Afterwards, the chromatography column was washed with equilibration buffer (50 mL of 50 mM of K-phosphate buffer including 1 mM of EDTA and 1 mM of DTT, at a pH of 6.0) and washing buffers (25 mL of 0.1 M K-phosphate + 0.1 M of K-acetate at a pH of 6.0 and 25 mL of 0.1 M K-phosphate + 0.1 M of K-acetate at a pH of 7.85). The enzyme was eluted with 1 mM of GSH + 0.5 mM of NADPH + 1 mM of EDTA in 50 mM of K-phosphate, at a pH of 7.5. One milliliter samples of the eluates were placed into Eppendorf tubes and the activity was individually measured. Active fractions were collected. All the procedures were performed at 4°C (Le Trang et al., 1983).

Protein Determination

Protein concentration was determined at a wavelength of 595 nm using the Bradford method (Bradford, 1976). Bovine serum albumin was used as the standard during this process.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out using the Laemmli method to check the purity of the enzyme (Laemmli, 1970). Ten milliliters of the sample were placed on each electrophoresis gel stick. The acrylamide concentration of the stacking and the separating gels were 3% and 8% containing 1% SDS, respectively. The gel image obtained after electrophoresis was photographed (Figure 1).

In vitro Effects of Metal Ions

The inhibitory effects of Ag^{+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+} were investigated at five different concentrations for the fish gill, kidney and liver GR enzyme activities. The control cuvette with 100% enzyme activity did not contain any metal ions. GR activity was measured in the presence of different metal ion concentrations. The IC_{50} values were obtained from activity percentage versus metal ion concentration plots (Figure 2).

GSSG was used as a substrate for the analysis of the GR enzyme activities. In this study, substrate concentrations were determined to be 0.3, 0.8, 1.4, 2 and 3 mM. Metal ions were added to the reaction medium to provide inhibitors at three different constant concentrations. Lineweaver–Burk (Lineweaver and Burk, 1934) plots for fish gill, kidney and liver GR enzyme activities were drawn by using three different metal ion concentrations and five different substrate (GSSG) concentrations. After this, the *Ki* values and inhibition types were calculated using the Lineweaver–Burk curves (Figure 2).

Results

In this research, GR enzyme was first purified from *C. umbla* gill, kidney and liver tissue. The purification procedure consisted of three steps: preparation of the homogenate, ammonium sulfate precipitation and finally, affinity gel chromatography. The purity of the enzymes were determined by SDS-PAGE and showed single bands on the gel (Figure 1). R_f values were calculated for standard proteins and GR according to Laemlli's procedure from R_f-LogMW plots. Molecular weights for the gill, kidney and liver GR enzymes were 50, 53 and 55 kDa, respectively.

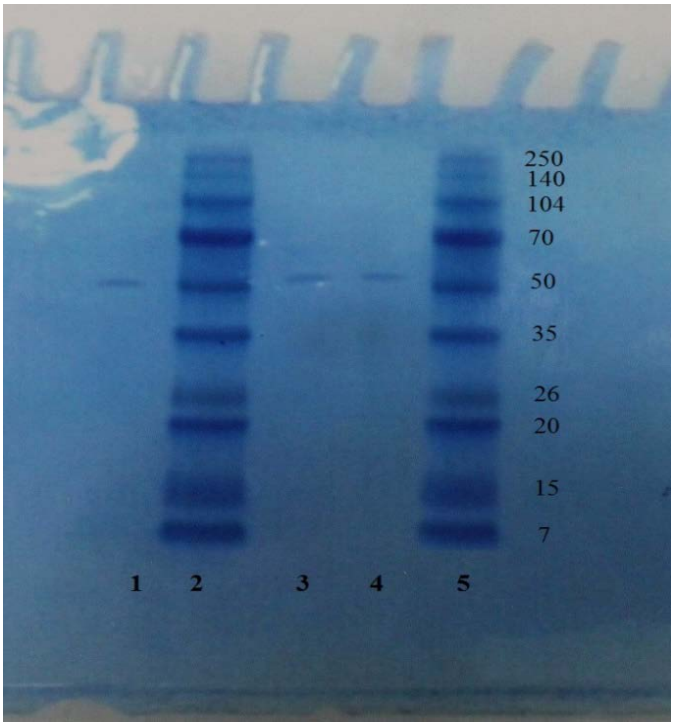

Figure 1. SDS-PAGE photograph SDS-PAGE analysis of purified liver G6PD. Lane 1: molecular-mass markers (kDa): *Escherichia coli* β-galactosidase (116), rabbit phosphorylase B (97.4), bovine serum albumin (66), chicken ovalbumin (45) and bovine carbonic anhydrase (29) (Sigma: MW-SDS-200) (Lane 1: Gill; Lane 2, 5: Standard proteins; Lane 3: Kidney; Lane 4: Liver).

C. umbla gill GR enzyme was purified by using 2′,5′-ADP Sepharose 4B affinity chromatography and found to have a specific activity of 13.54 U/mg proteins, a 1167-fold purity improvement and a yield of 48.74% (Table 1).

Afterwards, the *in vitro* inhibitory activities of Ag^{+}, Co^{2+}, Cu^{2+}, Pb^{2+} and Zn^{2+} metal ions were evaluated for the fish gill GR enzyme. IC_{50} values were found to be 0.0097, 0.844, 11.18, 0.944 and 1.313 mM for Ag^{+}, Co^{2+}, Cu^{2+}, Pb^{2+} and Zn^{2+}, respectively, and their K_i constants were 0.006 ± 0.001,

Table 1. Purification scheme of GR enzyme from gill, kidney and liver tissues of *C. umbla*

Tissue	Purification step	Activity (U/mL)	Protein (mg/mL)	Total volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification factor	Yield (%)
Gill	Hemolysate	0.193	16.64	29.5	5.694	490.9	0.012	1	100
	Ammonium sulfate precipitation	0.328	4.27	9.6	3.149	40.99	0.077	6.621	55.30
	2', 5'-ADP Sepharose 4B affinity chromatography	0.555	0.041	5	2.775	0.205	13.54	1167	48.74
Kidney	Hemolysate	0.276	7.28	25	6.9	182	0.038	1	100
	Ammonium sulfate precipitation	0.334	1.06	6.5	2.171	6.89	0.315	8.31	31.46
	2', 5'-ADP Sepharose 4B affinity chromatography	0.531	0.036	3	1.593	0.108	14.75	389.2	23.09
Liver	Hemolysate	0.463	43.81	31	14.35	1358	0.011	1	100
	Ammonium sulfate precipitation	0.609	33.46	11.3	6.882	378.1	0.018	1.72	47.95
	2', 5'-ADP Sepharose 4B affinity chromatography	0.825	0.049	3	2.475	0.147	16.84	925.1	17.24

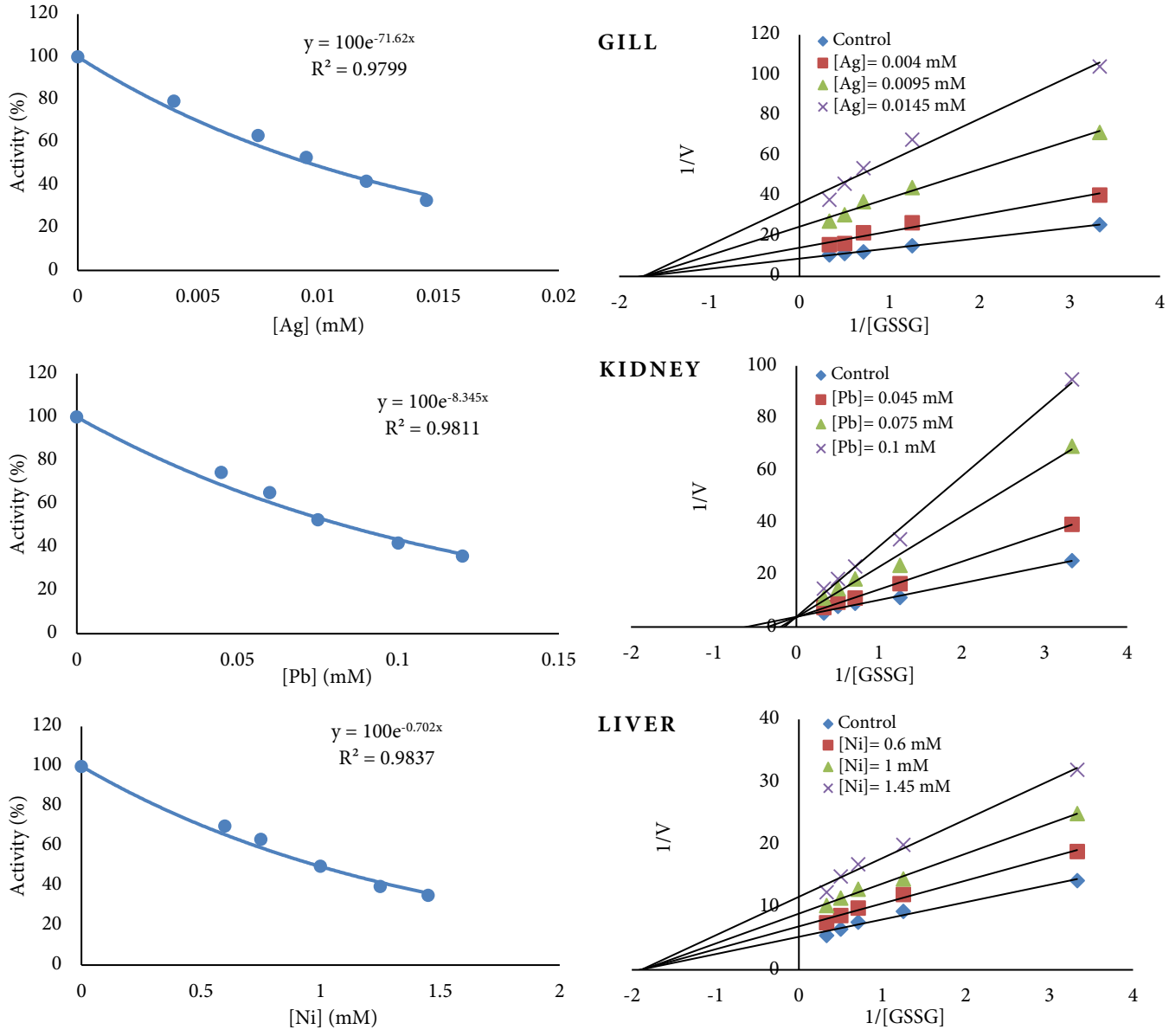

Figure 2. Activity (%) ±[Metal] regression analysis graphs for some fish tissues GR in the presence of five different metal concentrations

0.921 ± 0.196, 4.493 ± 0.806, 0.533 ± 0.377 and 1.850 ± 1.034 mM, respectively. From the results obtained, the sequence of the inhibitors was $Ag^{+} > Co^{2+} > Pb^{2+} > Zn^{2+} > Cu^{2+}$ (Table 2 and Figure 2). While Cu^{2+} and Pb^{2+} showed competitive inhibition, Ag^{+}, Co^{2+} and Zn^{2+} inhibited fish gill GR in a non-competitive manner.

Table 2. IC_{50} values, K_i constants and inhibition types of some metal ions GR obtained from *C. umbla* gill

Metal ions	IC_{50} (mM)	K_i (mM)	Inhibition type
Ag^{+}	0.0097	0.006 ± 0.001	Non-competitive
Co^{2+}	0.844	0.921 ± 0.196	Non-competitive
Cu^{2+}	11.18	4.493 ± 0.806	Competitive
Pb^{2+}	0.944	0.533 ± 0.377	Competitive
Zn^{2+}	1.313	1.850 ± 1.034	Non-competitive

C. umbla kidney GR enzyme was purified by using 2′,5′-ADP Sepharose 4B affinity chromatography and was found to have a specific activity of 14.75 U/mg proteins, a 389.2-fold purity improvement, and a yield of 23.1% (Table 1). Afterwards, the *in vitro* inhibitory activities of Ag^{+}, Cd^{2+}, Co^{2+}, Pb^{2+} and Zn^{2+} metal ions were evaluated for fish kidney GR enzyme. IC_{50} values were found to be 0.00087, 0.559, 0.569, 0.083 and 0.487 mM for Ag^{+}, Cd^{2+}, Co^{2+}, Pb^{2+} and Zn^{2+}, respectively, and their K_i constants were 0.0009 ± 0.0005, 0.824 ± 0.124, 1.203 ± 0.210, 0.043 ± 0.017 and 0.124 ± 0.018 mM, respectively. From the results obtained, the sequence of the inhibitors was $Ag^{+} > Pb^{2+} > Zn^{2+} > Cd^{2+} > Co^{2+}$ (Table 3 and Figure 2). Zn^{2+} and Pb^{2+} showed competitive inhibition, while Ag^{+}, Cd^{2+} and Co^{2+} inhibited fish kidney GR in a non-competitive inhibition manner.

Table 3. IC_{50} values, K_i constants and inhibition types of some metal ions GR obtained from *C. umbla* kidney

Metal ions	IC_{50} (mM)	K_i (mM)	Inhibition type
Ag^{+}	0.00087	0.0009 ± 0.0005	Non-competitive
Cd^{2+}	0.559	0.824 ± 0.124	Non-competitive
Co^{2+}	0.569	1.203 ± 0.210	Non-competitive
Pb^{2+}	0.083	0.043 ± 0.017	Competitive
Zn^{2+}	0.487	0.124 ± 0.018	Competitive

C. umbla liver GR enzyme was also purified using 2′,5′-ADP Sepharose 4B affinity chromatography and found to have a specific activity of 16.84 U/mg proteins, a 925.1-fold purity improvement and a yield of 17.24% (Table 1). Afterwards, the *in vitro* inhibitory activities of Ag^{+}, Co^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+} metal ions were evaluated for the fish liver GR enzyme. IC_{50} values were found to be 0.0006, 0.881, 0.987, 0.092 and 3.194 mM for Ag^{+}, Co^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+}, respectively, and their K_i constants were 0.0005 ± 0.0002, 0.408 ± 0.009, 1.506 ± 0.359, 0.061 ± 0.016 and 2.304 ± 0.486 mM, respectively. From the results obtained, the sequence of the inhibitors was $Ag^{+} > Pb^{2+} > Co^{2+} > Ni^{2+} > Zn^{2+}$ (Table 4 and Figure 2). Ag^{2+} and Pb^{2+} showed competitive inhibition, while Ni^{+}, Co^{2+} and Zn^{2+} inhibited fish gill GR in a non-competitive inhibitory manner.

Table 4. IC_{50} values, K_i constants and inhibition types of some metal ions GR obtained from *C. umbla* liver

Metal ions	IC_{50} (mM)	K_i (mM)	Inhibition type
Ag^{+}	0.0006	0.0005 ± 0.0002	Competitive
Co^{2+}	0.881	0.408 ± 0.009	Non-competitive
Ni^{2+}	0.987	1.506 ± 0.359	Non-competitive
Pb^{2+}	0.092	0.061 ± 0.016	Competitive
Zn^{2+}	3.194	2.304 ± 0.486	Non-competitive

Discussion

There are many chemicals that have harmful or beneficial effects on the metabolic reactions that occur in the living body, especially enzymes. The toxicological effects of metals are known as usually enzyme denaturation and inhibition (Ekinci & Beydemir, 2010). There are some enzymes that play a crucial role in metabolic pathways. Some chemicals, especially metals, cause some metabolic diseases by increasing or decreasing the activity of these enzymes, such as Alzheimer's, diabetes and Parkinson's disease (Tchaikovskaya et al., 2005; Gironi et al., 2011; Raza, 2011). Usually, it inhibits the enzyme by binding the metal to the protein. Inhibition of enzymes can be deadly for the metabolism of all living organisms, especially fish (Innocenti et al., 2010).

The harmful effects of metals on the living body are prevented by enzymatic and non-enzymatic antioxidant defense systems. The enzymatic antioxidant defense system consists of many enzymes such as glutathione reductase (GR), superoxide dismutase, glutathione peroxidase, catalase and glutathione s-transferase. On the other hand, the non-enzymatic antioxidant defense system consists of different agents such as vitamins, transferrin, lactoferrin, taurine and glutathione. In particular, one of the most important protective systems in cells is glutathione metabolism (Knapen et al., 1999). GR is especially required for the protection of reduced cellular glutathione, which is highly nucleophilic for many reactive electrophiles. The GR enzyme protects many vital functions such as detoxification of free radicals and reactive oxygen species in the cell (such as H_2O_2, $O_2^{\bullet}$ and $^{\bullet}OH$), by maintaining a high ratio of GSH/GSSG (Schirmer et al., 1989).

GR has been purified from many different animal, plant and microorganism sources using various procedures: techniques like affinity chromatography, size-exclusive chromatography,

hydrophobic interaction and reversed phase chromatography, ion-exchange chromatography and combinations of these have been used as purifying steps (Carlberg et al., 1981; Le Trang et al., 1983; Akkemik et al., 2011; Taser & Ciftci, 2012; Yadav et al., 2013; Kuzu et al., 2016; Kırıcı et al., 2017c). In the study conducted by Tekman et al. (2008) GR from rainbow trout liver was purified and some of the kinetic features were determined. The study reported that the enzyme was purified 1654 times with a yield of 41% and 27.45 U/mg protein specific activity using the techniques of ammonium sulfate precipitation, 2′,5′-ADP Sepharose 4B affinity chromatography and Sephadex G-200 gel Sepharose filtration chromatography. The researchers reported that the molecular weight of the rainbow trout liver GR was 53 kDa (Tekman et al., 2008). Similarly, it was also reported that the enzyme from *Chalcalburnus tarichi* liver and erythrocyte were purified 4552 and 7619 fold, respectively and the specific activity of the enzyme were 122 EU/mg proteins for liver and 96 EU/mg proteins for erythrocyte. The molecular weight of *Chalcalburnus tarichi* liver and erythrocyte GR were calculated to be 55 kDa using SDS-PAGE (Altun et al., 2015). In this study, GR enzyme was purified from the gill, kidney and liver of *C. umbla* using ammonium sulfate precipitation and 2′,5′-ADP Sepharose 4B affinity chromatography.

Almost all of the chemicals, including metals, exhibit their functions on the mechanism of enzyme interaction (Innocenti et al., 2010). Therefore, the metabolism of all living species is affected by metal toxicity. In particular, it is known that metals are effectively some of the strongest naturally occurring enzyme inhibitors (Kucuk & Gulcin, 2016). The effects of different chemical pollutants, especially metal ions, drugs and pesticides, on GR, glucose 6-phosphate dehydrogenase, carbonic anhydrase, paraoxonase, glutathione s-transferase and aldose reductase enzymes have been investigated in many *in vitro* studies performed with various organisms. For example, Kucuk & Gulcin (2016) reported that Ag^{+}, Co^{2+}, Cu^{2+}, Fe^{2+} and Pb^{2+} inhibited the *Salmo coruhensis* kidney carbonic anhydrase enzyme under *in vitro* conditions. Tekman et al. (2008) demonstrated the *in vitro* inhibition of liver GR enzymes of rainbow trout by metal ions (Al^{3+}, Cd^{2+}, Cu^{2+}, Hg^{2+}, Fe^{3+} and Pb^{2+}). It was reported that Al^{3+}, Cd^{2+}, Cu^{2+}, Hg^{2+}, Fe^{3+} and Pb^{2+} caused inhibition on fish liver GR activity (Tekman et al., 2008). In a similar study, Ozaslan et al. (2017) investigated the *in vitro* inhibitory effects of Cd^{2+}, Cu^{2+}, Zn^{2+} and Ag^{+} on glutathione s-transferase from *Chalcalburnus tarichi* fish gills. They found that Cd^{2+}, Cu^{2+}, Zn^{2+} and Ag^{+} inhibit the *Chalcalburnus tarichi* fish gill glutathione s-transferase enzyme. *In vitro* results showed that metal ions inhibit fish gill glutathione s-transferase activity in the sequence $Ag^{+} > Cu^{2+} > Cd^{2+} > Zn^{2+}$.

Conclusion

In this study, GR enzyme was purified from *C. umbla* gill, kidney and liver tissue by preparing the homogenate and using ammonium sulfate precipitation and 2',5'-ADP Sepharose 4B affinity column chromatography methods. Enzyme purity and molecular weight were determined with SDS-PAGE. The inhibitory effects of metal ions (Ag^{+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+}) on GR activity were investigated. K_i constants, IC_{50} values and inhibition types for Ag^{+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+} were determined by plotting activity percentage versus [I] and Lineweaver–Burk plots.

Compliance with Ethical Standards

Authors' Contributions

MK and MK performed the research, analyzed the data and helped to draft the manuscript; MK, MK, MA and SB conceived and designed the work and wrote the manuscript. All authors contributed to and approved the final manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

For this type of study, formal consent is not required.

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RESEARCH ARTICLE

Adverse effects of *Ruditapes decussatus* (Linnaeus, 1758) diet on stomach tissues in rats

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ABSTRACT

Ruditapes decussatus (Linnaeus, 1758) belongs to the *Veneridae* family is given as nutrients to rats. *R. decussatus* was collected from three different locations from the Çanakkale Strait. Twenty-four male Wistar Albino rats (270-310 g) were used in the study. There were six rats for each experimental group. The first group fed with standard rat feed, the second group fed with 80% *R. decussatus* + 20% standard rat feed daily; the third group fed with 80% *R. decussatus* + 20% standard rat feed every other day, the fourth group fed with 80% *R. decussatus* + 80% standard rat feed every three days. After routine histopathological follow-up, gastric tissue samples of all subjects were stained and examined under the light microscope. There were no histopathological findings in the gastric tissues of rats in the control group of hematoxylin-eosin staining. In gastric tissues of rats fed with *R. decussatus*, chronic gastritis caused by mononuclear inflammation between lamina propria and occasional gastric glands was noted. It was observed that inflammation severity and distribution were high especially in tissues of rats fed with *R. decussatus* every day. It was observed that the most commonly consumed shellfish may cause pathological picture on the digestive system of rats. As a result, increasing environmental pollution threatens the life of water as well as land life, and the consumption of living organisms exposed to polluted environments continues to threaten and affect human life. It is important to pay attention to the conditions under which the consumed products are collected and how they are collected.

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Introduction

Seafood is nutritionally important all over the world and there has been a significant increase in fisheries in the last two decades. Asian countries cover 90% of the aquaculture industry. In Turkey, seafood constitutes an important part of the food sector. Shellfish consumption has become widespread in Turkey coastal regions. The issue of food safety in seafood is regionally dependent on environmental conditions and production methods. The microbial quality of seafood varies greatly depending on environmental conditions, microbial quality of water, water temperature, salinity, distance from settlements, natural bacterial flora in water, food consumed by fish, fishing methods and cooling conditions (Kolsarıcı & Ertaş, 1989; Küçüköner & Küçüköner, 1990; Garret et al., 1997; Feldhusen, 2000).

Environmental pollution has been increasing day by day and threatening the lives of many living species including humans. The rapid development in the industry not only threatens the lives of land and aquatic organisms but also causes toxicity in humans who consume these organisms as foodstuffs and other aquatic organisms, which are the food source of humans (Sánchez-Marín et al., 2019). The Çanakkale Strait, an important route for trade ship crossings, has been exposed to environmental pollution for years. In fact, the Bosporus is an important region where both fish migration routes and crustaceans are cultivated. The fish and shellfish obtained here are consumed in other regions as well as the people of the region. There is not much data on the toxicity of seafood exposed to environmental pollution in other organisms. For this reason, the study was planned to determine the effects of clams which are very popular and consumed as a food source, on the stomach tissue of rats.

Ruditapes decussatus (Linnaeus, 1758) [Synonym: *Tapes decussatus* (Linnaeus 1758)] is a bivalve aquatic animal living from 1 to 12 m depth in the Çanakkale Strait (Lapseki, Çardak, Çamburnu) which belongs to *Veneridae* family. It is called Chequered carpet shell and Cross-cut carpet shell in English, Geruite tapijtschelp in Dutch, and Palourde in French. It is known that as a result of pollution in the Çanakkale Strait with environmental factors, heavy metals accumulate in its internal organs, as in other bivalves. In a study conducted on the Umurbey coast in the Çanakkale Strait about some mollusks growing in the region, zinc and manganese were found higher than the acceptable values in the clam (Gezen et al., 2011). In the sea water of the same region, the zinc level was higher than the acceptable values of heavy metals. This study aimed to reveal the histopathological effects of *R. decussatus* diet, which is delicious and has nutritious properties consumed due to human eating habits, on the rat digestive system. The aim of this experimental study is to determine the amount of heavy metals in bivalves and seawater, and compare the bioaccumulation of toxic metals in seawater and detect the relationship between heavy metal levels of bivalves and parameters of the seawater quality; to assess human health risk from heavy metal and determine the maximum amount to be consumed by humans.

Material and Methods

Chemicals and Reagents

The following chemicals were used for histopathological evaluation. 10% neutral buffered formaldehyde solution (Bio Optica, dilution: 1/7), Ethyl Alcohol Absolut (%99.8 Sigma-Aldrich, LB.SA.32221), Paraffin (MERCK, LB.M.107337.9020), xylenes (Honeywell, 16446).

Experimental Animal Design

Twenty-four male Wistar albino rats (270-300±10 g weight) were used in this study. All rats were housed in Experimental Animal (rat) shelter of Çanakkale Onsekiz Mart University, Faculty of Medicine, with an average temperature of 22±1°C, a humidity of 55±5, and a ventilation and air conditioning system for 12 hours of light and 12 hours of darkness. The rats were given as much water as they could drink. Standard rat food and *R. decussatus* were given 15% of the weight of each rat in the feeding planning.

- Group 1 (6); given standard rat feed.
- Group 2 (6); given 80% *R. decussatus* + 20% standard rat feed daily continuously.
- Group 3 (6); given 80% *R. decussatus* + 20% standard rat feed every two days; standard rat feed was given on the other days.
- Group 4 (6); given 80% *R. decussatus* + 20% standard rat feed three days apart; Standard rat feed was given for the other two days. Subjects in each group were fed for 30 days.

Histopathological Examination

Stomach tissue samples taken from all groups were detected in 10% neutral buffered formaldehyde solution (Bio Optica, dilution: 1/7) for 24 hours. The detected tissue samples were passed through graded alcohols (70%, 80%, 90%, 96%, Absolute) alcohol and their water was removed. Later, the tissues that were passed through xylene were made transparent and the tissues were removed from alcohol. Paraffin was allowed to enter into the tissue samples that were passed through xylene + paraffin and paraffin stages in a 60°C oven

(Core 500). Tissue samples extracted from paraffin were blocked using a tissue embedding device. Tissue samples taken into blocks were cut at 4-5 µm thickness in microtome for routine histopathological staining and placed in a water bath. Tissue samples opened here were taken on a normal slide and hematoxylin-eosin staining was applied.

Experiment Material Collecting and Preparing

The samples were collected from 10 to 40 m depth by scuba diving from Lapseki (40°21'6.9840" N, 26°41´31.6248" E), Çardak (40°22'45" N, 26° 42' 50" E) Çamburnu (40°10´1.1460" N, 26°22´18.0372" E). Then the samples were dried in an oven at 60 to 65 °C until a constant weight and then was ground with electric spice grinder (MRC, WSG60E) into powder. The samples were analyzed for cadmium, lead, copper and zinc by inductively coupled plasma-optical emission spectrometry (ICP-OES, Perkin Elmer Optima 8000) (Støving et al., 2013). The material used in the study was stored at -20°C. It was added to the weekly prepared feed mixture after it was dissolved.

Results and Discussion

There was no significant change in the staining of the stomach tissues of the rats in the control group (Figure 1) with hematoxylin-eosin staining. Tunica mucosa, submucosa, lamina propria and muscularis mucosa layers were observed in the normal histological structure. There were signs of active chronic gastritis in the gastric mucosa of rats in the second group (Figure 2). It was observed that the inflammation was distributed between the lamina propria and gastric glands. Among the groups, the most severe inflammation table was seen in this group. While chronic gastritis was observed in the third group (Figure 3), inflammation was observed to be less severe, and mononuclear inflammatory cells were observed rarely in the fourth group (Figure 4). In the semi-quantitative scoring made in terms of inflammation, it was seen that the meaning of gastritis increased in the second group compared to the other groups.

Heavy Metal Analysis

According to the results, considering the Cadmium (Cd), Copper (Cu), Lead (Pb), and Zinc (Zn) level in *R. decussatus* the location where the heavy metal concentration is high are Lapseki and Çardak (Table 1). As heavy metal analysis results of the seawater from which the clam samples were taken, the values of the Lapseki and Çardak region were found to be in parallel with the heavy metal values in the muscle tissue of the clam samples obtained from these regions. Heavy metal accumulation in clam tissue suggests that it may trigger toxicity in tissues with the consumption of marine based food frequently (Table 1).

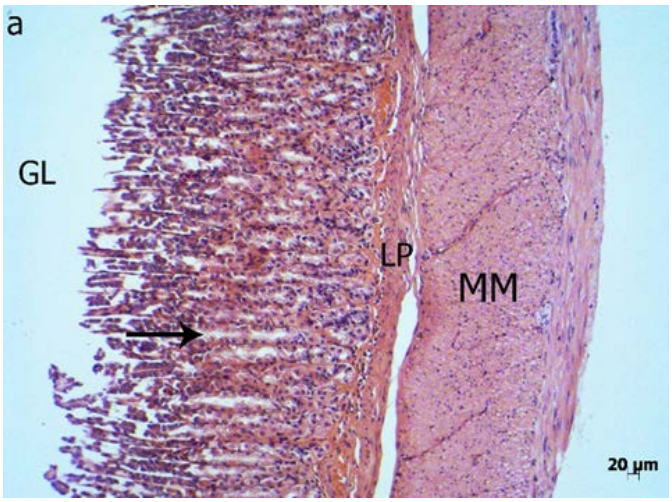

Figure 1. Group 1 Hematoxylin-Eosin staining of the gastric mucosa (X10) (GL: Gastric lumen, LP: Lamina propria, MM, Muscularis mucosa, arrow: Gastric pit)

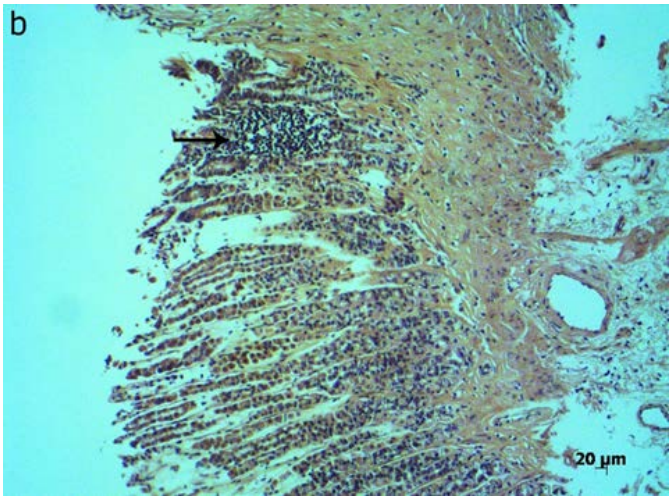

Figure 2. Group 2 Hematoxylin-Eosin (X10) (Arrow: inflammation in the lamina propria)

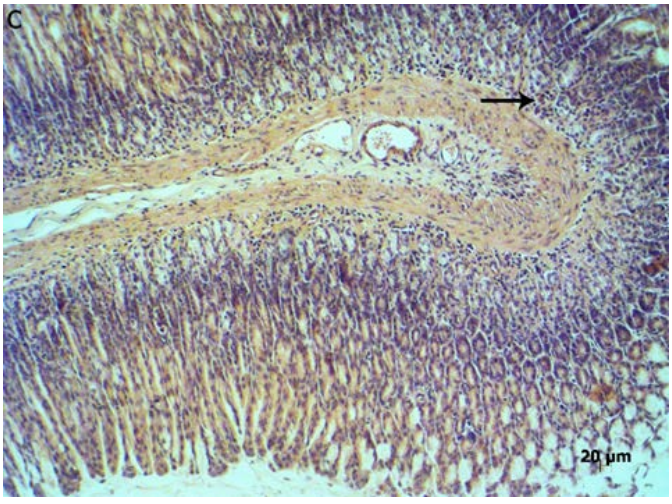

Figure 3. Group 3 Hematoxylin-Eosin staining (X10) (Arrow: inflammation)

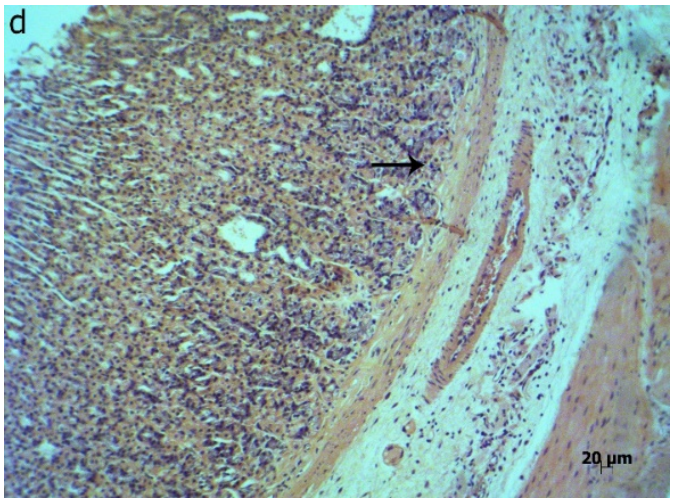

Figure 4. Group 4 Hematoxylin-Eosin staining (X10) (Arrow: inflammation)

As a result of the analyzes, it was determined that Cd, Cu, Pb, and Zn values in sea water were higher than the upper limit values determined by the Turkish Standards Institution (TSI). Cd, Cu, Pb, and Zn levels in sea water samples were found to be higher in the Çardak region and Zn in the Çamburnu region. The upper limit values determined by the Turkish Standards Institution and the World Health Organization are shown in Table 2.

Currently, there are no adequate medical epidemiological studies on the negative effects of marine pollution on human health (Allen, 2011). Various studies have shown that there is an accumulation of elements such as copper, nickel, zinc and lead in cancerous tissue samples (Yaman et al., 2007). Free radicals are electron acceptor molecules of the biological system. Ustunada et al. (2011) determined the values of the metals such as Cu, Zn, Pb and Cd in *C. fragile* at the highest level, respectively. Algae are among the foods consumed in many parts of the world, especially as a salad. They can produce different effects in organisms that consume depending on the pollution of their environment such as sand mussels and other sea creatures. Especially heavy metal accumulation is an inevitable fact in aquatic organisms. Crustaceans have a good filter system and feed by a filtering system. It has a high potential for heavy metal deposition. In the present study, it was found that the amount of lead accumulated in bivalves *in vivo* is quite high (Sánchez-Marín et al., 2019). *In vivo* experimental study shows that cadmium poisoning causes a serious degeneration in kidney epithelial cells in addition to the expansion and adhesion of secondary lamellae in the gills, as well as in fish fed with cadmium-contaminated fish food (Beširović et al., 2011). These studies show crustaceans that store toxic products increase oxidative stress parameters and trigger apoptosis, such as bacteria, infectious agents, and heavy metal, and can pass to other organisms after consumption. In this study, it was determined that the crustacean consumption of the rats was two weeks and 20%, therefore fewer toxic values were found than our findings. However, toxic effects were found to be considerably high in the stomach tissue of rats that we fed with long-term and shelled feed.

Table 1. Heavy metal concentrations of *R. decussatus* muscle tissue (µg/g dry weight, *Turkish Food Codex, **World Health Organization)

Heavy metals and Region	Cd	Pb	Cu	Zn
Camburnu	1.32±0.23	0.68±0.45	1.47±0.28	20.74±2.6
Lapseki	1.54±0.5	0.81±0.47	1.53±0.3	23.74±3.87
Cardak	0.94±0.24	0.45±0.38	0.82±0.33	18.24±4.22
Average value	1.26±0.32	0.64±0.43	1.27±0.3	20.90±3.56
Limit value (µg/g) **WHO	1	1	5	20
Limit values (mg/L) *TFC	0.5	0.5	3	10

Table 2. Heavy metal analysis of seawater for different region in Çanakkale Strait (*Turkish Standards Institution, **World Health Organization, United States Environmental Protection Agency)

Heavy metals (µg/L)	Region			Average value	Limit values (mg/L) *TSI	Limit values (mg/L) **WHO and USEPA
	Çamburnu	Çardak	Lapseki			
Cu	1.94±0.28	2.24±0.65	1.88±0.54	2.02±0.49	0.01	0.01
Cd	1.75±0.28	1.72±0.45	1.74±0.66	1.73±0.46	0.01	0.01
Pb	0.85±0.22	0.86±0.35	0.94±0.44	0.88±0.36	0.1	0.10
Zn	40.14±3.84	42.62±4.02	42.35±5.20	41.70±4.35	0.1	0.10

Active oxygen derivatives are called oxidants (Erken, 2012). They cause cell death by causing protein modifications, lipid peroxidation and DNA fragmentation (Bakonyi and Radak, 2004). It is known that there are different defense mechanisms against free radicals such as repair, physical defense, and antioxidant defense. A small part of the reactive oxygen varieties produced in the body escape from the antioxidant defense system and cause some systemic diseases and aging (Berger, 2005). Heavy metals and infectious agents that pass into tissues with foods disrupt the oxidant/antioxidant balance and cause cell death along with oxidative stress. In this study, it has been shown that excessive consumption of *R. decussatus* can cause serious damage to the rat digestive system, especially these creatures that are not supplied from a clean environment.

The data obtained in this study revealed that *R. decussatus* grown in Lapseki-Çardak region of the Çanakkale Strait may cause histopathological changes in digestive system of organisms fed with these animals frequently. Because of it should be ensured that water resources are kept clean. The number of researches on aquatic organism in polluted waters should be increased and these foods obtained from areas considered to be contaminated should be consumed after a strict control.

Conclusion

It is possible that crustaceans can transmit harmful factors such as heavy metals, pesticides, viral and bacterial organisms collected by filtering from their diet to the mammals consuming these animals. However, in vivo studies are scarce in the literature. Therefore, the findings obtained from the present study may shed light on experimental diet studies. It is required to make a more comprehensive study, to develop concurrent nutritional models and to obtain clinical results. An experimental feeding model will provide an idea about whether the histopathological findings that we think may be acute will cause chronic disease. The scarce of the available reports caused limitations in our interpretation of the clinical picture to compare the findings. Food safety has a great importance for health. Habitat conditions in which aquatic organsims consumed as food sources obtained are important. For this reason, importance should be attached to the studies determining environmental pollution and international measures should be increased on what can be done to eliminate the negative effects caused by this situation.

Compliance with Ethical Standards

Authors' Contributions

Author LCİ designed the study. ŞÖ and LCİ wrote the first draft of the manuscript, LCİ and ŞÖ performed and managed statistical analyses. Both authors read and approved the final manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

A total of 24 male Wistar albino rats were used in the study. The study protocol was approved by the Çanakkale Onsekiz Mart University Ethics Committee for Animal Research (Protocol number: 2021/02-01).

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RESEARCH ARTICLE

Investigation of the use of zeolite (Clinoptilolite) as aquarium filtration material for electric blue hap (*Sciaenochromis ahli*)

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ABSTRACT

In this study, the effects of using zeolite, both inside and outside the filter, on water quality and the growth of electric blue hap (*Sciaenochromis ahli*) were investigated. The 3-month study consisted of 7 groups in triplicate. One of the groups was designated as the control, and zeolite was not used in this group. For the remaining 6 groups, zeolite was placed in tulle bags and used both inside and outside the filter in 3 various ratios (0.35, 0.70, 1.05 g l^{-1}). The statistical differences between groups for certain water quality parameters pH, NH_3) were significant ($P<0.05$), while the statistical differences between groups for other parameters (water temperature, dissolved oxygen) were found to be insignificant ($P>0.05$). Furthermore, the growth parameters, feed conversion ratio, and survival rate of the electric blue hap were determined. At the end of the study, the differences between weight gain, specific growth rate and feed conversion ratio were determined as statistically insignificant ($P>0.05$). At the end of the study, when the data were evaluated, no negative effects on the growth parameters and water parameters of the ahli cichlid fish were determined. Therefore, it is advisable to place zeolite in mesh bags at the bottom of the aquarium and in the aquarium filter to prevent ammonia from reaching high concentrations. Thus, when the findings on pH and NH_3 of the present study were evaluated, it can be suggested that low ratios as 0.35 g l^{-1} of zeolite may be used in tulle bags on floor or inside the filter to prevent ammonia rising to high concentrations.

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Introduction

Intensive aquaculture facilities frequently produce organic wastes such as feed residue and metabolic discharge. Ammonia, usually originating from organic waste, must be monitored and controlled in fish culture. Ammonia is difficult to detect as it is colorless and odorless in lower concentrations (Hagreaves & Tucker, 2004; Yıldırım & Korkut, 2004).

It has been reported that the toxicity of un-ionized ammonia (NH_3) begins at 0.05 mg l^{-1}, and causes mortality at 2 mg l^{-1} (Swann, 1992; Jorgersen, 2002; Deng, 2004; Hargreaves & Tucker, 2004; Floyd & Watson, 2005; Hekimoğlu, 2009).

In the physicochemical treatment process conducted with ion exchange, a type of zeolite; clinoptilolite, capable of keeping nitrogen compound away from aquatic habitats and maintaining ammonium (NH_4) adsorb around 90%, has caught attention as an environment-friendly, economical, and efficient material. Zeolites are hydrated crystalline aluminosilicates. The cage-like microporous structure of zeolite provides large internal and external surface areas for ion exchange (Uğurlu & Pınar, 2004; Surmeli (Sava) et al., 2019).

In the chemical treatment process, among nearly 40 natural zeolites, clinoptilolite is commonly used for this purpose due to its efficiency and low-cost (Emadi et al., 2001; Yörükoğulları, 2005; Deng, 2014). Ammonia concentrations in especially domestic and industrial wastewaters are much higher than those in aquaculture systems. It has been reported that while the ammonium concentration in aquaculture wastewaters should be as low as 1 mg l^{-1}, it may be 10 times higher in municipal wastewaters, and may exceed 100 mg l^{-1} in industrial wastewaters (Jorgersen & Weatherley, 2003). Furthermore, the ammonia adsorption efficiency of zeolites in aquaculture is affected by the chemical and physical properties of both the zeolite to be used, and the water to be treated (Şahin et al., 2018a; Şahin et al., 2019). It has been reported that the organic and suspended solids in the water may affect the charge density on the zeolite surface by providing cation exchange sites for NH_4^+ adsorption, or block the pores of zeolite and thus, block the NH_4 adsorption sites of the zeolite (Nguyen & Tanner, 1998). Therefore, increasing the number of studies regarding the use of zeolite in aquaculture systems is important for both the aquaculture industry and the utilization of zeolite. Zeolites can be used inside the filtration systems, as well as by placing at the bottom of aquariums (Öz et al., 2017; Skleničková et al., 2020). There are several studies conducted on the growth, reproduction, and water quality of the electric blue hap (*Sciaenochromis ahli*), a popular ornamental fish species (Trewavas, 1935) (Güllü et al., 2008; Erdoğan et al., 2012; Karslı et al., 2014). However, there is no research on the effect of zeolite on aquaculture conditions of the electric blue hap.

According to Zain et al. (2018), even though there were many research conducted on the application of zeolite in fish culture, there is no specific dosage for specific fish culture. On top of that, it is considered to be an obstacle in application of zeolite for large-scale aquaculture (Ghasemi et al., 2016). The specific dose of zeolite is impossible to be recommended especially in fish rearing system. The dose of zeolite is depending on such factors as the stocking density of fish, protein content in feed, feed stability and definitely the quality of water (Abdel-Rahim, 2017).

In this study, the effects of using zeolite, inside and outside the filter, on water quality and the growth of the electric blue hap were investigated.

Material and Methods

The study was conducted at Sinop University Fisheries Faculty Aquarium Fish Culture Unit. The trial consisted of 7 groups in triplicate, and zeolite was not used in the control (C) group. 1-3 mm sized zeolites were placed in tulle bags for the other 6 groups in 3 varying ratios (0.35, 0.70, 1.05 g l^{-1}). After zeolite into net bags were placed on the bottom of the aquarium and into aquarium filter. 3 of the bags were placed inside the filter (IF) and the other 3 at the bottom of the aquaria (OF). In the study, internal (canister) filters were used, which are appropriate for physical and biological filtration, functioning in 50 cm at 500 l $hour^{-1}$ capacity (Skleničková et al., 2020). Electric blue hap, an interesting species among aquarium fish, was used in the experiment. Fish were obtained from Akdeniz Fisheries Research, Production, and Education Institute (Kepez, Antalya, Turkey). In this study, a total of 273 fish were used, with 13 fish in each aquarium randomly for each repetition. The aquarium used in the study were 30×45×50 cm sized glass tanks with 20 l capacity. The uneaten feed and feces were removed by a siphon method and, 10% volume of water in each aquarium was changed twice a month.

The filtration material (commercially known as Filter-Clino) was obtained from Enli Mining as 1-3 mm sized granules. Zeolites, prior to being placed in tulle bags, were washed until clear and dried at 105°C (Nguyen & Tanner, 1998, Öz et al., 2017). The study was conducted for 3 months, and the water quality parameters were regularly monitored at 3-day intervals. Dissolved oxygen, pH, ammonium, and temperature were measured with the YSI Professional Plus multiparameter. Daylight photoperiod (10 hours light – 14 hours dark) was applied in the experiment.

The fish weight was measured monthly, and they were starved on the measuring day. The fish were fed twice a day *ad-*

libitum. The trial feed was commercial cichlid feed containing 40.3% crude protein, 6.1% crude fat, 2.2% cellulose, and 9.9% ash.

Data Evaluation

The following variables were calculated as:

$$Weight\ Gain\ (g) = Final\ weight\ (g) - Initial\ weight\ (g) \quad (1)$$

$$\text{SGR} = \frac{\ln W_2 - \ln W_1}{\text{Number of experiment days}} \times 100 \quad (2)$$

SGR: Specific growth rate (%day^{-1})

W_2: Final weight (g)

W_1: Initial weight (g)

$$\text{FCR} = \frac{\text{Total weight of given dry feed (g)}}{\text{Total weight gain by fish (g)}} \quad (3)$$

FCR: Feed conversion ratio

$$\text{SR} = \frac{\text{Final number of fish}}{\text{Inital number of fish}} \times 100 \quad (4)$$

SR: Survival rate (%)

During the study, NH_3 and TAN levels were calculated from NH_4^+, water temperature and pH values (Emerson et al., 1975; Chow et al., 1997; EPA, 1999). The calculation of the ammonium concentration was given below:

The dissociation constant, K_a, of ammonium ion was expressed as:

$$K_a = [NH_3][H^+][NH_4^+] \quad (5)$$

Eq (5) can be further arranged as:

$$[NH_3][H^+] = K[H^+] \quad (6)$$

Thus, the relationship between ammonia and ammonium concentrations may be described by:

$$\log 10\,[NH_3][NH_4^+] = pH - pK_a \quad (7)$$

pK_a varies with solution temperature. This temperature dependence is given by Emerson et al. (1975) as follows:

$$pK_a = 0.09018 + 2729.22(273.2 + T) \quad (8)$$

Where T is the solution temperature in °C. Also;

$$[NH_4^+] = [NH_3]T - [NH_3] \quad (9)$$

$[NH_3]T$ being the total concentration of ammonia forms. Rearrangement of this equation yields can be explained as follows:

$$\log 10\,[NH_3][NH_3]T - [NH_3] = pH - [0.09018 + 2729.92(273.2 + T)] \quad (10)$$

Statistical Analysis

The results obtained from the study were statistically analyzed using *Minitab Release 17 for Windows* software at 5% level of significance. To data meeting the prior conditions of variant analysis, parametric (ANOVA), to those that did not, non-parametric tests (Kruskal-Wallis) were used. All data on growth, FCR and survival of fish are expressed as mean ± standard error (SE).

Results

At the beginning of the study, water temperature, dissolved oxygen, pH and ammonium were arranged as 26.5°C, 6.69 mg l^{-1}, 8.7 and 0.2 mg l^{-1}, respectively suitably for ahli cichlid culture and were same for all groups (P>0.05). Throughout the ninety-day experiment period, measurements were made periodically, and certain water quality parameters were determined. The water quality parameters determined at the end of the experiment are shown in Table 1.

Table 1. Water quality parameters in the 3-month experiment (mean±SE)*

Experimental Groups	Water Quality Parameters			
	Water Temperature (°C)	pH	Oxygen (mg l^{-1})	NH_3 (mg l^{-1})
0.35 OF	24.9±0.13	8.79±0.02^b	7.09±0.07	0.28±0.015^b
0.70 OF	24.9±0.13	8.78±0.02^b	7.18±0.09	0.29±0.015^b
1.05 OF	24.8±0.12	8.91±0.02^a	7.13±0.08	0.43±0.023^a
0.35 IF	24.8±0.12	8.81±0.02^b	7.05±0.08	0.30±0.015^b
0.70 IF	24.8±0.12	8.90±0.02^a	7.20±0.07	0.39±0.020^a
1.05 IF	24.8±0.15	8.89±0.02^a	7.16±0.11	0.42±0.026^a
Control	24.8±0.12	8.80±0.02^b	7.12±0.09	0.29±0.016^b
P	**0.99**	**0.00**	**0.76**	**0.00**

Note: SE: Standard error; *Different letters in the same column indicate significant differences between experimental groups (P<0.05). OF: Outside the filter; IF: Inside the filter

Table 2. Growth parameters, feed conversion ratio (FCR), and survival rate of the electric blue hap in the 3-month trial (mean±SE)*

Experimental Groups	Growth Parameters*					
	W_1 (g)	W_2 (g)	WG (g)	SGR (% day^{-1})	FCR	SR (%)
0.35 OF	1.35±0.07	3.98±0.22	2.64±0.01	1.49±0.00	1.29±0.01	100±0.00
0.70 OF	1.35±0.07	3.74±0.27	2.39±0.24	1.40±0.05	1.42±0.01	100±0.00
1.05 OF	1.33±0.07	3.84±0.27	2.51±0.21	1.45±0.04	1.37±0.01	100±0.00
0.35 IF	1.35±0.06	3.79±0.25	2.44±0.14	1.41±0.03	1.40±0.00	100±0.00
0.70 IF	1.34±0.06	3.79±0.24	2.44±0.14	1.42±0.02	1.38±0.00	100±0.00
1.05 IF	1.36±0.06	3.90±0.29	2.54±0.07	1.44±0.01	1.32±0.00	96.16±3.84
Control	1.34±0.06	3.98±0.24	2.64±0.22	1.49±0.04	1.28±0.01	94.87±2.56
P	**1**	**0.99**	**0.47**	**0.38**	**0.33**	**0.07**

Note: SE: Standard error; W1: Initial weight; W2: Final weight; WG: Weight gain; SGR: Specific growth rate; FCR: Feed conversion rate; SR: survival rate; OF: Outside the filter; IF: Inside the filter

It was determined that the pH and NH_3 values given in Table 1 were statistically different ($P<0.05$), and the water temperature and dissolved oxygen values were insignificant ($P>0.05$).

Growth parameters, FCR and survival rates obtained at the end of the study are presented in Table 2. At the end of the study, the difference between weight gain, specific growth rate, feed conversion ratio and survival rate were found to be statistically insignificant ($P>0.05$).

Discussion

The specific growth rate, feed conversion ratio and survival rate results obtained at the end of the study are similar to those of others conducted on blue electric hap (Güllü et al., 2008; Erdogan et al., 2012; Karslı et al., 2014).

When the results obtained at the end of the study were examined, it was determined that there were no differences among groups in terms of weight gain, water temperature, and dissolved oxygen. The results of this study are similar to a study conducted on rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792), wherein effects of zeolite addition in different ratios (0, 1, 2, 3 mg l^{-1}) were examined on the growth rate of the fish and the water quality parameters (Danabaş & Altun, 2011). On the other hand, in the present study, pH and NH_3 values were found to be different between experimental groups. Moreover, pH and NH_3 values increased in parallel with the zeolite amount in all groups. This finding regarding pH is supported by another study in which the ammonium retention capacity of clinoptilolite in various concentrations was investigated (Zabochnicha and Malinska, 2010; Şahin et al., 2018a; Şahin et al., 2018b; Şahin et al., 2019). The pH increases in zeolite treated groups are considered to be caused by the hydrolysis of the calcium and magnesium carbonates ($CaCO_3$ and $MgCO_3$) in the zeolite (Mazeikiene et al., 2008).

Although the pH values of all experimental groups were affected by zeolite addition, they were determined to be within the acceptable limits (6.5-9.0) specified for aquaculture (Swann, 1992).

Since zeolite can only adsorb NH_4, the efficiency of zeolite decreases in high pH levels (Deng, 2014). In this study, likewise, pH levels increased in parallel with the zeolite amount resulting the increase in the amount of ammonia (NH_3) compared to the amount of ammonium (NH_4) thus, the ammonium adsorption capacity of the zeolite decreased. Although the NH_3 values increased in parallel with the zeolite amount, they were determined to be within the acceptable limits for aquaculture in all groups. In the further studies, ammonia adsorbing of zeolite (clinoptilolite) will can be modified using by different natural adsorbent besides zeolite for lower pH.

The ammonium adsorption capacity of zeolite decreases in lower concentrations of ammonia. Booker et al. (1996) investigated the effect of natural zeolite on ammonium adsorption and reported that the ammonium adsorption capacity decreased in values lower than 1 mg NH_4-N l^{-1}. In the present study, the ammonia values varied between 0.29 and 0.43 mg l^{-1}. Thus, the NH_4^+ concentration did not increase to a level at which the zeolite is effective. It can be suggested that it would be beneficial to conduct further studies to increase the ammonium adsorption capacity of zeolite in lower concentrations of ammonia, by also considering the necessity of low levels of ammonia presence (below 1 mg l^{-1}) in aquaculture systems.

Conclusion

Ammonia is among the potential risk factors in aquaculture as its concentrations can unexpectedly rise and it does not display indications available to sensory perception such as odor, color, etc. Thus, when the findings of the present study were evaluated, it can be suggested that low levels of zeolite may be

provided in tulle bags or inside the filter to prevent ammonia rising to high concentrations.

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Compliance with Ethical Standards

Authors' Contributions

MÖ planned the experiments, performed the statistical analyses and managed the project. DŞ collected data, contributed to the writing and preparation of the manuscript. ZK collected data, performed the aquarium laboratory work. OA checked the results. MB performed the aquarium laboratory work.

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

The study protocol was approved by ethics committee of Sinop University (Protocol No: 2014/08) and experiments were carried out in accordance with the ethical guidelines and regulations declared by the Sinop University and the international principles of laboratory animal use and care.

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RESEARCH ARTICLE

The Effect of Aging on Ship Values: An Econometric Analysis on Major Ship Types

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ABSTRACT

Ships are investments that require significant capital and therefore the factors affecting their value must be analyzed carefully. The study in the paper is determined to what extent the effect of age, which is one of the most influential factors in sales value, differs in terms of ship type. The reports including the ship sale activities cover the period between January 2013 and December 2019 and consist of 84 monthly reports. Regression analysis was performed as the sales price as the dependent variable, age, freight, and size as independent variables. According to the results, it was determined that the ship types whose value decreases the most due to the age change are those used in gas transportation, while the least affected ship type by age is those used in bulk transportation. In addition, it has been determined that the ship type most affected by freight is those used in gas transportation and that the ship type most affected by the size is those used in container transportation. It can also be said that the ship type with the lowest risk of investment is bulk ships and the ship type with the greatest risk is gas ships. It is hoped that these results provide important information especially for players conducting their commercial activities upon sale & purchase transactions in the market.

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Introduction

Ships are assets with high values and therefore, careful analysis is required when making investment decisions (Ma, 2020). Although the secondhand market is a liquid market compared to the newbuilding market (Pehlivanoğlu & İnce, 2018), it may take a long time for the ships to be sold under several market conditions. The most important factor determining this duration is the demand for the ship. However, the demand for the ship is actually related to the demand for

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the cargo carried by that ship since the maritime market has a derived demand structure (Vermeulen, 2010). As developments in the world economy affect the demand for commodities, the demand for shipping is also affected (Başer & Açık, 2019a). Ship types in the maritime market are often called according to the types of cargo carried, such as dry cargo, liquid cargo, gas, container, general cargo, etc. (Branch, 2007).

There are some important factors affecting the demand for these ships by investors. Stopford (2009) expresses these factors in terms of cargo type, ship operation type, and commercial philosophy. The type of cargo intended to be transported constitutes an important factor in the selection of the ship since it often means abandoning the transportation of other types of cargoes. While some ships provide flexibility in the choice of cargo, some ships do not. The type of shipping operation is related to how the investor is interested in evaluating the ship; long-term chartering, operating in the spot market or operating in a liner network. For example, if long-term chartering will be held, the needs of the charterer can be more prioritized, and if it will be operated in the spot market, short-term resale values can be considered. Commercial philosophy is about how shipping investor looks at the maritime business. This philosophy determines whether the invested ship is flexible or not and the risk-taking courage. Shipping costs per unit are low on specialized ships to carry a single cargo in maritime transport, but different types of cargoes are unlikely to be transported when there is no specialized cargo. In ships that can carry more than one type of cargo, the cost of transportation per unit is high, but they are less dependent on a specific cargo. Considering these factors, the ship investor can make his investment by choosing a specific ship type.

Ship investors can be divided into two groups; the investors who purchase ships for transport activities (operators), and the investors who, as asset-players (speculators), purchase and sell ships in a short period (Kavussanos & Alizadeh, 2002). Although the investor has bought his ship for transportation activities, he can sell his ship with such a high-profit rate when market conditions revive, he cannot earn this profit rate for years only with transportation activities (Açık & Başer, 2018). For this reason, each factor affecting the value of ships is of vital importance for investors. These factors are stated as new building price, order book size, freight rates, fuel prices, age, and ship size in the literature (Pruyn et al., 2011). As the cycles in the maritime market are irregular and difficult to predict, it is unclear how soon favorable market conditions can occur when an investor purchases a ship. Consequently, the time-related depreciation rate of the ship's value stands out as an important factor. Since the types of ships used in maritime transport have a heterogeneous structure among themselves (Dickie, 2014), time-related value losses are likely to differ by ship type. Therefore, it is of great importance for ship investors whether the impact of time on ship values differs or not. However, it has been observed that there is no comprehensive empirical study with a satisfactory data set size in the literature.

In this study is examined monthly sale & purchase reports between 2013 and 2020 and collected information on the included ships. The size of the sample used is 2741 bulk carriers, 1238 oil tankers, 678 container ships, and 195 gas carriers. Although is analyzed the impact of age on values by ship types as a basic research question, the scope of analysis is expanded using factors such as freight and ship size that affect ship value as variables. As a result of the analysis, it is found that there was a differentiation in the impact of age, but this differentiation was lower than impact other variables that affect ship value. It is hoped that these results will be useful for investors, regardless of their commercial purposes, in reducing the risks arising from uncertainty. In addition, it is thought that an original contribution has been made to the literature with a comprehensive data set. In the second section of the study, the relevant literature is reviewed, and the framework of the study is formed.

Literature Review

To enrich the content of this study, which examines whether the effect of ship age on the prices of different ship types varies or not, the studies examined the secondhand market from various angles investigated during the literature review. Pruyn et al. (2011) conducted a study in the form of a literature review on which variables are used in studies on the secondhand ship value recently. The researchers revealed that new building price, order book size, freight rates, fuel prices, age, and ship size variables were used, and significant results were obtained mostly. However, to handle the market multidimensional, other related studies that are thought to be original are also included.

The purpose of the use of ships is one of the biggest determining factors in determining their size. According to these purposes, the size of the ships and their number in the market are formed. Some ships are used to transport volumes of trans-ocean cargo and their number is very low (Capsize, VLCC, etc.) while some ships are used to transport small volumes of cargo over short distances and their number is quite high (Handysize, Panamax, etc.). Based on this situation, it can be questioned whether the size of the ship makes a difference in the secondhand price volatility. Kavussanos (1997) conducted a study examining the effects of the size of secondhand dry bulk cargo ship on price volatility. According to the results of the study, which uses ARCH model, the price of small size ships is

more volatile than the price of large size ships. This could possibly be because the transaction volume is higher on smaller vessels. However, papers related to the price & transaction volume indicated opposite inference. Alizadeh & Nomikos (2003) focused on the relationship between the prices in the secondhand dry bulk ship market and the trade volume in their research. In this research, a number of different methods are used to investigate the structure of the price-volume relationship in the sale & purchase markets for different sizes of dry cargo ships. The results revealed that price volatility can be a useful way to make predictions on trade volume, as high capital gains trigger more transactions in the market. On the other hand, the impact of trade volume on price volatility was found to be negative. Increased sale & purchase activities in the market supported price stability. Syriopoulos & Roumpis (2006) expanded the scope of the same subject and explored it in both dry bulk cargo and tanker markets. Their results are identical to those of Alizadeh & Nomikos (2003) and confirmed their accuracy in defining the market structure.

One of the factors that can affect the value of secondhand ships the most can be said to be the new building prices. In times of alive market, secondhand ship prices rise high and close new building prices. New building prices affect the demand for secondhand or new ships. However, the question of which market leads which one is an important research question. Kou et al. (2014) conducted research on lead–lag relationships between the second hand and new build ship prices. As a result of the research, they found that there is an inverse lead-lag relationship between dry bulk and tanker ships. While secondhand ship prices in the dry bulk cargo sector led to the prices of new building ships, the findings in the tanker cargo market point to the opposite. The main reason for this distinction was the differentiation in competition and commercial philosophy in the sectors.

Significant incomes in the maritime market are not only provided by transportation. Simultaneously, the sale & purchase market offers opportunities for shipowners to generate high incomes. In fact, some investors earn a significant income by building their business philosophy solely on sale & purchase activities. These types of investors may have different characteristics, and the effect of this differentiation on secondhand prices may be different. Alizadeh et al. (2017) considered two major heterogeneous investor groups in the secondhand ship markets momentum and contrarian investors. While the first group determines their investment strategies by following the market momentum and trends, the second group determines their strategies by believing in the circularity in the market. With the cycle-based approach, they purchase when prices fall below the fundamental value and sell when prices rise above the fundamental value. As a result of their analysis using the heterogeneous agent model, the authors determined that an increase in the participation of momentum investors tends to increase price volatility. However, high demand from contrarian investors has been said to reduce price variability.

Heterogeneity can be seen not only in investors but also in ship-pricing periods. The effect degree of the factors that affect the value of the ship may differ in different periods. In the study by Merika et al. (2019), heterogeneity in the prices of dry bulk ships was investigated by applying the quantile analysis using the data of 5,591 ships that were traded in the dry bulk market. They used vessel size, age, new building price, scrap value, LIBOR, the time charter level, and technology dummy variable. When the researchers applied the analysis of prices in different distribution regions, they found that the factors that most affect heterogeneity were the age of the ship, 3-month LIBOR, and annual charter level.

Since maritime transport has a derived demand structure, demand for goods transported may affect ship value. With the demand-led approach, under the assumption that the price of a goodwill increase/decrease when the demand for it good increases/decreases, significant results were obtained when the relationship between the commodity price and the ship value is analyzed. Başer & Açık (2019b) focused on the relationship between the secondhand values of capesize ships and the iron ore price, which is the most basic load of these ships. In this direction, the asymmetric causality test was used to determine the causality relationship between the shocks contained in the series. As a result, negative shocks in iron ore were found to be the cause of the negative shocks in the 5-year-old capesize vessel value, and increasing shocks in the commodity price trigger the increasing shocks in the value of the vessel. So, following the commodity prices while implementing investment strategies can reduce the risks arising from uncertainties and even provide critical profit opportunities.

Our study differs from the studies in the literature in that it covers many ship types. In our research, separate models are estimated for dry bulk, tanker, container, and gas type vessels. Although we have an age-focused research question, variables such as freight and ship size are also included. Thus, both the explanatory powers of the models increase and a comparison between other factors affecting the value becomes possible. In the next section, the method we used is introduced.

Methodology

In this study, we decided to use linear regression analysis to determine the relationship between variables. Regression analysis is one of the most widely used statistical analyses and its application areas cover many fields such as medicine,

biology, agriculture, economics, engineering, sociology, geology, etc. (Yan & Su, 2009). This method examines functional relationships between variables to reveal statistical and theoretical relationships (Chatterjee & Hadi, 2015) and it consists of many different kinds according to the purpose of use, variables, and data types. The paper is used multiple linear regression analysis in our study, which can be expressed as in Equation (1):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \cdots + \beta_i X_i + \varepsilon \quad (1)$$

In Equation (1), Y is the dependent variable while X's are independent ones. The variable ε, on the other hand, refers to the unexplained parts of the model, namely, the residuals (Gordon, 2015). This equation is defined as multiple linear regression because it is constructed with more than one independent variable (Allen, 2004), if it included only one independent variable it would be expressed as simple linear regression (Gaurav, 2011).

After the regression model is estimated, situations such as whether the model is significant, how much the independent variables explain the dependent variable, which independent variable has a significant effect, whether the effect of the independent variable is negative or positive, and how much the effect of the independent variable is can be determined. The β's obtained from the model contain important information about the effect of independent variables. They show to what extent the independent variables affect the dependent variable and in which direction this effect is (Esquerdo & Welc, 2018). When there is more than one independent variable in the model, the βs enable us to determine the effect of each independent variable separately (Archdeacon, 1994).

After the model has been estimated, some assumptions should be tested before interpreting the coefficients such as the conditional mean of ε is zero, coefficient constancy, which reveals that both β and ε are fixed over the sample period, serial independence in the disturbances of ε, and a distributional assumption of normality for ε (Pagan & Hall, 1983). Obtaining appropriate findings from the assumptions supports the validity of the model and interpretation of the results becomes more reliable (Menard, 2002). In case some of these assumptions are not met, the model can be used after applying some correction methods.

The regression model in the study is constructed as presented in Equation (2):

$$\ln SP_i = \ln \beta_0 + \beta_1 \ln AGE_i + \beta_2 \ln FREIGHT_i + \beta_3 \ln SIZE_i + \varepsilon_i \quad (2)$$

where ship sale price variable (SP_i) is dependent variable while variables age (AGE_i), freight ($FREIGHT_i$) and size ($SIZE_i$) are independent. The aim was to obtain significant findings by estimating this model for selected ship types in the data sample and comparing their results. A log-log model was used in the regression coefficient estimation due to the opinion that in this way it is possible to compare the impact of age on ship value based on ship types. In the next section, the dataset used in the study is introduced and analyzed.

Data

The data used in the study were obtained from various available and relevant sources. Information including characteristics such as age, size and price of the ships was obtained from the Athenian Shipbrokers Monthly Sale & Purchase reports (Athenian Shipbrokers, 2020). The reports cover the period between January 2013 and December 2019 and consist of 84 monthly reports. Bulk, tanker, container, and gas types of ships are included in the sample. Ship type classifications are handled as in market reports. In addition to these ship types, Tween/MPP, Reef, RO-RO, Ferry, Cruise vessels are also available. However, statistically, they were excluded from the study because of their low volume.

The indices representing the freight levels according to the ship types consist of the Capital Link indices (Capital Link, 2020). The freight indices consist of the arithmetic average of the daily values in the month corresponding to the report month. Ship group types used in the study are dry bulk, tanker, container, and gas vessels. Capital Link Drybulk index for dry bulk ships, Capital Link Tanker index for tanker ships, Capital Link Container index for container ships, and Capital Link LNG/LPG index for gas ships are used as freight indices.

Descriptive statistics for bulk carriers are presented in Table 1. There are 2741 ships in the sample that make up the dry bulk ship type. This sample size is the largest of all ship types. The average age of this ship type is 10.8 years, and the average price is $ 13.6 million. In terms of size, DWT (Deadweight Tonnage), which expresses the cargo carrying capacity, is used and the ships sold are on average 67591 DWT.

The oil tankers' descriptive statistics are presented in Table 2. In the period under consideration, 1238 ships were sold. This tanker group includes crude oil, product, and chemical tanker ships. This type of ships have an average value of $23.8 million and their average age is 11 years. The size of the oil tankers is indicated by DWT as bulk carriers, and the tanker ships sold have an average size of 81440 DWT.

Table 1. Bulk market

	Price	Age	Freight	DWT
Mean	13.65879	10.85553	632.5176	67591.41
Median	10.00000	10.00000	666.2705	56734.00
Maximum	423.0000	40.00000	1051.693	402303.0
Minimum	0.450000	1.000000	152.7395	75.32100
Std. Dev.	16.81649	6.697172	234.3722	46659.10
Skewness	11.53961	0.449319	-0.390449	1.930111
Kurtosis	224.6174	2.649702	2.584860	8.183200
Jarque-Bera	5670092.	106.2432	89.32723	4770.125
Probability	0.000000	0.000000	0.000000	0.000000
Observations	2741	2741	2741	2741

Note: Source: Athenian S.A. (2020), Capital Link (2020)

Table 2. Tanker market

	Price	Age	Freight	DWT
Mean	23.80979	11.06785	1561.524	81440.87
Median	15.00000	11.00000	1204.705	47157.00
Maximum	685.0000	42.00000	3026.002	320051.0
Minimum	0.400000	1.000000	579.6037	1252.000
Std. Dev.	37.83948	6.161417	816.1023	87020.87
Skewness	9.958469	0.442944	0.409078	1.603877
Kurtosis	153.2586	3.632365	1.585152	4.633672
Jarque-Bera	1185093.	61.10995	137.7881	668.4461
Probability	0.000000	0.000000	0.000000	0.000000
Observations	1238	1238	1238	1238

Note: Source: Athenian S.A. (2020), Capital Link (2020)

Descriptive statistics for container ships are presented in Table 3. There are 678 container ships in our sample. Their average value is $13.8 million and their average age is 11.5 years. In terms of size, twenty-foot equivalent unit (TEU) is used differently from dry bulk and tanker ship types because it expresses the capacity better for container ships. The vessels in the sample are on average 2823.3 TEU in size.

Table 3. Container market

	Price	Age	Freight	TEU
Mean	13.88676	11.54277	1210.961	2823.363
Median	7.950000	11.00000	1252.019	2452.000
Maximum	280.0000	23.00000	2123.941	13806.00
Minimum	0.600000	1.000000	528.2529	272.0000
Std. Dev.	23.40684	4.618417	515.5810	2180.023
Skewness	6.336644	-0.034809	0.041419	1.914184
Kurtosis	58.02750	2.671722	1.445006	7.699473
Jarque-Bera	90079.03	3.181323	68.50255	1037.946
Probability	0.000000	0.203791	0.000000	0.000000
Observations	678	678	678	678

Note: Source: Athenian S.A. (2020), Capital Link (2020)

The gas carriers' descriptive statistics are presented in Table 4. In our study, the lowest sample belongs to this ship type and consists of 195 ships. Their average value is $35 million, and their average age is 14.9 years. Unlike other ships, cubic meter (CBM) values are used in terms of size, because it is a better measure for gas transportation. The average size of the vessels in the sample is 46463.3 CBM.

Table 4. GAS market

	Price	Age	Freight	CBM
Mean	35.06728	14.93333	2576.999	46463.37
Median	19.00000	15.00000	2715.561	22149.00
Maximum	310.0000	35.00000	4367.142	170234.0
Minimum	0.900000	1.000000	1303.960	1725.000
Std. Dev.	50.29824	8.614341	806.6976	48494.49
Skewness	2.761786	0.142159	0.169890	0.901438
Kurtosis	11.05677	1.962965	1.664295	2.652553
Jarque-Bera	775.2981	9.394753	15.43391	27.39005
Probability	0.000000	0.009119	0.000445	0.000001
Observations	195	195	195	195

Note: Source: Athenian S.A. (2020), Capital Link (2020)

Age distribution charts of the ships sold are presented in Figure 1. According to the chart, it can be said that the sales transactions of old ships in the dry bulk and tanker markets are very low. Sales of young and middle-aged vessels are quite intense. In the container market, both young and old ships have low sales. In the gas market, sales of middle-aged vessels are low, while sales of young and old vessels are relatively high.

To reveal the linear relationships of the independent and dependent variables in the dataset, the distributions in the XY chart were examined and the regression line and equations of their relations were revealed. In the visuals presented in Figure 2 for tanker and bulk ships and Figure 3 for container and gas ships, the linear relationship between the independent variables and the dependent variable can be clearly seen. There is a naturally negative relationship between the age and the sale price, as the age increases, the value of the ship decreases since the remaining economic life of the ship decreases. On the other hand, the relationship of freight and size variables with the sales price is positive. However, their slopes are flatter than the age sample. The main reason for this is, of course, their effects are examined regardless of age, which can be regarded as the most important factor in the ship value. In this respect, the ability of the freight variable alone to explain the sale price is very low considering R^2 values. The next section presents the results obtained by estimating the regression equation for each ship type.

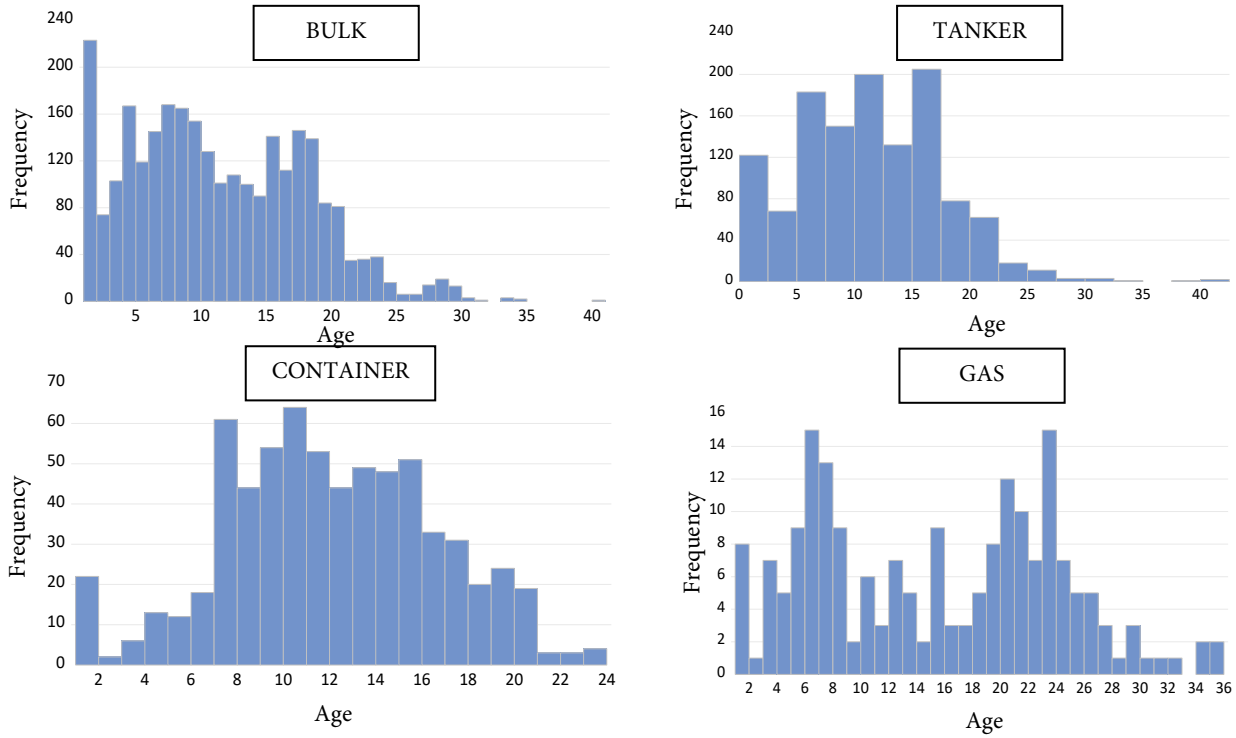

Figure 1. Age distribution in the markets (Source: Athenian S.A. (2020))

Table 5. Results of regression model (Probabilities are shown in square brackets "[]")

Model	Tanker	Tanker Robust	Bulker	Bulker Robust	Container	Container Robust	GAS	GAS Robust
Age	-0.59	-0.59	-0.53	-0.53	-0.56	-0.56	-0.72	-0.72
	[0.000]	[0.000]	[0.000]	[0.000]	[0.000]	[0.000]	[0.000]	[0.000]
Freight Index	0.11	0.11	0.46	0.46	0.26	0.26	0.58	0.58
	[0.000]	[0.000]	[0.000]	[0.000]	[0.000]	[0.000]	[0.000]	[0.000]
Size	0.44	0.44	0.53	0.53	0.77	0.77	0.55	0.55
	[0.000]	[0.000]	[0.000]	[0.000]	[0.000]	[0.000]	[0.000]	[0.000]
Constant	-1.59	-1.59	-5.42	-5.42	-4.31	-4.31	-5.471	-5.471
	[0.000]	[0.000]	[0.000]	[0.000]	[0.000]	[0.000]	[0.000]	[0.000]
F Stat.	1125	1125	2966	2966	457	457	206	206
	[0.000]	[0.000]	[0.000]	[0.000]	[0.000]	[0.000]	[0.000]	[0.000]
R-Squared	0.73	0.73	0.76	0.76	0.67	0.67	0.76	0.76
Adj. R-Squared	0.73	0.73	0.76	0.76	0.66	0.66	0.76	0.76
Durbin-Watson	1.48	1.48	1.19	1.19	1.04	1.04	1.65	1.65
Autocorrelation	Yes	-	Yes	-	Yes	-	Yes	-
Heterosc.	Yes	-	Yes	-	Yes	-	No	-
Normality (JB)	1170	-	14238	-	264	-	1159	-
	[0.000]		[0.000]		[0.000]		[0.000]	
Wald F Stat.		496		563		134		87.28
		[0.000]	-	[0.000]	-	[0.000]	-	[0.000]

Finally, the model estimated for gas carriers is significant and has a good R-squared value of 0.76. Since autocorrelation was detected in the residuals of the model, HAC correction was applied and the new results were presented as Gas Robust. 1% change in age causes 0.72% change in sale price, 1% change in freight causes 0.58% change in sale price, and 1% change in size causes 0.55% change in sale price. After the regression models were estimated for all ship types, the coefficients are visualized in Figure 4 to make the evaluations more appropriate.

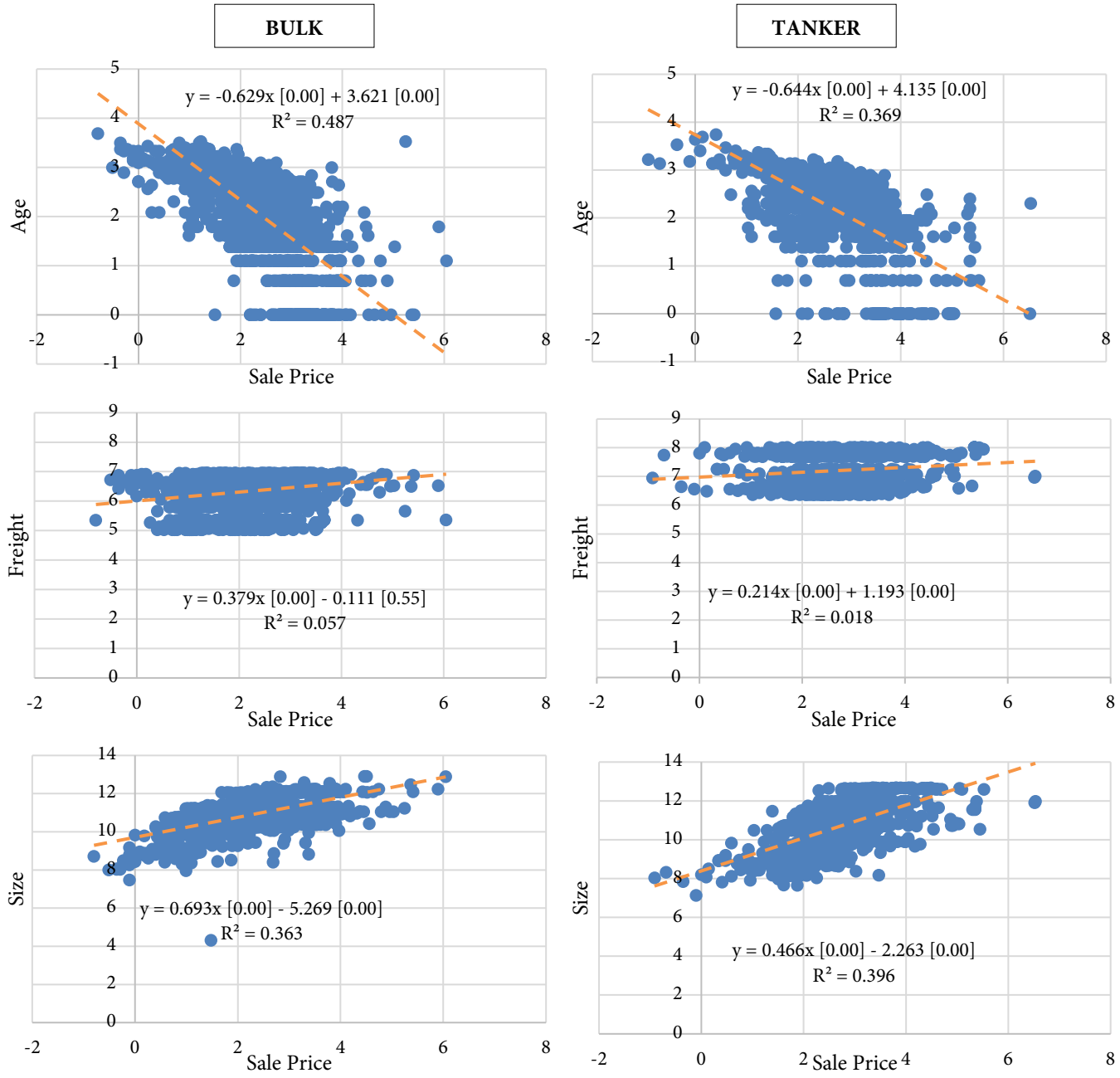

Figure 2. Relationship between dependent and independent variables in bulk and tanker ships (Probabilities are shown in square brackets "[]")

Results

The logarithms of the series were taken before the regression analysis. In this way, log-log models were established, and percentile changes indicated by the ship values against 1% changes in independent variables were determined. Multiple linear regression according to the expression 2 for each ship type is performed in EViews 10 econometric software. After the models are estimated and residuals are checked, Huber-White (White, 1980) correction is applied in the case of heteroscedasticity, HAC (Newey & West, 1987) in the case of autocorrelation, and HAC corrections in the case of both heteroscedasticity and autocorrelation.

Statistical results of the regression models for each ship type are summarized in Table 5. Theoretically, the signs of the variables were obtained logically. The coefficients of the age variables were obtained negative in all ships. As the ship ages, its technology gets old and its remaining economic life decreases. In addition, the signs of freight and size variables were obtained positive. It is normal for ship values to be higher in lively market conditions, as shipowners may be willing to pay higher prices to ships to take advantage of current and future higher revenue opportunities. In addition, since the increase in the size of the ship means more carrying capacity, the ships can naturally find purchasers at higher prices.

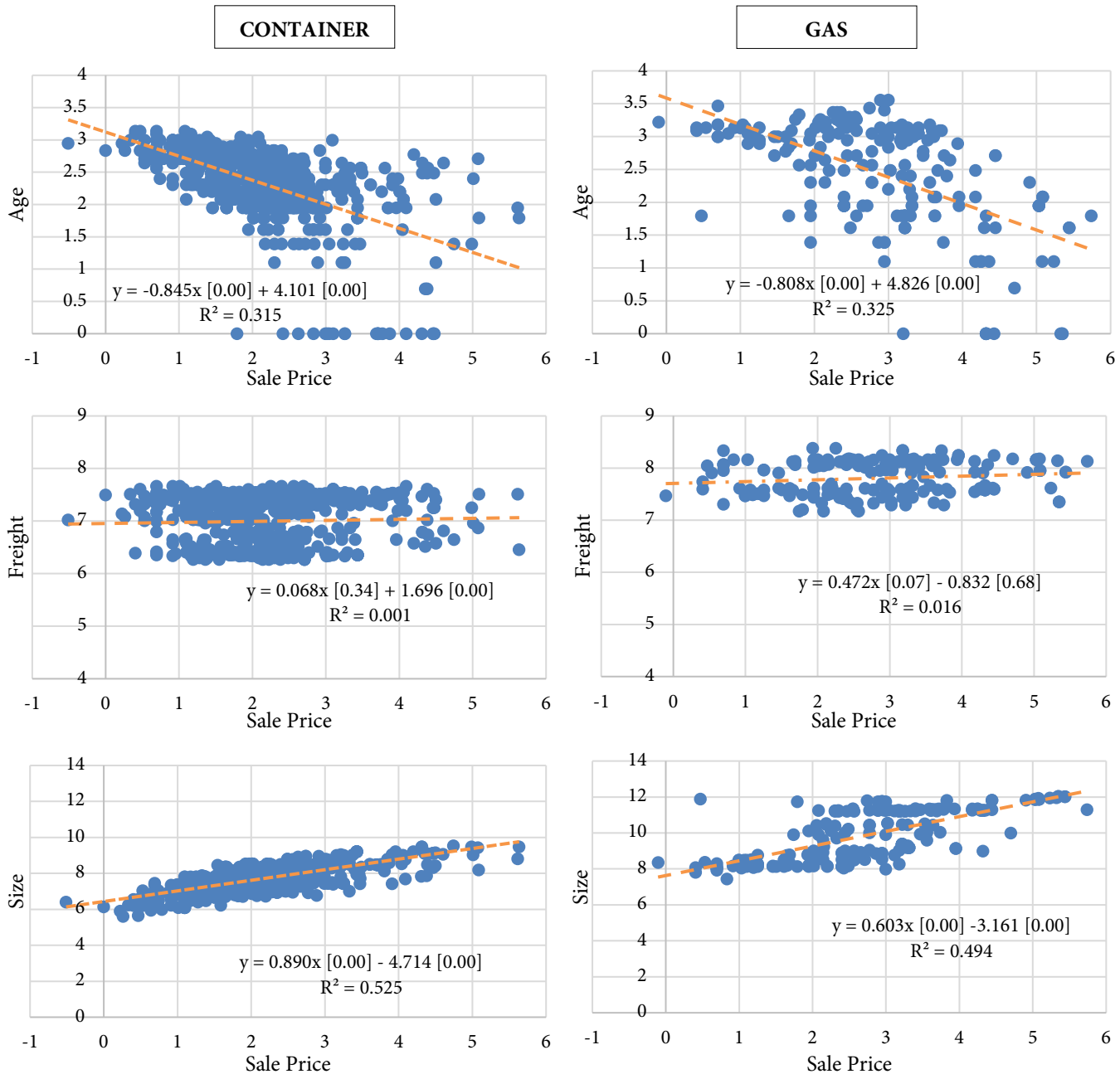

Figure 3. Relationship between dependent and independent variables in bulk and tanker ships (Probabilities are shown in square brackets "[]")

The estimated model for the oil tankers is significant and has a satisfactory R-squared value of 0.73. However, autocorrelation and heteroscedasticity were detected as a result of the diagnostic tests performed on the residuals of the model. For this reason, standard errors were recalculated by applying HAC correction and the new results are presented as Tanker Robust. 1% change in age causes 0.59% change in sale price, 1% change in freight causes 0.11% change in sale price, and 1% change in size causes 0.44% change in sale price.

According to the equation results estimated for bulk carriers, the model is significant and has a good R-squared value of 0.76. However, there is autocorrelation and heteroscedasticity considering diagnostic tests on residuals. For this reason, HAC correction has been applied to the model, and results are presented as Bulker Robust. 1% change in age causes 0.53% change in sale price, 1% change in freight causes 0.46% change in sale price, and 1% change in size causes 0.53% change in sale price.

The estimated regression model for container ships is significant and has a satisfactory R-squared value of 0.67. Diagnostic test results applied to residuals showed autocorrelation and heteroscedasticity in the model. Therefore, HAC correction has been applied and the results are presented as Container Robust. The results showed that 1% change in age causes 0.56% change in sale price, 1% change in freight causes 0.26% change in the sale price, and 1% change in size causes 0.77% change in sale price.

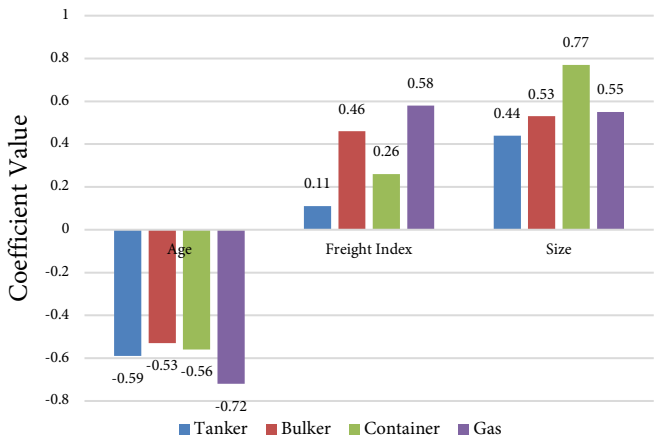

Figure 4. Comparison of the coefficients

According to the results, the ship type whose sale price is the most affected in terms of age stands out as gas carriers. A 1% change in age causes a 0.72% change in ship value. Next comes oil tankers and container ships, and the least affected one is the bulk carrier. It may be thought that the reason for this is the increased maintenance and operational costs of the vessels depending on age. It can also be said that the operational processes of tanker and gas vessels may have a more corrosive effect on these ships. To understand another possible cause, we should refer to Karl Marx's concept of moral depreciation. The fact that the new gas ships launched on the market are equipped with more advanced technologies compared to the old ones causes a more lowering effect on the prices of the old ships, which means that gas ships be exposed to moral depreciation more.

When the coefficients of the freight variable are examined, it is determined that there are big differences compared to age. In this variable, gas carriers come to the fore as ship type which is most affected by changes in freight rates. Next comes to bulk carriers and container ships and the least affected ship is oil tankers. The reason may be less affected by the freight due to their use as oil storage (Başer & Açık, 2018). The rate of response to freight rates may be low as demand for ships changes according to the changes in oil prices rather than freight levels. In addition, because people who want to transport and store oil prefer to charter tanker ships rather than purchase, the response of ship values to freight rates may be low.

In terms of size, container ships are the most affected ship type and have a very high value compared to other vessel types. This may be because container ships are used to transport high-value cargoes. The demand's response to freight may be limited, as the rate of transportation cost to cargo value is low. This structure may cause increases in earnings per unit and have led to the highest coefficient being observed in container ships. Bulk and gas carriers have very close coefficients, while the least affected ship was determined as oil tanker ships. Based on the use of oil tankers as storage, it could be expected to be the ship type most affected by size. However, in this case, the inventory cost brought by storing very large amounts of oil may be effective.

Discussion

Market cycles in the shipping industry are directly related to world trade volume since nearly 90% of world trade in terms of tonnage is carried out by ships (Rodrigue, 2013). Therefore, maritime transportation is very sensitive to economic ups and downs, which means that markets in maritime transportation have volatile structures and various regimes with different characteristics (Tarı & İnce, 2019). Under these circumstances, investments in the maritime industry are risky. Investors need to be able to accurately calculate market-related variables to minimize risks. Making sale & purchase decisions at the right time in the market cycle results in very profitable returns.However, just reading the market correctly may not be enough to gain profitable opportunities in the ship sale & purchase market. Because many different types of ships are operated in the secondhand market and there can be great heterogeneity even between ships of the same type. Our study can contribute to ship investors, who are grouped by Alizadeh et al. (2017) as operator, momentum, and contrarian, to follow the right strategies. As a result, although the operators maintain their commercial activities through transportation, they can earn an amount of income from ship sales in the peak periods of the market that they could not earn for years with transportation activities. Their commercial philosophies are important, that is, they will carry which cargo with which ship type, on which route, and in what amount, but it is also advantageous to consider the differences in the loss of value in the types of ships. As those in the momentum group follow market conditions, the loss of value due to age is important. In addition, the findings are valuable as we include the effect of freight rates in our models in addition to age. Contrarians, on the other hand, hope to make huge profits by aiming to sell at the highest point and to purchase at the bottom of the market, but the cycles in the maritime market are very irregular and heterogeneous. Similar to a cycle is unlikely to be seen in the further periods. Also, the lengths and sizes of the cycles are very different from each other. There have been recessions in the markets for a long time at some periods. It is of great importance that they consider the differentiation in value losses in different kinds of ships over time, especially for investments that coincide with such depressed periods, at least for loss-minimization.

When the value losses due to age are considered, it is seen that the highest loss of value is in the gas type ships. If investors

do not have the intention of making satisfactory profits from transport activities, such ships may be more likely to suffer from delays in sale & purchase transactions. On the other hand, they have the highest rate of gains in value due to freight rates. Considering this gain and the scarcity of sale & purchase transactions in our sample, it can be said that they are risky ships for investment, although there is a high probability of high return. The smallest loss of value due to age was detected in bulk ships. These types of vessels can be considered low-risk vessels for investment because as can be seen from our sample, there are several sales & purchase transactions and the loss in value due to age are the lowest. In addition, when considered with age, the value gains due to freight are quite high, and this may indicate that they are the suitable ship types for investors hoping to make big profits from their sale & purchase transactions. During peak market periods, their sellout can provide great profit opportunities. On the other hand, in tanker type ships, the value loss due to age is relatively high. In addition, value gains arising from freight have the lowest rate. This may be due to their being more sensitive to oil prices and being used as a storage house when necessary. In this respect, it seems very difficult to follow the maritime cycles and to make a profit by selling the ship during the peak periods of the market. On the other hand, in container ships, the value loses due to age is average and they stand out with their high coefficient in size. Considering the market structure of container transportation, this situation is reasonable, since the space occupied by the ship is more important than the content of the cargo in the container and the source of income is determined accordingly. In addition, since there is little concern for reaching somewhere and looking for another cargo contrary to bulkers, the cost increase due to speed may be low and the importance of size increases. In this respect, it is thought that original contributions are made to the literature and practical field.

In future studies, the scope can be expanded by including different variables representing market conditions during the trading of ships. Although freight rates represent the market as an indicator of income for ship investors, interest rates that show the cost of the capital required for investment are also an important factor. The return on freight rates and the cost of interest rates can be decisive in ship sale & purchase decisions. For example, in the study by Açık et al. (2020), it was determined that interest rates are also effective in new ship order and ship demolition decisions. These rates play an important role in balancing the transport capacity in the market. In addition to interest rates, shipbuilding costs can also be included in the analysis. Thus, a substitute option for purchasing the secondhand ship is included in the model. However, due to a large number of heterogeneous ship types in the dataset, it is difficult to add shipbuilding costs for all of them, and accessing such data requires bearing serious costs. Nevertheless, if such data can be obtained, it will greatly contribute to the development of the analysis.

Conclusion

We determined whether the effect of age, which is one of the most important factors affecting the values of ships, differs according to some major commercial ship types. Our results reveal that the effect of age differs slightly according to the ship type and it is greater in specialized ships. In the effects of freight rates and ship sizes, the differences between coefficients of ship types are higher. Studies have been conducted on ship valuations and the factors affecting these valuations in the literature. However, the lack of a study in the literature in which ships are analyzed based on market data by considering their ages, supports the originality of our study. It is hoped that our findings can guide ship and cargo owners in terms of their commercial strategies by revealing the risks arising from aging.

Compliance with Ethical Standards

Authors' Contributions

OHG designed the study, AA performed the statistical analysis, SÖB and KÖE reviewed the related literature. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

For this type of study, formal consent is not required.

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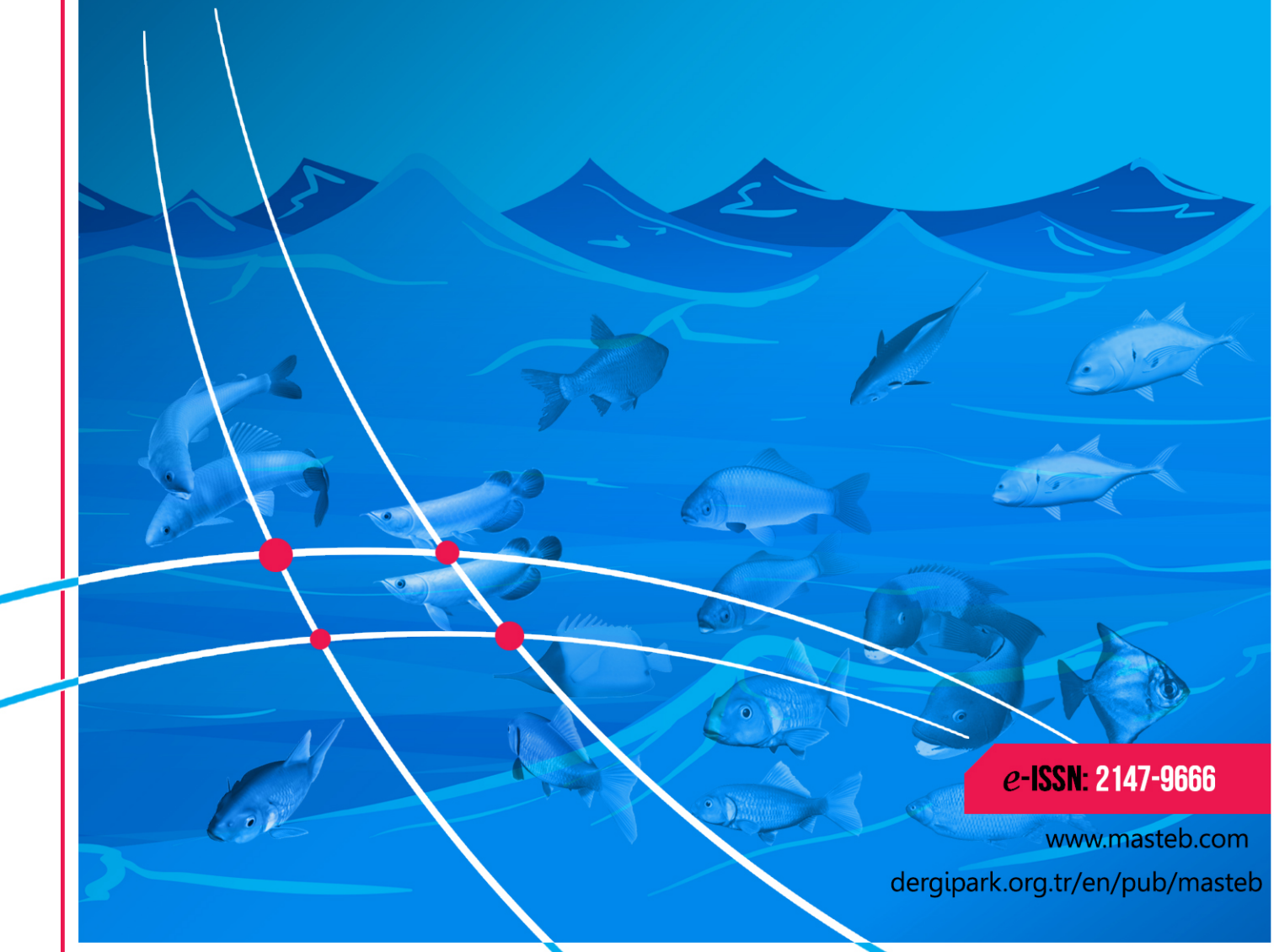

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