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Aims and Scope

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SHORT COMMUNICATION

AQUATIC RESEARCH

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Aquat Res 5(2), 99-109 (2022) • https://doi.org/10.3153/AR22009 *Research Article*

Effects of inorganic nutrient enrichment on the carrageenan yield, growth, and ice-ice disease occurrence of red alga *Kappaphycus striatus*

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ABSTRACT

One of the problems in *Kappaphycus* farming is the slow growth in some farms due to nutrient insufficiency caused by overstocking. In the southern Philippines, most seaweed farmers practice inorganic nutrient enrichment of *Kappaphycus* to boost growth and decrease ice-ice disease occurrence using ammonium phosphate at an average concentration of 8.82 g L^{-1}. In this study, experiments with *Kappaphycus striatus* enriched with inorganic nutrients were carried out at Pasiagan, Bongao, Tawi-Tawi, southern Philippines, using different inorganic nutrients (T_1=8.82 g L^{-1} of urea, T_2=8.82 g L^{-1} of phosphorus, and T_3=control) within 45 days. Seaweeds were enriched in these three inorganic solutions for 30 seconds, placed into a large mat, covered with canvas, and left overnight. After 15 days, findings showed that the specific growth rates of T_1 (6.99% day^{-1}) and T_3 (6.72% day^{-1}) groups were significantly higher than the T_2 (5.84% day^{-1}) group (p<0.05). Inorganic nutrient enrichment did not significantly influence the occurrence of ice-ice disease. Moreover, inorganic nutrient enrichment did not affect the carrageenan yield after 45 days. *K. striatus* nutrient-enriched with urea could increase growth at day 15, but no effect on the occurrence of ice-ice disease and carrageenan yield. Hence, inorganic nutrient enrichment using urea provides a positive effect to farmed *K. striatus* by enhancing its growth without affecting its health and carrageenan yield.

Keywords: Carrageenan yield, Ice-ice disease, *Kappaphycus striatus*, Nutrient enrichment, Specific growth rate

Introduction

Kappaphycus striatus is one of the many fishery resources that abound in Tawi-Tawi waters, southern Philippines, mostly of high commercial value in the national and international markets (Arupin, 1997). *Kappaphycus,* a red seaweed locally known as Guso (Cebuano) or Agar-agar (Tausug), is an important export product in Asia. It is one of the country's top three exports of marine-based products. France, China, and the USA are the main markets for seaweed products in the Philippines (BFAR, 2016). Red seaweeds are harvested globally (either from the farm-raised or wild) and have numerous applications as food for human consumption and as a source of two hydrocolloids: carrageenan and agar, which are widely utilized as an emulsifier, binder, gelling and thickening agents as well as food and non-food products (McHugh, 2003).

In the late 1960s, the line and stake method were utilized as the first commercial cultivation of *Kappaphycus* from the southern Philippines, and for over decades, the Philippines was the top producer of *Kappaphycus* until it was surpassed by Indonesia in 2008, although production from the Philippines has been on a downward trend since 2011 (Hurtado et al., 2015). However, in 2019, China was the top producer of aquatic plants, including seaweeds, where the Philippines ranked 4th (FAO, 2020). On the same year, the top fisheries performance in the Philippines was tuna having the export value at US$ 478 million, followed by seaweed, which went up 13% US$ 207 million in 2018 to US$ 250 million in 2019 or 22% total earnings for that year (BFAR, 2019).

The decreased material quality or overstocking is one of the main hurdles in seaweed production, which causes a decrease in nutrients and stunted seaweed growth (Luhan et al., 2015). Temperature, salinity, water movements, turbidity, and light intensity are abiotic factors that can cause ice-ice disease, epiphytes infestation, and poor seedling quality of grown seaweeds (Largo, 2002; Tahiluddin & Terzi, 2021a; Tahiluddin & Terzi, 2021b). One of the important factors in determining seaweed production sustainability and its yield is the fertility of water. The cultivation of *Kappaphycus* is primary dependent on the natural fertility of the water (Hurtado et al., 2001; Munoz et al., 2004; Hayashi et al., 2007a). One of the control measures to reduce the occurrence of ice-ice disease in *Eucheuma* and *Kappaphycus* species is by nutrient enrichment before out-planting (Tahiluddin & Terzi, 2021a). Two nutrients, nitrogen, and phosphorus, are vital supplements for the growth and production of seaweeds (Harrison & Hurd, 2001). Nitrogen combines biologically with carbon, hydrogen, oxygen, and sulfur to form amino acids, which are the protein building blocks and are utilized for the development of the plant and its growth (Uchida, 2000). Increased source nitrate or ammonium concentrations supplies can result in high nitrogen accumulation, increased growth as well as increased nitrogen sufficiency of the seaweed *Fucus spiralis* (Topinka & Robbins, 1976).

In addition, phosphorus plays a major role in energy storage. It helps to improve plant growth, reduces the incidence of diseases, and improves the quality of some plants (Uchida, 2000). Phosphorus application in agriculture substantially improved the relative water content of plant's leaf, including the rate of photosynthesis of *Alnus cremastogyne* seedlings even under drought period (Tariq et al., 2018). Enrichment of phosphorus significantly increased the photosynthetic rates and growth of *Sargassum fluitans* and *S. natans* (Lapointe, 1986). Sekar et al. (1995) showed that the seaweed liquid fertilizer at 0.25% concentration increased seaweed growth and increased total nitrogen and phosphorus accumulation. In Tawi-Tawi, southern Philippines, farmers are using inorganic nutrients such as ammonium phosphate with an estimated average concentration of 8.82 g L^{-1} to reduce ice-ice disease occurrence and to enhance the growth of *Kappaphycus*, which likewise proven effective in the field experiment (Tahiluddin, 2018; Tahiluddin et al., 2021a). However, it is still unclear which of the two important nutrients is more essential for *K. striatus*. Thus, this study aimed to determine the effects of urea and phosphorus on carrageenan yield, growth rate, and occurrence of ice-ice disease on the red alga *K. striatus*.

Material and Methods

Study Site and Duration

The study was carried out at the seaweed farm of Pasiagan, Bongao, Tawi-Tawi, southern Philippines (05° 00.424' N, 199° 45.39' E) from February to March 2019 for 45 days.

Preparation of Seedlings

Untreated and healthy *K. striatus* seedlings were purchased from the farmer in the field. Seedlings were placed in styrofoam with *Sargassum* sp. on the top and bottom of seaweeds to maintain the moisture and temperature and transported to the study site via a small boat. After the seedlings were transferred from the source to the study area, the seedlings were conditioned. The styrofoam with seaweeds was gently dipped into the farm area until the seaweeds were completely submerged. The seedlings were planted for three (3) days for acclimatization using the fixed-off bottom method. Seedlings were prepared by cutting with the help of a knife to 50 g per bunch. These were tied into a rope line (5 m) using

a soft straw with a distance of 25 cm (Hurtado et al., 2008). Each line consisted of 20 bunches, and 9 lines were prepared.

Inorganic Nutrient Enrichment

Inorganic nutrient enrichment was carried out late in the afternoon using the method previously reported (Tahiluddin, 2018). Two nutrient solutions were prepared: T_1=8.82 g L^{-1} of urea, T_2=8.82 g L^{-1} of phosphorus, and the control group (T_3= control). Simultaneously, all 3 lines were immersed in solutions for 30 seconds, placed into a large mat, and covered with canvas overnight. Seedlings were immersed in seawater for less than 30 minutes. Re-application of nutrient enrichment was done every 15 days (day 0, day 15, and day 30).

Planting

Seedlings were transported to the farm area using a small boat. Wooden poles were placed under the substrate as stakes. Seedlings were planted in Randomized Complete Block Design (RCBD) using the fixed-off bottom method (Trono, 1992). The distance from the seedlings to the bottom was 30 cm.

Farm Maintenance

The farm site was visited every seven days to maintain the cleanliness of the farm by removing epiphytes and debris attached to the seaweeds. The monitoring water parameters such as salinity, temperature, pH, as well as water depth were recorded every seven days using the refractometer (Atago Master), thermometer, pH meter (Smart Sensor), and meter stick, respectively. Water current was determined every seven days using improvised drogue.

Ice-Ice Disease Monitoring

Monitoring of occurrence of ice-ice disease was done every 15 days (day 0, day 15, and day 30). One or more soft white branches were labeled as an ice-ice disease (Luhan et al., 2015; Tahiluddin & Terzi, 2021a). Seaweeds with soft white branches were summed up and divided by the number of planted seaweeds per line. The occurrence of ice-ice disease was computed using the following formula (Largo et al., 1995a).

$$\text{Percent of ice} - \text{ice disease} = \frac{\text{number of infected bunches}}{\text{total number of bunches}} \times 100$$

Growth Sampling

Sampling was done every 15 days of the culture period. Five random subsamples or 25% of seedlings samples per line were taken. To remove excess water, seaweeds were patted with a smooth cloth and weighed using a weighing scale. The specific growth rate (μ) was computed using the formula below (Luhan et al., 2015).

$$\mu = \frac{\ln(\text{Wf}) - \ln(\text{Wi})}{\text{DOC}} \times 100$$

Where:

DOC = days of culture

Wf = final weight

Wi = initial weight

Analysis of Carrageenan Yield

Carrageenan yield was determined every 15 days. Seaweeds were cleaned by removing silt, sand, and other foreign matter. Seaweeds were dried in a solar drier for 3-5 days. The dried seaweeds were brought to the Seaweed Post-harvest Laboratory of the Mindanao State University-Tawi-Tawi College of Technology and Oceanography. Carrageenan yield was determined following the method of Luhan et al. (2015) and calculated by dividing the weight of carrageenan seaweeds treated with an alkali solution to dry weight and times by 100.

Data Analysis

IBM SPSS software version 20 was used to analyze the data of carrageenan yield, growth rate, and occurrence of ice-ice disease of seaweed *K. striatus*. Determination of significant difference was computed through the One-way Analysis of Variance (ANOVA), and Post hoc (Duncan) was used to rank the mean.

Results and Discussion

Physicochemical Parameters

Table 1 shows the environmental status of the farmed area. The temperature ranged from 27.68 ±0.43 to 32.87 ±0.19 °C; pH was measured between 6.93 ±0.03 to 8.43 ±0.03; salinity of the farmed area was 30.17 ±0.44 to 35.00 ±0.29 ‰; water current ranged between 0.05 ±0.00 to 0.16 ±0.03 m s^{-1}; depth of farm area varied between 27.68 ±0.29 to 129.17 ±0.88 cm.

Growth

The specific growth rates (SGR) of T_1, T_2, and T_3 groups were 6.99 ±0.16 % day^{-1}, 5.84 ±0.30 % day^{-1}, and 6.72 ±0.17 % day^{-1}, respectively, at day 15 of the culture period (Figure 1). Statistical analysis revealed that SGR of T_3 and T_1 groups were significantly higher (p<0.05) than the T_2 group. At day 30, SGR of T_1 (5.58 ±0.53 % day^{-1}), T_2 (4.14 ±0.10 % day^{-1}), and T_3 (5.02 ±0.40 % day^{-1}) groups were not differ significantly (p>0.05). At 45 days of the culture period, T_1, T_2, and T_3 groups achieved SGR of 3.90 ±0.46 % day^{-1}, 2.41 ±1.41 %

day^{-1}, and 2.98 ±0.34 % day^{-1}, respectively, and no significant difference between treatments was found (p>0.05).

Ice-Ice Disease Occurrence

Occurrence of ice-ice disease of farmed *K. striatus* was observed in all treatments throughout the sampling period (Figure 2). On day 15, the ice-ice disease occurrence of T_1, T_2, and T_3 groups were 24.12 ±11.77 %, 34.83±8.74%, and 32.22 ±5.02 %, respectively. On day 30, the incidence of ice-ice disease of T_1, T_2, and T_3 groups were 44.48 ±4.66 %, 64.08 ±4.59 %, and 37.85±12.04%, respectively. On day 45, the ice-ice disease occurrence of T_1, T_2, and T_3 groups were 39.95 ±2.53 %, 45.90 ±5.33 %, and 61.48 ±15.59 %, respectively. Throughout the sampling period, there was no significant difference (p>0.05) between treatments, suggesting that the use of fertilizers (urea and phosphorus) did not affect the *K. striatus* in terms of ice-ice disease occurrence.

Table 1. Physico-chemical parameters of the farm

	Sampling period						
Parameters	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Temperature (°C)	27.68±0.43	32.87±0.19	28.08±0.26	29.8±0.56	28.2±0.29	27.8±0.08	28.33±0.12
pH	7.72±0.01	7.44±0.03	6.93±0.03	8.43±0.03	7.59±0.11	8.25±0.10	8.00±0.06
Salinity (‰)	33.50±0.58	30.17±0.44	31.00±1.04	35.00±0.29	34.83±0.17	35.00±0.00	34.67±0.17
Current (m s^{-1})	0.06±0.00	0.07±0.00	0.05±0.00	0.07±0.00	0.05±0.00	0.16±0.03	0.09±0.01
Depth (cm)	27.68±0.29	59.17±0.67	103.50±0.58	129.17±0.88	103.50±0.29	104.83±0.17	77.17±1.67

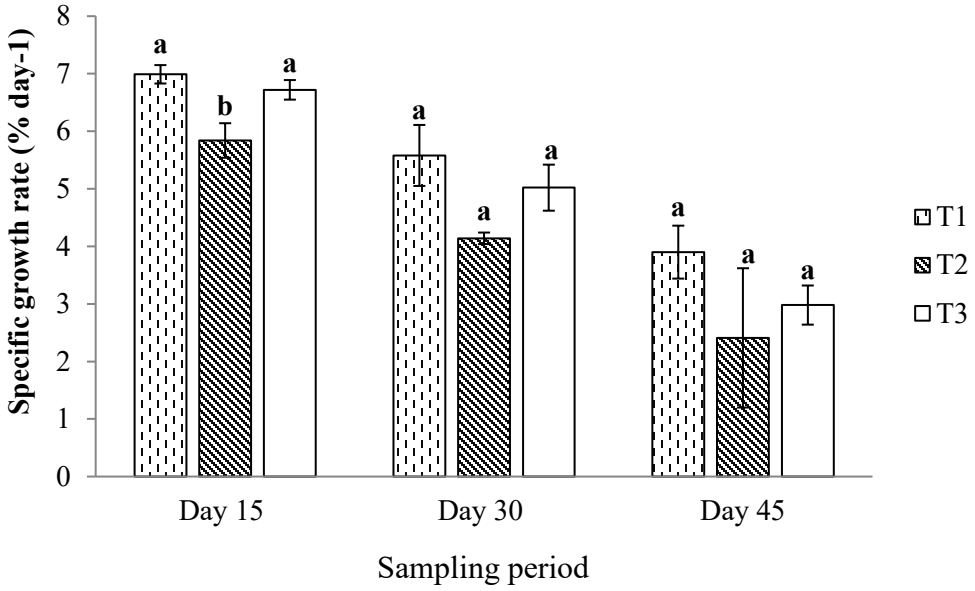

Figure 1. Specific growth rate of *K. striatus* in every sampling. T_1=8.82 g L^{-1} of urea, T_2=8.82 g L^{-1} of phosphorus, and T_3=control. Bars with the same letters are not significantly different (p>0.05). Error bars in SEM (standard error mean), n=5-15.

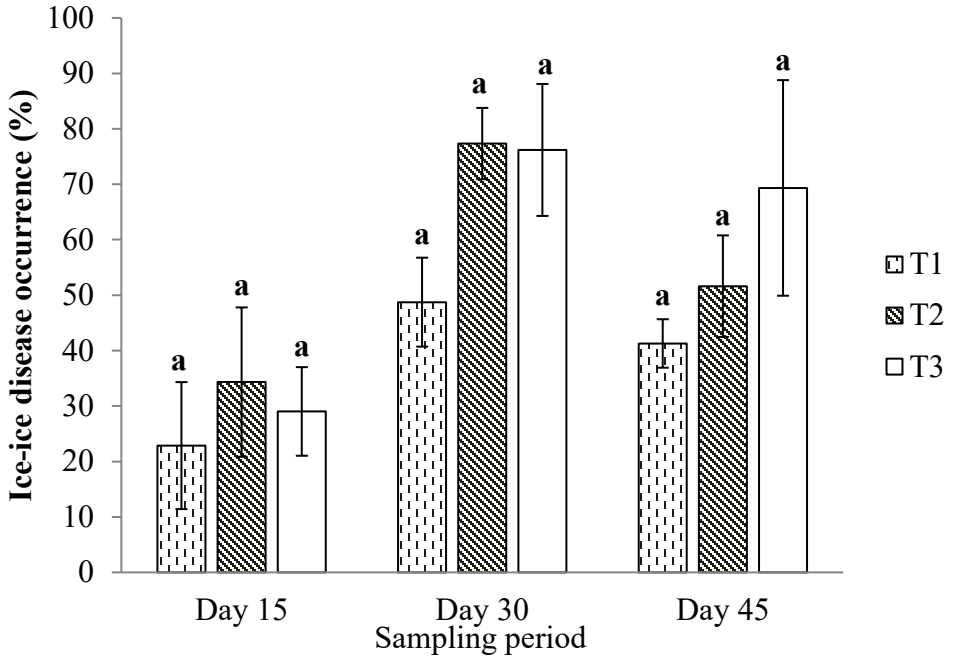

Figure 2. Ice-ice disease occurrence of *K. striatus* in every sampling. T_1=8.82 g L^{-1} of urea, T_2=8.82 g L^{-1} of phosphorus, and T_3=controlBars with the same letters are not significantly different (p>0.05). Error bars in SEM (standard error mean), n=5-20.

Carrageenan Yield

Carrageenan yields of alkali-treated seaweeds *K. striatus* in T_1, T_2, and T_3 groups were 28.33 ±0.29 %, 31.53 ±1.07%, and 29.27 ±0.54%, respectively on day 15. One-way ANOVA revealed that the T_2 group was significantly higher (p<0.05) than the T_1 group but not significantly different (p>0.05) from the T_3 group. On day 30, carrageenan yields of T_1, T_2, and T_3 groups were 25.65 ±0.63 %, 25.78 ±0.19 %, and 27.11±0.8 %, respectively. On day 45, carrageenan yields of T_1, T_2, and T_3 groups were 33.71±0.83 %, 36.20 ±0.10 %, and 31.04 ±2.49 %, respectively. There was no significant difference (p>0.05) between treatments as revealed by One-way ANOVA on days 30 and 45 (Figure 3). In terms of change in culture period, carrageenan yield of T_1 significantly dropped (p<0.05) from day 15 to day 30 and significantly increased (p<0.05) from day 30 to day 45. Carrageenan yield of the T_2 group significantly decreased (p<0.05) from day 15 to day 30 and significantly increased (p<0.05) from day 30 to day 45. In the T_3 group, carrageenan yield significantly dropped (p<0.05) from day 15 to day 30. However, there was no significant change (p>0.05) from day 30 to day 45 (Figure 4).

Growth

Phosphorus and nitrogen, which are mostly found in a natural environment, are important nutrients for the growth of seaweeds (Harrison & Hurd, 2001). Many researchers have stated that the cultivation of *Kappaphycus* spp. is mainly dependent on the natural enrichment of the sea (Hurtado et al., 2001; Munoz et al., 2004; Hayashi et al., 2007a). Fertilization of the water is very important in order to determine the sustainability, yield, and productivity of seaweeds (Luhan et al., 2015). Thus, the addition of nutrients can be beneficial to seaweeds depending on the fertilizer used as well as its concentration. In this study, *K. striatus* nutrient enriched with urea increased the growth (6.99 % day^{-1}) on day 15 and obtained higher growth (3.90% day^{-1}) after 45 days, although not significantly different from the control. According to Luhan et al. (2015), seaweed *K. alvarezii* enriched with sodium nitrate (0.01 g L^{-1}) showed an increase in growth (2.34% day^{-1}) after day 45 of culture period in a grow-out cage. They also stated that a lower nitrogen concentration resulted in slower growth, and a higher nitrogen concentration exhibited faster growth. A similar study used nitrate (1mM NO_3-N) to enhance the growth (0.97 % day^{-1}) of *K. alvarezii* cultured at the laboratory (Sahoo & Ohno, 2003). The used nitrate (35 µg NO_3 L^{-1}) to *Fucus spiralis* enhanced the growth (0.83 % day^{-1}) after 12 days of culture period in plastic regime rack (Topinka & Robbins, 1976). Uchida (2000) stated that nitrogen is vital because it is the main component of chlorophyll and necessary for photosynthesis. Hence, the enrichment of urea provided an additional nitrogen source to *K. striatus,* thereby enhancing its growth.

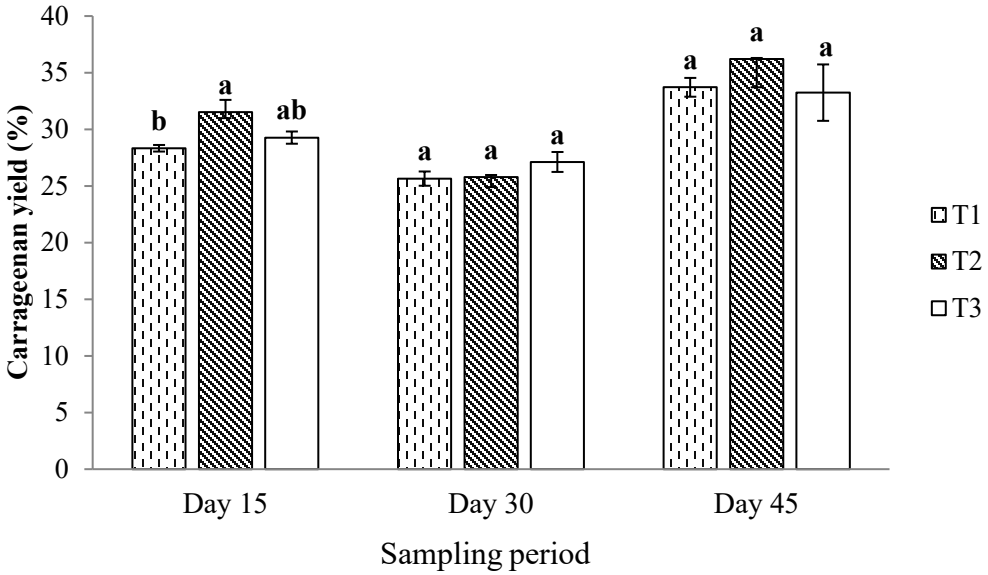

Figure 3. Carrageenan yield of *K. striatus* in every sampling. T_1=8.82 g L^{-1} of urea, T_2=8.82 g L^{-1} of phosphorus, and T_3=control. Bars with the same letters are not significantly different (p>0.05). Error bars in SEM (standard error mean), n=9.

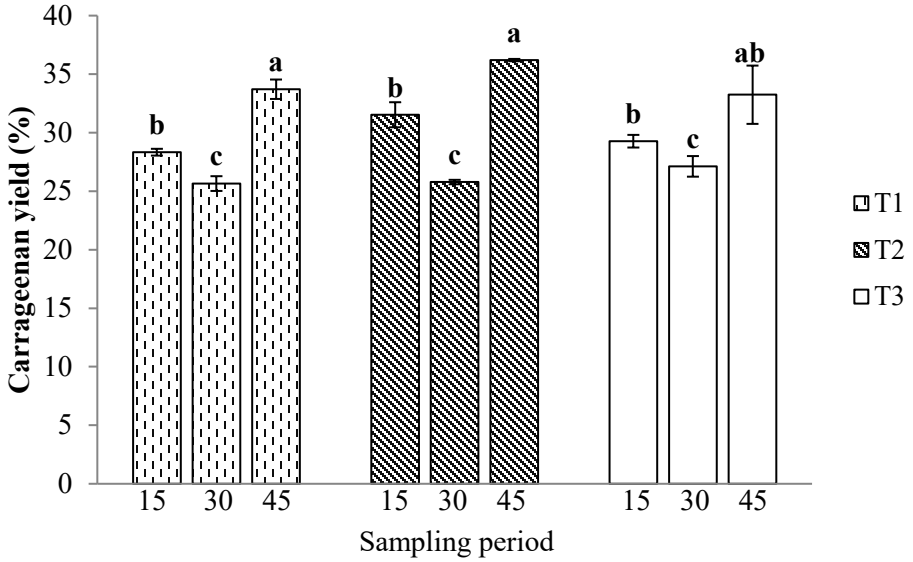

Figure 4. Change in alkali-treated carrageenan yield of *K. striatus* throughout the culture period. T_1=8.82 g L^{-1} of urea, T_2=8.82 g L^{-1} of phosphorus, and T_3=control. Bars with the same letters are not significantly different (p>0.05). Error bars in SEM (standard error mean), n=9.

On the other hand, stimulation of photosynthetic rates and growth of some algae can be improved by phosphorus enrichment (Lapointe, 1986; Villares et al., 1999; Martin et al., 2011). According to Xu et al. (2010), a high amount of carbon dioxide (720µl L^{-1}) and phosphorus (30µM) increased the growth of red alga *Gracilaria lemaneiformis* ranged from approximately 1.6 to 2.8% day^{-1} after 16 days cultured in the laboratory. Red alga *Agardhiella subulate* enriched with phosphorus (6 µM) obtained an SGR of 0.025% day^{-1} (Chopin, 1990). According to Uchida (2000), phosphorus plays a vital role in the transfer of energy and other components of genetic information found in plant photosynthesis and respiration. In our study, phosphorus-enriched *K. striatus* achieved 2.40% day^{-1} growth after 45 days and was lower than the control, indicating that higher phosphorus concentrations may lead to slow growth of seaweed *K. striatus*. Excess phosphorus reduces the plant's ability to take up essential micronutrients, particularly zinc and iron (Provin & Pitt, 2008). They also noted that phosphorus' overuse could become water-soluble and mobile, entering surface water and causing the growth of algae and other undesirable plants. The suggested concentration of the phosphorus fertilizer based on its prescription on the label is 4.5 g L^{-1}. However, this study used 100% phosphate fertilizer (Seachem) with a high concentration of 8.82 g L^{-1}, an average concentration of ammonium phosphate used by the seaweed farmers in Sibutu, Tawi-Tawi, Philippines (Tahiluddin, 2018) in *K. striatus,* which may be the reason of obtaining slow growth of the seaweed.

Ice-Ice Disease Occurrence

Urea (46-0-0) and phosphorus (pure) inorganic nutrient enrichment had no effect in cultured *K. striatus* in terms of ice-ice disease occurrence. However, in other studies, nutrient enrichment reduced ice-ice disease occurrence. Luhan et al. (2015) used sodium nitrate (0.01 g L^{-1}) to reduce the occurrence of *K. alvarezii* ice-ice disease to 8.75% compared to untreated (97%). Ammonium phosphate (8.82 g L^{-1}) used in *K. striatus* significantly lowered the incidence of ice-ice disease by up to 42% compared to untreated (78%) planted during the ice-ice season (Tahiluddin, 2018). Therefore, when there is a combination of these nutrients, seaweed *K. striatus* may lessen ice-ice disease occurrence. Loureiro et al. (2009) showed that Acadian Marine Plant Extract Powder (AMPEP) fertilizers effectively reduced the occurrence of ice-ice disease and epiphytes infestation of *K. alvarezii* cultured in raft method.

The primary cause of the occurrence of ice-ice disease is due to adverse environmental factors such as nutrient insufficiency and high or low salinity, light intensity, and temperature (Largo, 2002; Tahiluddin & Terzi, 2021a; Tahiluddin & Terzi, 2021b). The increased temperature of 33-35 °C resulted in the paling and whitening of seaweeds (Largo et al., 1995a). Similar to the current study, where on day 7, the temperature of the farmed area was about 33 °C which could cause the occurrence of ice-ice disease. Less than 50 µmol photon m^{-2} s^{-1} light intensity and less than 20‰ salinity could lead to the occurrence of ice-ice disease (Largo et al., 1995a). Pathogenic bacteria and fungi are other factors that cause ice-ice disease occurrence (Largo et al., 1995b; Solis et al., 2010; Tahiluddin et al., 2021a; Tahiluddin et al., 2021b). Slow water movement triggered the pathogenic bacteria to colonize the seaweed thalli can also cause ice-ice disease incidence (Largo, 2002).

The occurrence of ice-ice disease is high from May to August (Uyenco et al., 2019). In addition, seaweeds are also susceptible to ice-ice disease during the months of April, October, and December (Tisera & Naguit, 2009). This study was carried out between February and March, where the ice-ice disease appeared throughout the culture period. Intense heating and other environmental factors coupled with the presence of pathogenic microorganisms can cause the occurrence of ice-ice disease of cultivated *K. striatus.*

Production of seaweed, which has been affected by the ice-ice disease, has influenced seaweed farmers and the nation as a whole, particularly affected by the severe decline in production of aquaculture (Tisera & Naguit, 2009). The occurrence of ice-ice disease in seaweed farms could lead to a significant decline in seaweed production. (Doty & Alvares, 1975; Trono, 1993).

Carrageenan Yield

Carrageenan, extracted from red seaweeds and usually obtained by the extraction with water or alkaline water, is widely utilized in the food industry as thickening, gelling, and stabilizing agents, and as ingredients for pharmaceutical, cosmetic, personal care, and among others (Thirumaran et al., 2009; Hayashi et al., 2011; Ahmad, 2014; Husin, 2014). The main source of *kappa*-carrageenan is red alga *K. striatus* (Trono, 1997). Most *kappa*-carrageenan are produced by the presence of potassium ions under a process called potassium precipitation (McHugh, 1987). Inorganic nutrient enrichment used in the present study did not influence the carrageenan yield of *K. striatus* after 45 days of culture. In terms of the culture period, 45 days achieved the highest carrageenan yield compared to 30 and 15 days, but no significant differences were observed between treatments (p>0.05). On the contrary, Hurtado et al. (2008) obtained the highest carrageenan yield of *K. striatum* var. *sacol* on day 30 compared to 45 and 60 days. In addition, Hayashi et al. (2007b) revealed that the highest carrageenan yield was higher at day 28 compared

to 45 and 59 days. In this study, *K. striatus* nutrient enriched with urea obtained a carrageenan yield of 33.71% after 45 days. It was lower than the study of Luhan et al. (2015), where *K. alvarezii* enriched with 0.01 g L^{-1} of sodium nitrate obtained a carrageenan yield of 42.55% after 45 days. Neish et al. (1977) recorded a carrageenan yield of 35.9% in *Chondrus crispus* enriched with 6 µM nitrogen. A previous study demonstrated that nitrogen supply positively affects the phycocoloids in eucheumatoids (Rui et al., 1990; Chopin & Wagey, 1999; Sahoo & Ohno, 2003).

Moreover, phosphorus enrichment significantly increased the carrageenan yield of seaweeds and the vital mechanism of the flow of carbon in *C. crispus* towards carrageenan (Chopin et al., 1991). In this study, the *K. striatus* nutrient enriched with phosphorus obtained a carrageenan yield of 36.20% after 45 days and was higher than the carrageenan yield (30%) of red alga *K. striatus* enriched with 9 g L^{-1} ammonium phosphate after 35 days (Robles, 2020). In addition, 45 days of culture period achieved the highest yield of carrageenan in the present study compared to the study of Hurtado et al. (2008), in which *K. striatum* var. *sacol* yielded the highest carrageenan for a duration of 30 days, and they also stated that extension of cultivation duration from 45 to 60 days might result in the drop of carrageenan yield. Moreover, the present study coincided with Hayashi et al. (2007b), where the only duration significantly affected the highest carrageenan yield was 28 days of cultivation time.

Conclusion

Inorganic nutrient enrichment of *K. striatus* in a high concentration of urea could improve growth as early as 15 days, although not significantly different from the control, but did not affect the growth at 45 days of the culture period. On the other hand, both inorganic nutrient enrichments did not affect ice-ice disease occurrence throughout the culture period. In addition, both inorganic nutrient enrichment had no effect on the carrageenan yield of cultured *K. striatus*. However, in terms of the culture period, 45 days recorded the highest and better carrageenan yield. Refinement of application of enriched nutrients such as the time of dipping and concentration of nutrients still need to be studied and improved.

Compliance with Ethical Standard

Conflict of interests: The authors declare that for this article they have no actual, potential or perceived conflict of interests.

Ethics committee approval: Ethics committee approval is not required.

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The responses of cholinergic system in the brain tissue of Van Fish (*Alburnus tarichi*) exposed to antifungal tebuconazole compound toxicity

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ABSTRACT
Today, fungicide toxicity is quite common in aquatic ecosystems, and this situation adversely affects marine organisms. For this reason, it is essential to determine the effects of fungicides on aquatic organisms and to try to prevent organisms from being exposed to these toxic chemicals. In this study, changes in cholinergic system enzymes and (malondialdehyde) MDA levels as a result of exposure to acute fungicide toxicity in Van fish (*Alburnus tarichi,* Güldenstädt 1814) were investigated. Brain tissue was taken from Van fish exposed to 2.5 M Tebuconazole used in agriculture by sampling at 24, 48, 72, and 96 hours. Brain tissue acetylcholinesterase (AChE), butyrylcholinesterase (BChE) activities, and MDA levels were measured in this context. In the study, AChE (0.965± 0.03, 0.575±0.01) and BChE (0.421±0.02, 0,291±0.01) activities decreased in Van fish brain tissue due to exposure to Tebuconazole, but MDA (0.099±0.01, 0.192±0.01) level increased ($p < 0.05$).

Keywords: Van Fish (*Alburnus tarichi,* Güldenstädt 1814), Fungicide, Tebuconazole, AChE, BChE, MDA

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Introduction

One of the most critical factors threatening human, animal, and environmental health is pesticide residues. In chemical control, pesticides should not harm the environment and human health while protecting plants from diseases. However, due to the unconscious use of pesticides against pests, many problems such as deterioration of the natural balance, environmental pollution, and resistance of pests arise. Therefore, attention should be paid to the biological, toxic, and physical properties of fungicides. In the studies, it was determined that tebuconazole from the triazole group does not disappear in a short time due to its long half-life (Batta, 2005). In a study investigating its residue in soil and water 120 days after spraying with tebuconazole used in agriculture active ingredient fungicide, it was determined that the residual amounts of the said fungicide were still high (Nasr et al. 2003). Systemically effective tebuconazole also prevents the synthesis of ergosterol in fungi and can cause toxic effects on organisms in the aquatic environment for a long time (Bayer Crop Science Limited, 2005; Yeltekin et al. 2018). In other studies investigating the effects of tebuconazole, it was determined that tebuconazole residues persist for a long time and cause toxicity in living tissues (Siek and Paszko 2021).

Reactive oxygen species (ROS) originating from environmental pollutants cause structural and functional changes in the cells of aquatic organisms and can also cause changes in biochemical parameters (Parvez and Raisuddin, 2005). It is stated that in the presence of oxidative stress, the tissue and cell membranes of fish can be easily oxidized due to their high polyunsaturated fatty acid content (Mendes, 2009). Lipids in the membranes of intracellular organelles are highly susceptible to free radical damage. Lipid peroxidation, which occurs when free radicals react with lipids, can have highly damaging effects. Lipid peroxidation leads to the production of large quantities of toxic by-products. These produced by-products act as second messengers and exert their products in a region far from where they were produced. Damage from lipid peroxidation is highly detrimental to cell function (Devasagayam et al. 2003).

In general, terms, biomarkers are indicators of multiple toxic interactions such as physiological, biochemical, immunological, and histopathological effects caused by certain environmental pressures. Enzymes as biomarkers are usually associated with the first level of organization and can be considered an 'early warning sign. In this context, the enzymes to be regarded as biomarkers are esterases and oxidative stress enzymes. Cholinesterase enzymes are enzymes found in many tissues, body fluids, and plasma. They are divided into AChE and BChE according to their sensitivity to the inhibitor and substrate specificity. AChE enzyme is the main cholinesterase enzyme found in muscle, brain, and erythrocyte membrane. ACh is an enzyme that catalyzes various choline decomposition reactions, such as butyrylcholine and acetylthiocholiniodide. AChE and butyrylcholinesterase are the most well known cholinesterase enzymes. One AChE enzyme molecule hydrolyzes 4 x 10^5 ACh molecules per minute, and its 150 ms turnover time makes it the most effective hydrolytic enzyme. After the release of acetylcholine from the cholinergic synapses, the nerve transmissions are terminated due to its breakdown with the help of cholinesterases (Fetoui et al. 2010; Uçar et al. 2021; Yeltekin et al. 2020). They are among the acetylcholinesterase inhibitors with compounds such as pesticides and nerve gases. The AChE enzyme, which has a very high activity, breaks down approximately 25,000 acetylcholine (ACh) molecules per second. Chemicals inactivate the hydroxyl group of the serine amino acid in the enzyme's active site by phosphorylating it. As a result, the increase in acetylcholine in the cholinergic nerve junctions causes the smooth muscles to contract and the glands to secrete. The inhibitory effect on AChE activity shows that it also affects critical vital processes such as energy metabolism in nerve cells (Akdeniz, 2010). Therefore, studies on chemicals that cause cholinesterase inhibition are essential in terms of ecotoxicology. In addition to the studies on the activities of cholinesterases in serum or plasma, the relationship between brain acetylcholinesterase (AChE) inhibition and mortality is a very important point of view. Therefore, this study planned to investigate the effects of tebuconazole, which is widely used around Lake Van, on cholinergic enzymes (AChE, BChE) and malondialdehyde (MDA) in Van fish brain tissue.

Material and Methods

Fish

In the study, 80 Van fish of about 85-90 grams and 20-25 cm in length were used. The fish to be used in the study was obtained from Van Lake after obtaining the permission of the date 06.09.2018 and 08 number Van Yuzuncu Yil University Animal Research Ministry of Agriculture and the local ethics committee for animal experiments. After the fish were randomly distributed to 300 L water tanks, tebuconazole was applied after a one-week adaptation period. In the study, the water was constantly ventilated with oxygen stones, and the fish were fed twice a day, and the normal light process was applied. The tebuconazole concentration (2.5 M) to be administered Lutnicka et al. (2016). After the fish were kept in the anesthesia environment, they were separated into cranial incision tissues. Fish were sampled from both the concentration group and the control group at 24, 48, 72 and 96 hours.

Measuring AChE/BChE Enzyme Activity

In order to prepare for analysis, each tissue was homogenized for 5 minutes in a homogenizer by adjusting the pH to 7.4 in KH_2PO_4 buffer at 1/10 w/w. The obtained homogenates were centrifuged at 3000 rpm for 15 minutes. The obtained supernatant was used to determine the amount of MDA with acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzyme activities. AChE and BChE enzymes were determined spectrophotometrically according to the Ellman method. In the Ellman method, the thiol ester acetylthiocholine is used instead of the oxy ester acetylcholine as substrate. According to the principle of the Ellman method, acetylthiocholine is hydrolyzed by acetylcholinesterase, and the thiocholine released as a result of hydrolysis is combined with the Ellman reagent DTNB [5,5'-dithio-bis-(2-mtrobenzoic acid)] reacts. As a result of the reaction, yellow-colored chromophore TNB (5-thio-2-nitrobenzoic acid) is formed. The rate of formation (intensity of color) of this yellow compound formed at the end of the reaction is determined by measuring the absorbance at 412 nm (Ellman et al. 1961). The intensity of this yellow color is directly proportional to the AChE/BChE enzyme activity.

Measuring Lipid Peroxidation (MDA)

Homogenization of brain tissues was done according to Mis et al. (2018). This method was described by Placer et al. (1966) is based on the reaction of malondialdehyde (MDA), one of the aldehyde products of lipid peroxidation, with thiobarbituric acid (TBA). The resulting MDA forms a pink complex with TBA. The absorbance of this solution is measured at 532 nm with a spectrophotometer to determine the degree of lipid peroxidation.

Statistical Analysis

The one-way analysis of variance (ANOVA) and Duncan tests were performed to test statistically significant differences between the experimental groups using SPSS Software (version SPSS18.0). Statistical decisions were made with a significance level of $p<0.05$.

Results and Discussion

Oxidative stress occurs as a reaction to the stress caused by the effects of chemicals such as fungicides and pesticides, which damages the enzyme systems of all living things. It is of great importance to evaluate the oxidative stress parameter and the activity of neurotransmitter enzymes after exposure to a therapeutic agent or synthetic chemical compounds. The effect of oxidative stress biomarkers obtained in this study on its function is summarized in Figure 1, Figure 2 and Figure 3. The study's findings, the brain tissue AChE enzyme levels of Van fish decreased as the exposure time increased at the same tebuconazole concentration. It was observed that there was a significant decrease, especially at the 96th hour. These observed differences were also found to be statistically significant ($p<0.05$) (Fig.1).

According to the results obtained in the study, brain tissue BChE enzyme level of Van fish exposed to tebuconazole decreases as time progresses. It shows a statistically significant decrease especially after the 48th hour ($p<0.05$) (Fig. 2).

Malondialdehyde, one of the most important markers of oxidative stress, gave significant responses after tebuconazole application in the brain tissue of Van Fish. According to the results obtained, it was observed that the level of lipid peroxidation increased immediately after tebuconazole exposure started. It was determined that the MDA level increased as the exposure time increased. These observed differences were also found to be statistically significant ($p<0.05$) (Fig. 3)

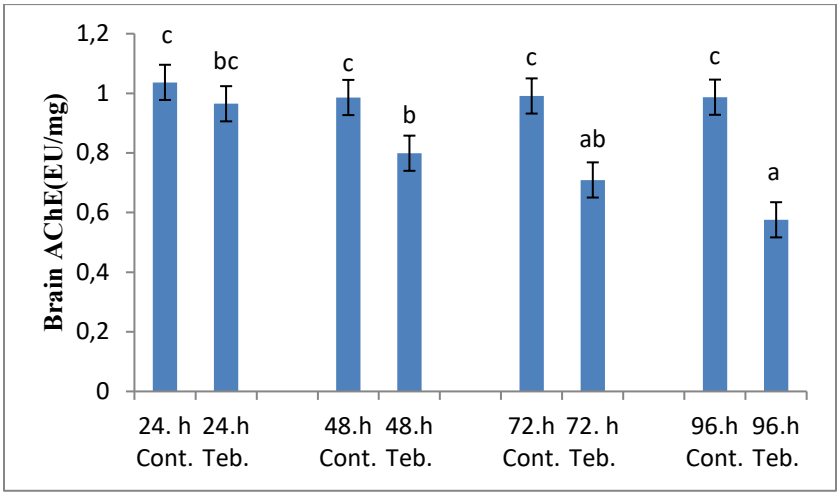

Figure 1. Van Fish brain tissue AChE activity exposed to tebuconazole

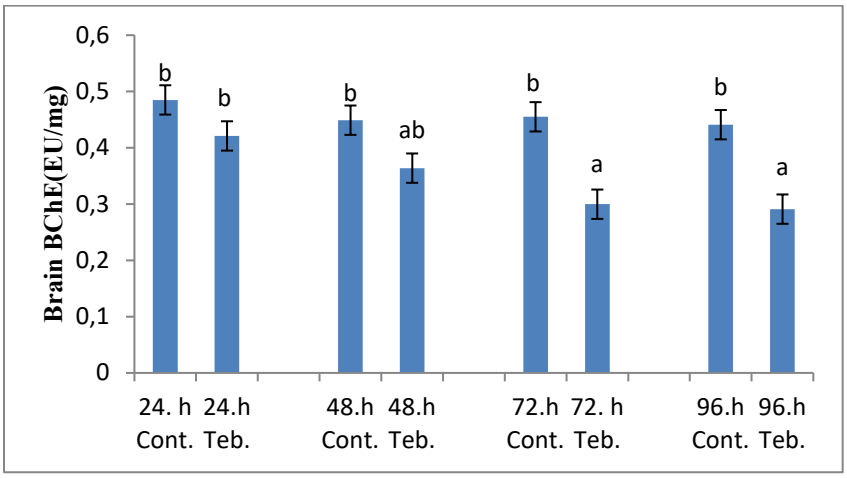

Figure 2. Van Fish brain tissue BChE activity exposed to tebuconazole

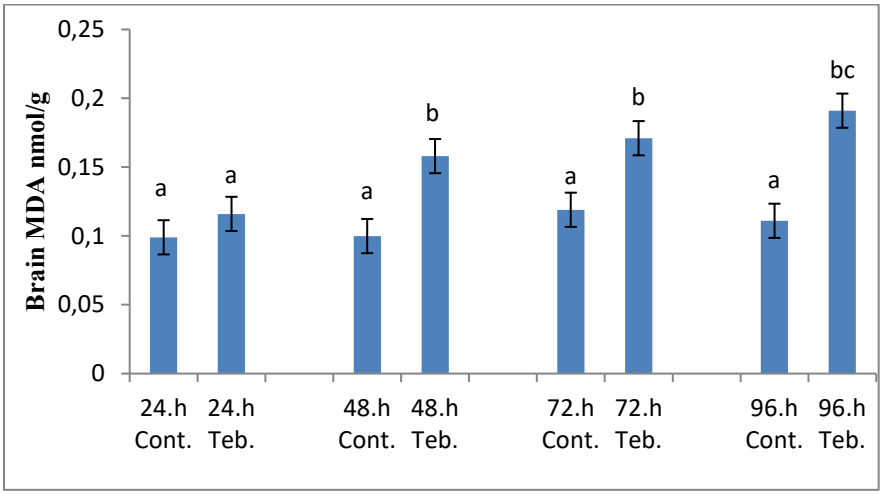

Figure 3. Van Fish brain tissue MDA level exposed to tebuconazole

Azole fungicides are a broad chemical class used to control molds and fungal infections on plants. These chemicals are also applied to ornamentals in commercial/residential applications. Triticonazole is one such triazole fungicide, but toxicity data are scarce on the potential for sublethal effects in nontarget aquatic organisms compared to other triazole fungicides. This study determined whether exposure to Tebuconazole would cause changes in Van Fish brain tissue AChE, BChE, and MDA levels. According to the findings, it was determined that Tebuconazole, an azole compound, decreased AChE and BChE levels by increasing oxidative stress in the brain tissue of Van fish. As the exposure time to the applied tebuconazole increases, the free radicals formed to increase and start the destruction in metabolism. As a result, AChE and BChE enzyme systems may be damaged, and their secretion may decrease. Similarly, in other studies with pesticides, AChE and BChE levels were found to decrease (Atamanalp et al. 2021). Santana et al. (2021) conducted a study examining the enzyme change by applying toxicity to fish with pesticides, herbicides and fungicides. In this study, it was determined that AChE and BChE levels of fish decreased in all three pesticide, fungicide and herbicide applications and even caused inhibition in some of them. It was determined that if the pesticide used was an organophosphate compound, it completely inhibited AChE and BChE enzymes, but the activation was significantly reduced in other pesticides and fungicides (Alak et al. 2019a, Alak et al. 2019b; Ramírez-Santana et al. 2020). It was determined that the oxidative stress levels in the larvae increased with the fungicide triticonazole compound applied to zebrafish larvae (Souders et al. 2020).

Again, a study was conducted in rats with tebuconazole fungicide. This study determined that Tebuconazole caused oxidative stress in tissues and triggered apoptosis (Nong et al. 2020). As with organophosphates, it has been determined that other pesticides- fungucide can inhibit the AChE enzyme. AChE is frequently used as toxic indicators of fungucide. The amount of neurotransmitter acetylcholine in sympathetic synapses, neuromuscular junctions and central nervous system. It has been reported that the inhibition of this enzyme, which regulates animals and humans, greatly affects (Glusczak et al., 2007). Acetylcholinesterase is an enzyme that controls impulse transmission by hydrolyzing acetylcholine in cholinergic synapses and terminating its function. Accumulation of acetylcholine as a result of enzyme inhibition causes excessive presynaptic stimulation, the continuation of the event results in paralysis and death (Sepici-Dincel et al., 2009).

It is known that free radicals increasing with pesticides and oxidative stress increasing with these reduce antioxidant enzymes. As a result, lipid peroxide formation (LPO) increases. Increasing LPO causes an increase in MDA, damaging the tissue cells and membrane structure (Fetouni et al. 2010). LPO formed in the membrane structure affects the permeability of the cell membrane and causes the disruption of intracellular balances (Gao et al. 2020). Our study determined that the brain tissue malondialdehyde level increased over time with tebuconazole application. This shows that the toxicity of azole compounds creates oxidative stress and increases the formation of free radicals. Our study determined that the brain tissue malondialdehyde level increased over time with tebuconazole application. This shows that the toxicity of azole compounds creates oxidative stress and increases the formation of free radicals. The free radicals formed can destroy the brain tissue, especially the lipid structure. In other studies, it was determined that exposure to fungicide increased the level of LPO (Das et al. 2020). Again, a study was conducted in which rainbow trout was exposed to azole compounds. In the study, oxidative stress and neurotoxic effects in fish were investigated. As a result, it was determined that azole compounds significantly increased oxidative stress and MDA levels (Rossi et al. 2020). Bartu et al. conducted a study investigating the inhibition of azole compounds on AChE and BChE. In the study, they revealed that AChE and BChE inhibitors are competitive inhibitors with enzyme kinetic experiments. Azole compounds have been reported to increase oxidative stress, increase MDA and inhibit the activity of AChE, and AChE measurement has been shown to be useful as a good biomarker. From this study and other studies, it is understood that ROS has an important role in fish tissue fungicide azole toxicity (Rafael et al. 2021).

Conclusion

Understanding this balance is essential to assess the complexity of toxicological effects in tissues. For aquatic toxicology, AChE and BChE enzymes are indicators that can be very effective in toxicology studies. Therefore, it is essential to investigate tissue-specific toxicity in elucidating toxic metabolism. In this study, the acute toxicity mechanism caused by tebuconazole fungicide in Van fish brain tissue was tried to be clarified by the approaching multi-biomarker (AChE, BChE activity, and MDA) parameters. When the data were interpreted, it was concluded that oxidative stress induced by tebuconazole fungicide in the brain tissue due to acute administration causes oxidative damage in the structural and functional activities of the cell by affecting the cholinergic system, inhibiting enzyme activities, and causing lipid peroxidation. Hence, to identify robust cause-effect relationships between fungucides and fish ChEs, future studies should turn their focus on filling the gaps found here.

Compliance with Ethical Standard

Conflict of interests: The authors declare that for this article they have no actual, potential or perceived conflict of interests.

Ethics committee approval: This study was conducted by Van Yüzüncü Yıl University Animal Experiments Ethics Committee (Ethics approval no: date 06.09.2018 and 08 number)

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Dose-dependent cytotoxic and proliferative effects of *Microcystis aeruginosa* extract and its fractions on human endothelial cells

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ABSTRACT

Microcystis aeruginosa, which spreads in five continents in the world and reported in drinking water resources in 257 countries, is a dangerous microalgae for human and animal health due to its toxins. The aim of current study was to evaluate the effects of *M. aeruginosa* extract and its chromatographically separated fractions on human endothelial cells. In this context, crude extract was prepared from *M. aeruginosa* cultured in BG-11 medium, and it was fractionated by an optimized HPLC method. Algae extract and its six fractions were then analyzed for their cytotoxic effects on ECV304 using MTT assay. The results revealed that algae extract inhibited ECV304 cells by around 72%, a higher percentage than all fractions. The most toxic fraction was the first fraction, which inhibited the cells by 55%. Other fractions, except the third one, were also toxic with 35-40% inhibition percentages. Third fraction and certain doses of some fractions showed proliferative activity on ECV304 cells. These results showed that the activities of the total extract and its fractions in promoting or inhibiting cell proliferation varied depending on not only the content but also the treatment dose.

Keywords: *Microcystis aeruginosa*, Human endothelial cells, ECV304, Cytotoxicity, Cell proliferation, Algae

Introduction

Cyanobacteria are organisms that are also called blue-green algae because of their photosynthetic pigments and have a wide habitat from water habitats that can freeze temporarily to hot water sources (Pearson et al., 2010; Harke et al., 2016). Due to their ability to perform photosynthesis, they increase the ratio of nutrients and O_2 in the water environment. Since cyanobacteria do not have nucleus and organelle membranes, their genetic material and pigmentous substances are free in the cytosol. They have a cell wall containing a small amount of peptidoglycans and 80S ribosomal RNA, similar to the cell wall of Gram (-) bacteria (Paiva et al., 2017). As the most primitive photosynthetic organism, they are described as "bacteria" because they do not contain a nucleus membrane, and as "algae" because they able to do photosynthesis. Cyanobacteria can form single-celled or multi-celled colonies. They can reproduce by vegetative division or spores, and they produce a large number of toxins (cyanotoxins) (Bryant, 1994).

Due to the increase in worlds' population, especially safety and quality of drinking water resources have become very important in recent years worldwide. The entity of cyanobacteria in water, and identification of their toxic components have become primary research subjects, since these data must be achieved to avoid their toxic or fatal effects on human and all living organisms. Furthermore, toxic substances and their mechanisms of action must be fully elucidated to develop efficient strategies for the prevention or treatment of pathological processes arising from cyanobacterial contamination (Carmichael, 1994; Campos and Vasconcelos, 2010).

It has been determined that at least 46 cyanobacterial strains are toxic to vertebrates worldwide. The most common cyanobacteria species in fresh waters are *Microcystis, Anabaena, Oscillatoria, Planktothrix, Chroococcus* and *Nostoc*. They synthesize a stable hepatotoxin molecule called microcystin (Kurmayer, 2011).

Studies with *Microcystis aeruginosa*, a microalgae living in almost all fresh water sources in all over the world, have revealed that this species has higher toxicity than other algae species. This toxicity threatens the lives of all living beings, especially humans and animals (Karjalainen et al., 2007). The toxic components participate to the plant circulation system through the absorption by the plants during the irrigation, and accordingly take part to food chain by not only the use of contaminated water, but also the consumption of the plants irrigated with this water (Lawton et al., 1994; Pearson et al., 2010).

Many peptides with high hepatotoxic activity have been described in *M. aeruginosa*. While these toxic peptides are generally retained in the cell, they are also released from the cell due to cell lysis, or by active transport systems (Babica et al., 2006). Dietary toxic peptides are transported to the liver by organic anion transport proteins and inhibit protein phosphatase 1 and protein phosphatase 2A enzymes, resulting in an increase of intracellular phosphoproteins, and associated intrahepatic bleeding, cell necrosis and tumor development in the liver (Lawton et al., 1994; Bagu et al., 1997; Tonk et al., 2005; Welker and von Dohren, 2006; Pearson et al., 2010).

M. aeruginosa contamination that has been reported in water resources in different parts of the world possess a vital threat to all living things in the region, especially humans, who come into contact with these waters. Reviews reporting the studies on the geographic distribution, toxins and genome of *M. aeruginosa* (Pearson et al., 2010; Harke et al., 2016), exert the seriousness of the subject, and draw attention to the importance of toxicity studies on *M. aeruginosa*. Those studies often appear to be a reference to the major toxin, microcystin (-leucine-arginine or -arginine-arginine forms) in total algae extract (Chong et al., 2000; Alverca et al., 2009; Dias et al., 2009; Piyathilaka, et al., 2015; Ramos et al., 2015; Herrera et al., 2018; Gutiérrez-Praena et al., 2019). However reports on the other toxins of *M. aeruginosa* are very limited in the literature (Kotak et al., 1995, Welker and von Dohren, 2006, Karjalainen et al., 2007, Yu et al., 2015, Entfellner et al., 2017).

Table 1. Some cellular peptides and proteins of *M. aeruginosa*.

	Peptide/protein	Molecular weight	Reference
TOXIC PEPTIDES	Microcystin -LR	995 Da	Chen et al., 2018
	Microcystin -RR	1038 Da	Zhong et al., 2017
	Microcystin -YR	1045 Da	Moreno et al., 2004
	Microcystin -LA	910 Da	Ramanan et al., 2000
	Microcystin -LY	1002 Da	Birungi and Li, 2009
	Microcystin -LW	1025 Da	Faassen and Lürling, 2013
	Microcystin -LF	986 Da	Faassen and Lürling, 2013
	Cyanopeptolin	957 Da	Kotak et al., 1995
	Anabaenopeptide	836 Da	Kotak et al., 1995
OTHER PEPTIDES/PROTEINS	Microcystin synthetase	116-205-402 kDa	Tillett et al., 2000
	Phosphoribulokinase	38.036 kDa	Wei et al., 2016
	Acetyl-Coa acetyltransferase family protein	41.396 kDa	Wei et al., 2016
	Phosphoglycerate kinase	42.811 kDa	Wei et al., 2016
	Fructose-bisphosphate aldolase, class II, Calvin Cycle subtype	39.156 kDa	Wei et al., 2016
	Glyceraldehyde-3-phosphate dehydrogenase	37.128 kDa	Wei et al., 2016
	60 kDa chaperonin	57.701 kDa	Wei et al., 2016
	ATP synthase subunit alpha	54.116 kDa	Wei et al., 2016
	ThiF family protein	42.979 kDa	Wei et al., 2016
	Oligo-ulvans	50-60 kDa	Kim and Chojnacka, 2015
	Akt substrate	160 kDa	Kim and Chojnacka, 2015
	Phlorogluquinol	162-650 kDa	Kim and Chojnacka, 2015
	Ulvan	189-8200 kDa	Kim and Chojnacka, 2015

M. aeruginosa contains several peptides and proteins, including toxic microcystins (Table 1). Among the microcystin derivatives, microcystin leucine-arginine (MC-LR) is the metabolite with the highest toxicity (Karan et al., 2015). For this reason, cytotoxicity studies in the literature have focused on this toxin. Studies on various cancer cells, such as kidney cancer, colon cancer hepatocellular carcinoma, breast cancer have shown that cell viability decreases depending on the MC-LR concentration (Dias et al., 2009; Ramos et al., 2015; Abdel-Rahman et al., 2020; Bittner et al., 2021). In addition to its cytotoxic properties, MC-LR is known to increase the effect of some inhibitors that block DNA repair, and intracellular reactive oxygen species. Besides, it damages the enzymes responsible for protecting DNA from oxidative stress, and causes DNA breaks (Zegura et al., 2003).

Apart from *M. aeruginosa*, the toxins belonging to other cyanobacteria also have various effects on endothelial cells. It is reported that cylindrospermopsin (CYN), produced by the *Anabaena* species, has a cytotoxic effect depending on the treatment dose, and 48-hour exposure, especially with 40 μg mL^{-1} CYN, reduces endothelial cell viability by 95% (Gutiérrez-Praena et al., 2012). In addition, another study in the literature shows that this cyanotoxin initiates apoptosis in endothelial cells (Wang et al., 2020). Despite its cytotoxic effects, it is reported that polysaccharides isolated from another cyanobacteria, *Nostoc* species, found in freshwaters, induce endothelial cell proliferation at some concentrations and may be used as a natural product for vascular repair in the future. (Foroh and Mahrouz, 2016).

In the present study, effects of crude algae extract and its chromatographic fractions on the cell viability of human endothelial cells were investigated in a dose-dependent manner, as human may be exposed to them by swallowing contaminated water or eating seafood contaminated with toxins. Main purpose was to make a prediction the effects of different constituents of *M. aeruginosa* on the veins, and on other tissues containing endothelial cells in general when they are taken into the body and transported to the organs/tissues through the veins.

Material and Methods

Preparation of Algae Culture, Algal Extraction and Measurement of Protein Concentration of Algal Lysate

Starting culture of *M. aeruginosa* (PCC7806) was obtained from Professor Reyhan Akçaalan Albay (Istanbul University, Faculty of Aquatic Sciences) as a gift, and cultivated in BG-11 medium in a shaking incubator under the conditions of 28°C, 110 rpm and continuous light (Stanier et al., 1971) for 28 days as determined by UTEX. The culture was centrifuged at 3901 x*g* for 50 min, the pellet was dried and suspended in PBS. The cell suspension was homogenized in a homogenizer at 5000 x*g* for 1 min, repeated 8 times. Cell disruption was confirmed by microscopic observations.

The protein concentration of the algae extract was determined by the SMART™ BCA Protein Assay Kit (iNtRON Biotechnology), according to manufacturer's instructions.

HPLC Analysis

Chromatographic fractionation of algae extract was carried out according to the method described by Lawton et al. (1994) previously, with some modifications.

Shimadzu Prominence UFLC System (Shimadzu Corporation, Kyoto, Japan) equipped with LC-20AD pumps, SPD-20A photodiode-array (PDA) detector, DGU-20A degasser, Inertsil® ODS-3 column (5 μm, 4.6 x 250 mm). The signal was recorded using Shimadzu LC Solution Software. The column temperature was maintained at 40°C and injection volume was 50 μL. The flow rate of the mobile phase was kept as 1 mL/min. Mobile phase A was composed of ultrapure water and 10% acetonitrile mixture containing 0.05% (v/v) trifluoroacetic acid (TFA) while mobile phase B was composed of acetonitrile containing 0.05% (v/v) TFA. The gradient conditions were as follows: 0-10 min (20→25% B), 10-40 min (25→80% B), 40-44 min (80→100% B), 44-46 min (100→20% B), 46-50 min (20% B). The chromatograms were monitored at 240 nm.

The algae extract was diluted with PBS to a protein concentration of 1 mg/mL before HPLC. Fractionation was maintained until no peak was observed, and repeated 13 times. Six fractions were collected separately by this process. Each fraction was lyophilized using a freeze drier (CHRIST/ALPHA 1-4 LD Plus). Lyophilized samples were dissolved in 100 μL of PBS and kept at -80°C until the cytotoxicity assays.

Mammalian Cell Culture and Cytotoxicity Assay

Cytotoxic activity of different concentrations of the algae extract and its fractions were assessed on human umbilical vein endothelial cell line (ECV304). DMEM/High Glucose medium (Gibco, 41966) supplemented with 10% fetal bovine serum (HyClone, SH3007003HI), 1% penicillin-streptomycin and 1% L-glutamine was used as growth medium. Cells were cultivated in 25 cm^2 polystyrene cell culture flasks, and incubated in a humidified atmosphere containing 5% CO_2 at 37°C. Adhesive ECV304 cells were detached by 0.5% trypsin–EDTA solution (HyClone™, SH30236.01), washed once with PBS and resuspended in DMEM at density of 1×10^5

cells/mL (Atasever-Arslan et al., 2016). The cytotoxic activity of *M. aeruginosa* extract and HPLC fractions on ECV304 cells was measured by using MTT (Sigma, M-5655) assay, as previously described (Pırıldar et al., 2010; Svobodova et al., 2012). The cell culture was incubated 24 h before each treatment.

Stock solution of the microalgae extract was prepared in PBS at a protein concentration of 13.06 mg/mL. Serial dilutions of the stock solution (6.53, 3.27, 1.63, 0.82, 0.41, 0.205, 0.102, 0.05 and 0.025 mg/mL) were prepared in PBS. Six fractions (No.1-6) obtained from HPLC having a dry weight of 9.6, 4.8, 4.9, 6.3, 6.2 and 6.1 mg, respectively, were diluted with PBS as 1:1, 1:2, 1:4, 1:8, 1:16 and 1:32 ratios.

On the mid-log phase of ECV304 cell growth (24th hour of the culture), 10 µL of each sample (algae extract, 6 HPLC fractions or their serial dilutions) was dispensed into 96-well round-bottom plates containing ECV304 cells. As a negative control, only 10 µL of sterile phosphate buffer saline (PBS) was used instead of algal extract and HPLC fractions, and cell viability for this sample was regarded as 100%.

After 48 h of incubation with samples, 10 µL MTT solution (5 mg/mL) in PBS was added to each well and the plates were incubated in a CO_2 incubator at 37°C for 3 h. Subsequently, 80 µL of supernatant was removed from each well and 100 µL of freshly prepared isopropanol-DMSO solution [1:1 (v/v)] was added. The microplates were stored at room temperature in the dark for 45 min, in order to dissolve the formazan crystals formed by reduction of MTT in living cells. Optical densities of the samples were measured at 570 nm wavelength in microplate reader (Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer). The cell viability was calculated as percentage of viable cells in experimental group (exp.) versus untreated (negative) control group (cont.) using the following formula, where A=absorbance of related groups:

Cell viability (%) = [$A_{exp.}/A_{cont.}$] x 100

Two independent experiments with at least three repeats were carried out, and the results were evaluated using GraphPad Prism® 7 program. One-way ANOVA with Dunnett's test was used in order to determine the differences between the groups. The limit of significance was accepted as $P<0.05$. Nonlinear regression analysis was also performed for calculating the half-maximal inhibitory concentration (IC_{50} in mg/mL) of algae extract.

Results and Discussion

Apart from the studies in the literature, here we separated *M. aeruginosa* total extract into 6 fractions by optimizing a RP-HPLC method. The effect of total extract and each fraction on the growth of endothelial cells (ECV304) was investigated. Different concentrations of total extract and fractions introduced to cells on mid-log phase for 48 hours, and their dose-dependent effects on cell viability were statistically evaluated.

HPLC Analysis of Cell Extract

According to the appearance of the peaks on the chromatogram, six fractions were collected, consisting of Fr.1-6 (Figure 1).

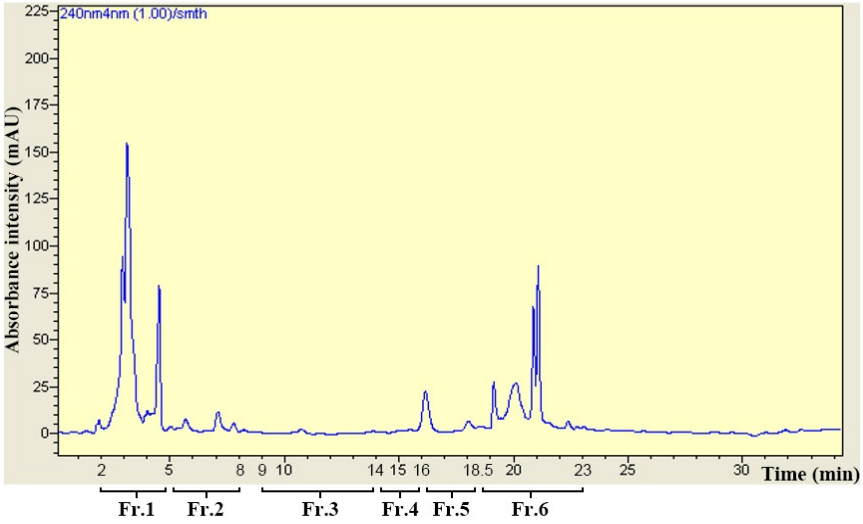


Figure 1. HPLC chromatogram of *M. aeruginosa* extract.

Dry weights of Fr.1, Fr.2, Fr.3, Fr.4, Fr.5 and Fr.6 collected at the end of 13 run were 9.6, 4.8, 4.9, 6.3, 6.2 and 6.1 mg, respectively, following the lyophilization.

Effects of Algae Extract and HPLC Fractions on ECV304 Cells

One-way ANOVA test was used to analyze the consistency between the data obtained from MTT tests to determine the effects of algae extract and its fractions on ECV304 cell viability.

The algae extract inhibited ECV304 cells in a dose-dependent manner. The highest inhibition percentage (72±12.99%) was detected in stock solution of the algae extract containing 13.06 milligram protein per milliliter (Figure 2). There was a correlation between the cytotoxic effect and protein concentration, up to 32 fold dilution (0.41 mg/mL), and statistically significant cytotoxic avtivity was detected in the samples containing 0.41-13.06 mg protein per milliliter compared to control (***P<0.001). However, dilutions with a protein concentration less than 0.41 mg/mL had no effect on cell viability (P>0.05). The IC_{50} value of algae extract on ECV304 cells was estimated as 2.737 mg/mL from nonlinear regression analysis.

The effects of different concentrations of the fractions (Fr.1-6) on ECV304 cells were presented comparatively in Figure 3. The cell viability was 55±5.04% when the cells were treated with the highest Fr.1 concentration obtained (96 µg/µL) (Figure 3). Very low inhibition percentages were detected for two dilutions of Fr.1 (20±4.98% for 48 µg/µL and 10±5.04% for 24 µg/µL) (***P<0.001). Neither cytotoxic nor proliferative activity was observed in other dilutions (P>0.05). This result showed that Fr.1 contains only moderately toxic substances (Figure 3a).

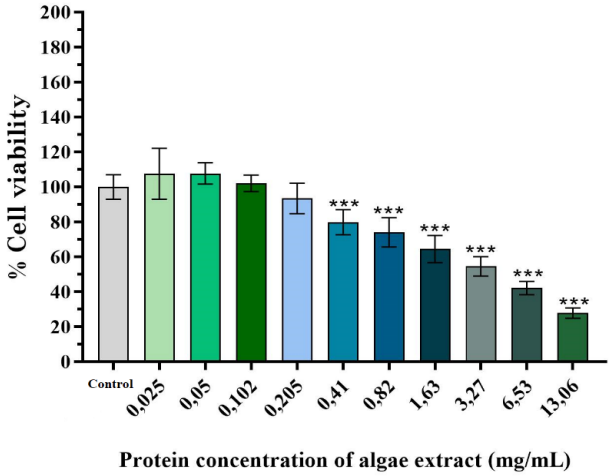
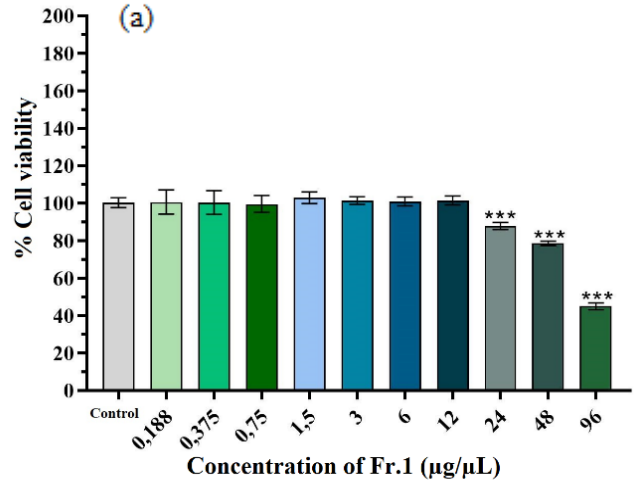
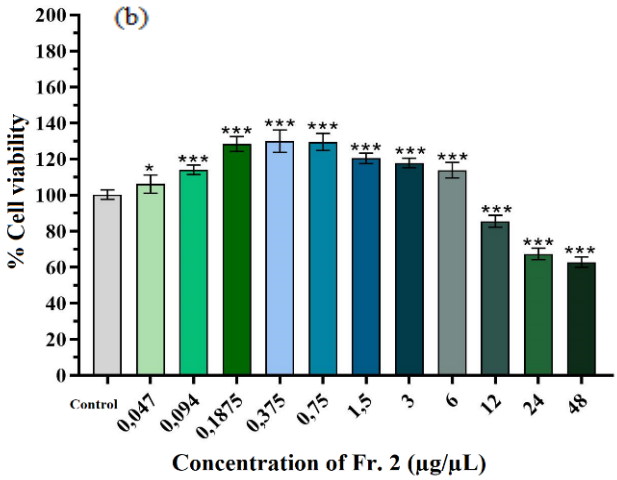


Figure 2. The effect of algae extract on the viability of ECV304 cells (***P<0.001, vertical bars show standard deviations)





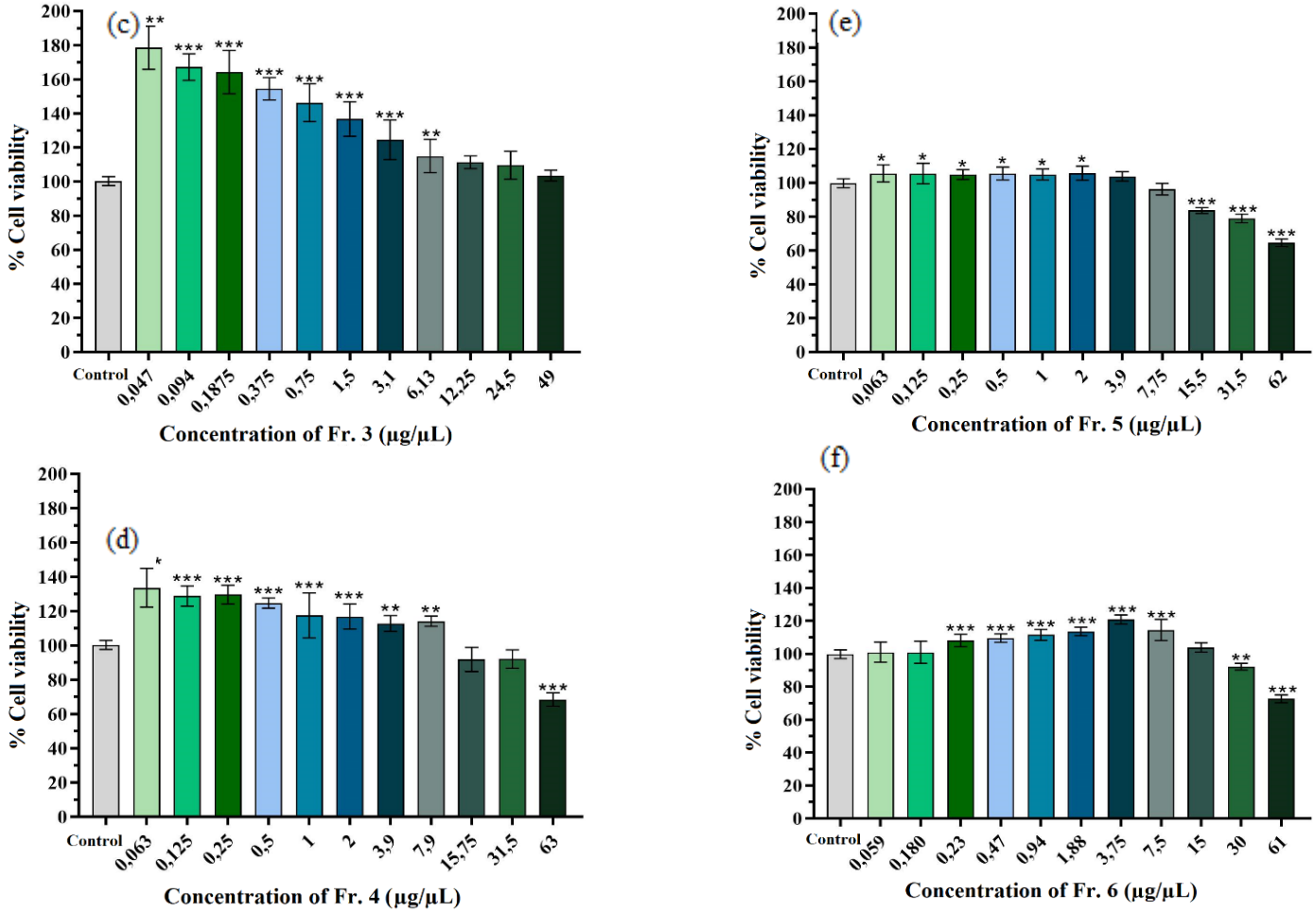


Figure 3. The effects of the fractions on the viability of ECV304 cells. (a) Fr.1, (b) Fr.2, (c) Fr.3, (d) Fr.4, (e) Fr.5, (f) Fr.6. (*P<0.05, **P <0.01, ***P<0.001, vertical bars show standard deviation values.

The highest test concentration was 48 µg/µL for Fr.2, and its inhibition percentage was 38±4.85% (Figure 3b). The cytotoxic effect of two dilutions (24 µg/µL and 12 µg/µL) were determined as 33±5.28% and 14±5.27%, respectively. Other concentrations (6, 3, 1.5 and 0.094 µg/µL) were observed to have a significant proliferative effect on ECV304; they induced the cell proliferation by 13±5.28%, 17±5.28%, 20±5.28%, respectively. Certain concentrations (0.75, 0.375 and 0.187 µg/µL) were more effective, with 29±5.62%, 29±6.09%, 28±6.09% proliferation, respectively. However, proliferative effect was not higher than approx. 29% (Figure 3b).

The highest application concentration (49 µg/µL) and subsequent two dilutions (24.5 and 12.25 µg/µL) of Fr.3 had no effect on the cell viability (Figure 3c). However, proliferative effect ranging from 14±12.36% to 78±13.16% was observed for lower concentrations. Interestingly, proliferative effect increased as the concentration decreased. The lowest concentration (0.047 µg/µL) exerted the highest proliferative activity (Figure 3c). This result showed that Fr.3 contains only proliferative substances.

The highest application concentration (63 µg/µL) of Fr.4 slightly (32±9.22%) inhibited the cell viability (Figure 3d). As detected in the lower doses of Fr.3, proliferative effect was also detected for two doses of Fr.4. The lowest dose (0.063 µg/µL) induced the cell proliferation by 33±10.64%.

The highest application concentration of Fr.5 (62 µg/µL) was found to inhibit the cell viability by 35±5.04% (Figure 3e).

Its two dilutions (31.5 and 15.55 µg/µL) also showed cytotoxic activity to a lesser extent while some dilutions (2-0.063 µg/µL) induced the cell viability by around 5%.

The highest application concentration of Fr.6 (61 µg/µL) and its 1:1 dilution (30 µg/µL) inhibited the cell viability by 27±5.68% and 8±5.67%, respectively (Figure 3f). In contrast, lower doses between 7.5 and 0.23 µg/µL had proliferative effect, and one dose (3.75 µg/µL), which causes proliferation by 21±5.67%, was the most effective one. Other concentrations less than 0.23 µg/µL were found to have no effect on cell growth.

The most interesting finding of the study was the variation of cell viability upon different treatment doses of the samples. There were several concentrations among all fractions, except Fr.1 and Fr.3, inducing or inhibiting the cell growth dose-dependently (Figure 3). Some concentrations of the Fr.1 exerted only inhibitory or no effect on cell growth, while Fr.3 induced the proliferation, or had no effect on cell growth. Especially lower concentrations of Fr.3 were very active. For example, 0.047 µg/µL of Fr.3 exerted significant proliferative effect (78%). However, the total extract containing all these fractions inhibited cell proliferation by 72 ±12.99%, the highest inhibition percentage within the all samples. Thus it seems that toxic constituents in total extract have a synergistic effect against the action of proliferative ones.

As a result, it was confirmed that proliferative substances are present aside from cytotoxic peptides/proteins in algae extract. Proliferation of endothelial cells is important in many aspects. First of all, endothelial cells form a single-cell layer called endothelium that lines all of blood vessels, and is critical for both vascular biology and endocrine system (Krüger-Genge et al., 2019). Endothelial cells originated from various tissues possess different functions under different microenvironments (Cines et al., 1998). Proliferation and survival of endothelial cells are of prime importance, since dysfunction of endothelial cells is associated with several diseases such as diabetes, pulmonary diseases, inflammatory diseases, cardiovascular diseases, immune diseases, cancer and currently COVID-19 (Rajendran et al., 2013; Fosse et al., 2021). Especially, prevention of coronary endothelial damage observed after ischemia and reperfusion is vital (Laude et al., 2001; Singhal et al. 2010). Today, various chemicals are tried to prohibit endothelial damage or accelerate healing. It is thought that the components detected in Fr.3 that cause the proliferative effect can be tested in future studies as a natural product as an alternative to the chemicals studied for vascular regeneration. However, it should be considered that this activity give rise to risk since endothelial cell proliferation is closely related to pathological angiogenesis in several diseases such as proliferative retinopathy, rheumatoid arthritis, psoriasis, and tumor angiogenesis (Plate et al., 1994).

On the other hand, some peaks in the HPLC chromatogram may refer various substances other than polypeptides. Thus it was concluded that total proteins precipitated from algae extract should be examined in order to identify toxic peptides in *M. aeruginosa* more accurately. Water-soluble organic substances other than proteins in algae extract should also be taken into consideration as bioactive constituents, and other biological activities of all constituents should be evaluated in the future, as in the previous reports (Singh et al. 2005; Khalid et al. 2010; Silva-Stenico et al., 2013). Studies on the exhibition of cytotoxic/proliferative peptides/metabolites in the separated fractions are in progress.

Conclusion

This study deals with the effects of *M. aeruginosa* total extract and its fractions separated by an optimized HPLC procedure on the viability of endothelial cells. Cell proliferation promoting or inhibiting activities of total extract and the fractions vary depending on the treatment dose. It is figured out that one fraction contains cytotoxic constituents while another contains only proliferative ones, at least for the test concentrations. Accordingly, *Microcystis aeruginosa* that is a famous organism with its toxic peptides, produces not only harmful but also potentially helpful constituents, which can be used as natural products in the future. Current study is expected to contribute fractionation of *M. aeruginosa* extract as well as evaluation of *in vitro* effects of total algae extract, and its fractions on the viability of healthy cells, and to provide a basis for related studies in the future.

Compliance with Ethical Standard

Conflict of interests: The authors declare that for this article they have no actual, potential or perceived conflict of interests.

Ethics committee approval: There is no need ethics committee approval.

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Disclosure: -

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Phylogenetic analysis of *Luciobarbus* Heckel, 1843 and *Barbus* Cuvier & Cloquet, 1816 species in the Euphrates River (Turkey) based on mtDNA COI gene sequences

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ABSTRACT

Natural fish species living in the Euphrates River System; It is subject to some pressures such as overfishing, competition with invasive species and habitat loss. As a result of these pressures, it leads to the decrease of endemic and native species. At the beginning of these species are the species belonging to the *Barbus* Cuvier & Cloquet, 1816 and *Luciobarbus* Heckel, 1843 genera, which have high economic importance. In this study, phylogenetic analysis of the species belonging to the genus *Barbus* Cuvier & Cloquet, 1816 and *Luciobarbus* Heckel, 1843, which live naturally in the Euphrates River, was carried out with mtDNA COI gene sequences. 17 fish samples belonging to five species from three localities belonging to the Euphrates river system (Turkey) were studied. Total DNA extraction was performed from muscle tissue using Commercial Kit. Then the mtDNA COI region was amplified by PCR and sequenced. Genetic distance values were calculated between 0.00201 and 0.15332, and it was determined that the closest species were *L. xanthopterus* and *L. esocinus*, and the most distant species were *B. lacerta* and *A. grypus*. In addition, phylogenetic analyzes of the target species were made and an phylogenetic tree was formed and the species were distinguished. In future studies, it is recommended to evaluate the data in this study, to determine the genetic characteristics of populations, and to carry out conservation studies at the population level.

Keywords: *Luciobarbus*, *Barbus*, mtDNA COI, Phylogenetic, Euphrates River

Introduction

Populations in aquatic habitats are often threatened by the effects of human activities such as pollution, harvesting, fishing, alien species, tourism and urban expansion (Cognetti and Maltagliati, 2000). The destruction or change of habitats can lead to decreases in populations and species diversity and even the extinction of some species. The decline of individuals in natural populations may cause the disappearance of unique genotypes that cannot be found anywhere else, and when this genetic information is lost, it is almost impossible to recover (Parmaksız, 2020; Parmaksız, 2021). Genetic diversity is estimated to decrease faster than species diversity under increasing threats, but its spatial distribution remains poorly documented on a global scale (Manel et al., 2020). Genetic diversity directly reflects the ability of species or populations to adapt to environmental factors of alien environments (Frankham et al., 2002; Spielman et al., 2004).

Natural fish species in the Euphrates River System are exposed to pressures increasing day by day due to factors such as overfishing, dominance of invasive species and habitat loss. Invasion of freshwater ecosystems by allien fishes can have significant consequences for natural biodiversity, including local extinctions of endemic and native species (Gozlan et al. 2010; Jackson et al. 2017; Mollot et al. 2017). Recently, invasive species such as *Carassius gibelio* (Bloch, 1782) and *Carassius auratus* (Linnaeus, 1758) pose a great threat to native species in the Euphrates River (Turkey). Due to these dangers, the number of individuals, especially in the populations of economic species, is decreasing which consequently causes species loss. These species which have high economic value mainly belong to the *Barbus* Cuvier & Cloquet, 1816 and *Luciobarbus* Heckel, 1843 genera. Some of these species found in the Euphrates River (Turkey) and the dam lakes built on it are caught and sold by the fishermen of the region and sent to the neighboring cities. Ensuring the continuity of the populations of these species is very important both in terms of biodiversity and economy. Therefore, the identification of the species and their genetic structure of the populations is a matter that needs to be addressed with the utmost urgency.

Since the genus *Barbus* Cuvier & Cloquet, 1816 was separated from the genus *Luciobarbus* Heckel, 1843 very recently, there are usually problems in naming the species (Korkmaz, 2017). Morphological characters are widely used in studies such as identifying differences in fish taxonomy. In addition, studies in the recent years demonstrate that molecular data has been very successful in identifying species and that DNA barcoding is an essential marker for species identification (Rock et al., 2008). Advances in sequencing techniques have popularized the mtDNA (Liu and Zhou, 2016) which is widely studied as a significant data for predicting the genetic makeup of living things (Xu et al., 2011). Analysis of the mtDNA-*COI* region can be used as a reliable marker to identify fish species (Ward et al., 2005).

The aim of this study is to determine *Barbus* and *Luciobarbus* species based on mtDNA COI in Euphrates river basin in Turkey and revealing the status of the species in the dendrogram created based on this information. To observe genetic similarity between species, a dendogram is usually prepared using a clustering algorithm. Being on the same branch in the phylogenetic tree reflects its genetic similarity.

Material and Methods

The fish samples used as material in this study were purchased from the fishermen of two locations on the Euphrates river system, and carried in an ice container when they were brought to the Zoology Laboratory of the Faculty of Science and Letters of Harran University. After the species were identified, muscle tissue was taken from the samples and placed in microcentrifuge tubes containing 90% ethanol and kept at -20°C until DNA was obtained.

Total DNA isolation was obtained from muscle tissue using the GeneJET Genomic DNA Purification Kit (Thermo Scientific). In order to check the presence of DNA after the protocol, DNA samples of all individuals were placed in the wells of 1% agarose gel added to SYBR Green, carried out in electrophoresis and visualized in a (UV) light-emitting device (Smart View Pro Imager System, Major Science). The primer used for amplification of the mtDNA *COI* gene region was adopted from Darabi et al. (2014) and PCR was applied.

PCR process was carried out with BIO-RAD T100TM Thermal Cycler device. For the PCR procedure, a total of 34 cycles were performed, including 3 minutes of initial denaturation at 95°C, 30 seconds of denaturation at 95°C, 30 seconds of bonding at 62°C, and 45 seconds of elongation at 72°C. The procedure was completed with keeping the samples at 72°C it for 10 minutes. The obtained PCR output was sent to a commercial firm for sequence analysis which was performed on the 3500 XL Genetic Analyzer (Thermo Fisher Scientific).

The raw data of the mtDNA *COI* sequences procured from the commercial company were evaluated using the ChromasPro v 2.0.1 (Technelysium Pty Ltd) program and converted into FASTA format. Sequences of all individuals in FASTA format were aligned using the BioEdit software version 7.2.5 program. Phylogenetic analyses between species

were carried out in the MEGA X program according to the Neighbor-joining tree model using the K2 parameter and a phylogenetic tree was created (Kumar et al., 2018). Bootstrap test (1000 replicates) was applied to test the reliability of tree branches (nodes).

Table 1. Information on the fish species studied in the research

Fish No	Species Name	Location	Date
1	*Luciobarbus xanthopterus* Heckel, 1843	Adıyaman	September 2020
2	*Luciobarbus xanthopterus* Heckel, 1843	Adıyaman	September 2020
3	*Luciobarbus xanthopterus* Heckel, 1843	Adıyaman	September 2020
4	*Luciobarbus kersin* (Heckel, 1843)	Adıyaman	September 2021
5	*Luciobarbus kersin* (Heckel, 1843)	Adıyaman	September 2021
6	*Luciobarbus kersin* (Heckel, 1843)	Adıyaman	September 2021
7	*Arabibarbus grypus* (Heckel, 1843)	Adıyaman	September 2020
8	*Arabibarbus grypus* (Heckel, 1843)	Adıyaman	September 2020
9	*Arabibarbus grypus* (Heckel, 1843)	Adıyaman	September 2020
14	*Luciobarbus esocinus* Heckel, 1843	Adıyaman	September 2020
15	*Luciobarbus esocinus* Heckel, 1843	Adıyaman	September 2020
16	*Luciobarbus esocinus* Heckel, 1843	Adıyaman	September 2020
17	*Luciobarbus esocinus* Heckel, 1843	Şanlıurfa-Bozova	October 2020
18	*Luciobarbus esocinus* Heckel, 1843	Şanlıurfa-Bozova	October 2020
19	*Luciobarbus esocinus* Heckel, 1843	Şanlıurfa-Bozova	October 2020
20	*Barbus lacerta* Heckel, 1843	Adıyaman-Gölbası	July 2020
21	*Barbus lacerta* Heckel, 1843	Adıyaman-Gölbası	July 2020

Results and Discussion

In this study, the mtDNA *COI* gene region of individuals of *Luciobarbus*, *Barbus* and outgroup *Arabibarbus grypus* species in the Euphrates River, whose number of individuals have decreased considerably, were studied by conducting an average of 603 bp region sequence analysis, and phylogenetic analysis of the species were imaged by using the "Finch TV" program (Figure 1). A total of 106 polymorphic regions were identified for this region. The mean genetic distances between the species were calculated in the MEGA X program (Kumar et al., 2018) and are shown in Table 2.

Table 2. Comparison of the sequences obtained in the study with the NCBI database

Species Name	Accession No	Per. Ident %
Luciobarbus xanthopterus	KM590446	99.83
Luciobarbus kersin	MF599072	100
Arabibarbus grypus	KM590450	100
Luciobarbus esocinus	MF599073	100
Barbus lacerta	MF106166	100

In Table 2, similarity values are given by comparing the haplotypes of the mtDNA COI region of different species obtained in this study with the haplotypes in the NCBI GenBank with Blast method. Information on species showing maximum similarity is presented. *Luciobarbus xanthopterus* species exhibits a different haplotype and the sequences of the other species studied are available in the GeneBank.

Genetic distance values of five species were calculated between 0.00201 and 0.15332 by analyzing according to the genetic distance estimation based on the Kimura parameter model. According to these calculations it was determined that the closest species were *L. xanthopterus* and *L. esocinus*, and the most distant species were *B. lacerta* and *A. grypus.*

In this study, the mtDNA *COI* region sequences and the neighbor joining tree were created with the MEGA X program as well (Kumar et al., 2018). The obtained NJ tree is given in Figure 2.

In Figure 2, it is seen that *A. grypus* is located on a separate branch, unlike other species. While *Barbus lacerta* species is located closer to *Luciobarbus* species, *Luciobarbus* species appear on separate branches at the species level. Although individuals belonging to the *Luciobarbus esocinus* species are close, they are divided among themselves because they were collected from two different localities and have different haplotypes

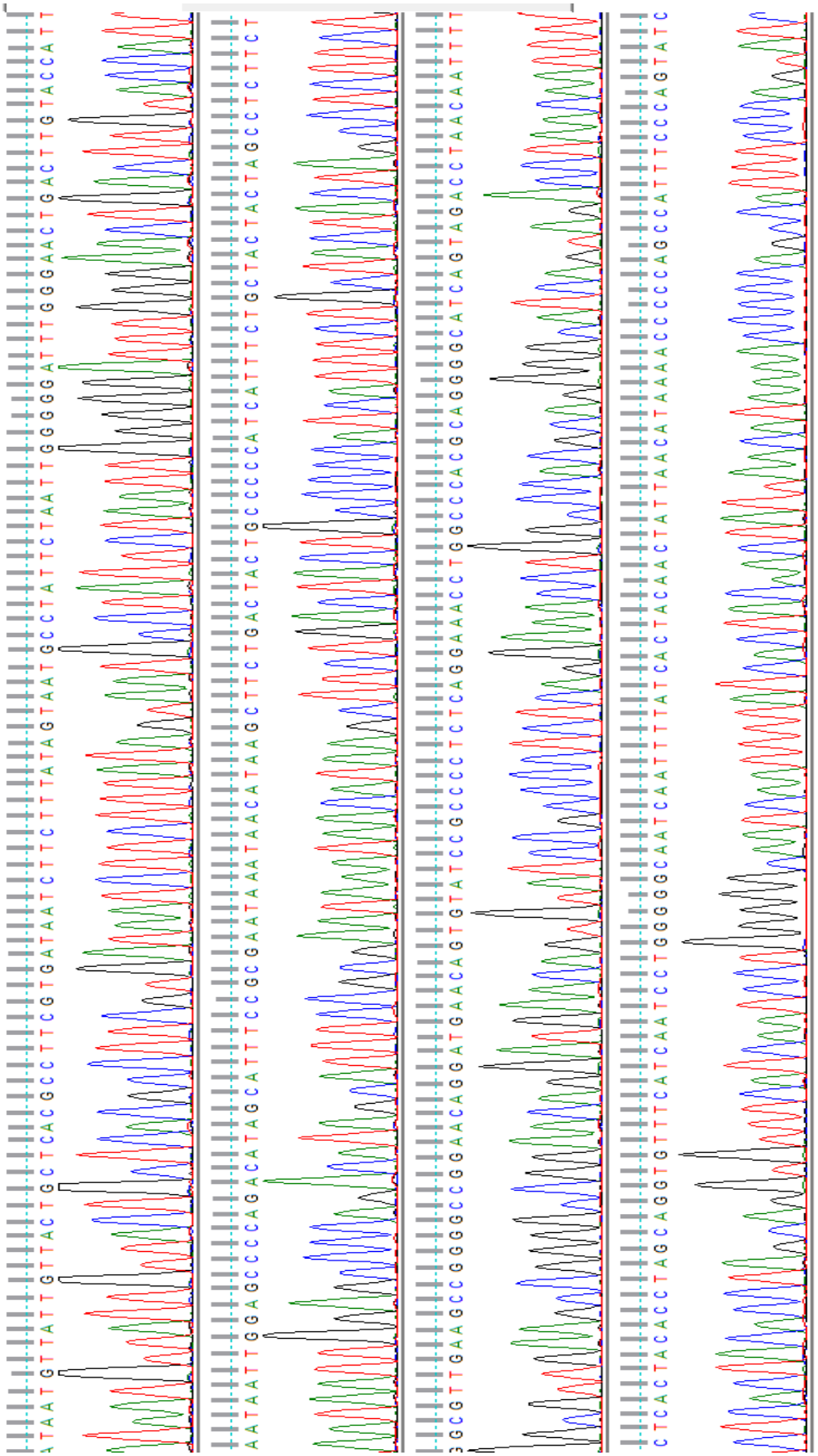

Figure 1. Chromatogram image of an exemplary sequence analysis of the mtDNA *COI* region.

Table 3. Means of genetic distance between studied species

Species	*L. xanthopterus*	*L. kersin*	*L. esocinus*	*B. lacerta*	*A. grypus*
L. xanthopterus	-				
L. kersin	0,02922	-			
L. esocinus	0,00201	0,02783	-		
B. lacerta	0,09258	0,10452	0,09108	-	
A. grypus	0,15107	0,13400	0,14947	0,15332	-

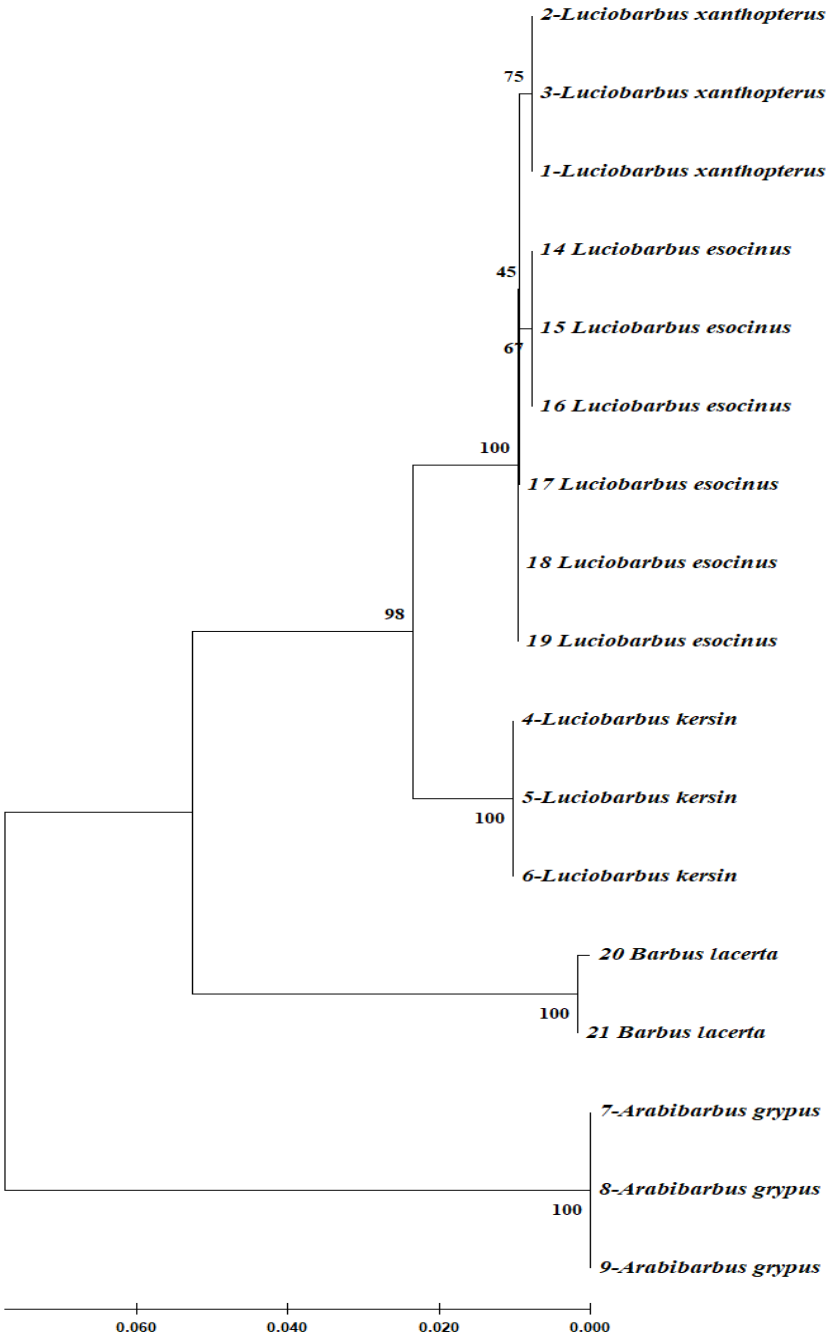

Figure 2. Neighbor Joining (NJ) tree of 5 species based on mtDNA *COI* sequences

Human activities have caused significant changes in the physical, chemical and biological composition of the Euphrates River systems. In addition, environmental factors such as industrial activities, intensive fishing and destruction of habitats will lead to the extinction of many species or the decrease of their populations (Kuru, 1986; Ünlü et al. 1997). Conservation of population size and genetic diversity is essential for the survival of the species. The decrease in the population results in deterioration of genetic diversity and poses a threat to survival of the population (Parmaksız, 2021). One of the most important things to be done in the study of populations is to differentiate the species genetically and morphologically. Once the species has been identified, the status of the populations should be determined and steps should be taken for future conservation strategies and habitat management of the target species. Especially in some localities unless the necessary precautions are taken, the level of genetic diversity will decrease, resulting in the degeneration of the feeding, reproduction, competition and adaptation abilities of the populations and the target organism will face the danger of extinction (Parmaksız, 2021).

Conclusion

In this study, species whose numbers of individuals have decreased considerably due to environmental factors such as overfishing and habitat degradation in the Euphrates River systems were targeted for phylogenetic analysis and species differentiation. In future studies, it is recommended to determine the genetic characteristics of populations and to carry out conservation studies at the population level.

Compliance with Ethical Standard

Conflict of interests: The authors declare that for this article they have no actual, potential or perceived conflict of interests.

Ethics committee approval: Ethics committee approval is not required for this study.

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Monitoring of growth and biochemical composition of *Dunaliella salina and Dunaliella polymorpha* in different photobioreactors

Zeliha DEMİREL

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ABSTRACT

In this study, the isolation of green algae were collected from two different stations of Aegean Sea and Seyfe Lake. The molecular identification of *Dunaliella* species using their 18S ribosomal DNA genes were sequenced and investigated with the BLAST program in the NCBI database. After the morphological and molecular identification, two different *Dunaliella* species were deposited in Ege University Microalgae Culture Collection. *D. salina* and *D. polymorpha* cells were firstly produce in both bubble column to monitor the growth profiles and then the species were cultivated in bubble column and stirred column photobioreactors (PBRs) under both high light intensity and different mixing conditions to investigate the total protein, carbohydrate, lipids and carotenoid concentrations. Moreover, this study aims to evaluate the production of β-carotene using two different PBRs. As a result of this study, *D. salina* in stirred PBR obtained the highest lipid (334.79 ±0.02 mg/L), total carotenoid (96.7 ±0.02 mg/L), and β-carotene content (21.18 ±0.03 µg/mL), while the maximum dry cell mass of 0.906 g/L was reached by *D. polymorpha* in bubble column PBR. The aim of this study was to investigate the nutritional values and β-carotene content of *Dunaliella salina* and *D. polymorpha* isolated from Turkey.

Keywords: *Dunaliella* salina, *Dunaliella polymorpha,* Isolation, Molecular identification, Carbohydrate content, β-carotene, Lipid content

Introduction

Microalgae have been long known for utilized of raw material for food products and feed animals. The three most important phyla of microalgae are to be in *Bacillariophyta* (diatoms), *Chlorophyta* (green algae), and *Chrysophyta* (golden algae). There are many trade applications of green microalgae products acquiring for carotenoids, lipids, proteins as used in various industries such as energy, cosmetics, pharmaceutical, bakery, and aquaculture. For instance, *Haematococcus pluvialis* is essential carotenoid as a source of astaxanthin, *Chlorella vulgaris* as a supplementary food product or food ingredient and the saline species of *Dunaliella* as a β-carotene resource. The β-carotene has a lipophilic terpenoid pigment, which can commercialize as food additives and provitamin A (Jesus and Filho, 2010). *Dunaliella* cells have a wide range of advantages in the production of chemicals such as carotenoids and xanthophylls with antioxidant, anticancer and anti-inflammatory activity, in bioremediation techniques, and in the production of biofuels used for *D. tertiolecta* biomass (da Silva et al., 2021). *D. salina* can grow up extensive open systems using raceway ponds in Australia, China, India, Chile, U.S., and Israel as a pigmenting agent (β-carotene) (Carvalho et al., 2006). Human bodies are not able to synthesize carotenoids; people must obtain enough amount from foods as a source of dietary supplements. More recent articles are determined the benefits of carotenoids to provide human healthcare such as the lower risk of inflammation, cardiovascular disease, neurodegenerative disease and diabetes, cancer prevention, improved ophthalmological diseases (Maoka, 2020). Although we provide carotenoids from nutrients of fruits and vegetables, a good alternative may be to use carotenoids from microalgae.

Dunaliella sp. is unicellular flagella green algae significantly found in halophilic environments world. *Dunaliella* is the richest resource of the carotenoid β-carotene and producing value compounds as high concentrations of glycerol and fatty acids (Elleuch et al., 2019). Halotolerant algae of *D. salina* has to accumulate large amounts of carotenoids (nearly 10-14% of the algal dry weight) and unicellular green microalgae of *D. salina* cells changes from red color under high light intensity, high salinity, limiting nutrient supplies (Zarandi-Miandoab et al., 2019). The morphologic taxonomy of *Dunaliella* has not openly defined under the different environmental conditions because *Dunaliella* do not own an apparent cell wall (Borowitza and Silva 2007). However, the taxonomy on morphological of the genus *Dunaliella* has significant differences in environmental conditions such as brackish lake and marine species. Anatolia, accommodating extensive saline areas, is the findable region as salt and brackish water lakes (Tuz Lake, Seyfe Lake and Sultansazlığı Lake) in Turkey. Sandy-clay-loam textured soils of the region were found to be light and strong alkaline, too salty, very calcareous and low organic matter content (Abaci-Bayar et al., 2020).

The aim of this study is to investigate the growth and biochemical composition of isolated and identified *D. salina* and D. *polymorpha* in both bubble column and stirred column photobioreactors (PBRs) under light intensity of 300 μmol photons/m^2s condition. Moreover, this study aims to evaluate the production of carotene especially β-carotene by indigenous *Dunaliella* species cultivated in two different PBRs.

Material and Methods

Isolation

Benthic samples were collected from the different stations of Aegean Sea/Burhaniye (located at 39°28'29.9"N 26°52'13.3"E) and Seyfe Lake/Kirsehir (located at 39°14'12.8"N 34°21'56.2"E) in Turkey. The samples were added to Daigo's IMK (FUJIFILM Wako Chemicals U.S.A. Corporation) liquid medium and incubated at 20 ±2 ºC in the incubator (IKa shaker) for three weeks. Green microalgae cells were diluted by transferring to fresh medium several times for two to four weeks. Single colonies obtained from singular cells by repeated sub-culturing on agar (1.5%) plates as described by Andersen (2005). After several re-cultivations, a single colony was transferred into sterile tube in liquid medium. The cultured isolate was maintained at 22ºC and a light intensity of 40 μmol photons/m^2 s.

Growth Condition

Dunaliella species were cultured in Daigo's IMK medium as inoculum in 300 mL erlenmeyer flasks containing 150 mL of a liquid medium with adding the sea salt, and pH of the medium was arranged to 7.5. Batch cultures were kept at 22°C on an orbital shaker (IKA KS 4000 ic) at 110 rpm under continuous light with an intensity of 40 μmol photons/m^2 s for 15 days.

Morphological Identification

Two different *Dunaliella* species were successfully isolated and continued in Daigo's IMK medium under laboratory conditions. *Dunaliella* species were discriminated by means of their morphological features cell shape, cell color, cell length, width, flagella length, and growth conditions. Bright-field microscopy photos from green microalgae were performed using a BX53 microscope (Olympus) equipped with a XC 30 camera.

Molecular Identification

DNA purification Isolation of chromosomal DNA of the species of *Dunaliella* was performed with the ZR Fungal/Bacterial DNA MiniPrep (ZymoResearch).

PCR amplification 18S rDNA amplification was performed in 50 µL reactions using primers SSUF-SSUR ([5'-TGGTTGATCCTGCCAGTAG-3']-[5'-TGATCCTTCCGCAGGTTCAC-3']; M1F-M2R ([5'-CGGGATCCGTAGTCATATGCTTGTCTC-3']- [5'-CG GAATTCCTTCTGCAGGTTCACC-3']) and M1F-M3R([5'-CGGGATCCGTAGTCATATGCTTGTCTC-3']-[5'-GGAATTCCGG AAACCTTGTTACGAC-3']) (Olmos *et al.* 2000). The amplification was fulfilled using 35 cycles in a BioRAD thermocycler, with an annealing temperature of 54°C for the reactions. One cycle consisted of 1 min at 95°C, 1 min at 54°C and 2 min at 72°C. DNA and PCR products were analyzed by 1 % agarose gel electrophoresis in TBE buffer (Tris-Boric acid-Ethylenediaminetetraacetic acid (EDTA)) and stained with SYBR safe and visualized under UV illumination. *Phylogenetic Analysis* The analysis of the PCR sequence was made by RefGen Biotechnology Company (http://www.refgen.com/) in Turkey.

Cultivation Conditions

Control of *Dunaliella salina* and *D. polymorpha* were grown in bubble column photobioreactor (PBR) (2 L), containing 1800 mL of Daigo's IMK medium including 21 g/L artificial sea salt at 20 ±2°C under using photoperiod at 18:6 h (Light:Dark) cycle for 18 days. The light intensity of 50 µmolphotons/m^2s provided by cool-white fluorescent and the aeration rate was at 2 L/min. *Dunaliella* species grown for control conditions under the light intensity of 50 µmolphotons/m^2 s were harvested during mid-log phase of growth. The microalgae cells were used as inoculants with a concentration of 20% (v/v) for photobioreactors experiments. The microalgae cell was counted using a Neubauer chamber, then specific growth rate and doubling time were measured from the logarithmic phase of growth curve as (specific growth rate) $\mu = (In\,X2 - INX1)/(t2 - t1)$

where Xn, cell numerousness on specific time point; tn, specific sample survey time (days). Doubling time (dt) was also calculated as $dt = 0.693/\mu$ (Sener et al., 2022).

The *Dunaliella* cultures were harvested at the beginning of stationary phase using centrifuged. Harvested *D. salina* and *D. polymorpha* were respectively grown to inoculate in the medium in two different (bubble column and stirred column photobioreactors) PBRs. Two different PBRs were used in the first 1 L only bubble column PBR and the second same volume of PBR having air bubbling aeration system with magnetic stirrer (IKA) at a stirring rate of 100 rpm (Figure 1). Two PBRs were prior autoclaved to use. Two different mixing systems was used with the ventilation rate of 1 L/min controlled using flow meter (RST electronic Ltd, LZM-6T Turkey). Illumination was provided under the continuous light on both side bottles by LED lamp (Cata 10W CT-5254, Velman Fixed Luminaires BG-T5001 9W linear) with a light intensity of 300 µmol photons/m^2s. For 18 days cultivated cultures were harvested and then biomasses were dried using freeze drying (Christ- Alpha 1-2 LDplus).

Figure 1. Microalgae cultivated in different Photobioreactors (PBRs) system

Analytic Methods

Two milliliters of the culture were centrifuged at 8000 rpm for 10 min. The pellets were extracted with 2 mL of 100% (v/v) methanol at 35 °C for 30 min in the ultrasonic bath (HYDRA ultrasonic). After the test tubes were centrifuged, the pigment contents (chlorophyll-a, chlorophyll-b and total carotenoids) were evaluated by spectrophotometre in methanol extracts at 480, 652, 665 and 750 nm. The amounts of the pigments were ultimately calculated by the following equations 1-3:

$$Chl-a\left(\frac{mg}{L}\right) = -8.10 \times (A652) + 16.57 \times (A665) - A750 \quad \text{Eq. 1}$$

$$Chl-b\left(\frac{mg}{L}\right) = 27.44 \times (A652) - 12.17 \times (A665) - A750 \quad \text{Eq.2}$$

$$Total\ Car\left(\frac{mg}{L}\right) = 4 \times A480 - A750 \quad \text{Eq.3}$$

The content of total carotenoids and chlorophylls were calculated according Wellburn method (Ajala and Alexander, 2020).

Total Protein, Carbohydrate, Lipid and B-Carotene Measurement

Total protein content was measured by the Lowry method (Lowry et al., 1951) using bovine serum albumin as a standard. Total carbohydrate content was measured based on the phenol-sulphuric acid reaction of carbohyrate (Dubois et al., 1956) by D-glucose as a standards ranging in concentration from 0 to 150 µg/mL. Lipid was extracted from lyophilized cell biomass using a modified Bligh and Dyer's method (Bligh and Dyer, 1959) as described by (Sahin et al., 2019). Total lipids were dosed gravimetrically.

β-carotene extraction: 0.01g dried weight of algal mass was extracted with 10 mL methanol (MeOH) sonicated in ultrasonic bath for 15 min. The extraction was cleared by centrifugation at 6000 rpm at 4°C for 10 min, then 2 mL of the supernatant was filtered through 0.45 µm syringe filter into HPLC vials. The β-carotene extraction solution was analyzed by an Agilent 1260 Infinity HPLC system with DAD detector an Agilent 5 µm, 250*4.6 mm C18 column (Figure 2). Carotenoids were extracted from lyophilized cells using MeOH as extraction solvent. In the mobile phase 100%, solvent A was as methanol and hexane (75:25, v/v). The flow rate was 1 ml/min, 0-14 min.

Each results were obtained with three biological replicates and all data were shown as mean ± standard deviation. It was considered as significant when $p < 0.05$.

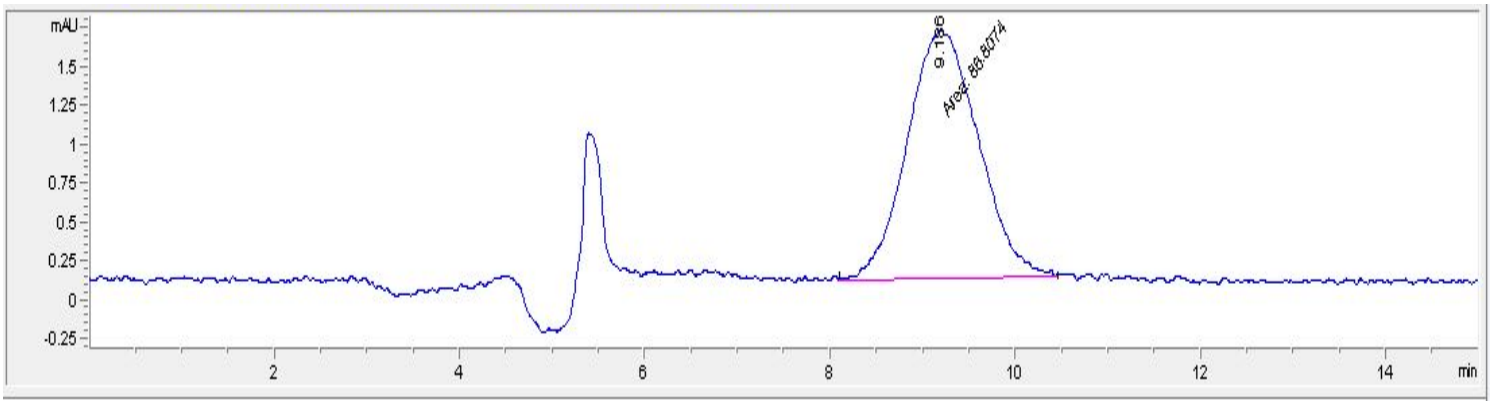

Figure 2. β-carotene detected using HPLC-DAD

Results and Discussion

Morphological and Molecular Identification

The morphologic taxonomy of *Dunaliella* has not been easily determined due to the cell morphological changeability and non-existing of the rigid polysaccharide cell wall (Emami et al., 2015 and Elleuch et al., 2019). The identification of species relying on only morphological characteristics is able to be troublesome. For this reason, reliable and accurate methods can be used to evaluate molecular variation.

Morphological identification; *Dunaliella salina* Teodoresco (1905); Cells oblong, pyriform, ellipsoidal to cylindrical with round anterior and posterior regions with two equal long flagella; chloroplast situated in the basal region; each cell 10.0-15.5 mm long and 6-9 mm wide; flagella 16-21 mm long. *Dunaliella polymorpha* Butcher (1959a); Cell generally green, radially symmetrical, mostly oval, ellipsoidal or cylindrical, 8-12 µm long, 5-8 µm wide. Flagella length about 1.5 times the cell length. Stigma small and medial.

Phylogenetic analysis of green algae evaluated using combined SSU rDNA gene sequence alignment and bright-field microscopical observations. Two different isolates of *Dunaliella* have been exposed to the comparison of 18 S rRNA regions for amplification. BLAST examined on NCBI-nucleotide database resulted in the highest similarity to *D. salina* (GenBank acc. no: KR340579, KR340580). Furthermore, the 18S rDNA gene sequence of *D. polymorpha* had been deposited in the GenBank with KR340581, KR340582 the accession numbers.

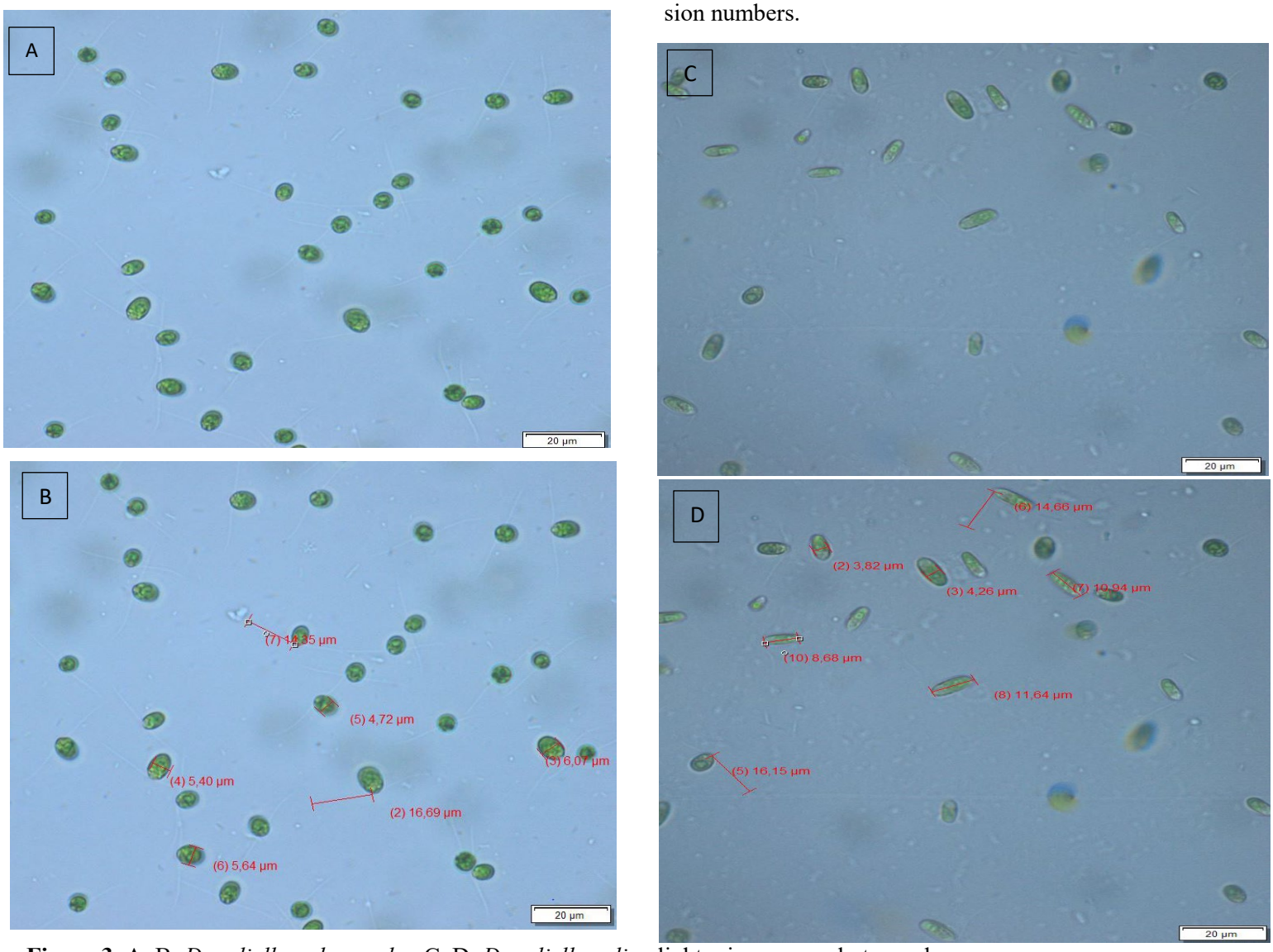

Figure 3. A, B: *Dunaliella polymorpha*; C, D: *Dunaliella salina* light microscope photographs

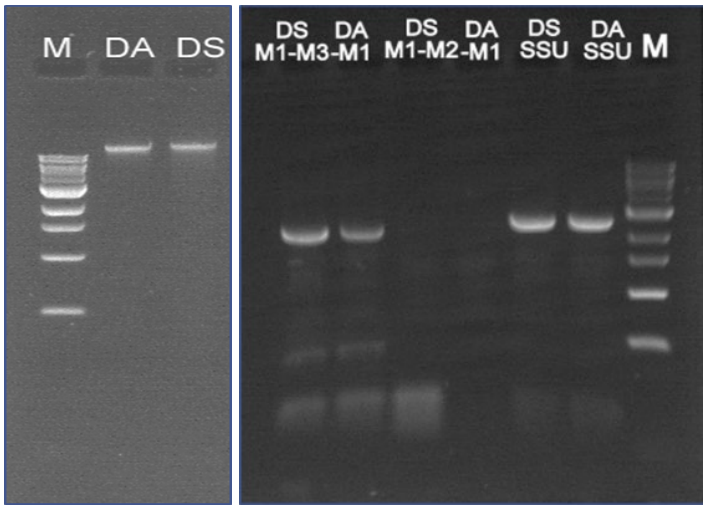

Figure 4. DNA and PCR products from *Dunaliella polymorpha* (DA) and *D. salina* (DS); M: size markers (1 kp DNA ladder) A: DNA isolation of DA and DS; B: 18S rDNA of DA and DS; (primers SSUF-SSUR, M1F-M2R, M1F-M3R)

Olmos et al., (2000) was reported that the primers pair (M1, M2) could let the amplification of the tallness of 18S rDNA in microalgae of *Dunaliella*. On the other hand, the report made M3 oligonucleotide analyzing from the 3′ termini of *D. salina* and then proven homological features with entire strains.

Phylogenetic analysis of green algae was evaluated using combined SSU rDNA gene sequence alignment and bright-field microscopically observations. In terms of the conspecific of the single-celled microalgae, stems from SSU rDNA sequence similarities could be not the whole time coherent with those from DNA base (mol % GC) values. Both of the species characteristic by their surprisingly range of DNA base composition values emerged SSU rDNA sequence the highest similarities among strains of *Prototheca zopfii* or *Chlorella sorokiniana* (Ueno et al., 2003 and Krienitz et al., 2011).

The isolated and identified indigenous strains of *Dunaliella salina* (EGEMACC 84) and *Dunaliella polymorpha* (EGEMACC 22) were joined to Ege University Microalgae Culture Collection (EGEMACC-http://www.egemacc.com/), Turkey. Cryopreservation of strains according to Day and Stacey (2007) will be applied in the future for alternative long-term storage by the culture collection.

Microalgae Growth Conditions

Dunaliella salina and *D. polymorpha* were respectively grown in the Daigo's IMK Medium for 18 days in bubble column PBR used to control. Kanamoto et al., 2021 reported that the marine microalgae *Pavlova* spp. cultivated the highest biomass production and highest fucoxanthin accumulation compared with f/2 and Walne's media, the use of grown in 50% seawater enriched with either 2× Daigo's IMK medium. The presence of seawater elements in Daigo's IMK medium was determined in the highest biomass (0.92 g dry cell weigh-dcw)/L) and the fucoxanthin concentration (2.62 mg/g dcw) after the cultivation. According to Colusse and colleagues (2020), the economical evaluation of media and biochemical analyses on biomass growth using different culture (F/2, Conway, and Johnson) media were investigated in *D. salina*. *D. salina* were grown in Daigo's IMK medium made with artificial seawater for dissolving 22 g/L sea salt (Sener et al., 2022). The cell cultivated under optimum conditions was illustrated in Figure 5. The specific growth rate of *D. polymorpha* in the growth phase was higher (μmax = 0.281 and dt=2.46 day^{-1}) than that of *D. salina* (μmax= 0.218 and dt=3.18 day^{-1}) at the light intensity of 50 μmolphotons/m^2s at 18:6 h (L:D). Khadim et al., 2018 used the same photoperiod of 16:8h L:D for *D. salina* inoculum preparation. Ricardo et al., 2018 showed that *D. salina* reached the highest densities at low salinities (100 and 500 mM NaCl) under a continuous light regimen. When exposed to 500 mM NaCl at 18:6 h L:D period, carotenes such as neoxanthin and violaxanthin obtained the furthest ample pigment. *Chlorella vulgaris* was cultivated at different light:dark periods. After, the maximum growth rate was 16:8 h L:D cycle (Kendirlioglu et al., 2015).

In the study of two-phase cultivation of carotenogenic microalgae *D. salina* and *D. polymorpha* in Turkey, their biochemical characteristics were studied and their production potential was determined. Given in Figure 5, the exponential phase cells removed from the medium and inoculated at irradiation 300 μmolphotons/m^2s in both bubble column and stirred column photobioreactors (PBRs). *D. salina* and *D. polymorpha* were cultivated under stress condition in bubble column and stirred column PBRs. The use of only a bubble could bring about weak mass transfer leading to the decreased contact area between liquid and gas (Kunjapur and Eldridge 2010). In each bioreactor configuration category, certain conditions for optimal cultivation are applied for the selected strain of microalgae. Also, green microalgae in different PBRs under 300 μmol photons/m^2s light intensity were evaluated to determine the protein, carbohydrate, lipid, carotenoid concentration and β-carotene content. *D. salina* in stirred PBR gave the highest lipid (334.79±0.02 mg/L), carbohydrate (40.94±0.04 mg/L), protein (137±0.013 mg/L), carotenoid (96.7±0.02 mg/L) and β-carotene content (21.18±0.03 μg/mL) by comparing with other cultivation systems given in Table 1. The maximum biomass concentration of *D. poly-*

morpha 0.906 mg/L was found at light intensity 50 µmol photons/m^2 while the lipid content (276.70±0.01 mg/L) and β-carotene content (17.51±0.02 µg/mL) was obtained under higher light intensity. Gharajeh et al., 2020 reported that the lipid, protein, carbohydrate, and pigment content of three isolates, *Dunaliella* sp. ABRIINW-B1, -G2/1 and -I1 were measured as produced respectively 42, 36 and 47% lipid content as well as the occurrence of high lipid and low carbohydrate (4–7%). The protein contains for *Dunaliella* species varies as about 40% *Dunaliella* (Hosseini Tafreshi & Shariati (2009)), 30–43% in *D. salina* (Muhaemin & Kaswadji (2010)) and 57% in *D. salina* (Berker, 2007). The lipid content for *Dunaliella* species varies as 23% in *D. primolecta*, 6-25% in *D. salina*, 17-67% in *Dunaliella* sp., 16-71% in *D. tertiolecta* (Ahmed et al.,2017). Ishika et al. 2018 reported that under high salinity (up to salt saturation (250 ppt)) cultivated and determined the average lipid and average carbohydrate content of *Dunaliella salina* 56.2% and 13.7%, respectively. Although total lipid, protein content was not very high, this study was nearly those of previous studies (Table 1).

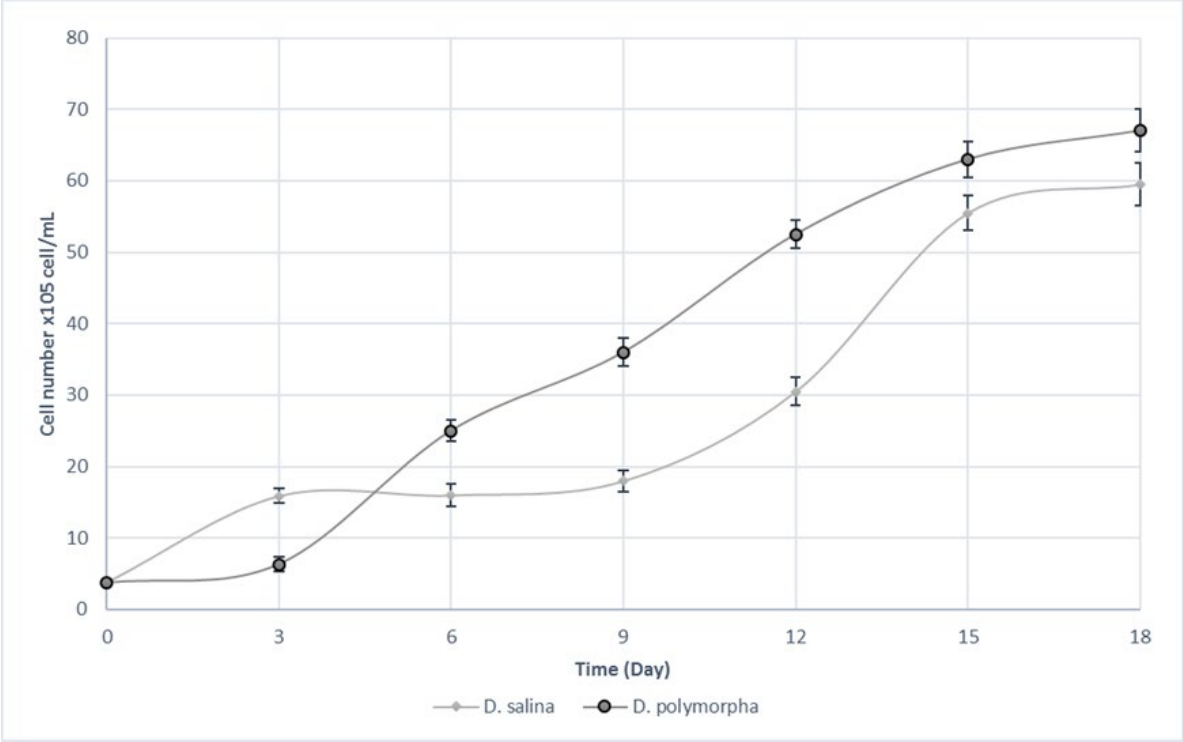

Figure 5. Control groups of the growth kinetic of *Dunaliella salina* and *D. polymorpha* cultivated under optimum condition by cell counting

Table 1. Effects of aeration and agitation in photobioreactors (PBR) on dry biomass, protein, carbohydrate, lipid, carotenoid and β–carotene content in *Dunaliella salina* and *D. polymorpha*

	Dry cell mass (g/L)	Protein content (mg/L)	Carbohydrate content (mg/L)	Total Lipid content (mg/L)	Carotenoid (mg/L)	β-carotene content (µg/mL)
Control *D. salina*	0.801	186 ±0.027	75.04 ±0.05	213.24 ±0.02	82.8 ±0.02	10.94 ±0.02
***D. salina* Bubble column PBR**	0.666	132 ±0.014	33.24 ±0.02	264.34 ±0.02	77.9 ±0.03	13.49 ±0.04
***D. salina* stirred PBR**	0.663	137 ±0.013	40.94 ±0.04	334.79 ±0.02	96.7 ±0.02	21.18 ±0.03
Control *D. polymorpha*	0.906	149 ±0.001	89.12 ±0.035	204.57 ±0.03	83 ±0.01	14.05 ±0.04
***D. polymorpha* Bubble column PBR**	0.799	110 ±0.016	39.65 ±0.02	276.70 ±0.01	94.6± 0.02	17.51 ±0.02
***D. polymorpha* stirred PBR**	0.697	113 ±0.01	30.13 ±0.016	268.58±0.04	88.6± 0.01	15.35 ±0.03

Mean ± standard deviation

This study showed that the variance of *Dunaliella* species and cultivation conditions significantly altered the metabolite concentrations in the cells. The effects of nitrogen, sulfur, and phosphorus limitations, different light intensities, and different CO_2 concentrations on growth and lipid accumulation were investigated for *D. salina*. According to Yuan et al., 2019 when high light intensity enhanced carbohydrate accumulation, low light intensity was beneficial to lipid accumulation under N-limited conditions. Ahmed et al., 2017 considered *D. salina* owned high lipid accumulation for the production of biofuel, industrial, and pharmaceutical purposes. *Dunaliella* cells have a lack of rigid cell walls made of cellulose; moreover, the disruption of cells is much speedier than that in green microalgae. However, *Dunaliella* cells can be easily damaged from stress-causing rupture of the air bubbles at the culture surface and mixing agitation of culture medium in PBRs. Ajala and Alexander 2020 reported that the productivity of algae affects the hydrodynamic effects of aeration and agitation in the PBR. In the current study, aeration and both stirring and aeration were performed to the *Dunaliella salina* and *D. polymorpha* cultures to state the biomass concentration and biochemical composition in different cultivation conditions.

Conclusion

This study has detected in two different photobioreactors how two identified indigenous *Dunaliella* strains play a role for the accumulation of carotenoids and biochemical compounds in two different photobioreactors. Carotenoid productivity in cells is known to enhance with high light intensity and different mixing systems in green microalgae. Nevertheless, among the newly isolated *D. salina* and *D. polymorpha*, much more productivity of β-carotene content was not determined under applied high light intensities and different mixing systems in this study. The biochemical composition performance of the newly isolated strains show a different cultivation strategy needed for all strains. In this study, strain selection from *Dunaliella* species emphasizes the investigation of their biochemical characteristic for the commercial production of carotenoids on human health products and animal feed.

Compliance with Ethical Standard

Conflict of interests: The authors declare that for this article they have no actual, potential or perceived conflict of interests.

Ethics committee approval: Ethics committee approval is not required for this study.

Funding disclosure: -

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Disclosure: -

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Cultivation of *Arthrospira platensis* in heterotrophic and mixotrophic conditions with different concentrations of whey

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ABSTRACT

Wastes left over from human food production is commonly used to produce feed for animals, which is an important issue for a rational utilization of food sources globally, and a topic that attracts researcher for the establishment of best food production management. Whey as a side product from cheese production has great potentials in terms of nutritional value for both human food and animal feed production. This study aimed to investigate the possible use of whey (1, 10 and 30%, v/v) as an external carbon source for mixotrophic and heterotrophic cultivation of the cyanobacterium *Arthrospira platensis*. The highest specific growth rate (μ = 0.2 day^{-1}), protein (3.76 ±0.14 mg/ g cell) and lipid (4.67 ±0.18 mg/g cell) contents were detected in heterotrophic culture while the highest chlorophyll-a (292.39 ±1.31 mg/ g cell) and total carbohydrate (1.42 ±0.07 mg/ g cell) contents were found in mixotrophic culture. In heterotrophic cultivation, it can be noted that the absorbed organic carbon source increased cell counts and triggered especially lipid production. In the mixotrophic cultivation, carbon absorbed from the culture medium or CO_2 captured with chlorophyll was utilized in the production of total carbohydrate. This study provides evidence that a cyanobacterium can adapt to heterotrophic conditions without light, creating an example for an economic and ecological production model for biochemical components.

Keywords: *Arthrospira platensis,* Biochemical composition, Heterotrophic cultivation, Mixotrophic cultivation, Whey

Introduction

Agricultural activities are remarkably influenced by the increasing environmental problems. The demand for high utility food for the increasing world population is a challenging issue for the food production industry. Whey is a side product of cheese production. Approximately 80-90 L of whey is formed from cheese produced from 100 L of milk (Božanić, Barukčić, and Lisak, 2014; Ghobrini et al., 2020). Nutritional composition of whey is dependent on cheese type. In average, it may contain lactose (46-52 %), protein (6-10 %), calcium (0.4-0.6 %), and phosphate (1-3 %). About 70 % of whey is used as raw material in different industries, while the remaining part is generally considered as waste (Božanić, Barukčić, and Lisak, 2014). This proportion of waste may have negative influences on the environment, due to its remarkably high biological oxygen demand (>35,000 ppm) and chemical oxygen demand (>60,000 ppm), which in fact can be further converted into a value product through microbial growth process (Bentahar et al., 2019; Smithers, 2008).

The cyanobacteria *Arthrospira* sp. has important nutritional properties with high protein, essential amino acid and vitamin contents (Rosas et al., 2018; Sivakumar et al., 2018). Production type for cyanobacteria is usually called phototrophic culture (Ozturk Urek & Kerimoglu, 2019). Heterotrophic cultivation is an alternative culture type with an organic carbon source but without light (Meireles et al., 2017). Another option for cyanobacteria production is mixotrophic cultivation that contains organic and inorganic carbon sources and also light (Joannesa et al., 2016; Velioglu Tosuner & Ozturk Urek, 2021). In our previous study the biomass, chlorophyll, and total lipid production of *A. platensis* was investigated with mixotrophic production in presence of sucrose (Velioğlu Tosuner & Öztürk Ürek, 2020).

Heterotrophic and mixotrophic cultures have some advantages over phototrophic cultivation in terms of better growth rate, higher biomass, protein, lipid production etc. Despite of many advantages of these culture types, there are some problems such as higher cost due to organic carbon source and contamination risk (Wang et al., 2017; Zhan et al., 2017). The cost for carbon source is approximately 50% of total microalgae cultivation medium (Chandra et al., 2014), hence it also affects the choice of carbon source type (Lutzu et al., 2016). Whey is seen as an important carbon source candidate due to its low cost, high amount and rich content.

In this study, *A. platensis* was grown in heterotrophic and mixotrophic cultivation conditions with different concentrations of whey. Effects of whey concentrations and trophic culture types on biomass increase chlorophyll, protein, total carbohydrate and total lipid were investigated. This study provides comparative beneficials from biotechnological application of mixotrophic and heterotrophic cultivations.

Material and Methods

Microalgae and Growth Media

The microalgae *Arthrospira platensis* (Gamont) Geitler 1952 was provided by Çukurova University, Faculty of Aquaculture, Türkiye. For the sustenance of cyanobacteria under photoautotrophic culture, it was grown in Zarrouk's Medium (pH 9.0) (Zarrouk, 1966). Batch cultivation was implemented in 750 mL working volume/1 L serum bottle with continuous illumination (2500 lux (33.75 µmol photon m^{-2} s^{-2}) by white fluorescent lamps), at 30°C and the cultures were mixed and aerated using filtered air continuously.

Mixotrophic and Heterotrophic Cultivation

Mixotrophic and heterotrophic cultures were applied in Zarrouk's Medium (pH 9.0) which contained different concentration of whey (1, 10 and 30%, v/v) as organic carbon source. Whey was provided by Balkan Süt Ürünleri, Izmir, Türkiye. Culture was inoculated to an initial optical density (OD= 600 nm) of 0.2. Since *A. platensis* is a filamentous microorganism, before reading, the OD the culture was transferred to spectrophotometer cuvette and the cuvette was turned upside down for three times (Velioğlu Tosuner & Öztürk Ürek, 2020).

Batch cultivation was operated in 100 mL working volume/ 250 mL Erlenmeyer at 100 rpm, 30°C for both cultivations. For mixotrophic culture, continuous illumination (1500 lux or 20.25 µmol photon m^{-2} s^{-2}) was provided by white fluorescent lamps.

Cyanobacteria was incubated in dark environment for heterotrophic culture. Specific growth rate (µ) was calculated according to the equation below.

$\mu = \ln \frac{X1-X0}{t1-t0}$ (X: amount of microorganism, t: time as day).

Determination of Total Lipid Content

Total lipid content of cyanobacteria was determined by using freshly prepared phospho-vanillin reagent and the absorbance was measured at 530 nm against a reference sample (Mishra et al., 2014).

Determination of Chlorophyll a and b Content

Chlorophyll a and b contents were measured as described by Lichtenthaler and Wellburn (1983). The algal suspension was collected by centrifuged (5000 rpm, 15 min, 4°C) and then

homogenized in absolute ethanol by 8000 rpm for 1 min and 9500 rpm for 1 min with 30 seconds intervals (Esen and Ozturk Urek, 2015). The obtained supernatant (12000 rpm, 10 min, 4°C) was measured at 664.2 and 648.6 nm. Chlorophyll contents were calculated according to the equations below.

$Chl\ a = 13.36 \times Abs_{664.2} - 5.19 \times Abs_{648.6}$

$Chl\ b = 27.43 \times Abs_{648.6} - 8.12 \times Abs_{664.2}$

Determination of Total Protein Content

Cells collected by centrifugation were homogenized with 50 mM, pH 7.0 phosphate buffer, followed by centrifugation (12000 rpm, 10 min, 4°C), and the supernatant was used for the analysis of protein content (Esen and Ozturk Urek 2015). Protein quantification was carried out by the Bradford method at 595 nm. Bovine serum albumin in concentrations ranging from 0-250 ppm is used as standard (Bradford, 1976). To prepare Bradford reagent, 100 mg of Coomassie Brillant Blue G-250 is dissolved in 50 mL of 95% ethanol. To the solution is added 100 mL of 85% phosphoric acid and complete with water to a total volume of 1000 mL. 100 µL of sample (100 µL of pure water as a reference) is mixed with 900 µL of reagent and allowed to stand at room temperature for 2 minutes and the absorbance is measured at 595 nm against the blank.

Determination of Total Carbohydrate Content

The supernatant of homogenized cell was used to determine total carbohydrate content by phenol-sulphuric acid method (Dubois et al., 1956). Homogenization procedure was applied as explained in the previous section. The absorbance was measured at 470 nm against a reference sample.

FTIR Analysis

The FTIR (Perkin Elmer Spectrum BX) spectra were recorded in the 4000- 400 cm^{-1} spectral region. Cells separated from growth medium were dried at 70°C overnight before analysis. Approximately 1 mg of dried cell sample was milled with approximately 100 mg of dried KBr and then pressed to form a pellet for measurement.

Statistical Analysis

All experiments were carried out in triplicates (n=3) and repeated 3 times. Each value is an average of 3 parallel replicates. Data were presented as mean ± standard deviation. The data were analyzed by analysis of variance (TUKEY) to identify the significantly different groups at ($p<0.05$) by one-way TUKEY test using SPSS software statistical program (SPSS for windows ver. 21.00, USA).

Results and Discussion

The cyanobacteria *A. platensis* was incubated under mixotrophic and heterotrophic cultivation conditions, in the presence of different concentrations of whey. The highest optical density value (2.737) was detected in mixotrophic medium containing 1% (v/v) whey and the highest specific growth rate ($\mu = 0.2\ day^{-1}$) was found in heterotrophic medium containing 30% (v/v) whey ($p<0.05$). The high organic carbon source concentration provides carbon skeleton and continuous energy supply for the maintenance of cyanobacteria (Chandra et al., 2014). Several earlier investigations reported that mixotrophic culture supports growth more than heterotrophic culture (Wang et al., 2017; Zhan et al., 2017). The heterotrophic medium with higher concentration of whey may have created the favorable condition for the growth of the cyanobacteria, resulting in higher specific growth rate. In the mixotrophic medium containing high whey concentration, high OD and specific growth rates have not been determined. In this medium, the required conditions for the simultaneous work of two metabolisms may not have been met. In mixotrophic cultivation, cells require a lower organic carbon source than heterotrophic cultivation because higher carbon source concentration can have an inhibition effect (Joannesa et al., 2016).

When chlorophyll change was examined during the incubation period, chlorophyll-a values increased in the last days of incubation in mixotrophic cultures containing 1% (v/v) and 10% (v/v) whey, but did not show a significant change in the medium containing 30% (v/v) whey (Figure 1). The highest chlorophyll-a (292.39 ±1.31 mg/ g cell) was determined on the 28^{th} day in the medium containing 10% (v/v) whey, and the chlorophyll-b (67.585 ±0.31 mg/ g cell) value was determined on the 21^{st} day of the incubation in the heterotrophic medium containing 1% (v/v) whey ($p<0.05$). The reason for the increase in the amount of chlorophyll in the mixotrophic medium containing 10% (v/v) whey in the last days of incubation could be attributed to its use of organic carbon source in the medium in the first days of incubation and then activated its phototrophic metabolism. This is also supported by the total carbohydrate content data (Figure 3). Chlorophyll content was determined at higher values in mixotrophic culture as expected. In mixotrophic cultures, CO_2, fixed by chlorophyll, in addition to the external carbon source, provides a carbon source that can be used in biochemical components production (Zhu et al., 2016). The low amount of chlorophyll-a in heterotrophic cultivation indicates that the cell is adapted to this type of cultivation and that only the heterotrophic metabolism is active. In the dark environment chlorophyll molecules oxidized and degradation occurs (Maroneze et al., 2019). The cell uses energy to biomass growth instead of

chlorophyll production. Different studies show that more chlorophyll-a degradation occurs while chlorophyll-b oxidation and degradation less happen (Maroneze et al., 2019).

The amount of protein did not change significantly during the incubation in mixotrophic media containing 10% and 30% whey whereas an increase was observed in the medium containing 1% (v/v) whey (Figure 2). This result is also supported by the OD data. In the mixotrophic cultures, the protein content has been detected to be very low. The highest protein value (1.51 ±0.68 mg/ g cell) was determined on the 21st day of incubation in a mixotrophic medium containing 1% (v/v) whey ($p<0.05$). The highest protein content was detected as 3.76 ±0.14 mg/ g cell in heterotrophic culture with 1% (v/v) whey (Figure 2) ($p<0.05$). The high protein content of whey might have triggered this result. The protein content in the heterotrophic cultivation (1% (v/v) whey) is 2.49 fold higher than the protein content in the mixotrophic cultivation (1% (v/v) whey) ($p<0.05$). Furthermore, it can be stated that the cells in this medium use the carbon they take from the growth medium in the production of protein causing the lipid level remain low.

When the total carbohydrate change in the growth medium was examined, an increasing trend was observed during the incubation period (Fig 3). The highest value (1.42 ± 0.07 mg/ g cell) was detected on 21st day of incubation in a mixotrophic medium containing 10% (v/v) whey ($p<0.05$). In the heterotrophic cultures, the maximum total carbohydrate content was detected as 0.72 ± 0.08 mg/ g cell on the 14th day in the presence of 1% (v/v) whey. The total carbohydrate content in the mixotrophic cultivation (10% (v/v) whey) is 1.92 fold higher than the total carbohydrate content in the heterotrophic cultivation (1% (v/v) whey) ($p<0.05$). While the carbohydrate contents remained low values, the protein level reached higher values in the heterotrophic medium containing whey. The presence of both organic and inorganic carbon sources in the mixotrophic culture caused both metabolisms to work. For this reason, the total carbohydrate amount was determined at higher levels than in the heterotrophic medium. However, 30% (v/v) whey creates a high carbon concentration for the mixotrophic medium. Based on the chlorophyll, protein and total carbohydrate values that the cells could not adapt to this medium (Figure 1, 2 and 3).

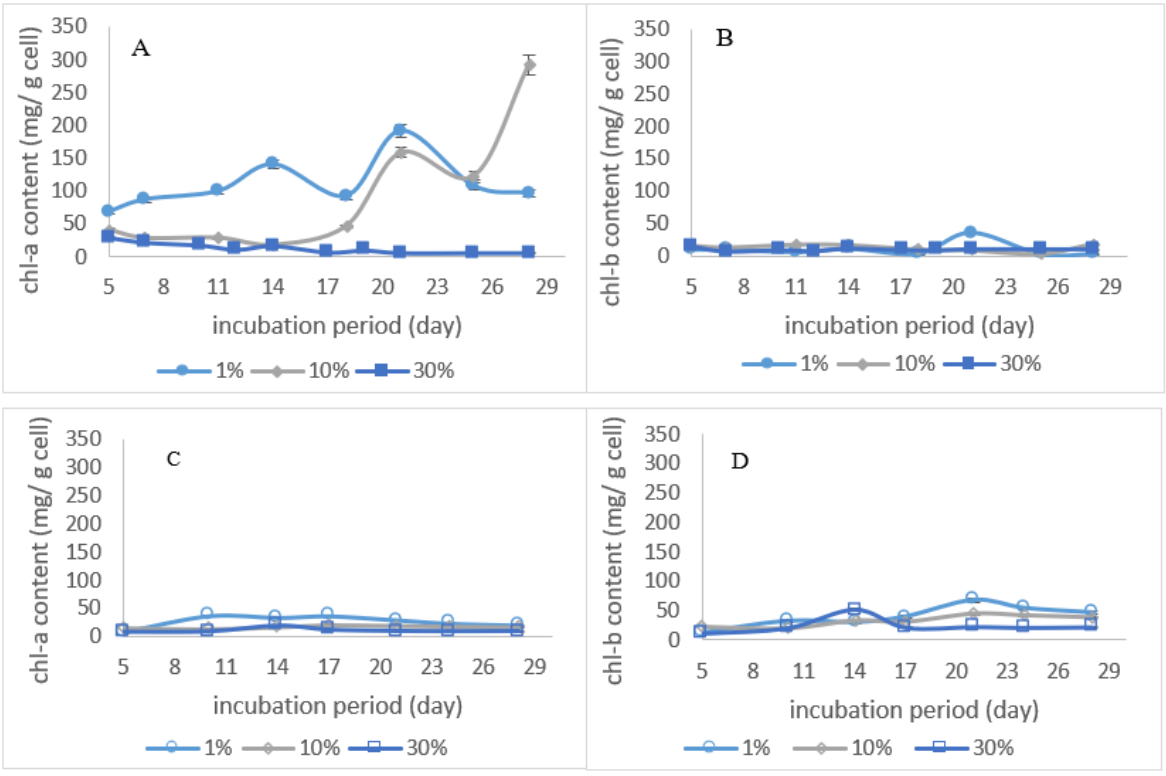

Figure 1. Chlorophyll-a and chlorophyll-b content changes depending on the incubation period of *A. platensis* grown in mixotrophic and heterotrophic cultures containing whey at varying concentrations (1, 10 and 30%, v/v) (A: Chlorophyll-a in mixotrophic culture, B: Chlorophyll-b in mixotrophic culture, C: Chlorophyll-a in heterotrophic culture, D: Chlorophyll-b in heterotrophic culture). The values are the mean ±SD for experiments of three separate experiments

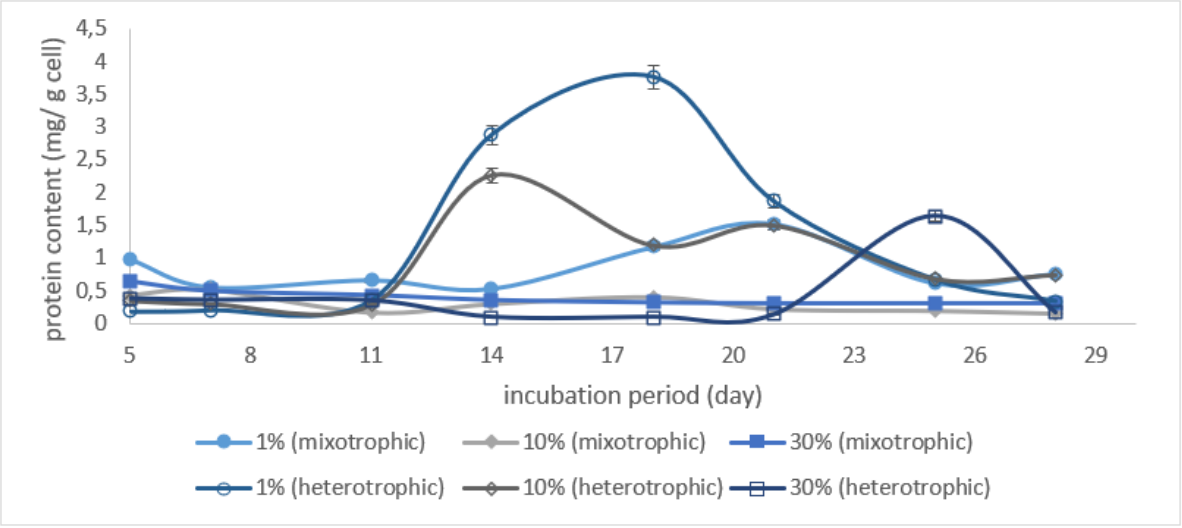

Figure 2. Protein content changes according to the incubation period of *A. platensis* grown in mixotrophic and heterotrophic cultures containing whey at varying concentrations (1, 10 and 30%, v/v). The values are the mean ±SD for experiments of three separate experiments

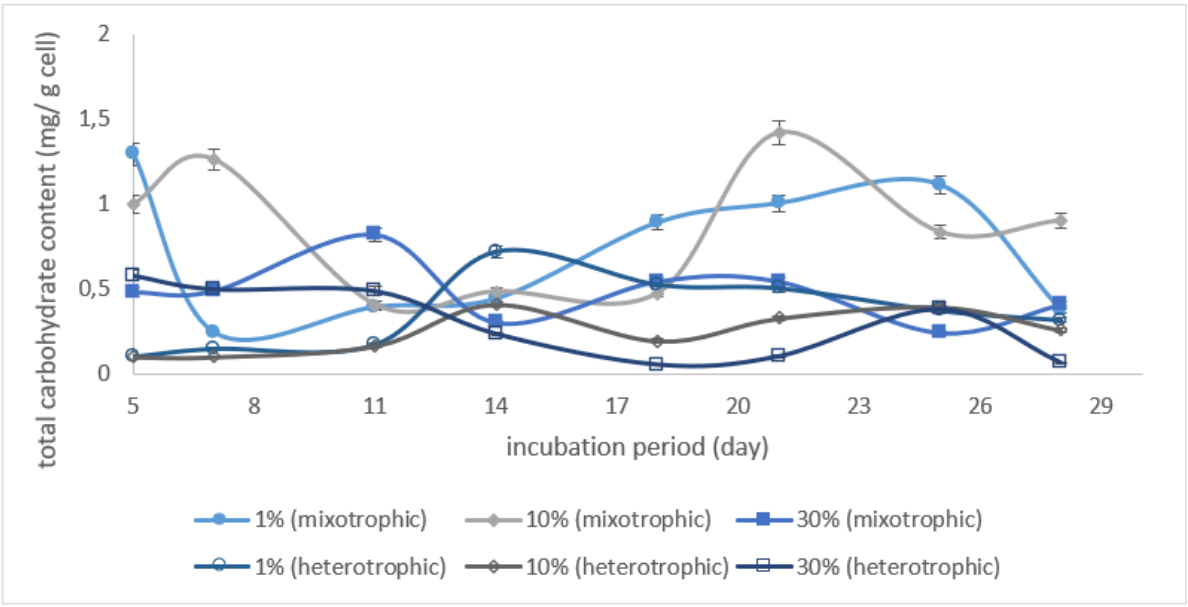

Figure 3. Total carbohydrate content changes depending on the incubation period of *A. platensis* grown in mixotrophic and heterotrophic cultures containing whey at varying concentrations (1, 10 and 30%, v/v). The values are the mean ±SD for experiments of three separate experiments

In terms of total lipid content, the highest values were detected in heterotrophic cultures (Figure 4). The highest lipid content was detected as 4.67 ±0.18 mg/g cell with 1% (v/v) whey in heterotrophic cultivation on 18th day (Figure 4) ($p<0.05$). In the higher whey concentrations, the maximum lipid production was observed in the later days of incubation. In mixotrophic cultures, the maximum lipid production (3.76 ±0.16 mg/ g cell) was detected with 10% (v/v) whey on the 14th day of incubation. The highest lipid values were obtained at the beginning of the stationary phase (Figure 4). The total lipid content in the heterotrophic cultivation (1% (v/v) whey) is 1.24 fold higher than the total lipid content in the mixotrophic cultivation (10% (v/v) whey) ($p<0.05$). The low chlorophyll production in the heterotrophic culture provides more acetyl CoA which are used in lipid synthesis pathway. Additionally, the amount of produced lipid is 1.27 fold higher than our previous study in which sucrose was used as organic carbon source (Velioğlu Tosuner & Öztürk Ürek, 2020).

In a study where *A. platensis* was grown mixotrophically in the presence of whey, increased protein contents and decrease carbohydrates were recorded with the increase of whey concentrations, however no significant changes were found for the lipid levels (Pereira et al., 2019). Although these results are similar to our results, higher whey concentration was tested in our study and substrate inhibition effect was observed (Figue 3). In addition, in our study it was shown that *A. platensis* can adapt to heterotrophic conditions and moreover, it can synthesize lipid and protein at a higher rate.

According to the FTIR data, -CH_2OH, -CH_3 peaks of carbohydrate structure were determined in cells grown in mixotrophic medium. The cells grown in heterotrophic cultures shows N-H and C-N stretching on protein structure and C=O and CH_2 peaks on lipid structure. These results are supported by the spectroscopic analysis results of total carbohydrate, lipid and protein.

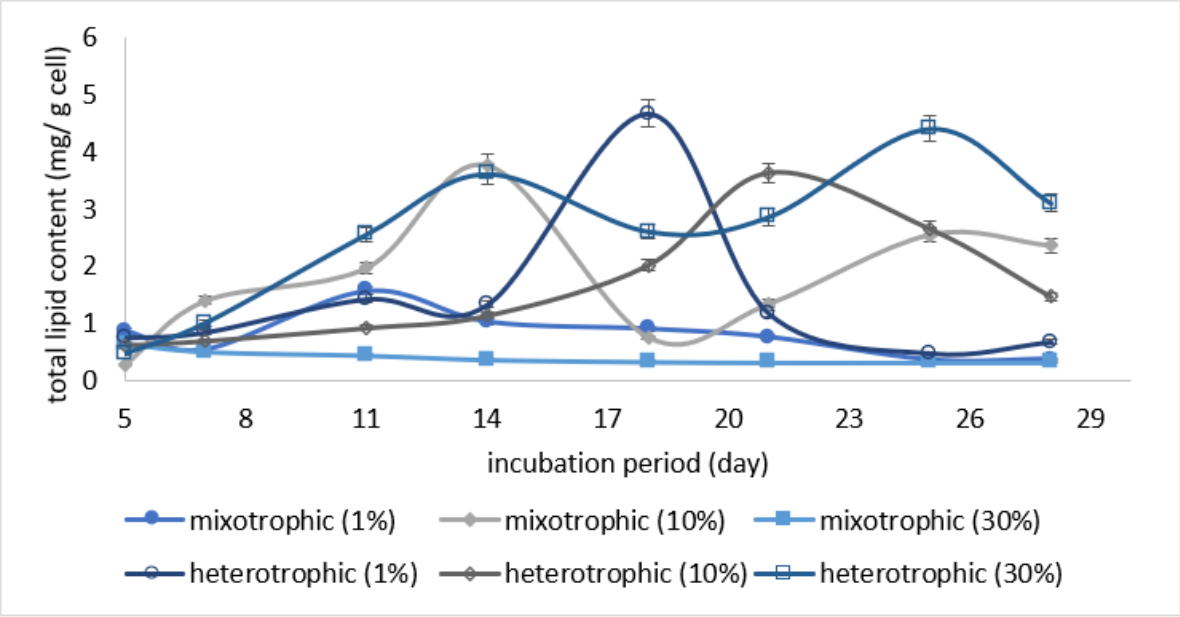

Figure 4. Total lipid content of *A. platensis* cell with different concentration of whey (1, 10 and 30 %, v/v) in mixotrophic or heterotrophic cultures. The values are the mean ±SD for experiments of three separate experiments

Conclusion

This study provided an alternative way for the disposal of a waste material by turning it into a value-added product. Not all microalgae cells could adapt in mixotrophic and especially heterotrophic cultures. This study shows that *A. platensis* is adapted to mixotrophic and heterotrophic conditions with different whey concentrations. Valuable materials such as protein, lipid and carbohydrate have been produced by using whey in the microbial growth medium as a carbon source. Whey is mentioned as a waste which is difficult to treat and comes out in high amounts.

It is very difficult for cyanobacteria to survive in the absence of light. However, heterotrophic cultivation type, which does not need light, is more economical and easier to implement for large-scale productions. This study has shown that, *A. platensis* adapted to the heterotrophic medium in the presence of whey and produced protein and lipid. It can be concluded that assimilation of organic carbon source by *A. platensis* in mixotrophic and heterotrophic cultures cell growth and biochemical content is not rigidly reliant on photosynthetic pathway. The organic carbon was transferred into cell and redirected towards carbohydrate synthesis in mixotrophic cultivation while it was used in protein and lipid synthesis pathway in heterotrophic cultivation. In the heterotrophic conditions the produced lipid and protein levels were higher than mixotrophic culture 1.24 and 2.49 fold, respectively. Produced lipid and protein are value-added products that can be evaluated in different fields, including human and animal healthy nutrition. Thus, the potential of their both economic and an ecological production system were revealed.

Compliance with Ethical Standard

Conflict of interests: The authors declare that for this article they have no actual, potential or perceived conflict of interests.

Ethics committee approval: Ethics committee approval is not required for this study.

Funding disclosure: -

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Disclosure: -

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Crustacean and Protozoan parasites of some Cyprinid fish living in the Murat River (Bingöl-Türkiye), with new host records

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ABSTRACT

Some Cyprinid fish species: *Cyprinion macrostomum* (Heckel, 1843), *Capoeta umbla* (Heckel, 1843), *Chondrostoma regium* (Heckel, 1843), and *Squalius cephalus* (Linnaeus, 1758) living naturally in the Murat River, were investigated for Protozoan and Crustacean parasite fauna and their distribution. Fish samples were collected from different stations between July 2017 - June 2019, examined in the Bingöl University Zoology Research Laboratory, and the data were explained with various variables. The normality test revealed that the data were not normally distributed ($p< 0.05$), as with large samples, so non-parametric tests explained the data. A total of 365 fish were examined, and 100 fish (27.4%) were infected with at least one Protozoan or Crustacean parasite. Four different parasite species were recorded on the examined fish, namely *Ichthyophthirius multifiliis* and *Trichodina* sp. belonging to the phylum Ciliophora (Protozoan), *Ergasilus sieboldi*, and *Lamproglena pulchella* belonging to the phylum Arthropoda (Crustacean). As a result of this study, for the first time, Protozoan and Crustacean parasites of different cyprinid fish were examined according to the host species, seasonal distribution, host size, and new host records were reported for three parasites.

Keywords: Crustacean, Protozoan, Fish parasites, Cyprinid, Murat River

Introduction

Fishing has a vital place to provide the animal product needs of a country. It is also imperative to know the parasites that cause severe economic losses in the fish population. Investigation of fish diseases, parasites, and treatments are essential for today's fish industry and fish farming. The importance of fish parasites is directly related to the economic value of the fish species they affect. Diseases caused by parasites reduce fish immunity against dangerous infections and negatively affect growth, development, egg production, and meat quality. They can also cause infectious diseases and mass death of fish (Grabda, 1991).

It is known that approximately 10 thousand species of parasites live in fish. They are 27% Crustacea, 18% Protozoa, 17% Digenea, 15% Monogenea, 10% Cestoda, 7% Nematoda, 4% Acanthocephala and 1% Huridinea (Cengizler, 2000). Parasitic creatures in nature indicate biological events such as feeding and migration in their host and give some ideas about their environment. By identifying the hosts in the life cycle of parasites, information about the properties of different biotopes can be obtained. It is necessary to know the ecological characteristics of the parasite species, their geographical distribution, densities, and their relations with their host to determine the relationships between parasite faunas.

In this study, it was aimed to examine the Crustacean and Protozoan parasite fauna of fish species *C. macrostomum* (Heckel, 1843), *C. regium* (Heckel, 1843), *C. umbla* (Heckel, 1843), and *S. cephalus* (Linnaeus, 1758) living naturally in the Murat River. The study aims to detect Crustacean and Protozoan parasites in the mentioned fish species and contribute to the studies on fish parasites in their natural and breeding environments throughout the country. In addition, it is aimed to contribute to the precautions to be taken against the parasites to be detected in these fish that have commercial importance for Bingöl Province.

Material and Methods

Study Area and Sampling

The study was conducted between July 2017 and June 2019 in Murat River and Göynük Stream (Figure 1). The fish samples were caught by the various nets, and then the material was kept in the fish cage for the living stock in the catchment area. The fish caught were brought from the field to the laboratory with a transport tank and dissected within 24 hours by keeping them alive throughout the study with oxygen supplementation. The fish's total, fork, and standard-length measurements were recorded in millimeters (mm) and their weights in grams (g).

A total of 365 fish from the *C. macrostomum* (Heckel, 1843) (N=91, 130,88±28,61 mm), *C. umbla* (Heckel, 1843) (N=109, 133,67±26,25 mm), *C. regium* (Heckel, 1843) (N=80, 136,83±28,95 mm) and *S. cephalus* (Linnaeus, 1758) (N=85, 140,47±33,56 mm) fish species were examined, and 100 fish (27.4%) were infected with at least one Protozoan or Crustacean parasite.

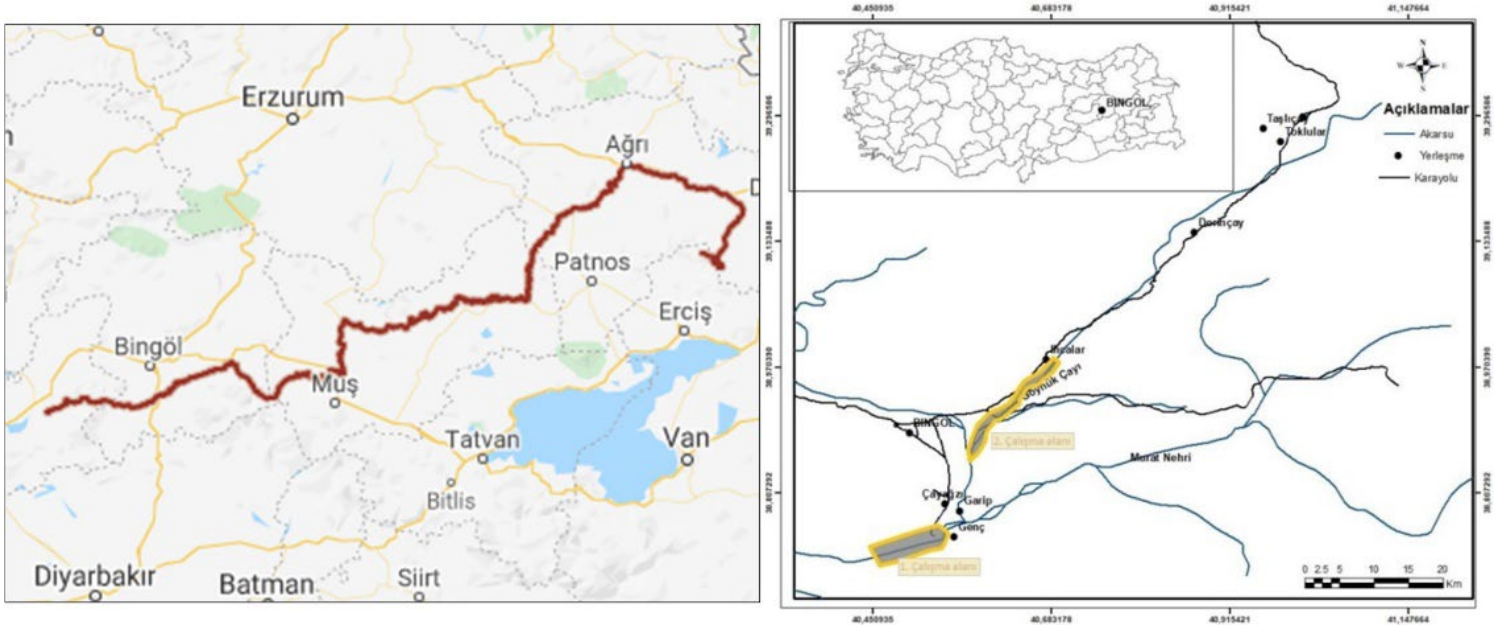

Figure 1. Murat River and the study area

Searching for Parasites

The skin, fins, nasal and oral cavities, gill lamellae were examined macroscopically. The gills were taken with forceps or scissors, placed in a petri dish containing physiological water, and examined under a stereomicroscope. The specimens were mounted unstained, photographed under the light microscope, and the number of parasites was recorded separately.

Statistical Analysis

The SPSS (version 25.0.0) program was used to calculate the prevalence, mean intensity, and mean rank of the parasites. The prevalence is the percentage of infested fish out of the total number of fish examined, the number of parasites per fish in the total number of infected fish is the mean intensity, and the mean rank is the average of the ranks for all observations within each sample. Kruskal-Wallis analysis was applied to the data to determine the significant differences between more than two groups (fish size or seasons, e.g.), and multiple comparison tests (Post Hoc analysis- Tamhane's T2) were applied determine which groups were different from each other.

The size of the fish; To facilitate the examination and to have sufficient information about the distribution, the number of groups was determined as four according to the classification rules, to best represent the groups for each fish species.

Results and Discussion

A total of four different parasite species were recorded on the examined fish, namely *I. multifiliis* and *Trichodina* sp. belonging to the phylum Ciliophora (Protozoan), *E. sieboldi*, and *L. pulchella* belonging to the phylum Arthropoda (Crustacean) (Table 1).

***I. multifiliis* Fouquet, 1876**

Host fish: *C. macrostomum*, *C. regium, C. umbla, S. cephalus*

It is a large ciliated Protozoan with a prominent comma-shaped nucleus. The size of these ciliates usually ranges from 0.02 mm to about 1 mm, and these different sizes are used to distinguish between young and old. On the outer surface of the organism, which appears in color brownish under a light microscope, ciliates activate the protozoa and gently push them forward (Noga, 2010) (Figure 2).

The ciliate *I. multifiliis*, widely "Ich," is probably the most common parasite of freshwater teleosts with an extensive geographic range from the tropics to the temperate regions, north in Europe, to the Arctic Circle. The main factors in the current worldwide distribution of *I. multifiliis*, which infects freshwater teleosts, including cold water and tropical species, are its low host specificity, natural life cycle, and wide temperature tolerance (Matthews, 2005).

Table 1. Descriptive statistics of the parasites

Host Fish (N)	Parasite	Infected (n)	Prevalence (%)	Mean±SD	Min.-Max.	Total
***C. macrostomum* (N=91)**	*E. sieboldi*	9	9.9	1.0±0.0	1	9
	I. multifiliis	7	7.7	4.6±5.3	1-15	32
	Total	15	16.5	2.7±3.9	1-15	41
***C. regium* (N=80)**	*I. multifiliis*	14	17.5	14.2±3.6	1-42	199
	L. pulchella	6	7.5	2.5±1.3	1-9	15
	Trichodina sp.	2	2.5	1.0±0.0	1	2
	Total	17	21.3	12.7±12.8	1-42	216
***C. umbla* (N=109)**	*L. pulchella*	41	37.6	1.3±0.1	1-3	54
	I. multifiliis	4	3.7	21.8±11.0	3-49	87
	Total	44	40.4	3.2±8.3	1-49	141
***S. cephalus* (N=85)**	*L. pulchella*	16	18.8	1.1±0.1	1-2	18
	I. multifiliis	7	8.2	17.0±7.6	1-42	119
	E. sieboldi	3	3.5	1.3±0.3	1-2	4
	Trichodina sp.	1	1.2	1.0±0.0	1	1
	Total	24	28.2	5.9±12.6	1-42	142
Total (N=365)		100	27.4	5.4±10.4	1-49	540

N= Number, Mean±SD: Parasite/Infected fish±Standart Deviation

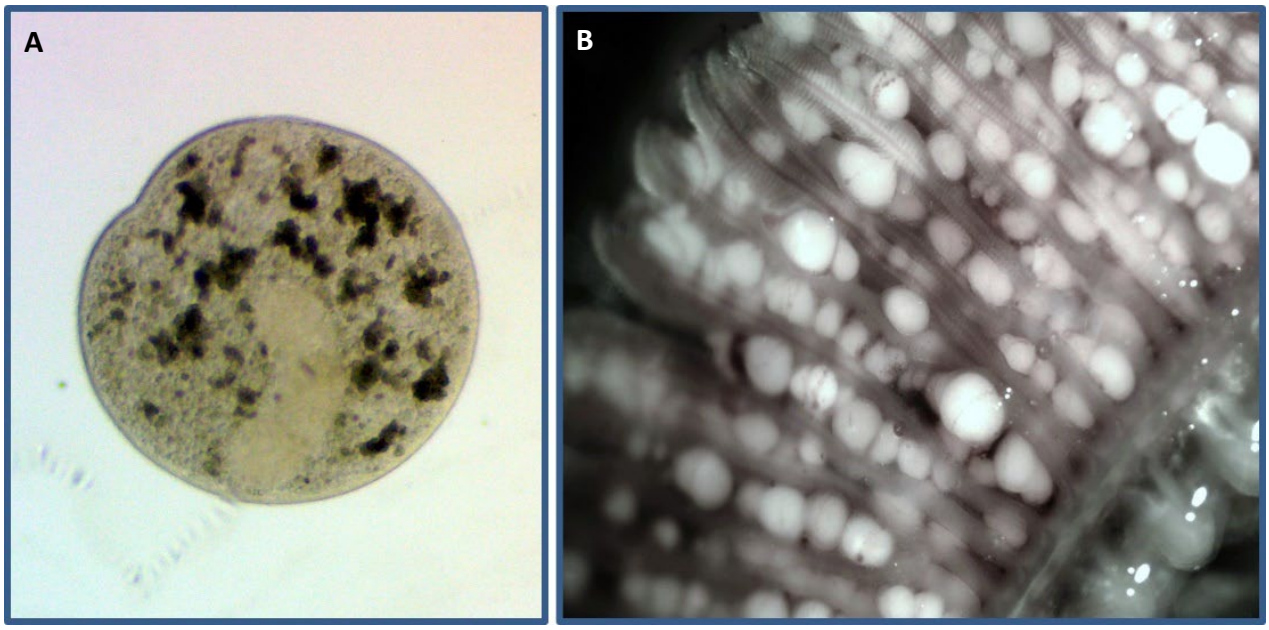

Figure 2. *I. multifiliis* A: Tomont stage B: Trophont stage

This parasite, which can live on the body, fins, and fish gills, causes White Spot Disease (Ich), one of the common and permanent diseases. Each white point is an encapsulated parasite. The parasite can be transmitted easily and quickly from one host to another or from an aquarium to another. Due to the natural life cycle of the parasite, it is not easy to control it when it enters a fish culture facility. When not controlled, a mortality rate of almost 100% on the host is possible. With careful treatment, the disease can be controlled. Due to the inflammation on the skin and gills of the host, mucus occurs in the areas where it is seen. The white speck that penetrates the tissue of the fish causes significant damage. As a result of the injuries, the fish become unable to control their movements and lose their swimming ability (Noga, 2010).

Host Distribution

The Kruskal-Wallis test indicates that there is no statistically significant difference in the *I. multifiliis* infestation levels of four different fish species [X^2(3, N=365) =4.392, p>0.05]. Descriptive statistics demonstrate that *I. multifiliis* is widespread on *C. regium* while concentrated in a small number of fish on *C. umbla* (Table 2).

Seasonal Distribution

The Kruskal-Wallis test states that there is no statistically significant difference in terms of seasonal infestation levels of *I. multifiliis* among the host fish [X^2 (3, N=365) =0.766, p> 0.05]. Prevalence reached high levels in autumn and mean intensity in spring (Table 3).

Distribution by Length

The Kruskal-Wallis test indicates that there is no statistically significant difference in *I. multifiliis* infestation levels between different sizes [X^2(3, N=365) =4.766, p>0.05] (Table 4). Although the test results do not evaluate the difference as acceptable (p>0.05), it is seen that there are variations between the host length groups. Mean intensity and mean ranks show that the larger the host size the higher the infestation rate. (Figure 3).

Table 2. Descriptive statistics of *I. multifiliis* and Kruskal-Wallis test results (Host type)

Host type	Infected (n)	Prev. (%)	Mean±SD	Mean rank	Test Statistics [a.b] *I. multifiliis*	
***C. macrostomum* (N=91)**	7	7.7	4.6±2.0	10.6	Kruskal-Wallis H	4.392
***C. regium* (N=80)**	14	17.5	14.2±3.6	18.6	df	3
***C. umbla* (N=109)**	4	3.7	21.8±11.0	20.8	Asymp. Sig.	0.222
***S. cephalus* (N=85)**	7	8.2	17.0±7.6	15.6	a. Kruskal Wallis Test	
Total (N=365)	32	8.8	13.7±15.3		b. Grouping Var.: Host type	

N= Number, Prev.: Prevalence, Mean±SD: Parasite/Infected fish±Standart Deviation

Table 3. Descriptive statistics of *I. multifiliis* and Kruskal-Wallis test results (Seasonal)

Seasons	Infected (n)	Prev. (%)	Mean±SD	Mean rank	Test Statistics [a.b] *I. multifiliis*	
Spring (N=108)	6	5.6	16.8±8.0	16.5	Kruskal-Wallis H	0.766
Summer (N=84)	8	9.5	12.8±6.2	15.6	df	3
Autumn (N=82)	10	12.2	14.1±4.6	18.5	Asymptotic Sig.(2-sided t.)	0.858
Winter (N=91)	8	8.8	11.6±4.7	14.9	a. Kruskal Wallis Test	
Total (N=365)	32	8.8	13.7±15.3		b. Grouping Var.: Seasons	

N= Number, Prev.: Prevalence, Mean±SD: Parasite/Infected fish±Standart Deviation

Table 4. Descriptive statistics of *I. multifiliis* and Kruskal-Wallis test results (By length)

Host length	Infected (n)	Prev. (%)	Mean±SD	Mean rank	Test Statistics [a.b] *I. multifiliis*	
1. Group (N=74)	4	5.4	9.5±6.9	13.5	Kruskal-Wallis H	0.846
2. Group (N=103)	12	11.7	13.5±5.0	15.9	df	3
3. Group (N=92)	14	15.2	14.6±4.0	18.0	Asymp. Sig.	0.838
4. Group (N=64)	2	3.1	16.0±15.0	15.5	a. Kruskal Wallis Test	
Total (N=365)	32	8.8	13.7±15.3		b. Grouping Var.: Host length	

N= Number, Prev.: Prevalence, Mean±SD: Parasite/Infected fish±Standart Deviation

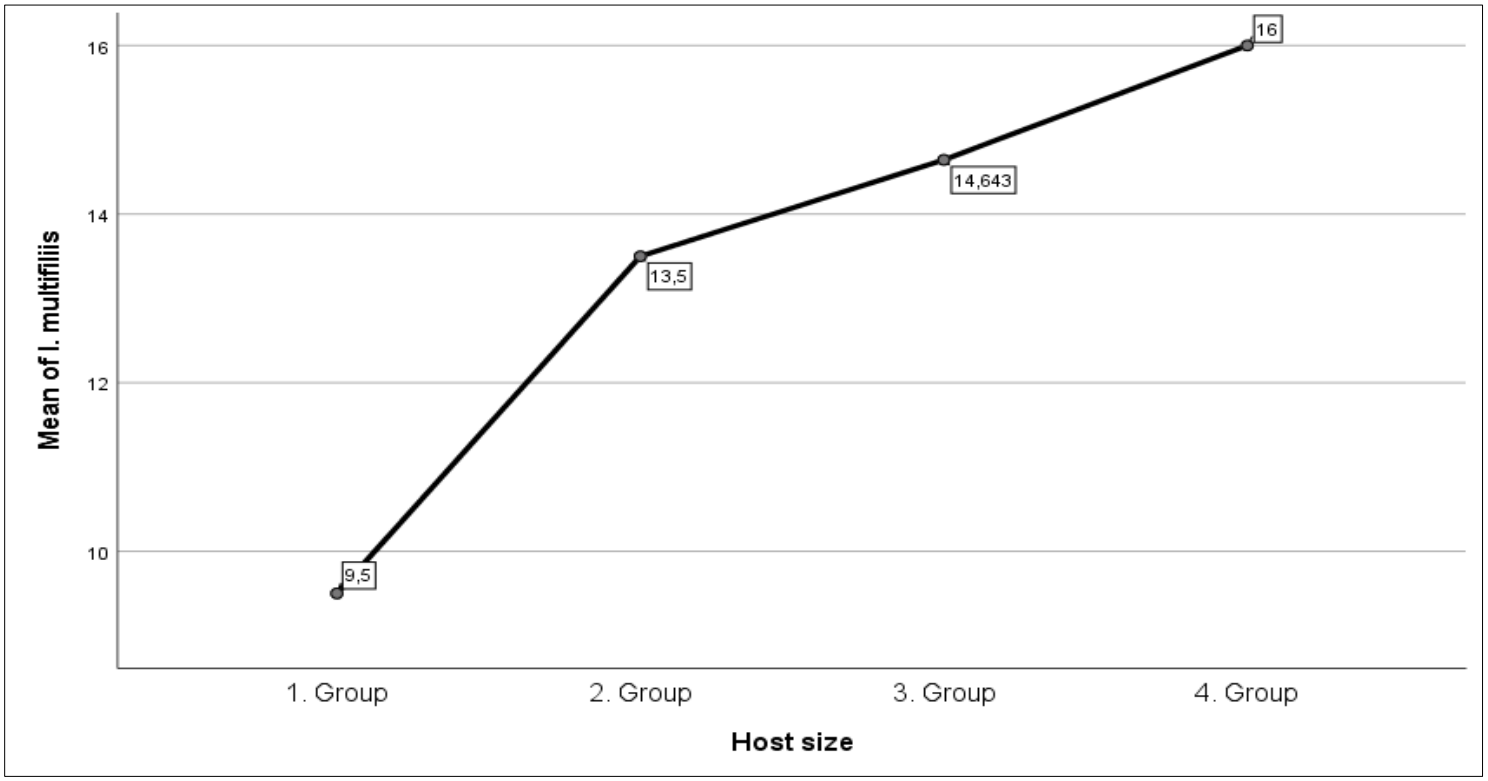

Figure 3. Mean intensity graph according to the host size of *I. multifiliis*

***Trichodina* sp.**

Host fish: *C. regium*, *S. cephalus*

Trichodinids are circular ciliates that can be disc-shaped or hemispherical. Cytostome (cell mouth) called the oral surface is on the surface of facing the host. There is a spiral of cilia leading to the cytostome and surrounding cells several rings of cilia, which are responsible for creating the absorbent for adhesion, the driving force for movement (Figure 4). In the taxonomy of trichodinids, the exact number, shape, and arrangement of cytoskeletal denticles are important for determining taxonomic relationships (Lom and Dyková, 1992).

Trichodinids, which can cause severe damage, especially in aquarium fish, are among the most common parasites of aquatic ecosystems and may prefer freshwater and marine fish as hosts (Çelik and Korun, 2018). Most trichodynides live ecto-commensal life as they feed on bacteria and only use their host fish as a substrate for attachment. However, certain species are primary pathogens because they can occur in sterile areas (e.g., urinary system) or provoke specific responses in host fish (e.g., *Tripartiella* on gills) (Lom and Dyková, 1992).

Statistics of Infestation with Trichodina sp.

It has been recorded on only three fish specimens from two different hosts. Since the data are not sufficient and only descriptive statistics are given in this section, statistical tests or comments are not made. (Table 1).

L. pulchella von Nordmann, 1832

Host fish: *C. regium*, *C. umbla*, *S. cephalus*

An adult female *L. pulchella* has an elongated body consisting of three separate parts: cephalothorax, thorax, and abdomen (Figure 5).

On the cephalothorax there are prominent antenna structures, eye spots and grabbing claws. There are intestinal structures in the thorax which have three segments, and a developed tail following the thorax. During the breeding times, a pair of eggs hatch from the third segment of the thorax and extend posteriorly on both sides of the tail (Figure 5-D). There are five pairs of legs in their bodies, which are quite distinct during the larval period, and it has seen that these legs do not develop in adults.

Host Distribution

According to the distribution of *L pulchella*, which is the dominant species among the parasites detected, there is no statistically significant difference in the infestation levels of three fish species among the hosts (Table 5) [$X^2(2, N=274) = 1.655$, $p>0.05$]. Since the parasite density is close to each other between hosts, it would be more accurate to interpret the prevalence from descriptive statistics than test results. Accordingly, it can be said that *L. pulchella* is more common on *C. umbla* than the other hosts.

Seasonal Distribution

The Kruskal-Wallis test indicates that there is no statistically significant difference in the infestation levels of *L.* pulchella according to the seasonal variations [X^2 (3, N = 274) = 2.583, $p>0.05$)]. *L. pulchella* reached the highest infestation rate in the Spring, which is the breeding season, and saw the lowest level in the Summer (Table 6).

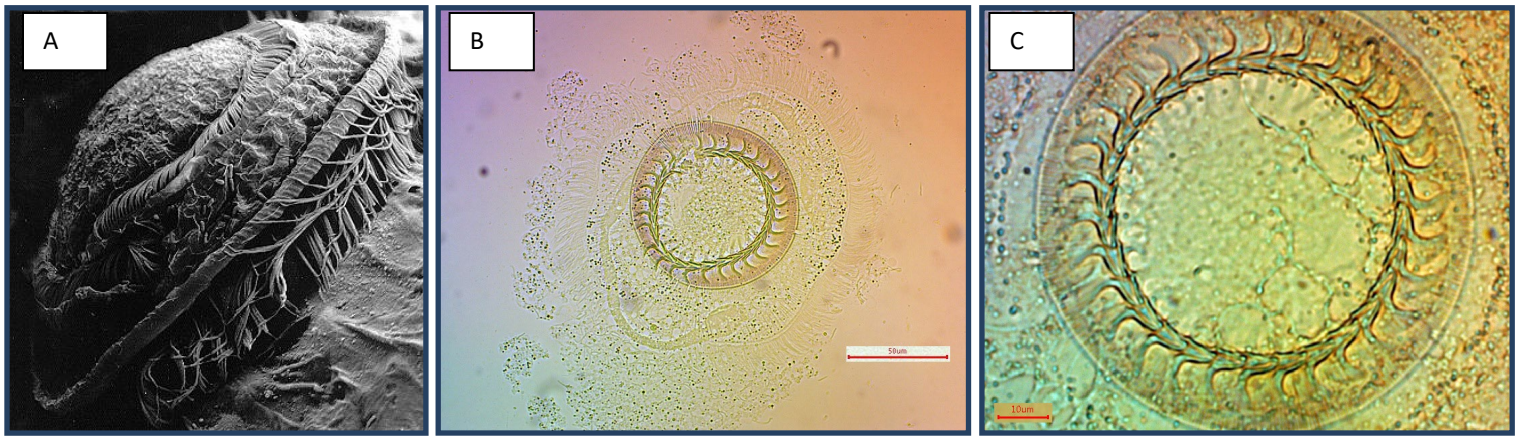

Figure 4. *Trichodina* sp. A: Scanning electron micrograph of a trichodinid ciliate attached to the gills of an Australian mullet (*Mugil cephalus*) (Dove, 2007), B-C: Image under a light microscope (Scale bars: 50 and 10 µm)

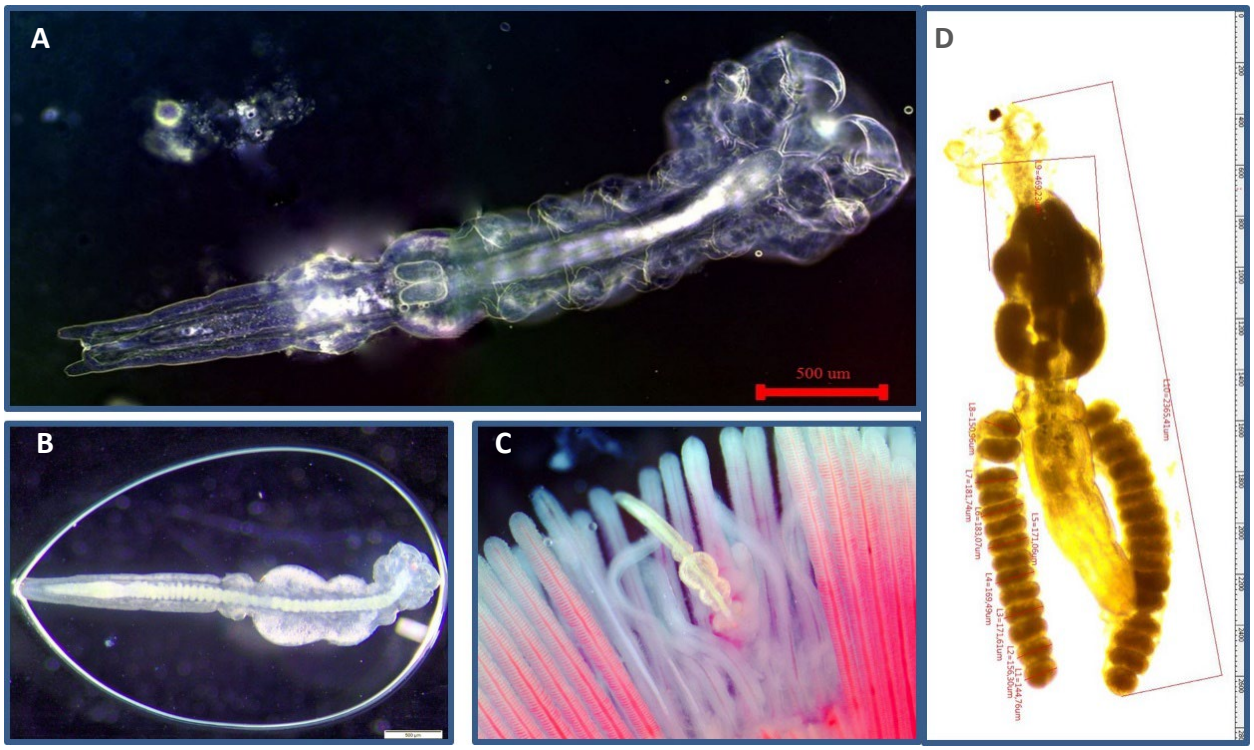

Figure 5. *L. pulchella* A: Juvenile form, B-C-D, Adult form

Table 5. Descriptive statistics of *L. pulchella* and Kruskal-Wallis test results (Host type)

Host type	Infected (n)	Prev. (%)	Mean±SD	Mean rank	Test Statistics a.b *L. pulchella*	
***C. regium* (N=80)**	6	7.5	1.3±1.2	34.7	Kruskal-Wallis H	1.655
***C. umbla* (N=109)**	41	37.6	1.3±0.9	33.1	df	2
***S. cephalus* (N=85)**	16	18.8	1.1±0.9	28.3	Asymp. Sig.	0.437
					a. Kruskal Wallis Test	
Total (N=274)	63	23.0	1.3±0.5		b. Grouping Var.: Host type	

N= Number, Prev.: Prevalence, Mean±SD: Parasite/Infected fish±Standart Deviation

Table 6. Descriptive statistics of *L. pulchella* and Kruskal-Wallis test results (Seasonal)

Seasons	Infected (n)	Prev. (%)	Mean±SD	Mean rank	Test Statistics [a.b] *L. pulchella*	
Spring (N=88)	21	23.9	1.2±0.9	30.3	Kruskal-Wallis H	2.583
Summer (N=61)	7	11.5	1.4±0.2	37.6	df	3
Autumn (N=60)	18	30	1.2±0.9	29.6	Asymptotic Sig.	0.46
Winter (N=65)	17	26.2	1.4±0.2	34.4	a. Kruskal Wallis Test	
Total (N=274)	63	23	1.3±0.5		b. Grouping Var. Seasons	

N= Number, Prev.: Prevalence, Mean±SD: Parasite/Infected fish±Standart Deviation

Distribution by Length

The Kruskal-Wallis test indicates that there is no statistically significant difference in *L. pulchella* infestation levels among the host fish of different sizes [X^2 (3, N = 274) = 1.364, p> 0.05]. Mean and mean ranking show that as the size of the host increases, the number of infestations increases (Table 7).

***E. sieboldi* von Nordmann, 1832**

Host fish: *C. macrostomum*, *S. cephalus*

Blue colour pigment is its characteristic. The blue pigment on its posterior can be seen scattered even with the bared eyes. Blue pigment appears more clearly in young and female individuals (Figure 6). As the parasite grows old, the colour of the pigment becomes lighter and age determination can be made according to this colour darkness. One pair of swimming legs is located on each of the thoracic segments. Adult males are like females, but they are much shorter and thinner.

E. sieboldi, a Crustacean ectoparasite, is known to be a common gill parasite on Cyprinid fish. Only female individuals of *E. sieboldi*, are parasitic and sometimes show a cosmopolitan distribution as a parasite in much freshwater fish and sometimes in free form.

Statistics of Infestation with E. sieboldi

It has been identified as 13 on 12 fish in two different hosts. Since the data are not sufficient, only descriptive statistics are given in this section, statistical tests or any comments are not made (Table 1)

Table 7. Descriptive statistics of *L. pulchella* and Kruskal-Wallis test results (By length)

Host length	Infected (n)	Prev. (%)	Mean ±SD	Mean rank	Test Statistics [a,b] *L. pulchella*	
1. Group (N=57)	11	19.3	1.3±0.2	30.7	Kruskal-Wallis H	1.364
2. Group (N=92)	22	23.9	1.3±0.1	33.2	df	3
3. Group (N=81)	16	19.8	1.3±0.1	34.0	Asymp. Sig.	0.714
4. Group (N=44)	14	31.8	1.4±0.2	28.9	a. Kruskal Wallis Test	
Total (N=274)	63	23.0	1.1±0.9		b. Grouping Var.: *Host length*	

N= Number, Prev.: Prevalence, Mean±SD: Parasite/Infected fish±Standart Deviation

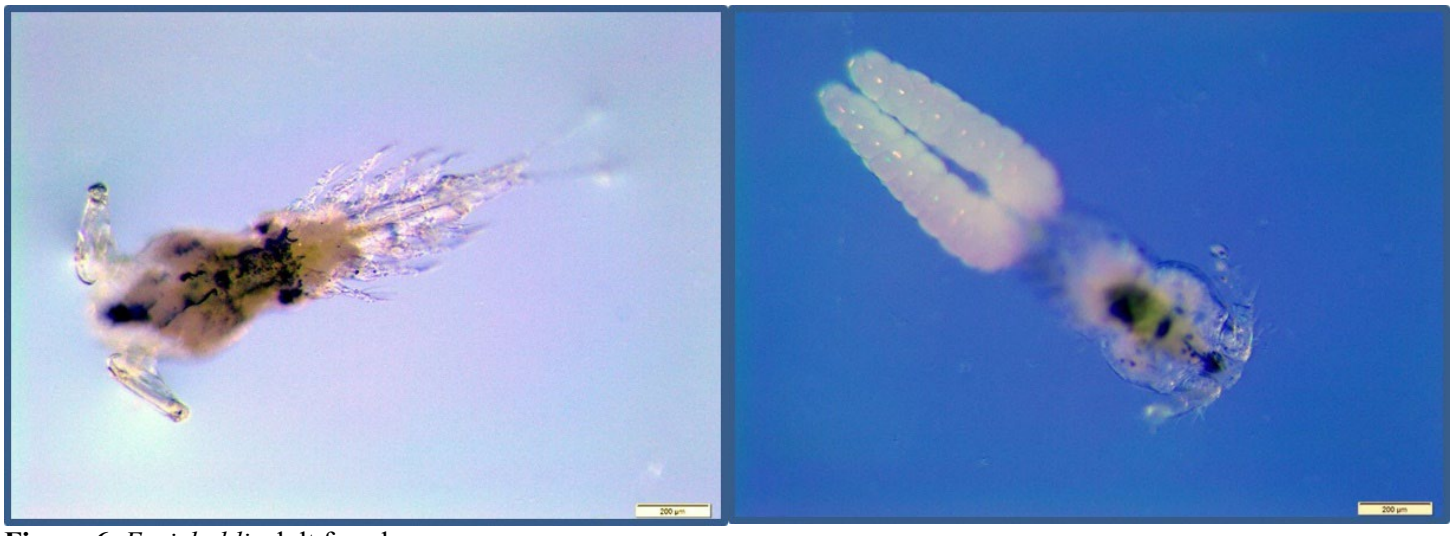

Figure 6. *E. sieboldi* adult female

Conclusion

This study was conducted between July 2017 and June 2019 in Murat River and Göynük Stream. A total of 365 fish from the *C. macrostomum*, *C. umbla*, *C. regium*, and *S. cephalus* fish species were examined, and 100 fish (27.4%) were infected with at least one Protozoan or Crustacean parasite. It was observed that there was a statistically significant difference in total parasitization levels of the two fish species (*C. regium* and *C. macrostomum* Tamhane's T2 $p<0.05$). The rate of infection with any parasite reached the highest level on *C. umbla* (40.4%), while it was followed by *S. cephalus* (28.2%), *C. regium* (21.3%), and *C. macrostomum* (16.5%), respectively. General infestation levels for all fish species have taken values close to each other in all seasons, and there was no statistically significant difference between the infestation amounts ($p>0.05$). It was determined that there was no statistically significant difference in total parasite infestation levels among host fish of different sizes ($p>0.05$); however, as the host size increased, parasite infrapopulations also increased. In this section, the detected parasites are discussed separately for each parasite species, first Protozoan and then Crustacean, within the framework of the effects and distributions reported in the previous studies.

***I. multifiliis* Fouquet, 1876**

Host: *C. macrostomum*, *C. regium, C. umbla, S. cephalus*

I. multifiliis was reported from skin and gills of *A. marmid* from Greater Zab river and Darbandikhan lake, *A. grypus* (reported as *B. grypus*), and *C. trutta* from Darbandikhan lake, *C. umbla* (reported as *V. umbla*) from Lesser Zab river, *Carasobarbus luteus* (reported as *Barbus luteus*) and *C. macrostomum* from Erbil's fish market and Greater Zab river, *C. luteus* from Darbandikhan lake, skin, fins, buccal cavity and gills of *C. regium* from Greater Zab river, *C. carpio* from Lesser Zab river, gills of *H. molitrix* from Darbandikhan lake, skin and gills of *L. barbulus* (reported as *Barbus barbulus*) from Lesser Zab and Greater Zab rivers, skin and gills of *L. esocinus* from Darbandikhan lake, *M. mastacembelus* from Darbandikhan lake, skin, fins and gills of *S. triostegus* from Greater Zab river, skin and gills of *S. lepidus* from Darbandikhan lake. A total of 35 fish host species are known for *I. multifiliis* in Iraq (Mhaisen and Abdullah, 2017).

Balta et al. (2008) found *Trichodina* sp. and *I. multifiliis* on *Oncorhynchus mykiss, Salvelinus fontinalis, Salmo trutta* fario. Kayış et al. (2018) reported at low densities *Trichodina* sp. on *Alburnoides fasciatus*, *Barbus artvinica*, *Capoeta banarescui*, *Capoeta ekmekciae*, *Capoeta sieboldii*, *Squalis orientalis*, and *I. multifiliis* on *C. banarescui*, *A. fasciatus* and *S. oriantalis.* Bingöl (2018) reported *Trichodina* sp. and *I. multifiliis* on *Oncorhynchus mykiss* and *Salmo coruhensis*. As a result, *I. multifiliis* reported for the first time on *S. cephalus.*

Especially the presence of *I. multifiliis*, which is common in aquaculture and is relatively more challenging to treat than other Protozoan parasites, carries a risk in the future for aquaculture activities in the region.

***Trichodina* sp.**

Host: *C. regium*, *S. cephalus*

There was no report on trichodinid species until 1998 in Türkiye. In 1998, *T. acuta*, *T. mutablis*, and *T. nigra* were reported from natural and cultured fish for the first time. Various parasite species infected a total of 204 out of 850 fish species in Türkiye, and only 31 fish species were found to be infested with 33 trichodinid parasites. Considering the total number of fish species in the Turkish fauna and the number of trichodinids identified, more extensive studies on unexamined fish species are required to obtain a complete picture in all Turkish waters (Özer and Öztürk, 2015).

Although the Protozoan mentioned above parasites reported in the study were reported from both aquaculture systems and aquarium fish (Kayış et al. 2013), no severe cases were encountered in the literature when considered in terms of mortality. In addition, since previous studies were checked, it can be said that *Trichodina* sp is a new record for these cyprinid fish (*C. regium* and *S. cephalus*).

***E. sieboldi* von Nordmann, 1832**

Host: *C. macrostomum, S. cephalus*

The first studies on *E. sieboldi* in Türkiye were the studies of Sarıeyyüpoğlu and Sağlam (1991). *E. sieboldi* has been reported on *C. carpio* from Dalyan Lagoon (Aydoğdu et al. 2001), *Platichthyes flesus* from Sarıkum Lagoon (Sinop) (Öztürk and Özer, 2008), *Tinca tinca* from Sapanca Lake (Akbeniz and Soylu, 2008), *Neogobius fluviatis*, *Proterorhinus marmoratus*, *Pomatoschistus marmoratus* from Bafra Fish Lakes (Çam, 2012), *Acathobrama marmid* from Göynük Stream (Koyun et al. 2019), *Barbus lacerta* (Koyun et al. 2015) and *Alburnus mossulensis* from Murat River (Tunç and Koyun, 2018).

In this study, 13 fish were detected in a total of 12 fish from two different hosts (*C. macrostomum-S. cephalus*). When studies in Türkiye and abroad were investigated, it was seen that *E. sieboldi* was not previously reported for neither *C. macrostomum* nor *S. cephalus*. In this study, *C. macrostomum* and *S. cephalus* were reported as new host records for *E. sieboldi*.

***L. pulchella* von Nordmann, 1832**

Host: *C. regium, C. umbla, S. cephalus*

The genus *Lamproglena*, which lives on freshwater fish families such as Cyprinidae, Cichlidae, Clariidae, and Channidae, contains more than 40 species. *L. pulchella* has previously been reported from South America, Europe, Asia, and Africa. The first record of *L. pulchella* was reported in *Chondrostoma nasus* from Romania by Angelescu (1974) (Stavrescu-Bedivan et al. 2008).

In Iraq, *L. pulchella* was firstly reported from gills of both *C. regium* and *C. trutta* (reported as *V. trutta*) from Tigris River at Mosul city. So far, *L. pulchella* has 20 fish host species in Iraq. *L. pulchella* was reported from gills of *C. regium* living in Lesser Zab River, *C. damascina* (reported as *B. belayewi*) *C. umbla* (reported as *V. umbla*), *C. luteus* (reported as *B. luteus*), *C. regium, G. rufa, L. vorax* (reported as *A. vorax*), *L. barbulus* (reported as *B. barbulus*), *L. esocinus* (reported as *B. esocinus*), *S. cephalus* (reported as *L. cephalus*), *S. lepidus* (reported as *L. lepidus*) and *S. spurius* (reported as *L. spurius*) living in Greater Zab River, *C. damascina* (reported as *B. belayewi*), *C. umbla* (reported as *V. umbla*), *C. macrostomum, L. barbulus* (reported as *B. barbulus*), *L. kersin* (reported as *B. kersin*) and *S. lepidus* (reported as *L. lepidus*) living in Bahdinan River, *C. regium* living in Bahdinan Lake, *C. luteus* (reported as *B. luteus*) living in Darbandikhan Lake, *L. esocinus* (reported as *B. esocinus)* and *L. xanthopterus* (reported as *B. xanthopterus*) living in Dokan Lake (Mhaisen and Abdullah, 2017).

In Türkiye, *L. pulchella* was reported from gills of *S. erythrophthalmus* from Sapanca Lake (Soylu, 2012) (Kuş and Soylu, 2013), *C.* trutta and *C. regium* from Keban Dam Lake (Sağlam, 1998), *C. trutta* from Balıklıgöl (Şanlıurfa) (Öktener et al. 2008), and *C. trutta* (Koyun et al. 2019), *B. lacerta* (Koyun et al. 2015), *A. mossulensis* (Tunç and Koyun, 2018) from Göynük Stream and Murat River (Bingöl).

In this study, *L. pulchella* was detected on *C. regium* (7.5%), *C. umbla* (37.6%), and *S. cephalus* (18.8%). As seen in previous studies and this study, this parasite appears to be common among Cyprinid fish species.

Compliance with Ethical Standard

Conflict of interests: The authors declare that for this article they have no actual, potential or perceived conflict of interests.

Ethics committee approval: The use of fish was approved by Bingöl University Animal Experimentation Ethics Committee (Bingöl, Türkiye) 08.11.2021-E.33221.

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Biological indicator of warming events: Presence of the Cortez angelfish *Pomacanthus zonipectus* at temperate conditions of Bahía de San Quintín, Baja California, México

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ABSTRACT

An individual of the Cortez angelfish, *Pomacanthus zonipectus* (Gill, 1862) was obtained at the temperate Bahía de San Quintín, Baja California, México (Lat. 30.428343, Long. 115.987014), on May 15, 2021. All counts, morphological description and colors of the specimen caught agree with the previous descriptions for the species *P. zonipectus*. The Cortez angelfish is a tropical fish species, and its presence at the temperate environment may possibly be associated with warming events, The Blob-El Niño 2013-2016, or the most recent moderate El Niño 2018-2019. This occurrence constitutes the first record for the Cortez angelfish in the San Quintín area, northern Pacific off Baja California, and after thousands of samplings with different fishing gears beginning in 1993. The Cortez angelfish was recently recorded in August 25, 2016 as new occurrence in Laguna Ojo de Liebre, Baja California Sur, México, also in the same warming event of The Blob-El Niño 2013-2016; from Ojo de Liebre to Bahía de San Quintín represents a northward movement of 355 kilometers and a new record in its distribution.

Keywords: Cortez angelfish, Northward distribution, Temperate lagoon, The Blob, El Niño

Introduction

The Bahía de San Quintín is located in the temperate zone of the Pacific of Baja California, México. However, since 1997 fish species with tropical or subtropical affinity have been recorded in the interior of the lagoon during warming events such as El Niño or The Blob (Rosales-Casián and Ruiz-Campos, 1999; Rosales-Casián, 2004b; Rosales-Casián, 2017).

The fish known as angelfishes belong to the Pomacanthidae family, which contains seven genera with 90 species (Fricke, Eschmeyer, and Van der Laan, 2021), all inhabitants of warm tropical or subtropical waters. According to Allen and Robertson (1998), in the eastern Pacific only four species of angelfishes are known *Holacanthus clarionensis* Gilbert, 1890, *Holacanthus limbaugui*, 1963, *Holacanthus passer* Valenciennes, 1846, and *Pomacanthus zonipectus* (Gill, 1862).

The Cortez angelfish, *P. zonipectus* occurs at depths of 6-12 m to 50 m, and is distributed in the eastern Pacific Ocean, from the northern Gulf of California (México) to Peru in the south, with affinity to Provinces of Cortez, Mexican and Panamic (Horn, Allen, and Lea, 2006; Pyle et al., 2010). As an indicator of its northward movement, in a previous study conducted in 2015-2016, its northernmost new record was in Laguna Ojo de Liebre (Lat. 27°51'28.14" N, Long. 114°14'2.40" W), at the middle of the Pacific of Baja California, México, and was registered during the warming events of The Blob (2013-2015) and the El Niño 2014-2016 (Civico-Collados and Rosales-Casián, 2021; Dorantes-Gilardi and Rivas-Camargo, 2019). The Blob was a mass of warm water observed first in Gulf of Alaska during October 2013, shifted east and extended south to coasts of California (USA and Baja California (México), this event was unprecedented with temperature anomalies reaching +3ºC and ending in 2015 (Peterson et al., 2015). The "El Niño" began at the equator in December 2014, extended north through the northeastern Pacific, peaked in November 2015, and ending in June 2016, with positive temperature anomalies from 0.5 ºC to 2.6 ºC (NOAA's El Niño, available at http://www.elnino.noaa.gov/, last accessed 20 September, 2021; Rupic *et al*., 2018).

The aim of the present study is to document a northward movement of the Cortez angelfish to the temperate environment of the Pacific of Baja California, which represents the first occurrence of this species of the family Pomacanthidae in Bahía de San Quintín, México.

Material and Methods

The Bahía de San Quintín is located 320 km south of the California (USA)–Baja California (México) border (Lat. 30° 28' 59.99" N, Long. 115° 58' 38.60" W), this coastal lagoon is made up of two arms, Bahía Falsa and Bahía San Quintín (Figure 1), the first is shallow with oyster cultures develop there, and in the second arm is the site of El Molino Viejo (The Old Mill), place of departures and arrivals of commercial artisanal fishing and sportfishing boats. It is a coastal lagoon with the presence of a permanent upwelling through the rocky point that forms the mouth; the upwelling fertilizes with nutrients the interior of the lagoon during high tide and in produces a refuge and feeding area for temperate fishes (Gracia-Escobar *et al*., 2015: Rosales-Casián, 2004a).

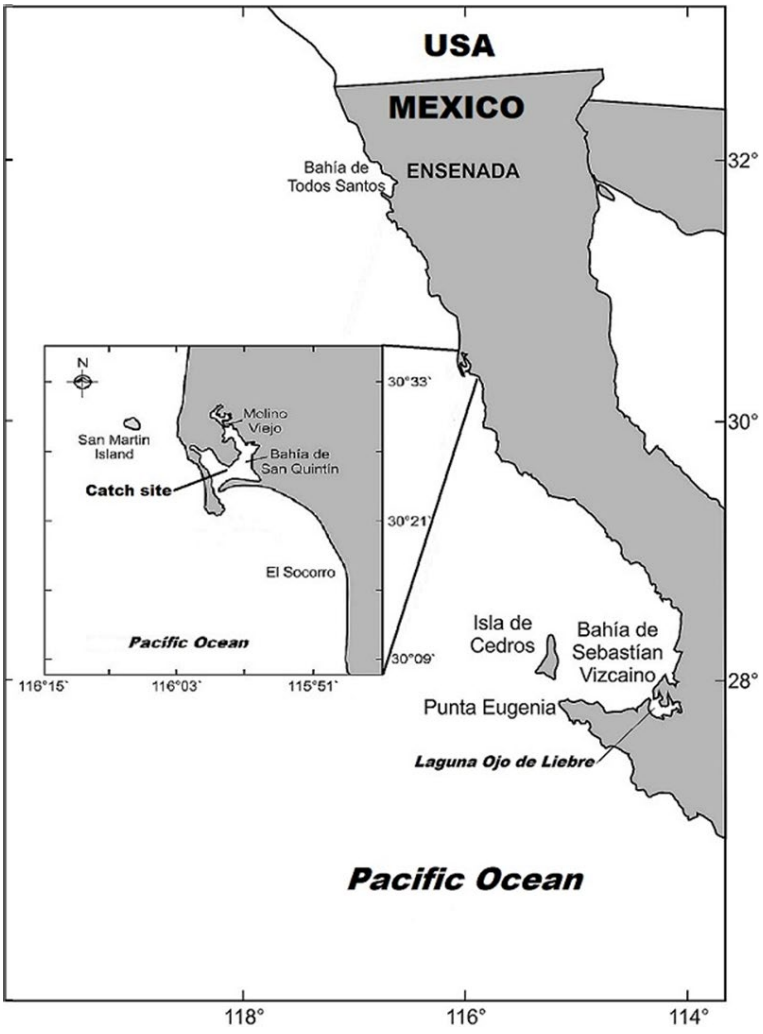

Figure 1. Localization of the Bahía de San Quintín, Baja California, México, and capture site of the Cortez angelfish, *Pomacanthus zonipectus*.

Since 1993, monitoring of the fish in the bay and coast of San Quintín has been carried out, with direct collections using five different fishing gears at sites close to the beach, 5 and 10 meters depth (Rosales-Casián, 1996: Rosales-Casián, 2004a), and also evaluating the commercial (Rosales-Casián and Gonzalez-Camacho, 2003) and sportfishing catch (Rodriguez-Santiago y Rosales-Casián, 2008). These last two activities are carried out on the adjacent external coast, on rocky spots located at distances outside the lagoon mouth from 3 km to sites as far as 80 km.

Before of the sportfishing activity, fishers catch juveniles of Pacific mackerels (*Scomber japonicus*) inside the lagoon to use them as live bait for pelagic fish or on the rocky bottom of the spots; for this, fishermen use the Sabiki hooks formed by a line of five small hooks type lures with plastic "bristles".

In addition, the commercial or sportfishing fishers also report when a species is unknown to this temperate zone, and in this way the presence of several species that widen their distributions northward during events such as El Niño (Rosales-Casián, 2017), or southward during La Niña (Rosales-Casián and Almeda-Jauregui 2009), have been documented.

Results and Discussion

On May 15, 2021, during our monthly monitoring of the sportfishing catch upon arrival of the boats at the site El Molino Viejo in Bahía de San Quintín, a boat fisher showed a fish species individual unknown to the temperate area. The individual was caught with Sabiki hooks at the beginning of the Bahía Falsa (Lat. 30.42287 N, Long. 115.98496 W), one of the two arms that form Bahía de San Quintín.

This fish species was identified as the Cortez angelfish, *P. zonipectus* (Gill, 1862), and was easy to identify due to its body shape and coloration (Figure 2). The description coincides with what is established for this species: 11 dorsal spines and 25 dorsal rays; 3 anal spines and 20 anal rays; 19 pectoral rays; it has a bump on the head, the dorsal fin ends in a point, and it has a strong spine on the lower edge of the operculum; the part of the anterior head grayish in color, has a broad yellow band that crosses the operculum from top to bottom, and a second yellow band between two black bands behind the pectoral fin; the caudal fin pale yellowish color (Allen and Robertson, 1994). Total length (TL) of the individual of Cortez angelfish was 25.5 cm, standard length (SL) was 22.9 cm, and with a weight of 748 g. Sea surface temperature at the coastal zone was 12.8-12.9ºC and inside of the bay was 16.1ºC.

Figure 2. Individual of the Cortez angelfish, *Pomacanthus zonipectus* (25.5 cm total length) from Bahía de San Quintín, Eastern Pacific of Baja California, México.

The taxonomic classification for this species is: CLASS ACTINOPTERYGII, ORDER PERCIFORMES, Family Pomacanthidae, *Pomacanthus zonipectus* (Gill, 1862), considering the work of Page *et al*., 2013.

Bahía de San Quintín is an important lagoon due to its high productivity (Lara-Lara and Alvarez-Borrego 1975; Gracia-Escobar *et al*., 2015); it functions as a nursery ground for different species of fish, and until 1995, a total of 69 species of temperate fish had been identified in the interior of the bay, and 71 species on the external coast at depths less than 10 meters (Rosales-Casián, 1996).

In a previous study that we conducted in 2015-2016, its northernmost record of the Cortez angelfish was in Laguna Ojo de Liebre, Baja California Sur, on August 25, 2016, as a new occurrence during the same warming event 2014-2016 (Civico-Collados and Rosales-Casián, 2021). This new movement of the Cortez angelfish to Bahía de San Quintín in a straight line represents an extension in its northward distribution of 355 kilometers from Laguna Ojo de Liebre.

The Cortez angelfish is considered an omnivorous, generalist and opportunistic species; it can feed on a large number of plant and animal species associated with the bottom (Perez-España, 1994). However, small planktonic crustaceans have also been identified in its stomach contents (Reynolds and Reynolds, 1977), and this may possibly explain why this specimen was caught with Sabiki hooks; with this method, several individuals of Mexican lookdown (*Selene brevoortii*), a Panamic fish species, were captured within the Bahía de San Quintín during the warming event in 2014-2016 (Rosales-Casián, 2017).

But how to explain the presence of the Cortez angelfish in the Bahía de San Quintín? A lagoon considered a cold site due to the presence of a permanent upwelling near the mouth (Rosales-Casián, 2004a). When reviewing the recently history of temperature anomalies, from July 2020 to June 2021, a La Niña event was presented in our area with anomalies from -0.5 ºC to -1.3 ºC, and the most recent warming events occurred in August 2018 to July 2019 with positive temperature anomalies from 0.5 up to 0.9 ºC, and from September 2014 to May 2016 with positive temperature anomalies from 0.5 ºC to 2.6 ºC (NOAA's El Niño, available at http://www.elnino.noaa.gov/, last accessed 20 September, 2021); this last warming was formed by two overlapping events, "The Blob" that began at the Gulf of Alaska and extended to south from 2013 to 2015, and the "El Niño" from 2014-2016 (Civico-Collados and Rosales-Casián, 2021; Dorantes-Gilardi and Rivas, 2019; Robinson, 2016).

Possibly those warming events promoted the angelfish movement towards the temperate zone, similar to movements of others tropical fish species like the Mexican lookdown (*Selene brevoortii*) that was caught in the interior of Bahía de San Quintín by the sportfishing (23 October, 2014 and 11 July, 2015), and also the Cortez bonefish (*Albula gilberti*), and the Pacific tripletail (*Lobotes pacificus*) both caught just off the adjacent shore of the bay with gillnets by the commercial artisanal fishing on 28 March, 2015 (Rosales-Casián, 2017).

It is important to mention that in 36 years of monitoring coastal fishes on the Pacific coast of Baja California, México, (Islas Coronado at border with California, USA to Laguna Ojo de Liebre, Baja California Sur), in a distance of 660 km, only in the Ojo de Liebre Lagoon the species *P. zonipectus* was identified during the warming event of The Blob-El Niño 2013-2016, also as a new extension in its distribution towards the north (Civico-Collados and Rosales-Casián, 2021).

Regarding its conservation and according to the IUCN Red List category and criteria, the Cortez angelfish is classified as Least Concern (Pyle *et al.,* 2010). They are "catch and release" when caught for recreational anglers and is occasionally retained for subsistence by fishermen (Snow, 2021). However, The Cortez angelfish *P. zonipectus* is an important fish species for the aquarium market, its cultivation can supply the demand, and it will be useful to establish strategies for its fishery management (Arellano-Martinez *et al.,* 2006).

Conclusion

The occurrence of Cortez angelfish in a temperate environment such as Bahía de San Quintín, México is important as a biological indicator of warming signals. This angelfish joins a number of eight tropical fish species that have been recorded in the area during El Niño or The Blob events.

Compliance with Ethical Standard

Conflict of interests: The authors declare that for this article they have no actual, potential or perceived conflict of interests.

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

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Disclosure: -

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....(Crockatt, 1995).

Direct quote from the text

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Major Citations for a Reference List in Table 2.

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An eBook	**Millbower, L. (2003).** *Show biz training: Fun and effective business training techniques from the worlds of stage, screen, and song*. p. 92-90. Retrieved from http://www.amacombooks.org/ (accessed 10.10.15)
An article in a print journal	**Carter, S., Dunbar-Odom, D. (2009).** The converging literacies center: An integrated model for writing programs. *Kairos: A Journal of Rhetoric, Technology, and Pedagogy,* 14(1), 38-48.
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