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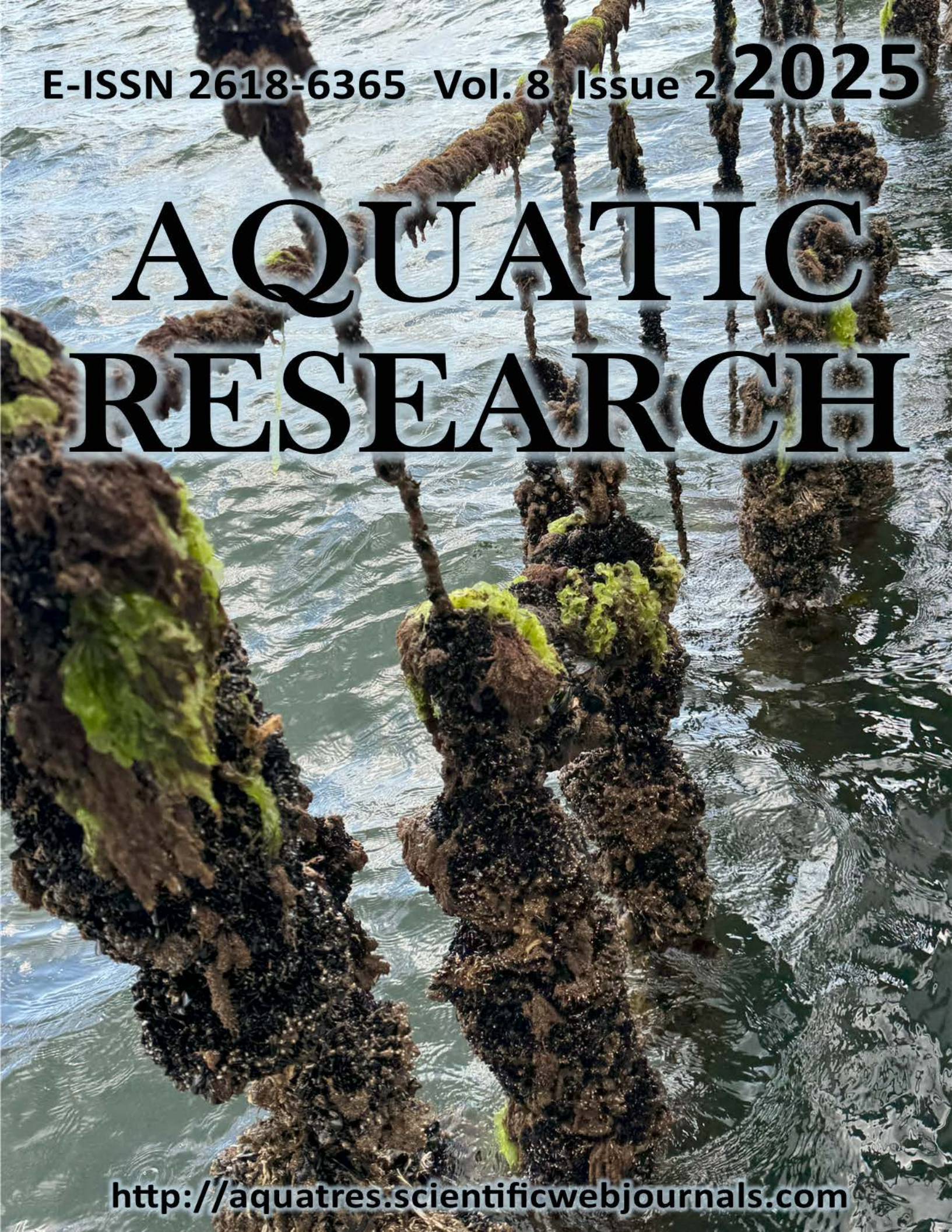
AQUATIC RESEARCH



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Content

Research Articles

Short Communication

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Length-weight relationship, condition factor dynamics, and feeding preference of *Clarias batrachus* (Linnaeus, 1758) from the rivers of Bataan, Luzon Island, Philippines

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ABSTRACT

There is a paucity of information on Philippine catfish (*Clarias batrachus*) thriving in fishery areas in Bataan, Luzon Island, and the Philippines. The study examined the length-weight relationship ($W = aL^b$), condition factor, and stomach content of *C. batrachus* collected from two major river systems in Bataan (Orani and Bagac Rivers), Philippines. A total of 60 fish specimens (12.4–25.5 cm) were collected using a 12-v electrofishing gear and fishing net. Although the specimens from Orani were significantly larger than those from Bagac, the latter exhibited an isometric growth rate ($b = 3$). Orani population displayed a negative allometric growth ($b < 3$). Female and male samples, regardless of site variability, showed a statistically isometric growth rate (b = 3). The condition factor of *C. batrachus* was K = 1.0, irrespective of site and sex variation, signifying that the populations are in good condition. Five food items were detected in Bagac, with the Gobiidae family emerging as the predominant prey items based on number (48.89%), frequency of occurrence (100%), and weight (72.83 %). Orani recorded three food items, primarily macroinvertebrates (Chironomidae), accounting for 75 %N, 86.67 %O, and a frequency of 91.19%. The Index of Preponderance and Index of Relative Importance recognized Gobiidae and Chironomidae as the two most important food items in Bagac and Orani, respectively. The baseline dataset generated from this study is hoped to provide insights into the current population status of this important fishery resource for improved riverine conservation management.

Keywords: Bagac, Growth coefficients, Index of preponderance, Orani, Prey items

Introduction

The *Clarias batrachus* (Linnaeus, 1758) populations are native to Southeast Asia and have been introduced worldwide for fish farming (Allen, 2011). This clariid species is an air-breathing and hardy fish that can thrive in areas where many other fish struggle to survive. It is mostly found in freshwater and brackish water rivers, lakes, ponds, streams, swamps, ditches, rice paddies, and reservoirs (Froese & Pauly, 2023; Allen, 2011). Successful aquaculture of this species provides socio-economic sustainability for rural communities (Debnath, 2011). Its economic importance stems from its attractiveness, taste, food conversion efficiency, ruggedness, and consumer popularity (Hossain et al., 2006; Debnath, 2011). This fish is also commonly referred to as Asian catfish and is considered an integral part of commercial fisheries, aquaculture, and home aquariums, particularly in Asia, where it is widely consumed. In the Philippines, the wild populations are now displaced (Paller, 2011) attributed to various factors such as drought periods, habitat destruction, and the uncontrolled introduction of a larger African catfish (*C. gariepinus*) that are known for fast growth and feral wild populations (Ahmad et al., 2012). In the Philippines, it is locally known as *hito* or *pantat* and supports communal and subsistence fisheries in the riverine fishery areas of Bataan, Philippines (Corpuz & Espaldon, 2023).

Despite the ecological and economic importance of *C. batrachus*, studies on fisheries biological tools, including length-weight relationships (LWR) and condition factors, have hitherto not been conducted at a local level to assess the ecological status of this important aquatic resource. Apart from LRW and condition factors, the gut analysis of the species provides important insight into feeding patterns and quantitative assessment of food habitats, which is also a key aspect of fisheries management (Hyslop, 1980). Moreover, the gut content analysis helps understand the food preference of fish species' natural history, nutritional requirements, trophic, material and energy dynamics, food webs, food chains, and material and energy transfers between and within ecosystems (Manko, 2016). It also reflects habitat separation in fish as the stomach content analysis can reveal the habitat where fish feed (Gümüş et al., 2002). Knowledge of morphometry, growth coefficients, food, and feeding is fundamental to understanding fish biology and trophic interactions between species in a fish community (Blaber, 2000; Corpuz et al., 2013; Corpuz, 2018). Similarly, these baseline datasets are yet known for *C. batrachus* populations thriving in Luzon Island, Philippines. Hence, the present study evaluated the LWR, condition factor, and feeding preference of *C. batrachus* populations based on the samples collected from Bataan's two separate river systems (Orani and Bagac).

Materials and Methods

Study Areas

The specimens were collected in the daytime from the east and west coasts of Bataan, Philippines. The *C. batrachus* specimens for the east portion of Bataan were collected in Orani River, Tagumpay, Orani (14º48'46" N and 120°30'56" E). The fish specimens for the west coast were collected in the Bagac River (Silahis-Pag-asa, Bagac, 14º35'45" N and 120º23'47" E). Both river systems serve as a communal fishing area for the local stream communities. Apart from fishing, the area is often used as a water source for agricultural purposes.

A total of 60 *C. batrachus* individuals (30 specimens from Orani, Bataan, and 30 specimens from Bagac, Bataan) were collected using 12-v electrofishing gear and scoop net from August to November 2023. The fish specimens were immersed in a phenoxyethanol solution (1 ml 5 L^{-1}) to induce sleep and immediately preserved in an ice box to avoid the digestion of food items. The specimens were brought to the laboratory of Bataan Peninsula State University for further examination.

Fish Analyses

In the laboratory, the fish specimens were immersed in phenoxyethanol solution (0.2 ml L^{-1}) to sleep prior to length and weight determination. The total length (TL, measured from the snout to the tip of the caudal fin) was measured using a vernier calliper (0.01 cm). The wet weight of specimens was determined using a digital weighing scale (0.01 g).

Using a scalpel, the fish specimens were incised from the anus up to the throat to reveal the alimentary canal. The stomach was exenterated from the whole alimentary canal by separating the attached organs from it and cutting it from the cardiac area to the pyloric section. The incision was performed in the lesser curvature of the stomach. The stomach contents of each specimen were extracted and transferred in gridded Petri dishes with tissue paper (no fixation). The sorted food items were counted and identified to the lowest possible taxonomic level under a simple microscope. The wet weight of the prey item was determined to the nearest 0.01 g using an analytical balance. The volume of each prey taxa was measured by water displacement in a graduated cylinder.

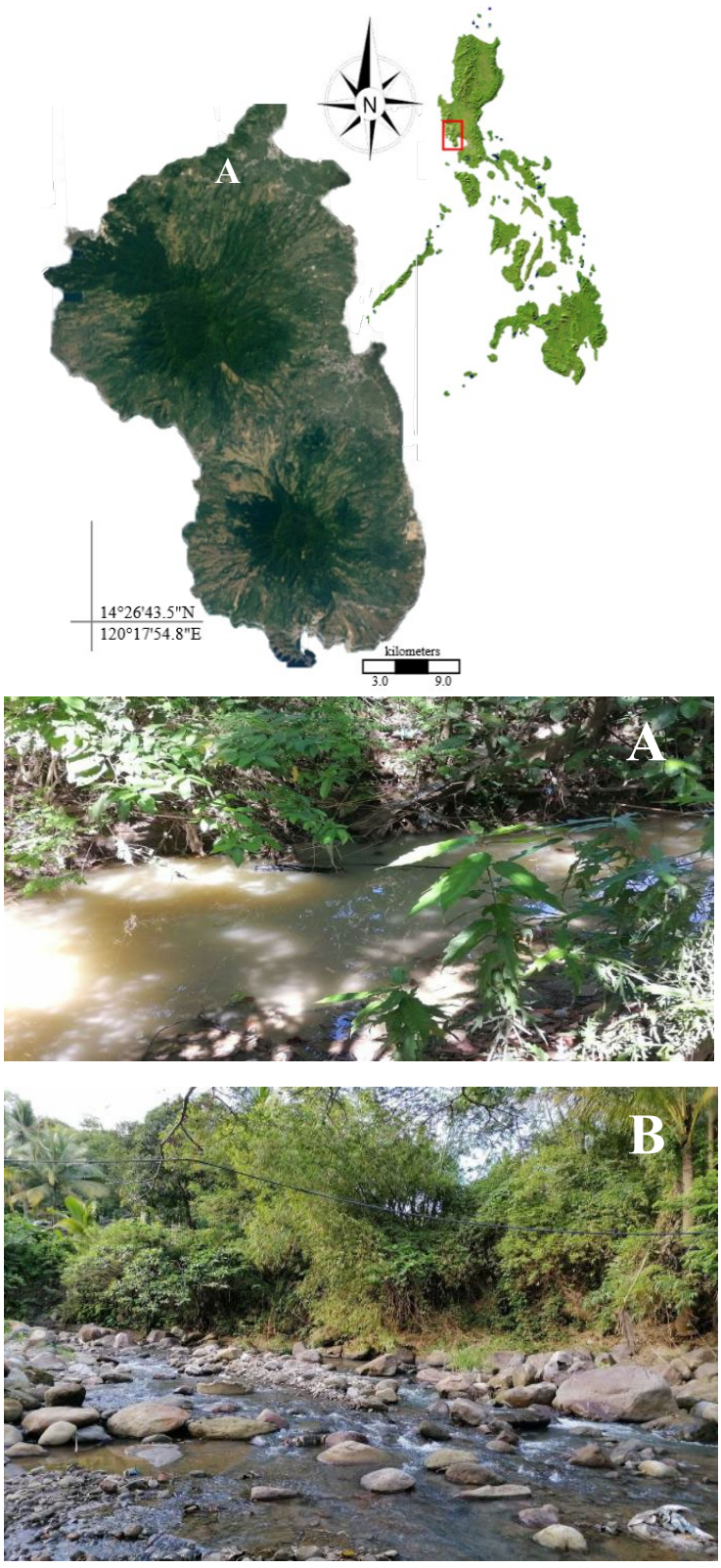

Figure 1. Map of Bataan, Philippines, showing the collection sites. (A) Tagumpay in Orani and (B) Parang in Bagac

Data Analyses

The equation expressed the allometric LWR:

$$W = a\ TL^{\ b}$$

Where W = is the weight (g) of an individual fish; TL = is the total length (cm) of fish individuals; *a* is the intercept, and *b* is the slope. The transformation of fish length and weight as Log w = log *a* + *b* x log (TL) was used to compute the *a* and *b* (Froese, 2006). The fish body condition factor was calculated using the equation by Fulton (1902).

$$K = W/LWR$$

K = condition factor, W = weight (g) of fish, and LWR = Length-weight relationship.

The growth rate pattern of the fish (allometric or isometric) as expressed in the value of *b* (slope) was tested for theoretical value for isometry when *b* was significantly equal to 3; growth was regarded as isometric if $b < 3$ is negative allometric, and $b > 3$ is positive allometric (Santos et al., 2020) (*t-test*, $P < 0.05$).

The relative measures of stomach content were evaluated quantitatively using three methods of occurrence (O%), defined as the number of stomach samples in which prey occurs expressed as a percentage of all stomachs; numeric percentage (N%) was defined as the number of individual in each prey categories recorded for all stomachs with the total expressed as a percentage of the total individuals in all prey categories and wet weight percentage (W%) defined as the wet weight of each prey recorded for all stomachs, with the total expressed as a percentage of a total wet weight of all prey categories (Hyslop, 1980). The partial fullness index was computed to compare the variation of the food found in the stomach among sampling sites. Two indices of dietary importance were also calculated to evaluate the prey importance through the equations:

- Index of Preponderance (IOP) (Natarajan and Jhingran 1962); IOP = %V.%F÷∑%V.%F*100;
- Index of Relative Importance (IRI) (Pinkas et al., 1971): IRI = %F (%N + %V); %IRI = (IRI/ΣIRI) *100.

The correlation between the prey item biomass and TL of *C. batrachus* was predicted using the transformed $\log_{10}(x+1)$, and the relationship was determined by a non-linear regression function: f = axb, where a = coefficient, x = TL, and *b* = slope.

Results and Discussion

Total Length and Weight

The mean and SD of the total length and weight of *C. batrachus* specimens are summarized in Table 1. The TL variation between *C. batrachus* populations was significant (t = 3.62; P < 0.01), with Orani (22.15 ±2.98 cm) having longer TL than Bagac (19.25 ±3.20 cm). The length of female samples in Orani (23.36 ±2.29 cm) was significantly larger than the male (20.56 ±2.29 cm). While in Bagac, the length of the female (19.56 ±3.11 cm) was slightly longer than the male (18.64 ± 3.46 cm). Inter-sexual variation in TL was not significant (t = 1.66; P < 0.05), albeit the site-sex factor was found to be statistically different (F = 7.2; P < 0.01).

The weight of specimens from Orani (82.65 ±30.81 g) was significantly heavier than in Bagac (52.06 ±24.79 cm) (t = 4.24; P < 0.01). The weight of female specimens in Orani (92.41 ±35.15 g) was heavier than the male specimens (69.89 ±19.66 g). While the weight of female specimens in Bagac (53.01 ±24.16 g) was slightly heavier than the male (48.82 ±28.51 g), sexual differentiation was not significant (P > 0.05).

Length-Weight Relationship and Condition Factor

The summarized dataset of LWR and the condition factor of *C. batrachus* is presented in Table 2. The observed growth coefficient of Bagac (b = 3.11, SE = 0.284) was isometric despite a slight deviation from b = 3.0. On the other hand, a negative allometric growth rate was observed in Orani populations (b = 2.55, SE = 0.200). Male and female populations exhibited isometric growth coefficients (male b = 2.963, SE = 0.285; female b = 2.9458, SE = 0.202). For Bagac, the male and female growth coefficients were isometric (male b = 3.40; female b = 3.04), while in Orani, male and female had negative allometric (male b = 2.45; female b = 2.73). Scatter plots of the relationship between the length and weight of specimens are illustrated in Figures 3 and 4.

Table 1. Descriptive statistics of *Clarias batrachus* in Orani and Bagac, Bataan, Philippines

Collection Sites	Sex	*n*	Total Length (cm)			Body Weight (g)		
			Min	Max	Mean ± SD	Min	Max	Mean ± SD
Orani	F	17	18.43	28.79	23.36 ±2.92[a]	51.05	163.49	92.41 ±34.59[a]
	M	13	15.76	23.27	20.56 ±2.29[a]	30.79	92.21	69.89 ±19.66[ab]
Bagac	F	20	14.14	23.27	19.56 ±3.11[b]	19.21	91.74	53.01 ±24.16[b]
	M	10	13.73	23.27	18.64 ±3.41[b]	19.24	100.72	48.82 ±28.51[b]

Similar letters indicate no statistical difference in each row at a 5% confidence level. a > b.

Table 2. The LWR parameters and condition factor of *C. batrachus* in Orani and Bagac, Bataan, Philippines

Collection Sites	Sexes	*n*	Growth coefficients			Condition Factor	
			a	*b*	r^2		
Orani	Female	17	0.02	2.73	0.83	0.99	1.01
	Male	13	0.04	2.45	0.85	1.04	
Bagac	Female	20	0.01	3.04	0.81	1.01	1.01
	Male	10	0.01	3.40	0.93	1.01	

The overall computed condition factor value showed no significant deviation from K = 1.0 (Table 2), signifying that the two populations are well-being. Moreover, regardless of sex, the K scores of specimens from the two sites were not significantly different (F = 0.002; $P > 0.05$). The result of the present study is similar to the results obtained by Rosli and Isa (2012), wherein the growth of *Plicofollis argyropleuron* from the Northern Part of Peninsular Malaysia was also observed to be isometric. A similar result was also reported by Fafioye and Ayodele (2018), where *Coptodon zillii*, *Brycinus nurse,* and *Oreochromis niloticus* from Oyan Lake, Nigeria, were observed to have negative allometric growth coefficients. Similarly, the condition factor (*K*) recorded in the above-stated fishes was greater than one (>1). Several factors have been noted to influence the LWR in fishes, including season, habitat, gonad maturity, sex, diet and stomach fullness, health, and preservation techniques, which can contribute to the different *b* values of the same species from various areas (Perez & Ignacio, 2019).

The LWR is usually used to determine the stock assessment, population dynamics, growth pattern, general health, habitat conditions, life history, fish condition, and morphological characteristics (Falsone et al., 2022; Jisr et al., 2018; Santos et al., 2020). A recent study revealed that the male and female *C. batrachus* in Orani had negative allometric ($b < 3$). Negative allometric occurs when the fish's body length increases faster than the fish's body weight, meaning these fish become lighter with increasing size (Mazumder et al., 2016). At the same time, the population from Bagac revealed that the male and female *C. batrachus* had isometric growth ($b = 3$), where the weight and length of the organism increased at the same rate.

The condition factor of a fish reflects physical and biological circumstances and fluctuations, such as feeding conditions and parasitic infections (Datta et al., 2013). It is an important tool that provides information on fish inter-population variation in growth patterns and condition factors (De Leon et al., 2017; Santos et al., 2020). The K values for *C. batrachus* in Orani and Bagac observed in the study are similar to the observation by Abobi (2015). This study also provides evidence of good overall health and welfare of *C. batrachus.*

Prey Composition and Food Analysis Indices

Sixty stomachs were examined in two sampling sites, and no empty stomach was recorded. In Bagac, five food categories were identified in the stomach: fish, gastropods, insects, plant materials, and crustaceans. Large prey items, particularly *Glossogobius giuris*, a common species of goby found in the river of Bagac, dominated the diet, comprising 48.89% by number (%N) and 100% by frequency of occurrence (%O). This was followed by crustaceans with 26.67 %N and 60 %O, plant materials with 11.11 %N and 33.33 %O, insects with 8.89 %N and 26.67 %O, and the least amount in the diet was gastropods with 4.44 %N and 6.67 %O. On the other hand, only three categories were identified in Orani comprising insects with 75 %N and 86.67 %O, gastropods with 16.67 %N and 33.33 %O, and plant materials with 8.33 %N and 13.33 %O.

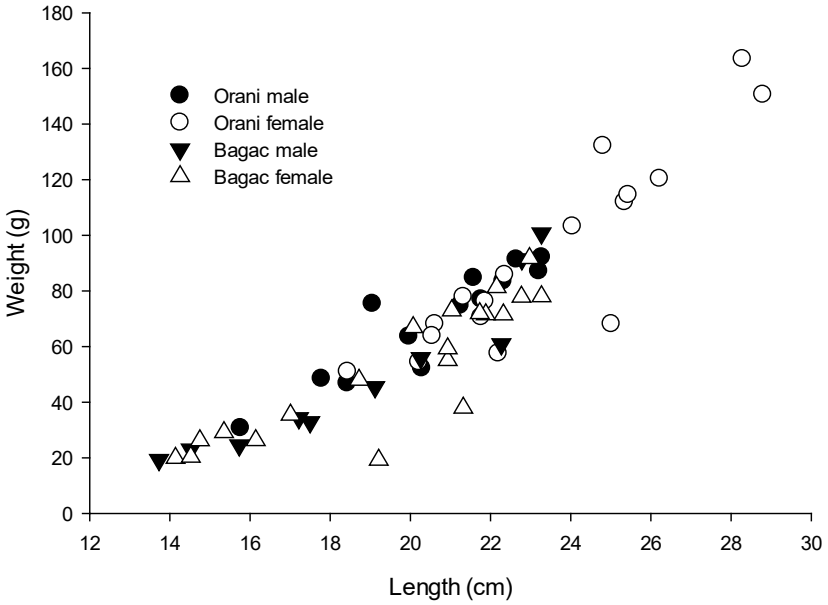

Figure 3. Scatter diagram plot of male and female *Clarias batrachus* length-weight relationship from Orani and Bagac, Bataan, Philippines.

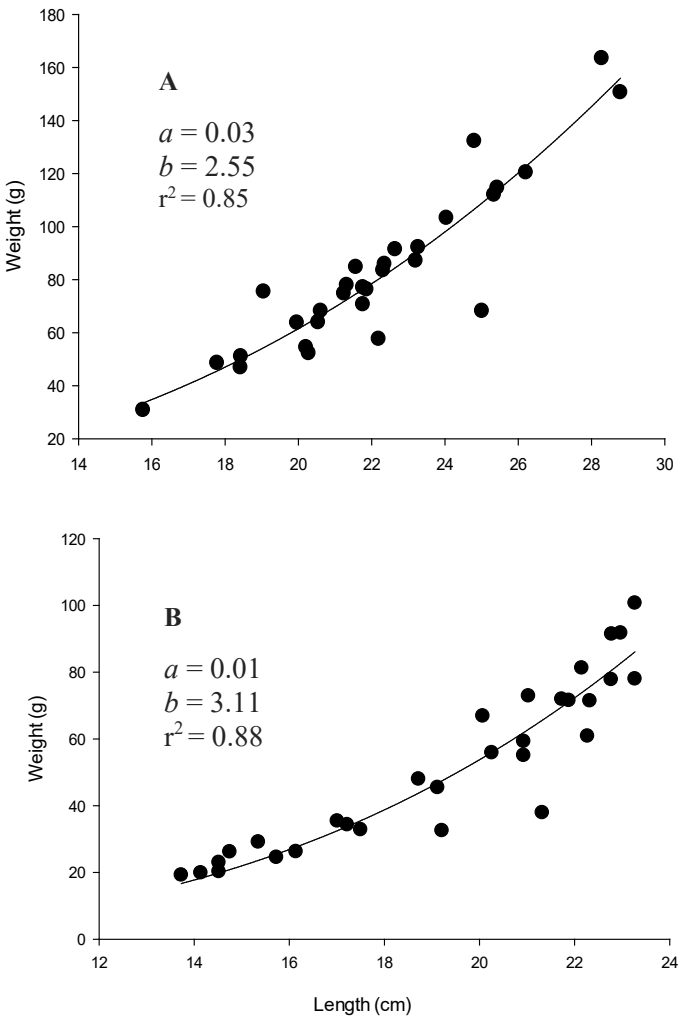

Figure 4. Scatter diagram plot of the length-weight relationship of *Clarias batrachus* in (A) Orani and (B) Bagac, Bataan, Philippines

The ranking of prey items based on IRI and IOP is presented in Table 3. The most important food item in Bagac, which comprised 76.61 %IRI and 83.13 %IOP, was fish prey item. While in Orani, insects were recognized as the most relevant food items comprising 94.26 %IRI; in terms of %IOP, it appeared with 94.26%.

Diet Variation

The relationship between number, volume, and occurrence displayed a degree of diet variation among the *C. batrachus* specimens (Table 5). In Bagac, fish was recognized as the major prey item by number and exhibited the highest occurrence and volume rate in the gut content. In Orani, insects were determined as the major prey item by number and exhibited the highest volume and occurrence rate.

A fish-based diet was the dominant prey item of Bagac populations (48.89 %PFI), but no fish consumption was recorded in Orani. The proportion of crustacean prey items was 26.67 %PFI, and there was no crustacean intake in Orani. On the other hand, insects were recorded as the major prey in Orani samples (75 %PFI); on the contrary, they ranked second to the least (8.89 %PFI) on the other site. Plant materials (11.11 %PFI) and gastropods (4.44 %PFI) were also recorded in Bagac. The Orani specimens also consumed gastropods (8.33 %PFI) and plant materials (16.67 %PFI). There was no significant relationship between the TL (cm) of the fish and prey item weight (g), as the amount of food consumed by the fish was not dependent on its length ($r^2 = 0.33$, $b = 10.24$) (Figure 6).

Table 3. Index of Preponderance (IOP) and Index of Relative Importance (IRI) of *C. batrachus* populations from Bagac and Orani

Prey items	IOP	Rank	IRI	Rank
Bagac				
fish	83.13	1	76.61	1
crustaceans	15.61	2	18.68	2
insects	0.57	5	1.81	4
gastropods	0.07	4	0.22	5
plant materials	0.63	3	2.68	3
Orani				
fish	0	0	0	0
crustaceans	0	0	0	0
insects	97.41	1	94.26	1
gastropods	0.69	3	1.09	3
plant materials	1.91	2	4.65	2

The feeding habit of *C. batrachus*, a carnivorous fish species, reveals a high preference for preying crustaceans, insects, and teleost fishes. A similar observation was reported by Sakhare and Chalak (2014), wherein *C. batrachus* populations in India preferred small fishes and insect larvae as the primary food items. The small fish prey items found in Bagac specimens signified the importance of small fishes in the river to sustain native catfish protein source requirements. The present findings agreed with the observation of Ramesh and Kiran (2016) and several authors in South Africa that indicated that the *C. gariepinus* did not rely only on offshore fishes and benthic invertebrates at high lake levels. However, they readily switched their feeding to littoral fishes and invertebrates when these became abundant (Wakil et al., 2014). On

the other hand, nourishment for the growth of Orani samples is highly dependent on insects. This change in feeding habits might be attributed to the absence of other fish species in the Orani River caused by anthropogenic activity such as the illegal use of chemical compounds (e.g. sodium) for fishing, including other environmental perturbations (Romero et al., 2016: Flores et al., 2015).

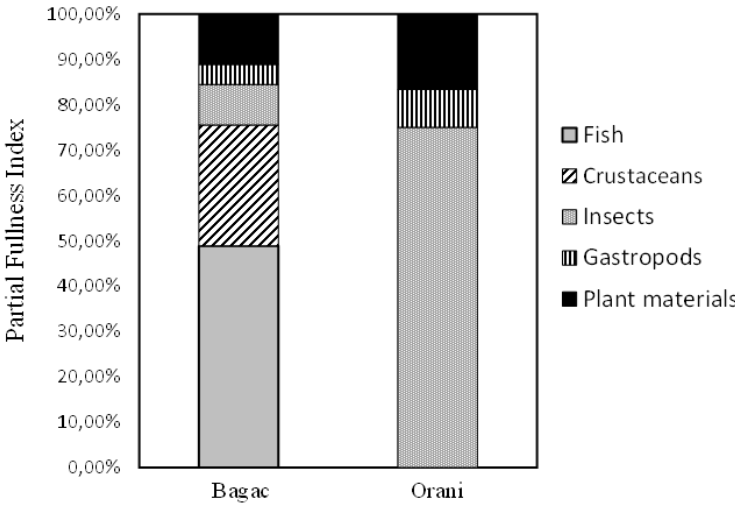

Figure 5. A stack bar representation of the main diet composition of *Clarias batrachu*s is expressed as a distribution of the Partial Fullness Index among sampling sites

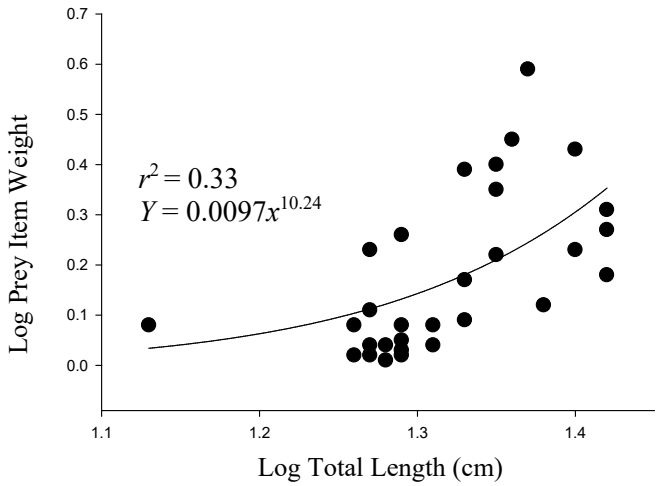

Figure 6. Relationship of prey item biomass with the total length of *Clarias batrachus*

The present study revealed that the amount of prey items ingested by *C. batrachus* is not size-dependent. It is highly evident that *C. batrachus* is a voracious feeder that consumes a large amount of food regardless of its size, as shown in Figure 6. This contradicts the study of Admassu et al. (2015), who stated that the proportion of insects and zooplankton decreased with the increase in fish size, while fish prey increased with the increase in Lake Babogaya.

Although the study is limited to a few individuals, to the best of our knowledge, it provided vital datasets of the population profile of *C. batrachus*, which can serve as a baseline reference for future investigation. There is a need to reduce fishing pressure (Romero et al., 2016) and other aquaculture activities (Flores et al., 2015) impacting the ecological integrity of the river ecosystems, which may eventually affect the *C. batrachus* and other native fish populations. This study hoped to enlighten the local community on the status and growth condition of *C. batrachus*. It could serve as a basis for the conservation management program for the two studied rivers.

Conclusion

Despite being larger in length and weight, the growth coefficient of Orani populations had negative allometry compared to the isometric growth rate of those from Bagac. However, the good condition factor of the two populations is similar, attributed to their biological capacity to adapt to varying habitat conditions. Both populations were in good condition, albeit their growth coefficients were dissimilar, indicating morphological plasticity to adjust to external environmental pressures.

Further investigations on potential factors (diet, physicochemical, and anthropogenic disturbances) affecting LWR and condition factors are necessary. Increasing sample size and inclusion of other populations from other river systems can improve the robustness of LWR and condition factor data. Further investigation is also suggested to assess the influence of sexes and seasonality on the feeding pattern dynamics of *C. batrachus.* An additional study can also be done to understand further the dietary aspects and feeding habits of *C. batrachus*. Given the importance of stomach content analysis, future investigation should also be extended to other fish species, especially indigenous ones, to provide scientific information for their management.

Compliance with Ethical Standards

Conflict of interest: The author(s) declare no actual, potential, or perceived conflict of interest for this article.

Ethics committee approval: The Animal Care and Use Committee of Peninsulares Ethics Review Board of the Bataan Peninsula State University (REDO.PROJ.OC026) approved protocols for animal experiments.

Data availability: Data will be made available on request.

Funding disclosure: No funding provided.

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Disclosure: -

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Protein and carbohydrate contents related to varying light levels and chlorophyll-a in selected freshwater and marine phytoplankton

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ABSTRACT

This study investigated correlations between chlorophyll-a (CHLa) and certain biomass parameters (protein and two forms of carbohydrates) under the influence of light intensity. These findings are applicable to the estimation of metabolizable biomass in water bodies, which is important for understanding the nutritional value of phytoplankton and their impact on aquatic food webs. Furthermore, these determined biomass relationships can also assist in the prediction of the generation of anoxia during and following algal blooms. That is, one could relate the standing crop of metabolizable organic matter (proteins and carbohydrates) to existing conditions of water depth, currents, dissolved oxygen trends and other parameters. Results from this study indicate that protein, colloidal carbohydrates, and storage carbohydrate concentrations in phytoplankton can be broadly estimated by multiplying chlorophyll-a amounts (pg/cell or mg/L) by 202.6, 17.7, and 144.9, respectively. The methodology presented can therefore serve as a means of approximating the standing crop of metabolizable phytoplankton organic matter (viz. protein and two forms of carbohydrates).

Keywords: Pigment analysis, Algal protein, Algal carbohydrates, Water column, Algal metabolizable organic matter, Irradiance levels, Algal bloom

Introduction

As primary producers, phytoplankton form the base of aquatic/marine food webs. Photosynthetically produced organic matter (OM) is grazed and consumed by zooplankton and other microconsumers (Schaffner et al., 2019) which are then consumed by macroconsumers, such as fish and even, via krill, to whales (Miller et al., 2019). The quality of the organic matter (~' food') produced by phytoplankton therefore has significant impacts on zooplankton and other consumers (Gulati and Demott, 1997).

Chlorophyll-a (CHLa) is the most widely used estimator of algal biomass. It is accepted as a fairly accurate and convenient measure of algal 'biomass' (weight and volume) and can serve to indicate interactions between nutrient concentration and several biological phenomena (Huot et al., 2007; Mineeva, 2011). The review by Mineeva (2011) concluded "*Taking into account high pigment diversity in algae, the high-performance liquid chromatography method seems to be the most perspective method for assessment of total biomass of algal cenoses and of the relative abundance of representatives of large taxonomic groups*." High performance liquid chromatography (HPLC) also allows the full separation and quantitation of chlorophylls and carotenoids and allows for taxon-specific carotenoids to be used in the pigment-based chemotaxonomic assessment of microalgal taxa present in water samples (Louda, 2008; Millie et al., 1993; Wright et al., 1996). Changes in algal physiology are not confined to ratios of CHLa to pigments but is also reflected in other indices of biomass such as proteins, carbohydrates, and organic carbon. The determination of the protein and carbohydrate content of microalgae can provide important information for phytoplankton biomass assessment, which can in turn be used to investigate protein and carbohydrate dependent physiological processes in cells as well as with studies of nutritional value of phytoplankton and their impacts on food webs (Bhavya et al., 2018; Liao, 2024).

Carbohydrates are major products of photosynthesis and include polysaccharides and storage structure compounds. Carbohydrates play important roles in biogeochemical cycles in the water column and water-sediment interface, in cellular metabolism and structure, and are major storage compounds in autotrophic organisms. Carbohydrates, particularly polysaccharides, contribute significantly to the organic matter of diatoms, green algae, and cyanobacteria (Fernandez et al., 1992). Carbohydrates have also been previously suggested as a measure of phytoplankton abundance (Marshall and Orr, 2009).

The two major groups of carbohydrates in microalgae are extracellular, loosely bound colloidal carbohydrates and intracellular storage polysaccharides (glucans and starch). Several groups of microalgae have been shown to secrete copious amounts of carbohydrates and are involved in the transfer of nutrients in lower food webs (Decho, 1990). Colloidal carbohydrate fractions have been shown to contain mucopolysaccharides, extracellular polymeric substances (EPS), transparent exopolymers (TEP), and others, each with their own function. However, these secretions have largely been ignored in studies regarding microalgal production and trophic energy transfer. Epipelic diatoms secrete mucopolysaccharides to facilitate movement. These secretions then represent sources of food for bacteria and invertebrates (Decho 1990). EPS also contributes to the aquatic flocculent organic matter (aka floc), such as in the coastal Everglades (Neto et al., 2006); and excreted polysaccharide EPS forms the basis of hydrogels that stabilize the fine-grained carbonate sediments in Florida Bay and elsewhere (Louda et al., 2004). In addition to function in the mucous matrix of diatoms, mucopolysaccharides have also been reported to serve as storage polysaccharides (Lancelot & Mathot, 1985). Few studies have been done to investigate the influence of light on mucopolysaccharide production in phytoplankton. Studies carried out on *Cyanospira capsulate* and *Synechococcus* strains grown under various light/dark cycles showed that both produced smaller amounts of mucopolysaccharides in comparison to control cultures grown under continuous light (Philips et al., 1989). Decreased production of mucopolysaccharides equated to shorter light periods. Therefore, it could be concluded that the synthesis and release of these polysaccharides is light dependent (Philips et al., 1989). The effect of light on the production of carbohydrates and proteins therefore forms the basis of this study.

Proteins are essential biomolecular components of cells that have the following roles: regulating metabolic activities, providing structural support and existing as pre-cursors as well as end-products of macromolecular synthesis and catabolism (Yun et al., 2015). Therefore, knowledge of the quantity of total protein present is important for understanding a broad range of biological processes in phytoplankton cells. With respect to biomass, the proteins in phytoplankton cells are also important to the secondary consumers (e.g. zooplankton, benthic microbes etc.) that feed on them (Gulati and Demott, 1997). The quantitative information of the protein content in phytoplankton cells, as well as their relationships to chlorophyll-a, is important to a variety of studies that are directly and indirectly related to various aspects of cellular ni-

trogen metabolism as well as predictors of phytoplankton dynamics and physiological state. Few studies have reported generalized relationships between algal protein and chlorophyll-a. For example, a weight- to- weight ratio for protein/CHLa of 8.57:1 has been reported and is often used in the literature (Meyers and Kratz, 1955). However, that reported work focused only on one species of a cyanobacterium, *Anacystis nidulans*. The study by Moal et al. (1987) examined many species across 5 major taxa and found protein/CHLa weight ratios between 20-385:1 and carbohydrate to protein weight ratios between 0.16-2.36:1. However, the two studies mentioned above did not examine the effects of light intensity on protein/CHLa relationships. We report herein, the effects of varying light intensities on the concentrations of protein and CHLa in freshwater and marine phytoplankton.

Traditionally, algal cell volume measurement via microscopy has been the method of choice and is a relatively good indicator of algal biomass. However, the method is laborious and highly dependent on the skills of the researchers (Dunker et al., 2018; Karlson et al., 2010). Moreover, samples become altered, and measurements are biased by the very preservative that the samples are fixed in prior to microscopic analysis (Zarauz & Irigoien, 2008). In the present study, correlations between CHLa and biomass parameters (protein and two forms of carbohydrates) under the influence of light intensity were investigated to assess if the determined relationships could aid biomass estimation since phytoplankton have significant nutritional contribution to food webs.

Therefore, the working hypothesis for this study is that chlorophyll-a *per se* is not the ultimate descriptor of phytoplankton biomass, especially 'food' biomass for transfer amongst food webs. Large variations in 'true' biomass, defined here as metabolisable organic matter (proteins, carbohydrates, lipids), exist between phytoplankton groups (taxa) and within each taxon by variations in light and/or nutrient availability. Thus, taxonomic information would also be required to convert chlorophyll-a to biomass. Alternately, the null hypothesis would be that chlorophyll-a alone works perfectly to estimate phytoplankton biomass.

Materials and Methods

The overall experimental design: algal growth, harvesting, pigment analysis, protein and carbohydrate analysis is shown as schematic 1.

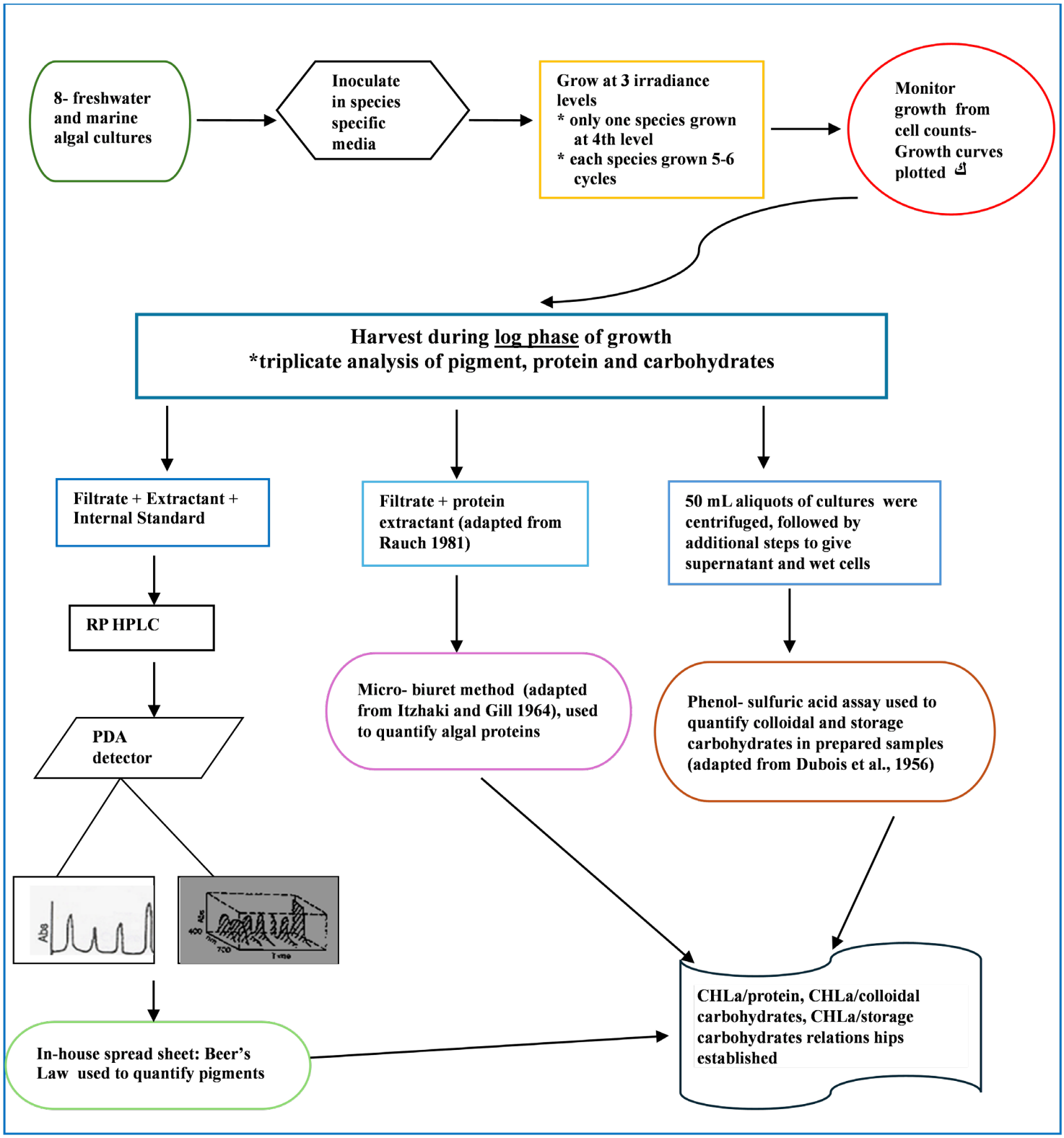
Experimental organisms: The following eight freshwater and marine microalgal species were purchased from the Carolina Biological Supply Company (Burlington, N.C.): Cyanobacteria; *Synechococcus elongatus* (marine), *Microcystis aeruginosa* (freshwater), Chrysophyta; *Thalassiosira weissflogii* (marine), *Cyclotella meneghiniana* (marine), Chlorophyta; *Scenedesmus sp.* (freshwater), Pyrrophyta, Dinophyceae; *Amphidinium carterae* (marine). The following species were purchased from the University of Texas (UTEX) algal culture collection (Austin, TX): Cryptophyta; *Rhodomonas salina* (marine), Chlorophyta; *Dunaliella tertiolecta* (marine).

Selected details of the experimental species: This section is included to stress certain characteristics of each species and include: (a) harmful algal bloom (HAB) formation leading to high inputs of metabolizable organic matter such as proteins and carbohydrates; and (b) importance as primary producers supporting heterotrophic growth in food chains/webs.

Synechococcus elongatus is a common cyanobacterium. *Synechococcus* is the main source of primary production in oligotrophic, pelagic marine, open, warm waters. They have been known to cause non-toxic harmful algal blooms in Florida Bay (Phlips et al., 1999). Harmful here includes blooms leading to anoxia, disruption of socio-economic function and environmental change. The dominance of this species in the center of the Florida Bay may be attributable to its physiochemical characteristics: small size, buoyancy, and tolerance to high light intensity (Phlips et al., 1999).

Microcystis aeruginosa is a common unicellular colonial cyanobacteria found in freshwater environments. The existence of intracellular structures such as gas vesicles provides cells with buoyancy. *M. aeruginosa*, used in this study, occurs in large amounts on the surface waters of lakes and reservoirs in the spring and summer months. This species synthesizes a variety of toxins of which the three most abundant are microcystin-LR, cyanopeptolin-A, and aerucyclamide-A (Ricca et al., 2024). It is one of the most damaging species, due to its toxicity to aquatic and terrestrial organisms and is known to occur worldwide (Ross et al., 2006).

Thalassiosira weissflogii grows primarily in marine waters with some species within the genus being found in estuaries, high-conductance waters and rivers, polluted ponds, and other aquatic systems that have been impacted by human activities (Spaulding and Edlund, 2009).



Schematic 1. Experimental design

Cyclotella meneghininana, synonymous with *Stephanocyclus meneghinianus* (Kützing) Kulikovskiy (Guiry, 2024), used in this study, is perhaps the best-known species of this genus and is widely used in growth experiments (Mitrovic et al, 2010). *C. meneghininana* is a very common freshwater diatom in many places across the world (Kiss & Nausch 1988, Murakami et al. 1994) and may predominate over other diatoms in silica-rich environments (Hori et al. 1969).

Scenedesmus quadricauda is very common in eutrophic freshwater ponds and has planktonic forms in rivers and lakes; it is reported worldwide in all climates and is rarely found in brackish water (Wehr and Sheath, 2003).

Amphidinium carterae is a brown tide organism forming harmful algal blooms, releasing toxins, and physical irritants and creating noxious events. Most species produce toxins that affect humans as well as fish (ichthyotoxic). This species, as well as others in the genus are CFP (ciguatera fish poisoning) producers (Hallegraeff, 1993).

Rhodomonas salina is a marine photoautotrophic flagellate. They occurs in marine and brackish water. This species contains fragile cell membranes and has the name hidden-plant (crypto-phyte). Cryptophytes are very fragile and are often lost in fixed samples (Jeffrey and Vesk, 1990).

Dunaliella tertiolecta is a unicellular, ovoid, biflagellate, naked green marine alga (Morais Jr. et al., 2020). Twenty-eight species of *Dunaliella* are presently recognized (Jayappriyan et al., 2010). The unique morphological feature of *Dunaliella* is that it lacks a cell wall. The cell is enclosed by a thin plasma membrane or periplast, which permits rapid changes in cell shape and volume in response to osmotic changes. To survive, these organisms have high concentrations of β-carotene to protect against the intense light and high concentrations of glycerol to provide protection against osmotic pressure.

Algal culturing: All species were grown in 2 L batches in 4L cylindrical polycarbonate containers (CAMBRO. Huntington Beach, CA). Zephyrhills ® Natural Spring Water was used for the freshwater cultures (*M. aeruginosa, Scenedesmus sp., C. meneghiniana)* and the media was prepared according to Guillard's (1975) f/2 medium recipe. The seawater for the marine cultures was collected from coastal water (FAU Gumbo Limbo Environmental Complex and Nature Center, Boca Raton, Florida). Media were autoclaved (122º C and 2 atm.) after filtering. The addition of nutrients, including vitamins and trace metals were also based on Guillard's (1975) f/2 medium for the marine species *S. elongatus, T. weissflogii,* and *A. carterae.* Erdschreiber's (Schreiber, 1927) marine medium was used to culture *Dunaliella tertiolecta* and *Rhodomonas salina*.

Culture conditions: Light intensity (photosynthetically active radiation (PAR) radiation: 400 – 700 nm) was measured with a 4π spherical radiometer and Li-Cor LI-250 Light Meter. PAR transmission through the polycarbonate flasks was measured at 90% and subsequent measurements were adjusted by 0.9. Light levels are defined here as: high (HL: 180-200 μmol photons·m^{-2}·s^{-1}), moderate (ML: 70-75 μmol photons·m^{-2}·s^{-1}), low (LL: 35-37 μmol photons·m^{-2}·s^{-1}), and dim (DL:10 μmol photons·m^{-2}·s^{-1}). It is noted here that "high light" in this context refers only to the conditions utilized in this study and is not meant to mimic natural environment light conditions in surface waters (~ 1,500+/- μmol photons·m^{-2}·s^{-1}). For this study, light conditions were achieved within three separate temperature-controlled (25°C) growth chambers: a Revco-Harris growth chamber was used for the high light experiments, while two Precision Illuminator 818 growth chambers were used for the remaining light levels. All growth was achieved at 25°C with a 12-hour light: 12-hour dark diurnal cycle. Temperature control was observed within + 1.5°C. The samples in the two Precision growth chambers were illuminated from the front only (fluorescent tubes vertically attached to the inside door) with two 34W Econo (Philips) 120 cm long fluorescent tubes, covered by a diffuser screen for the medium light experiments and without a diffuser screen for the low light experiments. Samples for the high light experiments (Revco-Harris growth chamber) were illuminated from the top as well as both sides with sunlight quality (Verilux Instant Sun™), full Spectrum™ (ValuTek) and "aquarium" quality (Sylvania Gro-Lux™) fluorescent tubes. Three 8W (Westwek 20121) cool white, fluorescent tubes were attached horizontally on the inside door of the Precision growth chamber and used for illumination in the dim light experiments. Only one of the species in the study: *Synechococcus elongatus*, grew at the dim light level, therefore, dim light experiments were discontinued for the remaining species. All species exhibited increasing specific growth rate constants with increasing light intensity. This indicated that the light intensities used did not limit or inhibit the growth of the algal cells.

Cell counting: Cell counts were taken with a Coulter Counter model ZM electronic cell counter. Cell counting was done every two to three days during and on the same day that the algal samples were to be harvested. ISOTON® II diluent (electrolyte solution) was pipetted (20 mL) into the counting vial (Fisher 'Accuvette') and 100 μL of suspended algal cells were added. The three most consistent counts out of six were averaged and used as the corrected count. The dilution factor (DF) was determined, and the number of cells per milliliter was calculated by multiplying the corrected count by the di-

lution factor (DF x corrected counts = cell mL^{-1}). The following equation was used for calculating the dilution factor: [DF = (S + E) ÷ (MS x S)], where S = mL sample, E = mL electrolyte, MS = manometer setting. The number of cells per milliliter was determined from cell counting and the concentration of each analyte (pigments, proteins, carbohydrates, organic carbon) in pg/cell or µg/L could then be calculated. Samples for the analyses of chlorophyll-a, proteins, colloidal and storage carbohydrates were taken from cultures in the log phase of growth.

Analyses overview: Relationships between protein-to-CHLa, colloidal CHO-to-CHLa, and storage CHO-to-CHLa, in relation to light treatments were analyzed for each species. Cellular concentrations of chlorophyll-a, protein, and the two functional classes of carbohydrates, as well as their relationship to biovolume for each species, at each light level were determined. Throughout this report and in figures, the acronyms DL, LL, ML and HL will be used for dim, low, medium, and high light levels, respectively.

Pigment Analyses: All pigment, notably chlorophyll-a, analyses were carried out under dim, yellow light conditions to prevent photo-oxidative alterations such as pigment isomerization. For harvesting and for pigment monitoring during growth, cultures were filtered onto glass microfiber filters (Whatman GF/F, 0.7-micron pore size borosilicate glass fiber). The filters were removed from the filter funnel, folded in half, and blotted between paper towels. The filters were then folded into quarters, re-blotted and wrapped in aluminum foil, then immersed in liquid nitrogen for quick freezing. The individual samples were removed from the liquid nitrogen and stored in a refrigerator at -80ºC until extraction. The filters were extracted in pre-chilled glass tissue grinders (Kontes "Duall" 15 mL) as reported previously (Grant and Louda, 2010). The UV/Vis absorption spectra (350-800 nm) of the extracts were recorded on a Perkin Elmer Lambda - 2 UV/Vis Spectrophotometer, calibrated for wavelength and absorbance vs. holmium oxide. Pigments were separated via reversed phase high performance liquid chromatography (RP-HPLC) as previously reported (Grant and Louda, 2010; Louda 2008). This allowed full quantitation of total chlorophyll-a (CHLa) as the sum of chlorophyll-a, chlorophyll-a-epimer, chlorophyll-a-allomer, chlorophyll-a-allomer-epimer, chlorophyllide-a, and pyro-chlorophyllide-a, all of which would be commeasured by normal spectrophotometric (UV/Vis, fluorescent) measurements.

Algal protein extraction: The procedure was adapted from Rausch (1981) with some modifications, as reported herein: 100 mL aliquots of algal culture were filtered onto pre-combusted glass fiber filters. The filters were then folded in halves, then quarters and refrigerated at -80˚C until analysis. Analyses were performed within one week of filtering. Samples were extracted in 0.5 M sodium hydroxide (NaOH), by grinding the filters in 12 mL tissue grinders (glass mortar with Teflon® pestle e.g. Kontes Duall). The tubes were next heated at 80°C for 10 minutes to further extract the proteins. After this step, the tubes were quickly cooled to room temperature, then centrifuged (Fisher Scientific, Centrific Model 228) for 5 minutes. The resulting supernatant was transferred to 10 mL graduated tubes for subsequent protein analysis. A second extraction was carried out on the remaining filter debris (extraction in 0.5 M NaOH at 80°C for 10 minutes, followed by cooling and centrifugation), and the supernatants were combined in the 10 mL graduated tubes. A third extraction was carried out (0.5 M NaOH at 100°C for 10 minutes) for green algae and cyanobacteria, as prescribed by Rausch (1981). The combined supernatants were then made up to a definite volume (6-10 mL) with 0.5 M NaOH and used for protein measurement. This methodology was applied to all eight species of this study.

Algal protein measurement: The micro-biuret method for estimating proteins as adapted from Itzhaki and Gill (1964) was slightly modified. The procedure used is as follows: 2 mL of algal protein extract was assayed with 1 mL of 0.21% $CuSO_4.5H_2O$ in 30% NaOH at 310 nm in a 1 cm quartz cuvette and another 2 mL of algal protein extract was assayed with 1 mL of 30% NaOH at 310 nm in a 1 cm quartz cuvette. The absorbance of the protein was obtained from the difference between the absorbance of the sample in 30% NaOH and that from the reaction in 0.21% $CuSO_4.5H_2O$ in 30% NaOH. All samples were measured against a distilled water reference. Bovine serum albumin was used as the calibration standard.

Algal colloidal and storage carbohydrate extraction: The following method was used: aliquots of approximately 50 mL of the algal cultures in the logarithmic stage of growth were collected in 50 mL centrifuge tubes and centrifuged (Dynac Centrifuge, Becton Dickinson and Co, Parsippany N.J.) at 3300 rpm for 30 minutes. The supernatant was decanted to leave ~ 0.5-1 mL of wet cells, plus 2 mL of the supernatant, for colloidal carbohydrate analysis. The wet cells, minus supernatant, were re-suspended in 30 mL ultra-pure water (Milli-Q® Ultra-pure water systems, Millipore Corporation) and heated in a water bath for an hour, with stirring at 10-minute intervals. The solutions were then sonicated for 5 minutes (Burdloff et al., 2001), followed by pelleting the cells via centrifugation for 30 minutes. The resulting supernatant containing mostly the water-soluble storage carbohydrates was then filtered through 0.22 µm membrane filters (Fisher Scientific).

The filtrates were then lyophilized, and the dried material used for carbohydrate analysis.

Algal colloidal and storage carbohydrate measurement: The two extracted carbohydrate fractions were analyzed using the phenol-sulfuric acid assay (Dubois et al, 1956). The lyophilized samples were dissolved in exactly 2 mL of ultra-pure water and pipetted into 10 mL disposable test tubes. Using an extracted colloidal carbohydrate fraction as an example: exactly 2 mL of the initial supernatant was pipetted into 10 mL disposable test tubes. Next, 0.05 mL of 80% phenol was added to each tube followed by the rapid addition of 5 mL concentrated H_2S0_4. The tubes were allowed to stand for 10 minutes, after which they were placed for approximately 20 minutes in a water bath at 25-30°C with occasional shaking. The resulting champagne-dark orange solutions were then measured at 485 nm against distilled water in a Perkin Elmer UV/Vis Lambda 2 spectrometer. Alpha-D (+)-Glucose was used as the calibration standard.

Results and Discussion

The appendix contains Figures A1 - A8 which detail the concentrations of chlorophyll-a (CHLa), protein (PROT), colloidal carbohydrates (C-CHO), and storage carbohydrates (S-CHO) versus light intensities and includes cross plots of PROT, C-CHO and S-CHO versus CHLa concentrations. It is the cross plots versus CHLa that are suggested for use in generating a method for estimating these organic matter types from total CHLa data gathered in the field and/or laboratory. Plots of CHLa, PROT, C-CHO, and S-CHO versus light intensity contain data points that are the mean plus standard deviations of triplicate trials.

Linear regressions of PROT, C-CHO and S-CHO relationships to CHLa gave R-squared (r^2) values greater than 0.8 – 0.9 for all species except *A. carterae* ($r^2 \sim 0.2$) and *D. tertiolecta* ($r^2 \sim 0.6$-0.7), likely from natural variability in the sample. Table 1 contains the linear regression equations for the estimation of protein, colloidal carbohydrates, and storage carbohydrates from chlorophyll-a data in these eight species. Additionally, the averaged values of each estimation regression are given for each compound classification estimation. Protein to carbohydrate ratios from Table 1 averaged 1.9. As a comparison to our reported data, the ratios of protein to carbohydrates in the twelve species reported by Sassi et al. (2019) grown at 150 mmol photons $m^{-2} \cdot sec^{-1}$ averaged 1.4.

The potential use of this method as a tool to aid in the estimation of metabolizable biomass can now be assessed. For example, if a water body was sampled and found to have a total chlorophyll-a concentration of 8.8 mg/L, then PROT, C-CHO and S-CHO could be estimated to be: PROT = (202.5 x 8.8 mg/L) – 9.2 = 1,863.9 mg/L; C-CHO = (17.7 x 8.8 mg/L) + 0.4 = 156.2 mg/L; and S-CHO = (144.9 x 8.8 mg/L) – 6.2 = 1,268.9 mg/L. Total carbohydrates would then equal the sum of C-CHO and S-CHO or 156.2 mg/L + 1,268.9 mg/L = 1,425.1 mg/L.

It must be noted that growth conditions may alter total chlorophyll-a, protein and carbohydrate contents and ratios. The constitution of microbiomes, also called the interactome, is known to affect many aspects of the growth and constitution of *M. aeruginosa* (Cook et al., 2020) and likely applies to other species as well.

Table 1. Data compendium: Estimation of protein, colloidal carbohydrates, and storage carbohydrates from chlorophyll-a concentrations.

		Averaged relations to chlorophyll-a.			
	High Light		y = Colloidal	y = Storage	
	CHL-a	y =Protein	Carbohydrates	Carbohydrtaes	Protein
Organism	~ pg / cell	pg / cell	pg / cell	pg / cell	to Carbs
Synechococcus elongatus	7.2	y = 188.0xC - 54.9	y = 12.3xC - 3.5	y = 57.1xC - 5.6	**2.7**
Microcystis aeruginosa	0.6	y = 87.1xC- 3.2	y = 7.4xC - 0.1	y = 107.3xC-2.6	**0.8**
Thalassiosira weissflogii	10.5	y = 194.6xC - 2.4	y = 16.1xC - 1.8	y = 229.5xC - 33.1	**0.8**
Cyclotella meneghiniana	1.5	y = 132.9xC - 10.2	y = 20.9xC - 0.1	y = 242.8xC - 26.6	**0.5**
Scendesmus quadricauda	5.8	y = 116.8xC + 46.6	y = 6.6xC + 3.9	y = 14.1xC + 28.4	**5.6**
Amphidinium carterae	0.34	y = 319.6xC + 26.1	y = 51.6xC + 5.6	y = 242.7xC + 8.9	**1.1**
Rhodomonas salina	0.48	y = 357.6xC - 5.2	y = 18.8xC + 0.6	y = 151.2xC - 2.2	**2.1**
Dunaliella tertio;ecta	0.9	y = 223.5xC - 70.4	y = 7.7xC - 1.1	y = 114.7xC - 16.9	**1.8**
Averaged values	N/A	**y = (202.6 xC) -9.2**	**y = (17.7 xC) +0.4**	**y = (144.9 xC)-6.2**	**1.9**
		C = Chlorophyll-a			

Conclusion

Correlations between CHLa and biomass parameters (protein and two forms of carbohydrates) under the influence of light intensity were investigated to ascertain if this could aid biomass estimation, especially as it potentially pertains to food chain considerations. This may also assist the prediction of the generation of anoxia during and following algal blooms. That is, one could relate the standing crop of metabolizable organic matter (proteins and carbohydrates) to existing conditions of water depth, currents, dissolved oxygen trends and other parameters.

The data given in the Appendix (Figures A1-A8) reveals that CHLa per cell increased linearly up to 200 mmoles photons m^{-2} sec^{-1} except for *Amphidinium carterae* (Fig. A8). In this species CHLa stayed at 0.30 – 0.35 pg/cell across all light levels. However, protein and the carbohydrate concentrations did increase with light. Previously, we found that CHLa per cell levels off or decreases at light levels above 200-300 mmoles photons m^{-2} sec^{-1} in several species of cyanobacteria, chlorophytes, chrysophytes and prymnesiophytes (Grant and Louda, 2010). The estimation of colloidal carbohydrates is most likely affected by sampling. That is, extracellular polymeric substances (EPS), such as polysaccharides, are likely sloughed away from cells during sampling and filtration prior to analysis.

We realize that, in nature, the presence of multiple species or a predominance of a single species, such as during harmful algal blooms, will affect the resultant estimation of the quantities of protein and carbohydrates present and available for food chain considerations. However, we offer these results as a broad-brush method by which to estimate the standing crop of metabolizable phytoplankton organic matter.

Compliance with Ethical Standards

Conflict of interest: The author(s) declare no actual, potential, or perceived conflict of interest for this article.

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

Data availability: Data will be made available on request.

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APPENDIX (Figures A1 – A8)

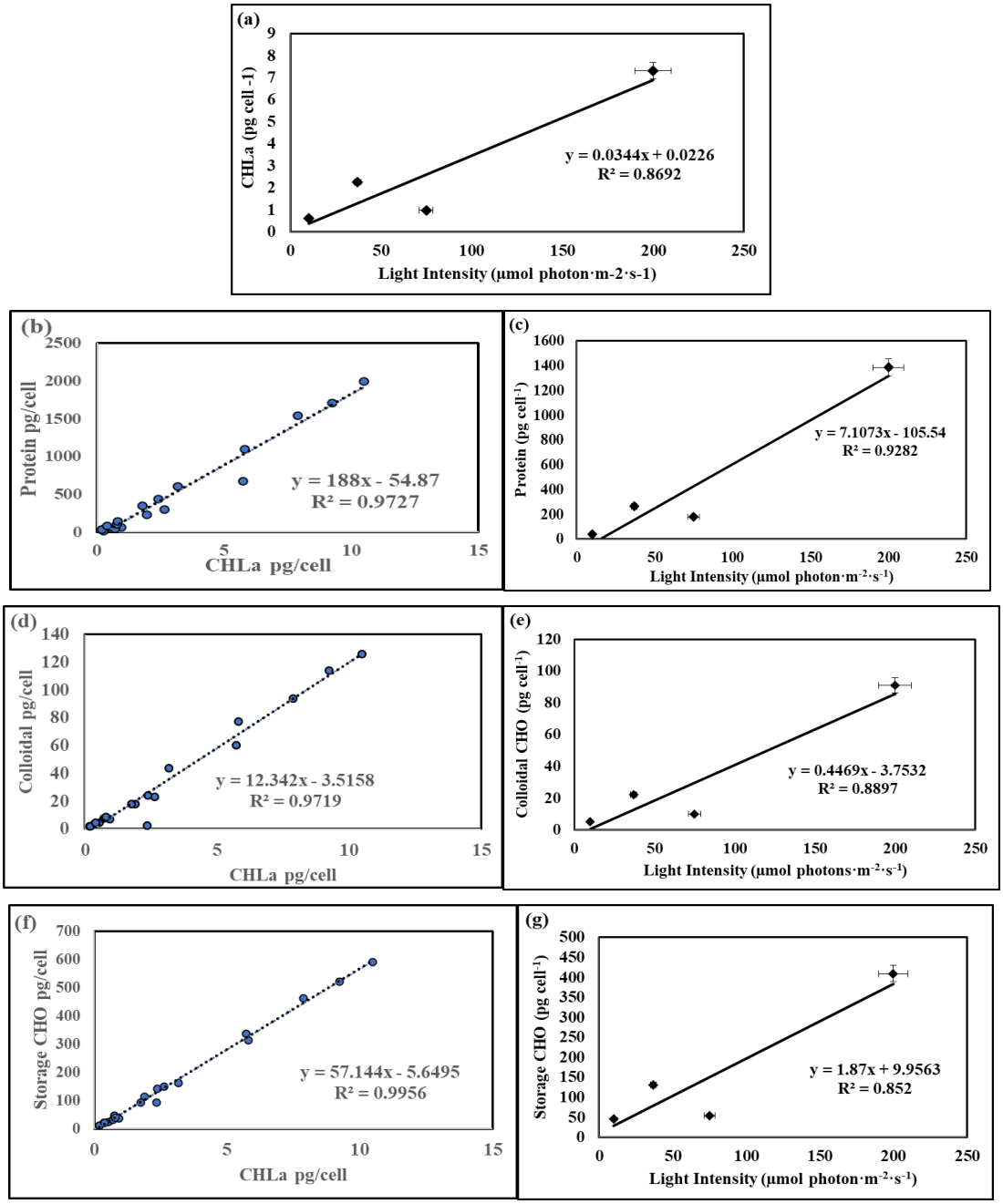

Figure A1: ***Synechococcus elongatus*** (a) Chlorophyll-a concentration per cell as a function of light intensity; (b) protein concentration related to CHLa; (c) Protein concentration as a function of light intensity; (d) Colloidal carbohydrates related to CHL-a; (e) Colloidal carbohydrate as a function of light intensity; (f) Storage carbohydrates related to CHL-a; (g) Storage carbohydrates as a function of light intensity.

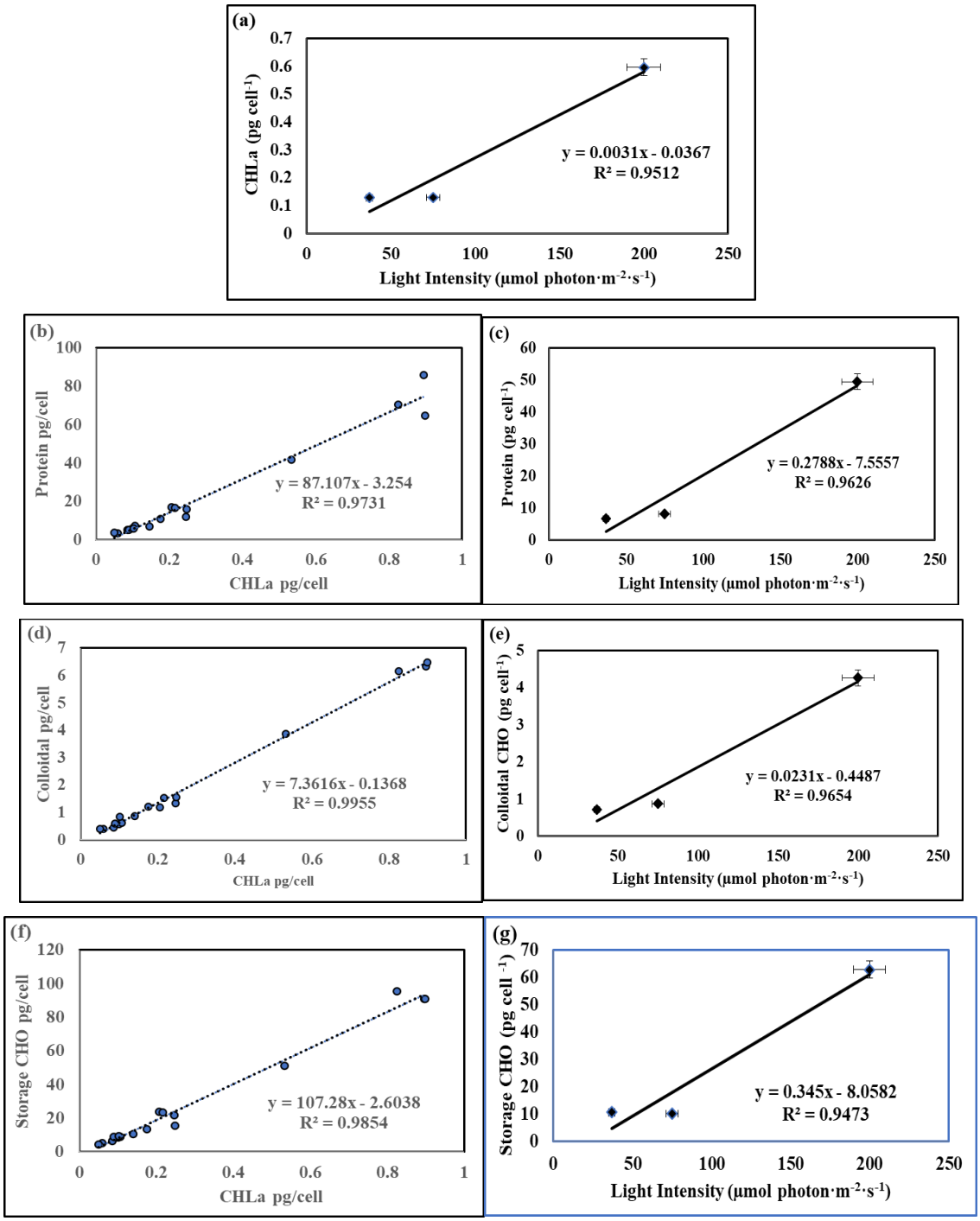

Figure A2. ***Microcystis aeruginosa*** (a) Chlorophyll-a concentration per cell as a function of light intensity. (b) protein concentration related to CHLa; (c) Protein concentration as a function of light intensity; (d) Colloidal carbohydrates related to CHL-a; (e) Colloidal carbohydrate as a function of light intensity; (f) Structural carbohydrates related to CHL-a; (g) Structural carbohydrates as a function of light intensity.

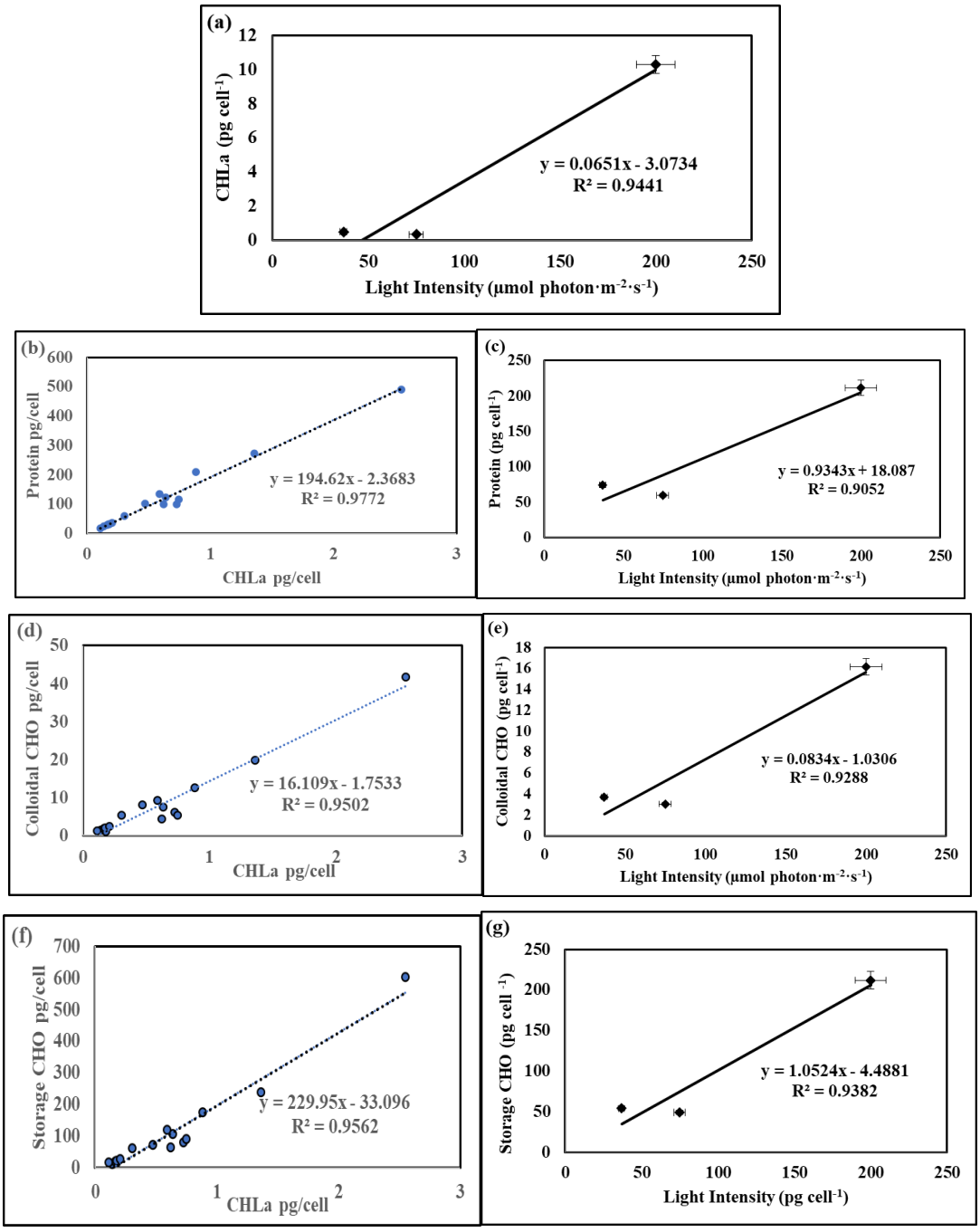

Figure A3. ***Thalassiosira weissflogii*** (a) Chlorophyll-a concentration per cell as a function of light intensity; (b) protein concentration related to CHLa; (c) Protein concentration as a function of light intensity; (d) Colloidal carbohydrates related to CHL-a; (e) Colloidal carbohydrate as a function of light intensity; (f) Structural carbohydrates related to CHL-a; (g) Structural carbohydrates as a function of light intensity.

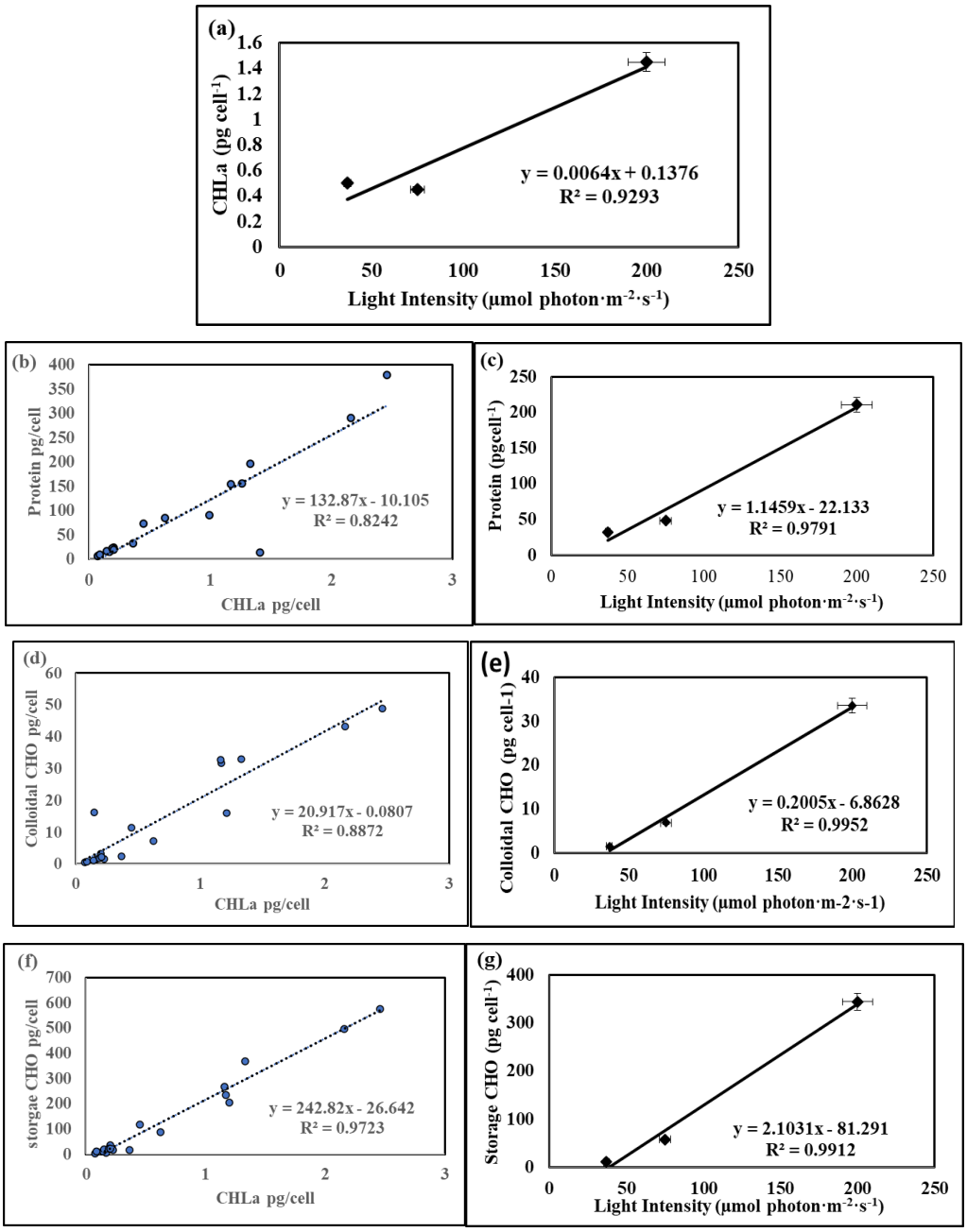

Figure A4. ***Cyclotella meneghininana*** (a) Chlorophyll-a concentration per cell as a function of light intensity; (b) protein concentration related to CHLa; (c) Protein concentration as a function of light intensity; (d) Colloidal carbohydrates related to CHL-a; (e) Colloidal carbohydrate as a function of light intensity; (f) Structural carbohydrates related to CHL-a; (g) Structural carbohydrates as a function of light intensity.

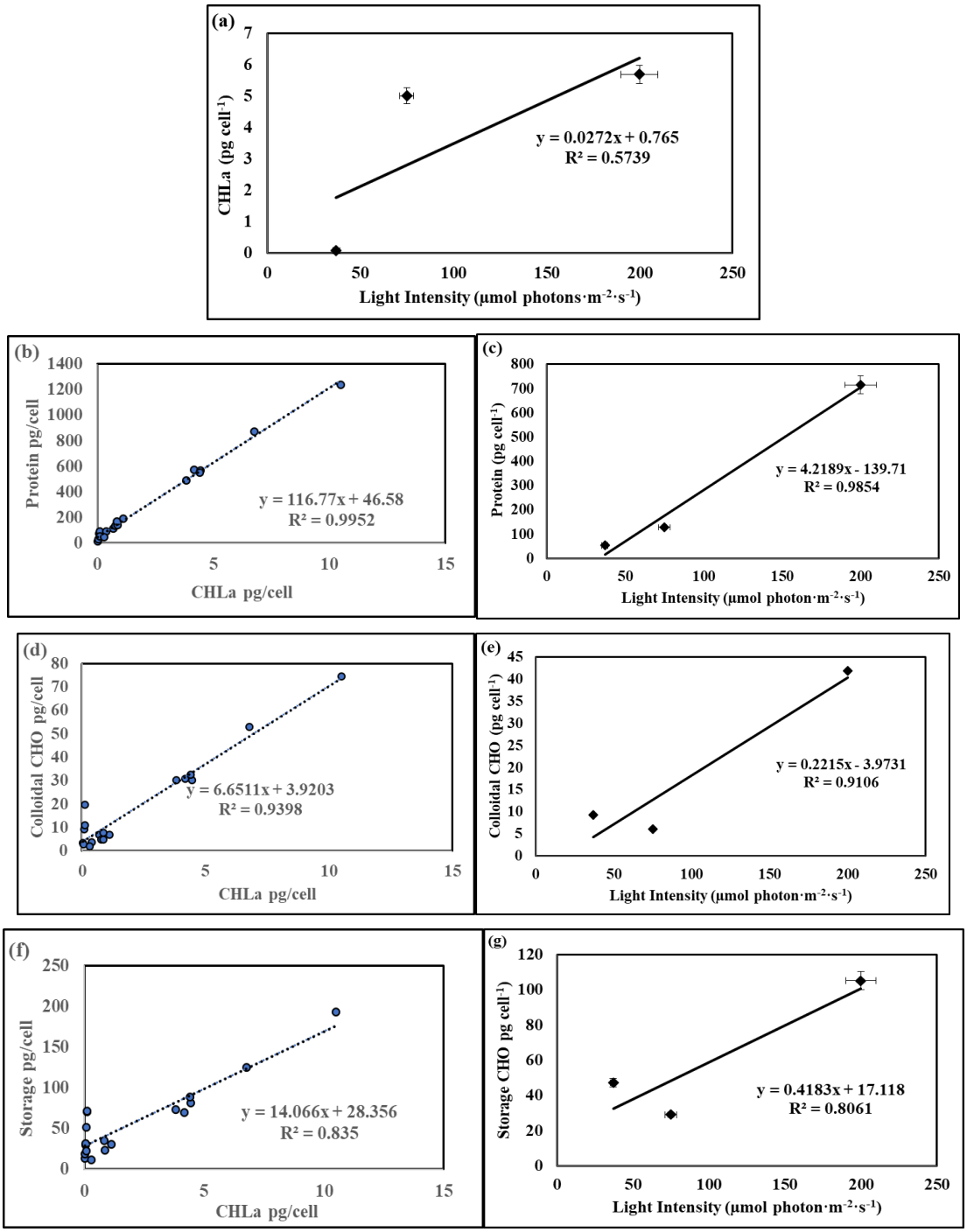

Figure A5. ***Scenedesmus quadricauda*** (a) Chlorophyll-a concentration per cell as a function of light intensity; (b) protein concentration related to CHLa; (c) Protein concentration as a function of light intensity; (d) Colloidal carbohydrates related to CHL-a; (e) Colloidal carbohydrate as a function of light intensity; (f) Structural carbohydrates related to CHL-a; (g) Structural carbohydrates as a function of light intensity.

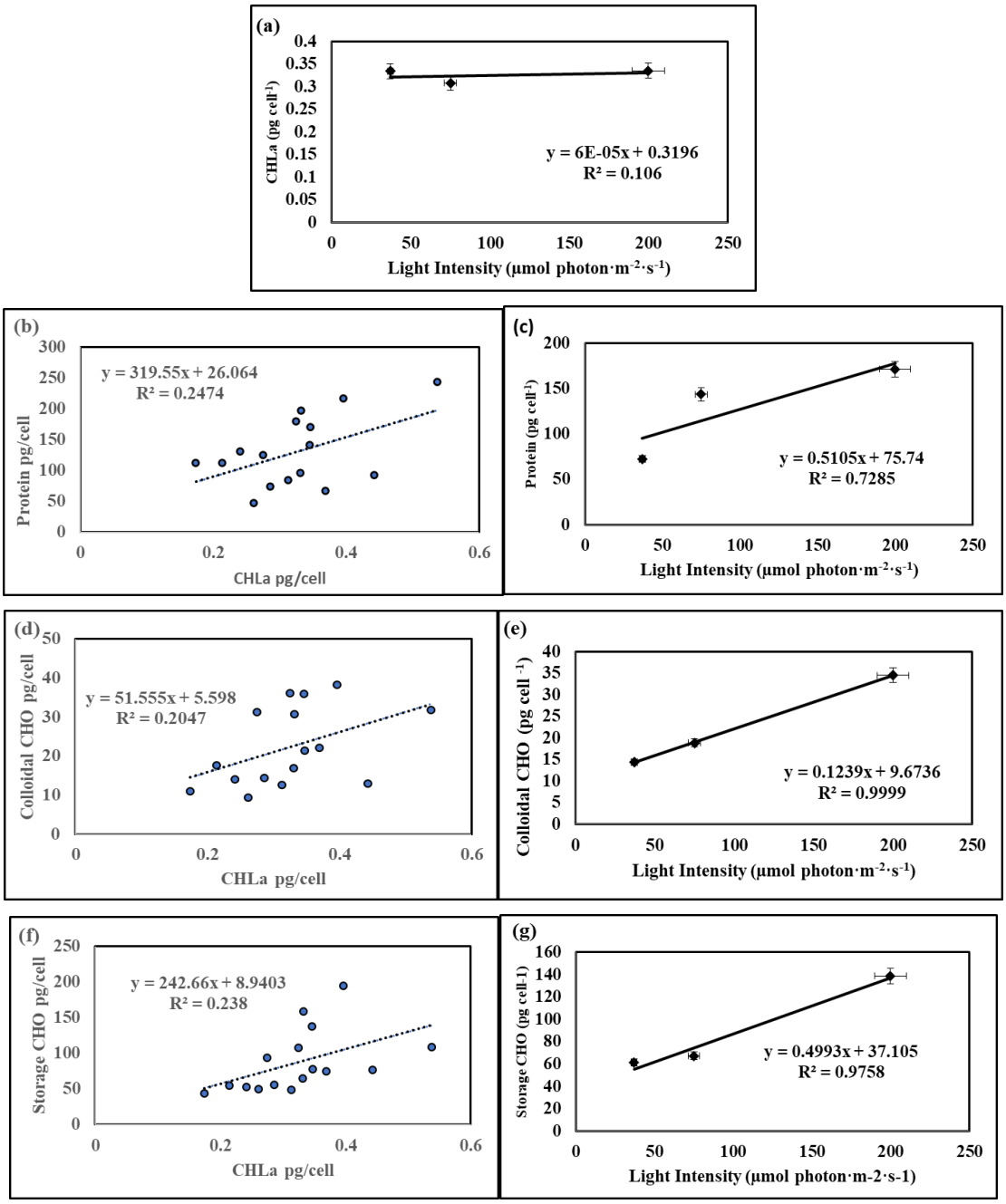

Figure A6. ***Amphidinium carterae*** (a) Chlorophyll-a concentration per cell as a function of light intensity; (b) protein concentration related to CHLa; (c) Protein concentration as a function of light intensity; (d) Colloidal carbohydrates related to CHL-a; (e) Colloidal carbohydrate as a function of light intensity; (f) Structural carbohydrates related to CHL-a; (g) Structural carbohydrates as a function of light intensity.

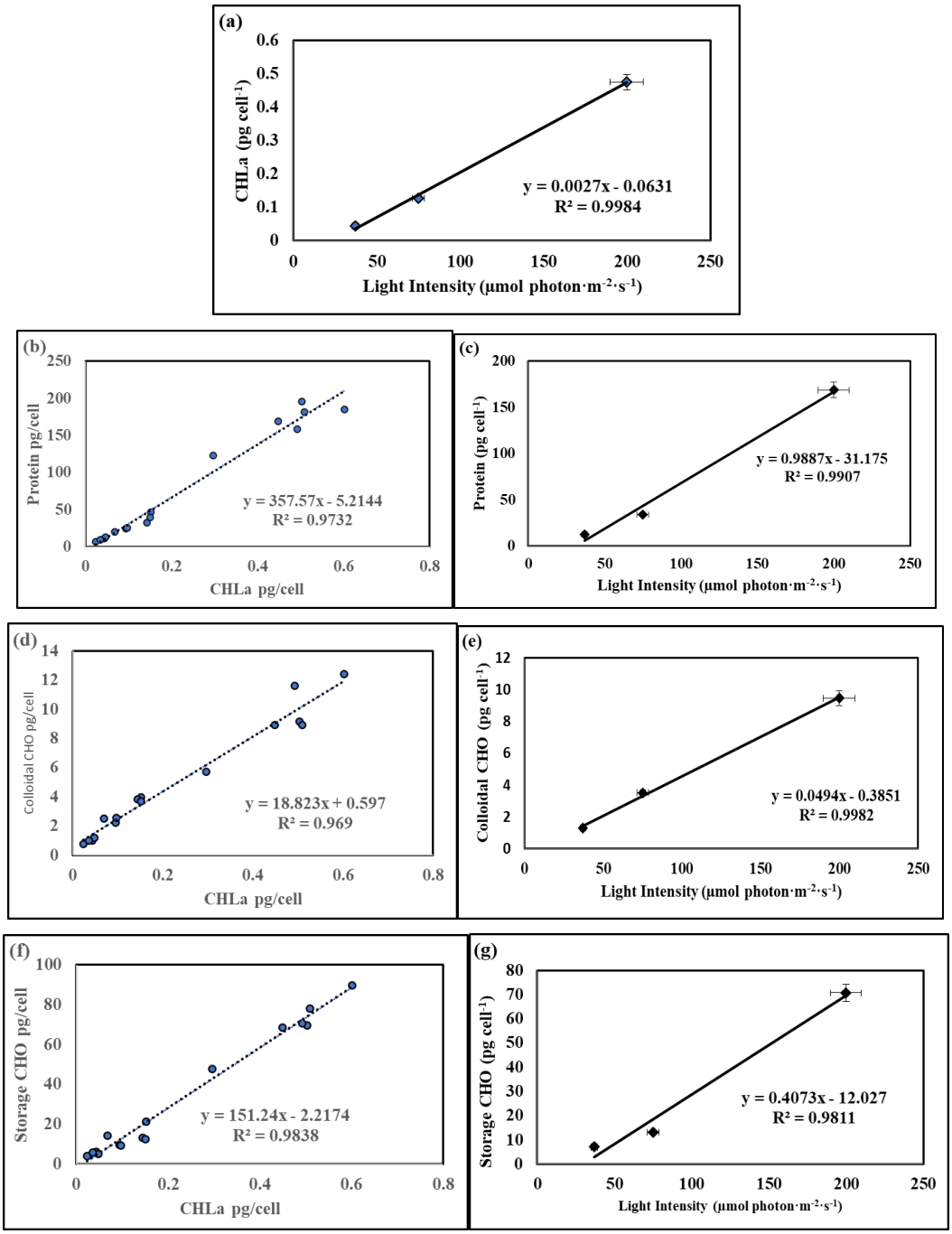

Figure A7. ***Rhodomonas salina*** (a) Chlorophyll-a concentration per cell as a function of light intensity; (b) protein concentration related to CHLa; (c) Protein concentration as a function of light intensity; (d) Colloidal carbohydrates related to CHL-a; (e) Colloidal carbohydrate as a function of light intensity; (f) Storage carbohydrates related to CHL-a; (g) Storage carbohydrates as a function of light intensity.

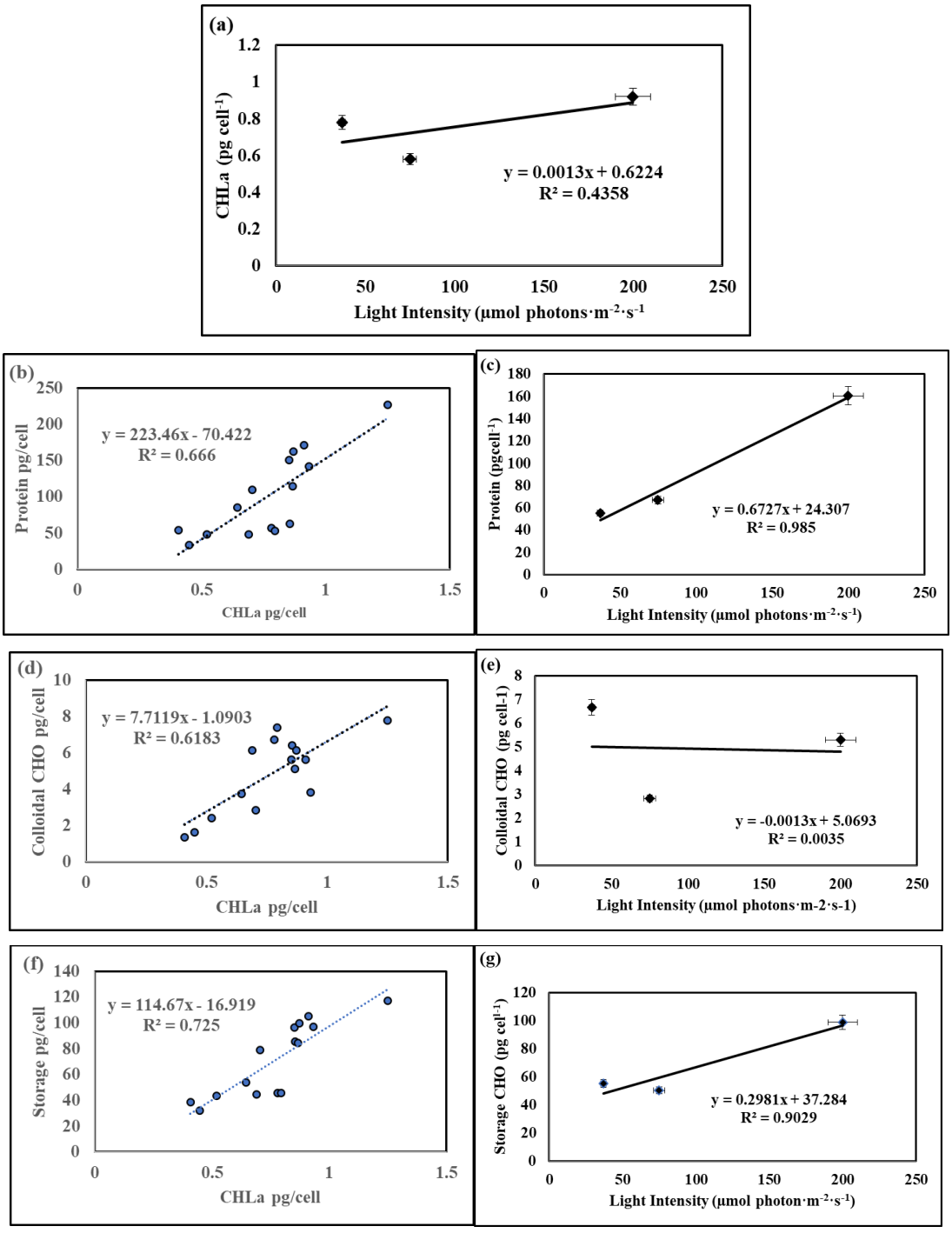

Figure A8. ***Dunaliella tertiolecta*** (a) Chlorophyll-a concentration per cell as a function of light intensity; (b) protein concentration related to CHLa; (c) Protein concentration as a function of light intensity; (d) Colloidal carbohydrates related to CHL-a; (e) Colloidal carbohydrate as a function of light intensity; (f) Structural carbohydrates related to CHL-a; (g) Structural carbohydrates as a function of light intensity.

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Assessing the water footprint of tea: Implications on Türkiye's freshwater ecosystems

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ABSTRACT

The relationships between freshwater systems and agriculture are complex, and they intersect in many ways. Human interference with nitrogen and phosphorus cycles has become so intense that may be the effect of nutrient enrichment in freshwaters. Thus, this study aims to assess current (2022) and future (2032) water footprint (WF) of tea production in Türkiye which is one of the major agricultural practices in the country and its effects on freshwater sources. The Water Footprint Network (WFN) suggested methodology for water footprinting was followed during the study. Results showed that rainwater (green water footprint) is the primary water source to grow the tea plant. The green water footprint (WF_{green} =877 m^3/ton) was followed by blue (WF_{blue}=142 m^3/ton) and grey water footprint (WF_{grey}=75 m^3/ton). This clarifies that there is no risk of producing tea in Türkiye in the near future due to the high green water footprint compared to blue and grey. Furthermore, freshwater systems have a low risk of nutrient pollution, as indicated by WF_{grey}. A further study with high-quality data including the amount and type of fertilizer used is therefore suggested.

Keywords: Agriculture, Freshwater, Tea cultivation, Water footprint, Water resource

Introduction

Life is dependent on water as a vital resource, maintaining ecological health and fostering socio-economic development (Barbosa & Cansino, 2022; D'Ambrosio et al., 2020). There are many pressures on water resources such as increasing population, uncontrolled discharges, excess water abstraction, global warming and climate change (D'Ambrosio et al., 2020). The increase in population triggers greater demand for food, thereby raising trade actions and escalating rivalry between sectors for water resources (Feng et al., 2023). Agriculture stands out as the primary sector using the largest portion of freshwater resources for irrigation and contributing to environmental degradation through diffuse pollution such as pesticides, fertilizers, etc. Humanity's pursuit of sustainability is significantly hindered by intense agricultural activities. Ensuring the preservation of both the amount and standards of water resources and setting a strategy for sustainable water management, it is pivotal to assess the sustainability of human activities (D'Ambrosio et al., 2020). To evaluate water use, Hoekstra & Hung (2002) have established the water footprint (WF) concept determining the use of total water resources including direct and indirect water consumption of producers or consumers. The water footprint has three components, which are green, blue, and grey water. The use of drinking water, irrigation water, and industrial water can be viewed as a blue water footprint. The green water footprint is the total volume of rainwater required to make a product. Grey water footprint refers to the amount of freshwater needed to absorb pollutants to ensure water quality meets standards (Hoekstra et al., 2011).

The leaves of the *Camellia sinensis* plant are the main source of tea and with significant historical, economic, and cultural significance tea is a commonly consumed drink in the world (Hu et al., 2019). Over the past decade, global tea production (including black, green, and others) has risen by an average of 3.2% annually, reaching 6.7 million tons in 2022. This growth has been predominantly by expansion in China, where production has surged by 5.9% annually, escalating from 1.92 million tons in 2013 to 3.34 million tons in 2022 (FAO, 2024). In the past ten years, global tea consumption has been rising at a rate of 3.3% annually, reaching 6.5 million tons in 2022. This increase has been driven by the swift growth in per capita income levels, particularly in China, India, and other Asian and emerging economies (FAO, 2024). Türkiye ranks as the fifth largest tea producer globally (Ozbayram, 2020). While the fresh tea crop yield varied between 1250 and 1400 tons on average in the last four years, the yield increased to over 1355 tons in 2023. In 2023, 1355 tons of fresh tea were processed yielding 270 thousand tons of dry tea (RTB, 2024). Under suitable climatic and soil conditions with effective management, the harvested leaf yield of tea can usually reach 4-5 t ha/year and could be higher (Hajiboland, 2017). Tea production is limited to 6-8 months in countries with relatively high latitudes, such as Türkiye, while tea cultivation is conducted for 12 months in tropical and equatorial regions.

The growing environmental awareness prompts people to inquire more frequently about the hidden natural resources within a product, especially water. This includes the water required for plant growth as well as the water needed to process the crop into its final form (Chapagain & Hoekstra, 2007). The annual freshwater consumption is around 54 x 10^9 m^3 (Altınbilek and Hatipoğlu, 2020) and Türkiye's total water footprint is dominated by the agricultural sector, which accounts for 74%. The majority of water usage in agriculture is used for crop production, with only 8% allocated for livestock grazing. Türkiye is considered to be a water-stressed country, with a water supply availability per person below 1500 m^3. This value will be reduced to 1120 m^3, which is lower than the average global water footprint of 1240 m^3 as the population of Türkiye is expected to reach 100 million by 2040 (Harmancioglu, 2020; Mekonnen and Hoekstra, 2011). Model projections indicate that water potentials in Türkiye will decrease by 16% and 27% by 2050 and 2075, respectively.

Effective water resource management requires the assessment of freshwater water contamination. The eutrophication of surface waters has become a worldwide problem with no end in sight. Biogeochemical flows, which include the nitrogen (N) and phosphorus (P) cycles, are going beyond the planetary boundaries (PBs) (Rockström et al., 2013). The phosphorus reserves will be depleted in less than 200 years (Schlesinger, 2009). Nitrogen fertilizer put on agricultural soils is transported through the environment, polluting water ecosystems, aquifers, rivers, and oceans and harming human health (Baldock et al., 2023). The degradation of freshwater, estuarine, and marine ecosystems is widespread, but it is our freshwaters that are most at risk due to their widespread use (Withers, et al., 2014). Staying within local or global freshwater boundaries can be achieved by diminishing water demand and increasing water use efficiency, which includes the choosing products that require less water and sustainable agriculture practices. Previous studies in the literature have calculated the water footprint of different crops in Türkiye (Muratoglu and Avanoz., 2021; Mekonnen MM, Hoekstra AY 2011) and tea production in various parts of the world (Jefferies et al. 2012 A.K.; Chapagain and A.Y. Hoekstra., 2003). However, they have not focused on the risk of nutrient pollution in freshwater systems due to crop production patterns.

The objective of this study is to evaluate the current (2022) and future (2032) water footprint of tea production in Türkiye and its effect on freshwater resources.

Materials and Methods

Study Area

The analysis of tea cultivation regions in Türkiye indicates that Rize holds the highest share at 74.9%, followed by Trabzon at 13.2%, Artvin at 8.9%, Giresun at 2.3%, and Ordu at 0.6% (Figure 1).

Calculation method and equations of Green and Blue Water Footprints

The present study was carried out in accordance with the Water Footprint Network (WFN) proposed Water footprint methodology (Hoekstra et al., 2011). Tea production, statistics on the climate data and soil parameters in Türkiye were used to calculate water footprint or crop water use CWU (m^3/yr) (Figure 2).

When producing a particular crop, the total water usage is known as CWU (m^3/yr). Measuring or estimation of evapotranspiration and irrigation amounts is required to accurately estimate CWU. Evapotranspiration is the process of water being transferred from the soil surface (evaporation) and plants (transpiration) to the atmosphere. According to the CWR model, the crop's blue water needs (irrigation) is zero if effective rainfall exceeds the total crop evapotranspiration. The blue water requirement is a term that defines how much water is required between crop evapotranspiration and effective rainfall. The water footprint of a product consists of the sum of freshwater utilized to produce the product, calculated across the various stages of the production chain. Water footprint has been calculated according to Hoekstra et al. (2011) in the Eqs. (1) – (2):

$$WFgreen = \frac{CWUgreen}{Yield} \quad (1)$$

$$WFblue = \frac{CWUblue}{Yield} \quad (2)$$

The green water requirement corresponds to the portion of the plant's water needs that is fulfilled by effective rainfall. When the effective precipitation (P_e) exceeds or matches the plant's water consumption, the green water footprint is equal to the plant's water consumption. Green and blue water footprint components of plant water consumption on a geographical basis (m^3/ ha) are values based on the sum of the daily plant water need/evapotranspiration (ET, mm / day) amounts of the plant in the growing season. The green water consumption of the plant represents the amount of rainwater lost from the plant through evapotranspiration during the growing season. Blue water consumption is the sum of irrigation water lost due to evapotranspiration Eqs. (3) - (5):

$$ETblue = \{ETc - Peff\ ;\ ETc > Peff\ 0;\ ETc < Peff \quad (3)$$

$$ETgreen = \{Peff\ ; ETc > Peff\ ETc; ETc < Peff \quad (4)$$

$$Peff = \{\frac{P(125-0.2P)}{125}; P \leq 250\ mm\ 125 + 0.1P; P > 250\ mm \quad (5)$$

Figure 1. Tea production in Türkiye (thousand tons per year for each province) (TSI)

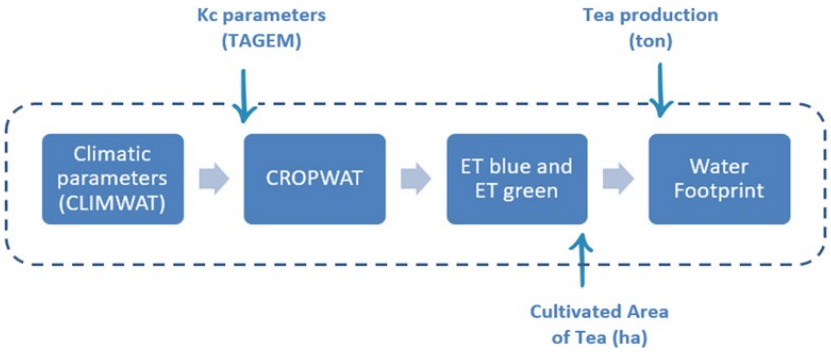

Figure 2. Calculation method of green and blue water footprints (WFN)

Data Inventory and Software

CLIMWAT and CROPWAT software were used for calculations involving blue water and green water. The publication CLIMWAT 2.0 for CROPWAT is jointly produced by FAO's Water Development and Management Unit and Climate Change and Bioenergy Unit. CLIMWAT 2.0 has a comprehensive climatic database that covers more than 5,000 stations worldwide (FAO, 1999a). CROPWAT 8.0 is a tool that can assess farmers' irrigation activities and assessing crop performance in both rainfed and irrigated situations (FAO, 1999b). In this study, the actual amount of irrigation water used in all tea was regarded as equivalent to the irrigation water need calculated by the CROPWAT model.

The ET_0 data used to calculate crop evapotranspiration was derived from long period averaged weather data including the temperatures, humidity, wind speed, solar radiation, and sunshine hours for a given day are listed as daily minimum, average, and maximum temperatures, relative humidity, wind speed, solar radiation, and sunshine hours (Table 1-2).

Table 1. Climate data (CLIMWAT)

	Artvin	Giresun	Ordu	Rize	Trabzon
	Annual Average				
Min Temp (°C)	7.7	11.5	10.6	11.2	10.8
Max Temp (°C)	16.9	17.9	18.6	17.9	17.8
Humidity (%)	65	74	73	80	77
Wind (km/day)	91	73	132	45	104
Sun (hours)	6	5.5	5.9	3.3	3.8
Rad (MJ/m²/day)	13.9	13.5	13.9	10.8	11.5
ET_o (mm/day)	**2.28**	**2.18**	**2.44**	**1.78**	**2.03**

Table 2. Effective rainfall (CLIMWAT)

	Artvin	Giresun	Ordu	Rize	Trabzon
	Eff rain (mm)				
January	65.2	97.9	81.9	143.4	62.9
February	59.8	76.5	67.5	124.2	48.5
March	51	76.3	66	111.9	50.2
April	55	70.5	60.6	85.4	51
May	48.5	59	46.8	86.7	47.7
June	45.2	68.3	65.2	100	47.7
July	23.1	66	62.2	105.3	32.2
August	25.8	77	59.8	130.6	40.9
September	32.2	92.4	66	143.4	62.2
October	51.8	118.8	100.6	152.2	90.6
November	66	110.1	95.7	150	78.5
December	84	103.7	98.8	147.4	72.7
Total	**607.6**	**1016.6**	**871.2**	**1480.5**	**685**

The purpose of CROPWAT software is to estimate crop water and irrigation needs of various plants using plant patterns, soil, and climate data. The Penman-Monteith method was used (CROPWAT 8.0 model) to calculate reference plant water consumption (ET_0) utilizing daily, 10-day, or monthly climate data Eq. (7):

$$ETo = \frac{0.408\Delta(Rn-G)+\gamma 900T+273u2(es-ea)\Delta}{\gamma(1+0.34u2)} \quad (7)$$

Estimating actual evapotranspiration is vital not only for the study of climate change but also for calculating crop water needs. ET_0 is the standard measure of evapotranspiration, R_n is the the crop surface's net radiation, the density of soil heat flow is G, T is the mean daily air temperature at 2 m height, u2 is the wind speed at 2 m, Δ represents the gradient of the saturation vapour pressure curve e_s is the saturation vapour pressure, e_a is the actual vapour pressure, (e_s – e_a) is the variance in vapor pressure , and γ is the psychrometric constant. Crop evapotranspiration (ET_C) is given in Eq. (8):

$$ET_C = K_C x ET_0 \quad (8)$$

ET_0 and crop coefficients (K_c) are the primary factors that affect water footprint, with blue water footprint being more sensitive than green water footprint (Zhou et al., 2014). The K_C is the crop coefficient for a particular crop and is typically determined by experimentation (Table 3).

Table 3. Tea coefficients (Kc) (TAGEM, 2017)

	Artvin	Giresun	Ordu	Rize	Trabzon
Kc-int	0.8	1	0.88	1.06	0.82
Kc-med	0.95	0.91	0.93	0.91	0.94
Kc-end	0.95	0.92	0.94	0.92	0.96

Turkish Statistical Institute (TSI) database was used to determine the provinces that produced tea in Türkiye in 2018 and 2022 in the study (Table 4). Then, based on the current regional information (tea production amount, precipitation data obtained from climate stations, irrigated cultivation area) for 2018 and 2022, covering the provinces in question, the blue water footprint (irrigation water) and green water footprint (rainfall) of tea production were determined. The only alteration made in 2032 was the quantity of tea production. The current global market situation and medium-term prospects indicate that tea production will increase by 2.4% in 2032 (FAO, 2024). The climatic conditions were assumed to be the same as those in 2022 for the CROPWAT software.

Grey Water Footprint

The greywater footprint calculations were determined by using general approximations and assumptions due to fertilizer application yields, leaching fractions, and maximum permissible concentrations of pollutants that are not point sources (A. Chapagain & Hoekstra, 2003; Yi et al., 2024). Nitrogen and phosphorus leaching have a significant impact on gray water footprints. Previous research that did not include pesticides underestimated the impact of crops on grey water footprints. Therefore, greywater was calculated using literature data that uses N, P, and various pesticide combinations for over a dozen major crops (including tea) in Chinese provinces (Yi et al., 2024) Eq. (9). Freshwater bodies were assumed to have a maximum acceptable concentration of 10 mg N/L and 0.2 mg P/L. The fertilizer N application of 2353 (kg N/ha) and the fertilizer P application of 23 (kg P_2O_5/ha) were assumed for tea. 0.1 mg N/L and 0.01 mg P/L were established as natural concentrations, while pesticides were set to zero. The leaching and runoff fractions for pesticides have been established at 0.001 (α_{min}) and 0.1 (α_{max}) respectively (Franke et al., 2013).

$$WFgrey = \frac{(\alpha\ x\ AR)/(Cmax-Cnat)}{Yield} \quad (9)$$

AR; Chemical implementation amount (kg/ha), α- fraction of leaching runoff, C_{max}-maximum permissible concentrations (kg/m^3), C_{nat} –natural concentration for the pollutant (kg/m^3), Y is the crop yield rate (ton/ha).

Table 4. Tea production, import and export (ton) (Türkiye, TSI)

Year	Fresh Tea (ton)	Import (ton)	Export(ton)	Hectare
2018	1,480,534	94,000	13,000	78,133
2019	1,407,448	59,000	17,000	78,569
2020	1,450,556	74,000	18,000	78,681
2021	1,453,964	101,000	23,000	78,900
2022	1,269,546	55,000	28,000	79,129
2032	1,300,015	56,320	28,672	79,129

* Water footprint calculations included the exported tea product that was produced using the country's water resources.

Results and Discussion

Water Footprint of Tea Production in Türkiye

The objective of calculating the water footprint geographically is to provide information on the potential of certain agricultural production practices to reduce water scarcity and improve water quality. These can be listed as plant characteristics, climate, plant growing plan (number of days grown), environmental variables such as soil properties, agricultural management practices and human impact such as irrigation.

The amount of water required to make a single unit of tea is known as the virtual water content of tea. Typically, the calculation and representation of tea is done in cubic meters of water per ton of tea. Agricultural water resource allocation varies between production and consumption perspectives in different regions due to differences in crop types, production capacities, and water needs. Trading virtual water between provinces is a key method for balancing the disparities between food production and consumption, and it significantly influences the agricultural water footprint (Cao et al., 2023). The tea produced in Türkiye is consumed extensively by its population. The water footprint of production (1.6×10^9 m^3/year) is significantly higher than that of exports (3.5×10^7 m^3/year) and imports (6.9×10^7 m^3/year) for this reason (Figure 3). Import and export water footprints were calculated solely on the amount of tea, and the effects of transportation and storage during the supply chain were not taken into account. Including these processes will result in higher values, but they will still be very low in comparison to the production water footprint.

Research on water usage in comparable products is limited. However, existing studies have varied in their scope. For instance, Chapagain and Hoekstra concentrated on water consumption during the agricultural phase and extended their scope to to encompass packaging and the consumer phase. In the Netherlands, coffee and tea consumption has beneficial effects on the economies of the producing countries. Developing countries, which are mostly producing countries, benefit economically from the utilization of a resource (rainwater) that has a reduced opportunity price relative to groundwater and surface water. The financial value of rainwater could be taken into account in the product price, even though it may not have a higher economic return when used for coffee or tea production. (Chapagain & Hoekstra, 2007).

Due to inter-regional virtual water flows, water scarcity can become more severe in crop-exporting regions while easing in crop-importing regions. A study carried out by Jefferies et al. 2012 showed that green water footprint of fresh tea was in the range of 880- 2214 m^3/tons. In a previous study, the virtual water of fresh tea in Türkiye was determined as 1828 m^3/ton for the period 1995–1999 (Chapagain & Hoekstra, 2007). According to Muratoglu and Avanoz (2021) tea production in Türkiye has a water footprint of 526 m^3/ton, which excludes grey water footprint. The total water footprint amount was found as 1094 m^3/ton in this study. While green water composed 80% (WF_{green} =877 m^3/ton) of the water content, the blue water and grey share were around 13% (WF_{green} =142 m^3/ton) and 7% (WF_{green} =75 m^3/ton) between 2018 and 2022. The results reveal that green water accounted for the greater share of fresh tea production in Türkiye.

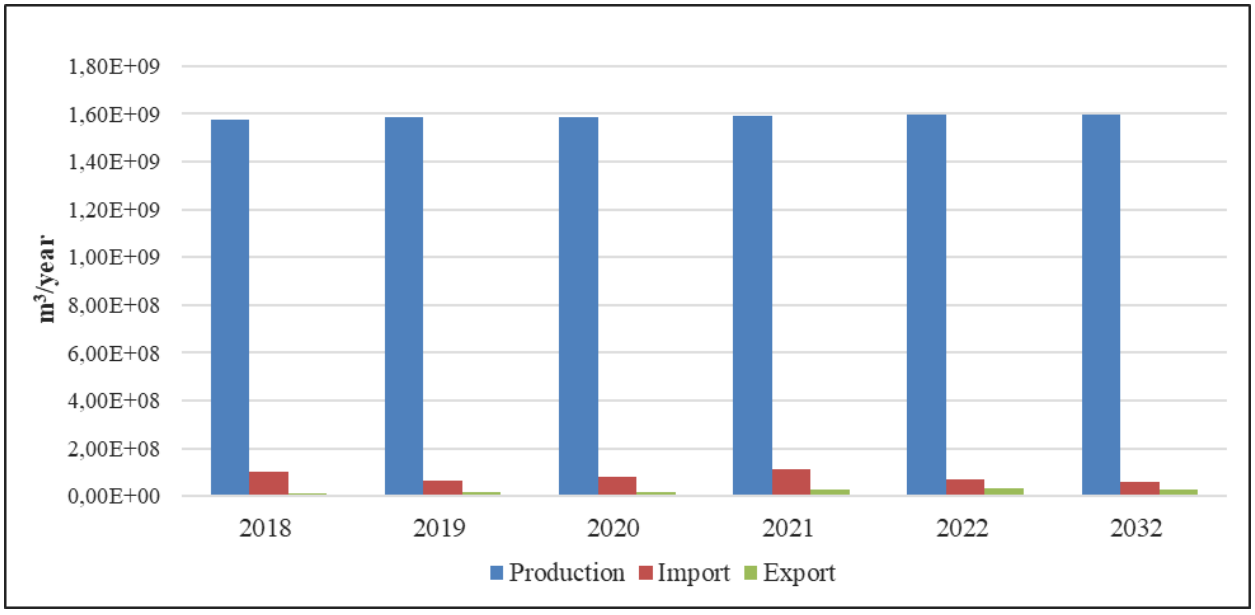

Figure 3. Water footprint **tea** by activities -Türkiye (m^3)

Green water is crucial in the water cycle and is essential for the production of rain-fed crops (D'Ambrosio et al., 2020). Crop cultivation relies more on green water from rainfall than on blue water from irrigation systems (Cao et al., 2023). Since tea is predominantly cultivated under rain-fed mono-cropping systems, weather conditions are crucial for optimal growth which makes it highly vulnerable to climate change, especially global warming. However, in 2032, the calculations revealed a slight difference between green and blue water in tea production. Total green, blue and grey water footprint were determined as 1.28 x 10^9 m^3/year, 2.06 x 10^8 m^3/year and 1.10 x 10^8 m^3/year in 2022 (Figure 4). There won't be any significant changes to the water footprint in 2032.

Implications on Türkiye's Freshwater Ecosystems

Developing water resources at a national scale requires integrated strategies to balance demand and available water, while also considering the declining trend in water availability. The use of irrigated agriculture in developing countries, where water extraction is often unregulated, puts more pressure on available freshwater resources (Sikka et al., 2022). Irrigated areas will increase in the future and more freshwater will be diverted from agriculture. The use of fertilizers in agricultural practice today is characterized by their misuse (Chartzoulakis & Bertaki, 2015). Water bodies have higher loads of nitrogen, phosphorus, and pesticides due to reduced water stream flows. This results in a change in the state of water quality, which means an increase in the concentration of pollutants in water ecosystems (Evans et al., 2019). The contamination of nitrogen and phosphorus by tea production, particularly in regions with intensive cultivation, can have a significant impact on freshwater systems.

Nitrogen and phosphorus are essential for the survival of life. However, they are being absorbed beyond their capacity. The world consumes twice as much nitrogen fertilizer as it requires, and the shortage of phosphorus is increasing at the same time (Helmholtz Centre for Environmental Research, 2022). Improving the chemical and ecological quality of numerous waterbodies impacted by farming remains a challenge for mitigating nutrient loadings from agriculture due to their diffuse nature (Withers et al., 2014). This could be a result of the inadequacy of controls on nutrient transfers from agricultural fields. The excessive use of nitrogen fertilizers results in nitrogen leaching, soil acidification, and runoff, while phosphorus fertilizers cause surface runoff and soil erosion. These pollutants also cause eutrophication, which results in excessive algae growth, oxygen depletion, and loss of biodiversity in aquatic ecosystems. The presence of phosphorus in environments leads to severe algal blooms, which can almost prevent freshwater sources from functioning. The growth of beneficial aquatic plants could be eliminated as a result of algal blooms reducing sunlight to freshwaters, particularly still waters such as lakes. Even without algal blooms, nutrient enrichment from nitrogen compounds can lead to problems such as blue baby syndrome at certain concentrations (Ohio EPA, 2011). To mitigate these effects, sustainable fertilizer practices, buffer zones, and improved soil management can be implemented to reduce nutrient runoff and leaching. Sustainable agriculture can be promoted, and the tea industry's environmental impact can be minimized by adopting these measures. In addition, water quality standards (including limit values for nitrogen and phosphorus) have been developed to protect not only human health but also freshwater sources and marine systems (Liu et al., 2017).

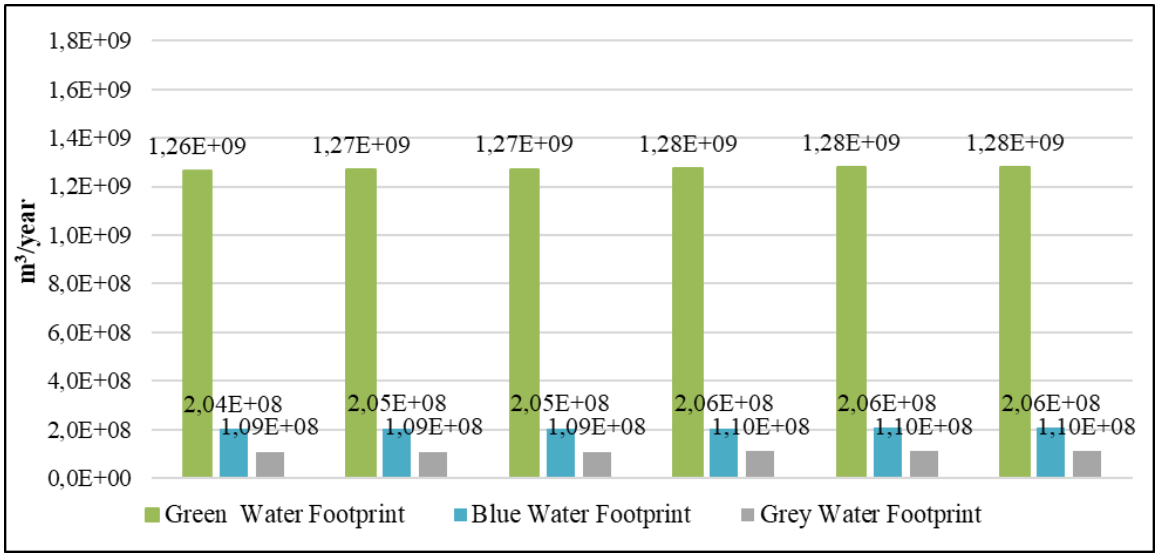

Figure 4. Total water footprint **tea** -Türkiye (m^3)

The majority of nitrogen and phosphorus contamination in Turkish freshwater sources comes from agricultural runoff, industrial discharges, and urbanization. Freshwater systems can be greatly affected by nitrogen and phosphorus pollution caused by tea production, especially in areas with intensive farming. Tea production has a water footprint of 1.6 x 10^9 m^3/year, which is equivalent to 3% of the country's total annual freshwater consumption in Türkiye. Water supply availability in Türkiye is projected to decrease according to models, while tea production patterns will increase. However, low WF_{grey} results compared to blue and green clarify that there is no risk of producing tea in Türkiye in the near future. Freshwater systems are less prone to being polluted with nutrients due to tea production.

Limitations of the Study

This research contributes to the existing literature by calculating the water footprint of tea production and its relations with freshwater resources. However, there are still some limitations, even though a detailed assessment has been conducted. Firstly, monthly data were used for modelling, but detailed reports on evapotranspiration, crop coefficients, and other meteorological variables on a daily basis will be more related in future studies (Nana et al., 2014). Secondly, the annual variations of WF_{grey} are mainly investigated using detailed temporal data of actual fertilizer application by farmers and runoff leaches. Due to the absence of necessary data, WF_{grey} was accepted as the constant throughout the year. Finally, WF_{grey} was calculated using maximum acceptable concentrations of 10 mg N/L and 0.2 mg P/L in freshwater bodies (Yi et al., 2024). Since, grey water calculation is based on the required water for assimilating the pollutants, to bring water pollution in the same unit as a consumptive use, the results of WF_{grey} will differ if a stricter water quality standard is implemented. For instance, these results are complied with Class II: water with low contamination, quality criteria of inland waters in Türkiye but not with Class I: high quality water.

Conclusion

The increasing water scarcity is becoming a more pressing issue, resulting in a shift towards more sustainable systems. The key to creating strategies for saving freshwater resources lies in developing robust results that estimate the future environmental impacts of nations' consumption. Türkiye is a country that is experiencing high water stress. Thus, the county has to manage its finite water resources effectively while protecting the environment and maintaining water quality. By changing local cropping patterns and altering global trade, significant amounts of water can be conserved. Türkiye's water consumption is mainly due to production, not exporting and/or importing. The production stage should be the priority rather than the trade. Thus, the good agricultural practices should be implemented in the field such as adoption of innovative irrigation techniques, using marginal waters (e.g. treated wastewater), optimizing water pricing policy. By providing information to local authorities about rainwater, irrigation, and freshwater usage, this study is expected to make an impact on agricultural water management studies.

Türkiye's tea production has a total water footprint of 1094 m^3/ton, whereas the grey water footprint only covers 75 m^3/ton, as revealed by this study. Grey water footprint results indicate that tea production for freshwaters in Türkiye will not be risky in the near future. However, a further study with high-quality data including the amount and type of fertilizer used will clarify how the freshwater systems effected by the risk of nutrient pollution coming from tea production. The impact of transportation and storage in the supply chain and the calculation of grey water footprint using high-quality data may be explored in future research.

Compliance with Ethical Standards

Conflict of interest: The author(s) declare no actual, potential, or perceived conflict of interest for this article.

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

Data availability: Data will be made available on request.

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Disclosure: -

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Biodiversity of marine macroalgae in Oran coast (Algerian west coast – Mediterranean Sea)

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ABSTRACT

This work aims to establish the current state of knowledge of the macroalgae on the Oran coast. This research used a survey method using three stations (Marsa El Hadjaj, Kristel and Ain Franine). Sampling was taken out in the intertidal zone of the coastal area. The study was conducted from April to May 2022. Floristic studies have been mainly focused on identifying the algae growing on rocky substrate. The results showed that there were 22 species of macroalgae grouped into 12 orders, 13 families, and 3 divisions: 5 Phaeophyceae, 8 Ulvophyceae and 9 Florideophyceae. The Ain Franine site is the most diverse, followed by Marsa El Hadjaj and Kristel with (21, 16 and 10 species) respectively. With a Global Average Cover of 47.80%, the Florideophyceae dominate the site of Marsa El Hadjaj, and the Chlorophyceae dominate at the sites of Ain Franine and Kristel with (52.53%; 48.35% and 48.35%) respectively.

Keywords: Macroalgae, Phaeophyceae, Ulvophyceae, Florideophyceae, Coastal ecosystems, Algerian west coast

Introduction

Macroalgae have long been used as biological indicators of marine ecosystem health worldwide due to their ecological importance and sensitivity to environmental stress (Su Jin et *al.,* 2023). Benthic macroalgae form a rather heterogeneous group of primary producers, including three major divisions based on the nature of their pigments and other fundamental characteristics: Phaeophyceae (brown algae), Ulvophyceae (green algae) and Florideophyceae (red algae), (Kokabi and Yousefzadi, 2015). Macroalgae play a key role in maintaining the ecological balance of the aquatic environment and are an indicator of the state of the marine environment. Macroalgae populations are influenced by various factors that define their spatial and temporal distribution in different habitats and regions (Hernández-Casas et *al.,* 2024). The diversity of algae in aquatic environments can help assess the health of ecosystems, provide information on invasions of new species and inform us about changes in species diversity based on environmental conditions (Birje, et *al.,* 1996; Ramdani et *al*., 2020). Macroalgae provides a habitat for several marine organisms, such as crustaceans, molluscs, echinoderms, small fish, and other small algae. The distribution of macroalgae in waters is influenced by various environmental factors such as human and anthropogenic factors (Mushlihah *et al.,* 2021). It is in this context that the study aims to determine the diversity and structure of macroalgae at 3 sensitive sites (Marsa El Hadjaj, Kristel and Ain Franine), in the Oran Coast (Algerian west coast). The ecological characterisation of communities is a fundamental part of using and exploiting resources, including seaweed.

Materials and Methods

Study Area

Our study area located on the coast of Oran, has 3 sites (stations) of ecological interest prospected over the period April - May (2022). Our study area located on the coast of Oran, has 3 sites (stations) of ecological interest prospected, in order to better understand the algal components and monitor the overall cover rate and by species (Fig. 1). The first Site (S1: Marsa El Hadjaj: 35°'49'928'; 0°9 35'756), characterized by siliceous clay sludge covering the area of a large mudflats (Kerfouf and Bouceta, 2022). It is an anthropogenic zone due to its proximity to a large petrochemical complex in Arzew and a large port in Bethioua (Dilem and *al.,* 2015). The second site (S2: Kristel: 35°50'40 N; 0°2 35'56 W), is more or less remote from pollution sources, with a bedrock with significant biodiversity (Hassani et *al*., 2015; Kies et *al.,* 2020). The third site (S 3: Ain Franine: 35°48'44.46 N; 0°35' 01), is considered not impacted due to its remoteness from the urban fabric and all industrial activities, and its difficult access. The bedrock of the resort, which is rocky and sandy, is bathed in radioactive water, rich in sulfur from the nearby thermal spring (Belhadj Tahar et *al.,* 2021).

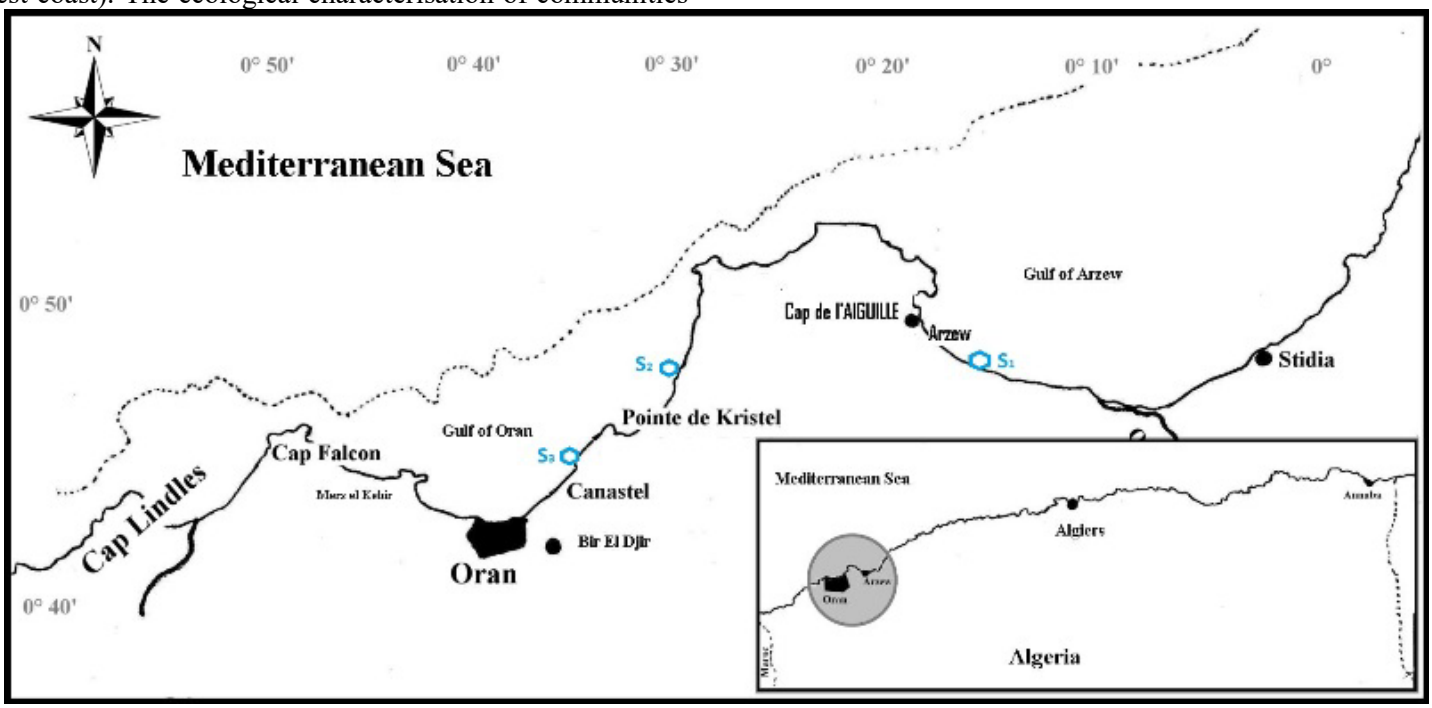

Figure 1. Geographical location of the study area

Sampling

The method used for the inventory of macroalgae flora is that of the minimum recommended area for phytosociological samples. Two major factors are taken into consideration when choosing the sampling period (season and pollution). Climatic conditions are favourable during the warm season for most floristic species (from May to August), and an increase in anthropogenic pressure (human use, flow of urban effluent discharges) is a determining factor in the proliferation or disappearance of sensitive species. The samples are taken on an elementary surface constituting a survey using a quadra with a surface of 50x50 cm^2, which corresponds to the minimum area adopted for the study of the macroalgae community (Boudouresque, 1971). Also, the complete scraping of the sampled surface is essential to continue its study in the laboratory. To establish an exhaustive floristic list of the studied stations, we conducted a random sampling of five surveys per station. The quadra is fixed on the surface to take samples between the upper mesolittoral and the upper infralittoral at depths between 1m and 3m. The collected material is sorted on the spot, in order to separate the thalli of the different species and then are kept separately in seawater (Ramdani et *al*., 2020) in bags kept cool in a cooler, for transport to the laboratory. In the laboratory, the species are separated and cleaned and dried as quickly as possible for quality samples. Macroalgae often lose their colour after drying. An identification number that appears on the outside of each folder of the paper, as well as data on the colour of the species and its habitat and recorded in a field notepad. The code of the macroalgae, the date and the place of harvest are indicated on a sheet. Several species identification keys are used, including the AlgaeBase database and Word Register of Marine Species (WORMS). Macroscopic characters such as colour, shape, size and also location facilitate the determination of species.

Data Analysis

Several classical and synthetic methods were used to evaluate the distribution and structure of macroalgae such as abundance, and species richness, as well as the Shannon and Weaver diversity index (H') and the index equity (J), (Shannon and Weaver, 1963), and the frequency of species. All statistical analyses were performed with R 3.5.2 (R Development Core Team 2019) provided with the FactoMineR package.

The cover rate for all individuals of a given species is calculated and estimated visually on a scale ranging from 5 to + (5: species covering more than ¾ (75%) of the surface; 4: from ½ to ¾ (50%-75%); 3: ½ to ¼ (50%-25%) ; 2: abundant species but covering less than ¼ (5%-25%); 1: species well represented but covering less than 5%; +: (species present but negligible).

The Ri cover rate is the first of the two main coefficients assigned to each species (Boudouresque, 1971). This is the approximate percentage of the substrate area projected by species i. Total survey cover over the total number of surveys: Ri n R=1 /Nr (n is the number of survey species, Nr: the number of surveys).

At each step (class) of the cover coefficient Ri assigned to the n species i of a survey corresponds a conventional mean value (class center) named average cover: Absence = 0; + = 0.1%; 1 = 2.5%; 2 = 15.0%; 3 = 37.5%; 4 = 62.5%; 5 = 87.5%. The GAC (Global Average Cover) for species i in a set of N records is, therefore, the average of its successive average cover:

GAC= Ri n p =1 / N

In a survey divided into qu adra, the frequency Fi of species I expressed as a percentage. It is the ratio of the number of quadra where it is present to the total number of quadra:

F (%) = ni/Nx100.

F is characterized by five classes: 0< F <20%: Class I: Very rare species; 20% <40%: Class II: Rare species; 40%< F < 60%: F Frequent species; 60%< F < 80%: Abundant species; 80%<F<100%.

Results and Discussion

Specific Diversity

Prospecting our study area has identified 22 species: 5 Phaeophyceae, 8 Ulvophyceae and 9 Florideophyceae (Table 1).

The largest number of algae was recorded at the stations of Ain Franine (18 species) followed by Marsa El Hadjaj (16 species). The lowest species richness is recorded at Kristel station (13 species) (Figure 2).

For the Marsa El Hadjaj station, 16 species of benthic macrophytes: 3 Phaeophyceae, 6 Ulvophyceae and 7 Florideophyceae were observed. Thus, the surveys show a dominance of Florideophyceae (red algae) (43.75%), followed by Ulvophyceae (green algae) (37.5%) and Pheophyceae (brown algae) (18.75%). Species with a coefficient of 5 and 4 are defined as very abundant: *Colpomenia sinuosa* (Phaeophyceae); *Ulva compressa*, *Ulva intestinaloides*, *Ulva lactuca* (Ulvophyceae); *Hypnea musciformis*, *Osmundea pinnatifida* (Florideophyceae). Most species are assigned an index of 2 and 1 where the cover rate is 25% to minus 5%, and are well represented by: *Dictyota dichotoma, Padina pavonica*

(Phaeophyceae); *Caulerpa prolifera, Caulerpa racemosa*, *Cladophoropsis membranacea* (Ulvophyceae); *Asparagopsis armata Harvey*, *Ellisolandia elongata*, *Gelidium sp*, *Gracilariopsis longissima* (Florideophyceae). Those indicated by the + sign are species present, but not quantified, it is *Peyssonnelia squamaria*. (Florideophyceae). The total absence of species marked by the sign - is represented by *Ericaria amentacea*, *Sargassum muticum* (Pheophyceus); *Codium decorticatum*, *Codium fragile* (Ulvophyceae), *Gelidium crinale*, *Palisa da perforata* (Florideophyceae).

Table 1. Floristic list according to the WORMS (World Register of Marine Species) database.

Class	Phylum (Division)	Order	Family	Genus	Species
Phaeophyceae	Ochrophyta	Dictyotales	Dictyotaceae	Padina	*Padina pavonica* (Linnaeus) Thivy*, 1960*
				Dictyota	*Dictyota dichotoma (*Hudson)
		Ectocarpales	Scytosiphonaceae	Colpomenia	J.V.Lamouroux, 1809
					Colpomenia sinuosa (Mertens ex Roth) Derbès & Solier, 1851
				Sargassum	*Sargassum muticum* (Yendo) Fensholt, 1955
				Ericaria	*Ericaria amentacea* (C.Agardh) Molinari & Guiry, 2020
Ulvophyceae	Chlorophyta	Bryopsidales	Codiaceae	Codium	*Codium decorticatum* (Woodward) M.A. Howe, 1911
					Codium fragile (Suringar) Hariot, 1889
			Caulerpaceae	Caulerpa	*Caulerpa racemosa* (Forsskål) J.Agardh, 1873
					Caulerpa prolifera (Forsskål) J.V.Lamouroux, 1809
		Cladophorales	Boodleaceae	Cladophoropsis	*Cladophoropsis membranacea* Bang ex C.Agardh) Børgesen, 1905
		Ulvales	Ulvaceae	Ulva	*Ulva compressa* Linnaeus, 1753
					Ulva intestinaloides (Koeman & Hoek) H.S. Hayden, Blomster, Maggs, P.C. Silva, Stanhope & Waaland, 2003
					Ulva lactuca (Linnaeus) 1753
Florideophyceae	Rhodophyta	Bonnemaisoniales	Bonnemaisoniaceae	Asparagopsis	*Asparagopsis armata* Harvey, 1855
		Gelidiales	Gelidiaceae	Gelidium	*Gelidium crinale* (Hare ex Turner) Gaillon, 1828
					Gelidium sp
		Gracilariales	Gracilariaceae	Gracilariopsis	*Gracilariopsis longissima* (S.G. Gmelin) Steentoft, L.M. Irvine & Farnham, 1995
		Gigartinales	Cystocloniaceae	Hypnea	*Hypnea musciformis* (Wulfen) J.V.Lamouroux, 1813
				Osmundea	*Osmundea pinnatifida* (Hudson) Stackhouse, 1809
		Peyssonneliales	Peyssonneliaceae	Peyssonnelia	*Peyssonnelia squamaria* (SGGmelin) Decaisne ex J. Agardh ،1842
		Corallinales	Corallinaceae	Ellisolandia	*Ellisolandia elongata* (J.Ellis & Solander)
		Ceramiales	Rhodomelaceae	Palisada	K.R.Hind & G.W.Saunders, 2013
					Palisada perforata (Bory) K.W.Nam, 2007

At the Kristel station, 13 listed species are divided into three groups (3 Phaeophyceae, 3 Ulvophyceae and 7 Florideophyceae), with a dominance of Florideophyceae compared to other groups. Red algae dominate (53.86%) more than double the two other groups, as well as a homogeneous distribution of brown algae (23.076%) and green algae (23.076%). The Pheophyceus *Ericaria amentacea* occupies the largest area in all the station's surveys, as well as the Florideophyceae, *Hypnea musciformis*, with a coefficient 4. These two species are defined as very abundant with a cover of more than 50%, followed by two o Florideophyceae with a coefficient 3 (*Ellisolandia elongata* and *Palisada perforata*), as well as Ulvophyceae, *Ulva lactuca* considered a frequent species. Most species are abundant or well represented and assigned an index of 2 and 1 where the cover rate is from 25% maximum to 5%. They are represented by two Phaeophyceae *Dictyota dichotoma*, *Padina pavonica*; one Ulvophyceae, *Codium fragile*, and four Florideophyceae, *Asparagopsis armata Harvey*, *Ellisolandia elongata*, *Gelidium sp*, and *Gracilariopsis longissima*. We note the total absence of species marked by the sign -, especially the Ulvophyceae, with five species *Caulerpa prolifera, Caulerpa racemosa*, *Cladophoropsis membranacea, Codium decorticatum, Ulva compressa*. The two Phaeophyceae *Colpomenia sinuosa* and *Sargassum muticum*, and the two Florideophyceae *Gracilariopsis longissima*, *Peyssonnelia squamaria* are absent.

22 species are harvested in all stations surveyed.

For the station of Ain Franine, 18 species of benthic macrophytes were collected including 4 Phaeophyceae, 7 Ulvophyceae and 7 Florideophyceae, with a dominance of Florideophyceae (red algae) and Ulvophyceae (green algae) with the same percentage of (38.9%), followed by Phaeophyceae (brown algae), with a percentage of (22.2%). Some species are considered very dominant by attributing to them the coefficient 5 or 4: *Cladophoropsis membranacea* and *Ulva compressa* (Ulvophyceae), and others with the coefficient 3, considered abundant species: *Padina pavonica*, (Pheophyceus) and *Ulva lactuca*, (Ulvophyceae), *Asparagopsis armata Harvey*, *Ellisolandia elongata*, *Hypnea musciformis*, *Palisada perforata*, (Florideophyceae). Many species are well represented, with an index of dominance coefficient 2 or 1, whose cover rate is 25% to less 5%. These are *Ericaria amentacea*, *Dictyota dichotoma* (Phaeophyceae) *Caulerpa racemosa*, *Codium decorticatum*, *Codium fragile*, *Ulva intestinaloides* (Ulvophyceae), *Gelidium sp.*, *Gracilariopsis longissima*, *Osmundea pinnatifida* (Florideophyceae). The least represented are denoted by the index +, and present but not quantified: Sargassum *muticum* (Pheophyceus). The total absence of species is marked by the sign -, case of *Colpomenia sinuosa* (Pheophyceae), *Caulerpa prolifera*, (Ulvophyceae), *Gelidium crinale* and *Peyssonnelia squamaria* (Florideophyceae).

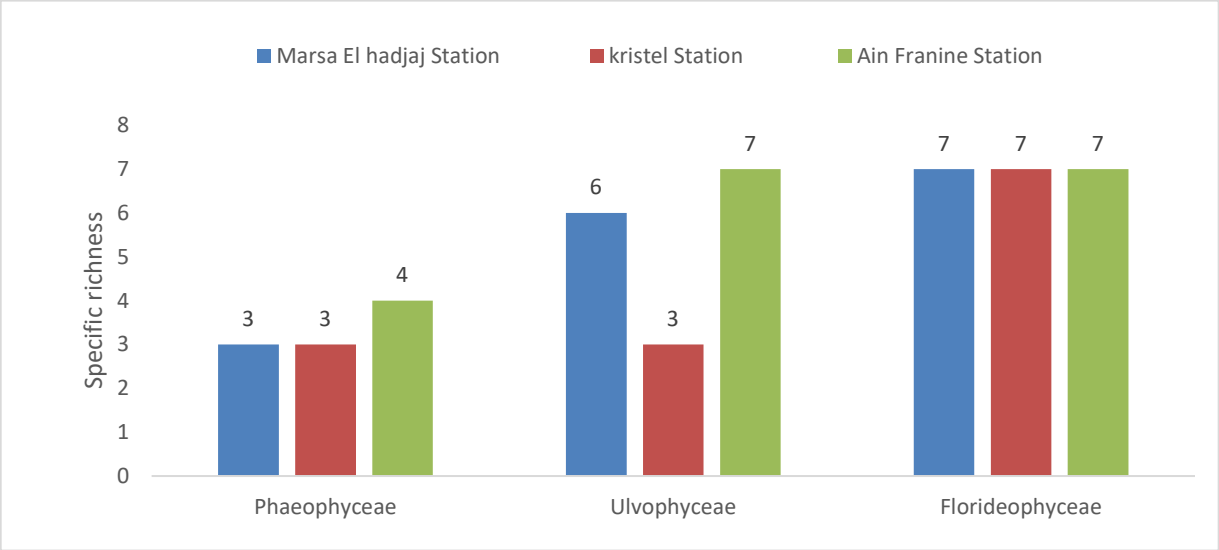

Figure 2. Specific richness of the three stations.

The Shannon index (H'), presents moderate to high diversity at the Ain Franin station and Mars el Hadjaj station, with an index of 2.84 and 2.72 respectively. The Kristel station with the lowest diversity with an index of 2.52, presents the highest equitability among the three stations, followed by Mars El Hadjaj and Ain Franin. The differences between (J) are very slight, suggesting a relatively balanced distribution of individuals between species at each station. These results indicate that the habitats of the three stations can support a similar diversity of species with comparable distributions (Figure 3).

Frequency of Species

The most frequent macroalgae at the Marsa el Hadjaj station are brown algae (Pheophyceae), including *Colpomenia sinuosa* and *Dictyota dichotoma* with a frequency of 100% each; they are therefore considered very constant species. Abundant species such as *Caulerpa prolifera*, *Caulerpa racemosa*, *Ulva compressa*, *Ulva intestinaloides, Ulva lactuca,* (Ulvophyceae), have a frequency of 80%, as well as *Gelidium sp*. *Gracilariopsis longissima* and *Osmundea pinnatifida* (Florideophyceae), with a frequency of 80%. The presence and development of the Ulve and Caulerpe threaten the ecological balance of the marine ecosystem. Other frequent species are found in this station, such as *Padina pavonica* (Pheophyceae), *Asparagopsis armata harvey*, *Ellisolandia elongata* (Florideophyceae). This last indicator of disturbed environment (Gramulin-Brida et *al*., 1967) and *Hypenea musciformis* (Florideophyceae) have a frequency of 60%, followed by other algae with a low frequency (40%) *Cladophoropsis membranacea* (Ulvophyceae) and *Peyssonnelia squamaria* (Florideophyceae) considered a very rare species with a frequency of 20%. However, the results of the analytical parameters reveal the presence of indicator species of pollution and eutrophication due to a remarkable proliferation of Ulvophyceae, as well as the total absence of algae indicative of the good state of the environment in particular, the Cystoseira. From this observation, we can consider the station of Marsa El Hadjaj as a disturbed area, subject to a serious anthropism. A sampling at the Kristel stations shows the massive presence of green algae (Ulvophyceae) such as *Ulva lactuca*, *Eneromorpha intestinalis* and *Codium fargile*. For this purpose, literature mentions the dominance of green algae in highly disturbed environments such as *Ulvales* (Golubic, 1970; Rodriguez-Prieto & Polo, 1996), or Enteromorpha (Ballesteros et *al*., 1984). Their presence is mainly due to coastal discharges of wastewater. According to previous work, the Kristel station is considered to be in relatively good condition and spared important sources of contaminants), with a high abundance (17.80%) of *Ericaria amentacea* in this station. The latter is very present. It was noted that the high percentage of *Ericaria amentacea* recorded in areas characterized by high hydrodynamic, a non-vertical substrate and good lighting emphasizes a good general environmental situation (Figure 4).

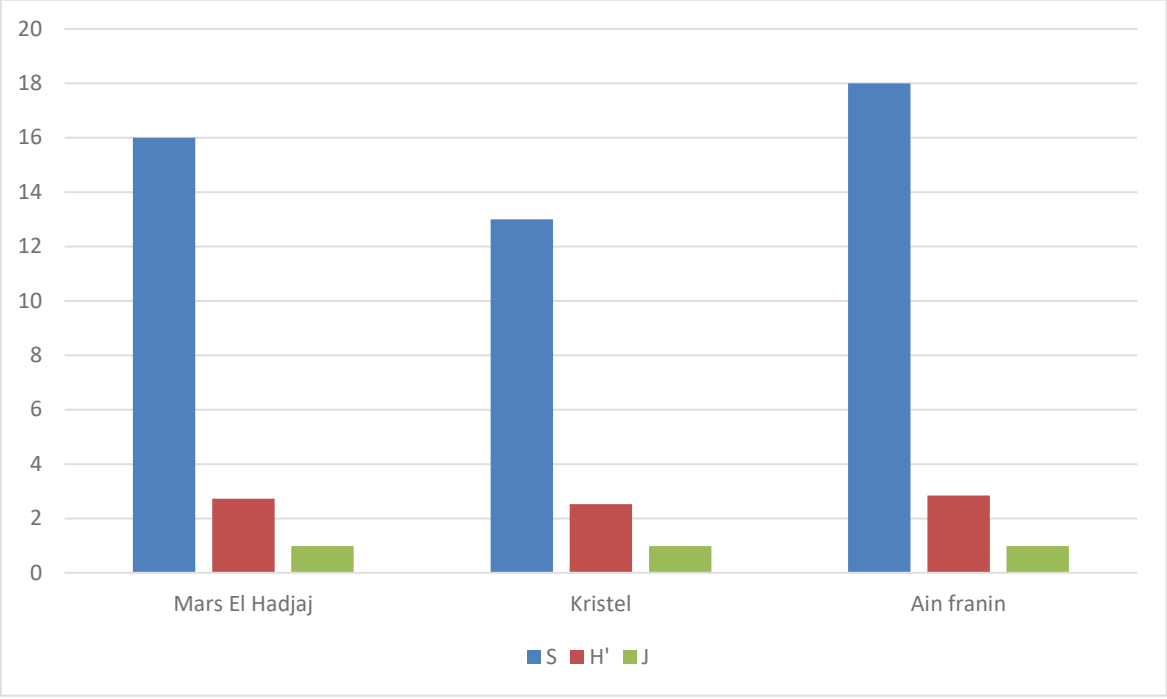

Figure 3. Diversity index and specific richness of sampled stations in the coast of Oran

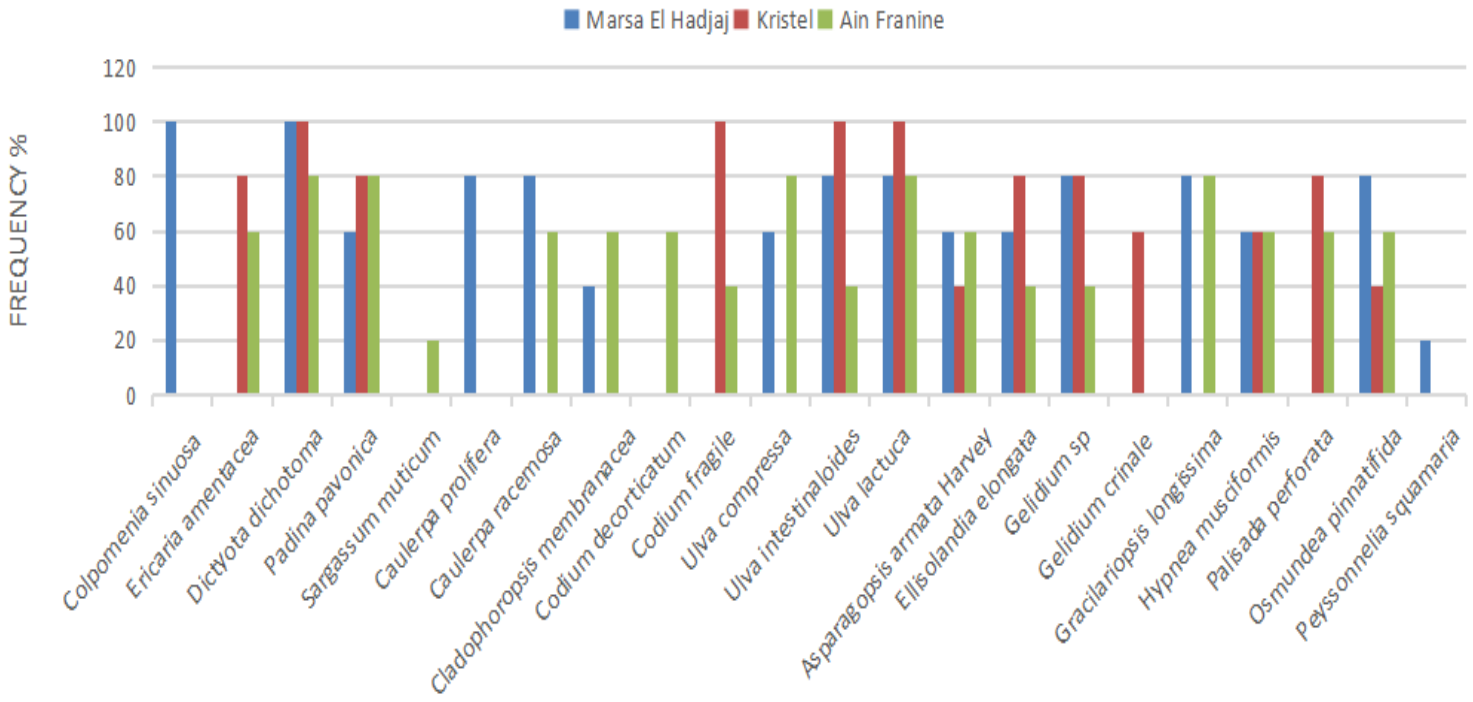

Figure 4. The frequency of algae at the three stations

Cover Rate

The rate of cover of each species at the three stations indicates a macroalgal diversity at the Marsa El Hadjedj station between a maximum cover rate per species of 30% and 0.04%, marked by the dominance of the red algae *Hypnea musciformis* (30%) then the green algae *Ulva lactuca* (23.8%), the red algae *Osmundea pinnatifida* (21.4%) and then *Padina pavonica* (alga bunes) (17%). Only one cover rate is minimum for the red alga *Peyssonnelia squamaria*. (0.04%). *Caulerpa racemosa* represents a cover rate of 12.8% (Fig. 5A). The diversity of machrophytes at the Kristel station shows that the majority species are *Ericaria amentacea*, (Phaeophyceae), *Ellisolandia elongata* (Florideophyceae), *Hypnea musciformis* (Florideophyceae), with a percentage of 17.8%, 20.4% and 23.2% respectively. *Caulerpa prolifera* and *Caulerpa racemosa*, are absent at this station, but present the station of Mars El Hadjadj with a cover rate respectively of 7.8% and 12.8%. The peculiarity of this station is the abundance of *Ulva lactuca* representing a sign of eutrophication and disruption of the environment (Borsali et *al*., 2020), like Caulerps with a 25% overlap rate, similar to station 1 which occupies a rate of 24%, which corresponds to similar results for this species (Bachir Bouiadjra et *al.,* 2021) (Figure 5). As with the Aïn Franine station, it is important to note the dominance of green algae according to their cover rates: *Ulva lactuca* (27%), (26.4%) and *Cladophoropsis membranacea* (22.8%) at the level of the various surveys, and a significant decrease in the overall average cover rate of brown algae indicating a good ecological state of the environment: *Ericaria amentacea* (3%). In this station, we can also notice the presence of invasive algae *Caulerpa racemosa* with a percentage of (3%). The brown alga *sargassum mitucum* (0.12%) absent in the other two stations, has a low overall mean overlap (Figure 5).

The Global Average Cover values of the three groups of algae are higher in Florideophyceae for Marsa el Hadjaj (47.8%) compared to Ulvophyceae (33.06%) and Phaeophyceae (19.14%) (Figure 6). Concerning the stations of Ain Franine, and of Kristel, the overall average cover rate is high respectively in the Ulvophyceae (52, 53%; 48.35%) compared to the Florideophyceae (33, 27%; 32.35%) and Phaeophyceae (14, 2%; 19.3%).

Noting that statistical analysis reveals a significant difference ($p < 0.05$) between all groups. F test (1.5468) is less than the critical value (1.8009) for a threshold of 0.05.

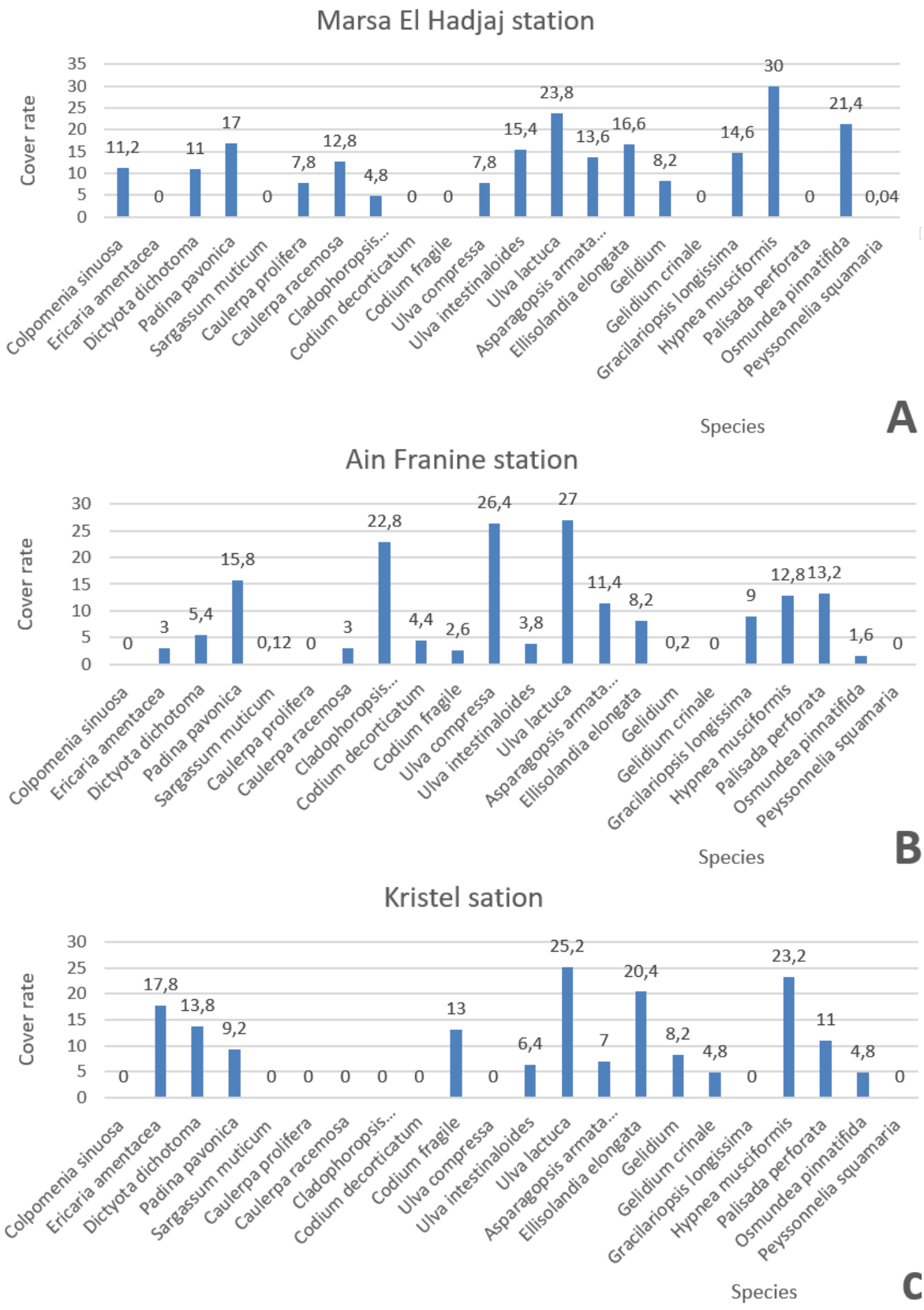

Figure 5. Cover rate of macroalgae in the three stations

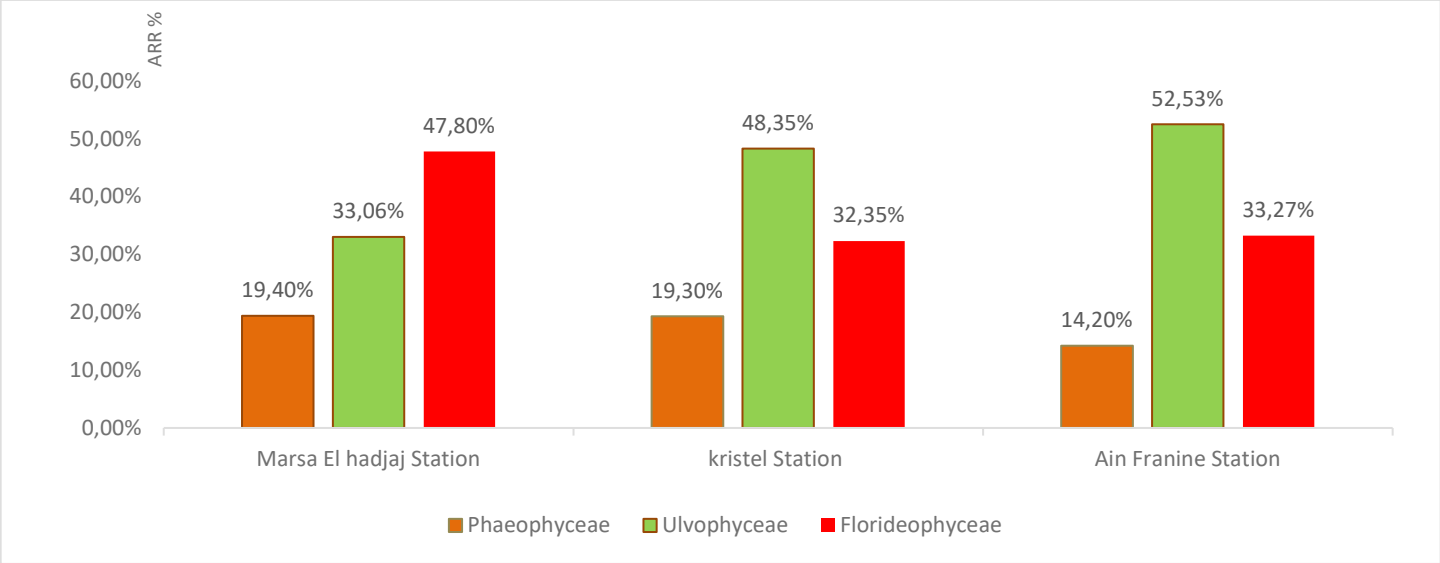

Figure 6. Global Average Cover of the three stations

The macroalgae encountered, called abundant, at the station of Ain Franine with a frequency of 80% are: *Dictyota dichotoma, Padina pavonica* (Pheophyceae) *Ulva compressa , Ulva lactuca* (Ulvophyceae) and *Gracilariopsis longissima* (Florideophyceae), and frequent species with a frequency of 80% such as *Ericaria amentacea* (Pheophyceae), *Caulerpa racemosa* , *Caudium decorticatum*, *Cladophoropsis membranacea* (Ulvophyceae) *Asparagopsis armata harvey*, *Palisada perforata* , *osmundea pinnatifida* (Florideophyceae). The presence of the invasive species *Caulerpa racemosa*, which appeared following climate change and the harmful effects of pollution in the Mediterranean basin (Boudouresque & Verlaque, 2002) at this site tends to colonize disturbed ecosystems, and decreases native algal biomass (Klein, 2007; Klein et *al*., 2008). Other rare species have a low frequency of 40%: *Codium fragile*, *e* (Ulvophyceae), *Gelidium sp*, *Osmundea pinnatifida* and finally *Sargassum muticum* with a low frequency of 20% said very rare. Previous work carried out on this station considers Ain Franine as a clean zone without impact (Djad *et al.,* 2015), due not only to the regression of the average overall cover rate of *Ericaria amentacea* but also to the evolution of Ulvophyceae, in particular, the overall average cover rate of Ulves and *Ulva compressa* which has progressed from the proliferation of *Caulerpa racemosa* which is constantly invading this relatively healthy coastal station. Finally, according to the results of the analytical parameters and the presence of pollution indicator species and a low presence of algae indicating the good state of the environment (*Ericaria amentacea*), we can consider that the Ain Franine Station is slightly impacted. The species identified were previously observed on the Algerian west coast (Hellal et *al.,* 2021; Mansouri et *al.,* 2021, Mehiaoui et *al.,* 2022), and macroalgae are excellent indicators of degradation in the Algerian coast (Khadidja et *al.,* 2018; Belhouari and Bezzina, 2019). The algae observed, are of economic interest, in particular *Ulva* used in pharmacy, animal feed and as fertilizer; as well as *Ericaria amentacea* used in the chemical industry and as fertilizer. However, the algae that we observed can also be used in the assessment of the ecological status index of coastal waters (Arevalo & Pinedo, 2007; Cavallo et *al*., 2016).

Macroalgae have long been used as biological indicators of marine ecosystem health worldwide due to their ecological importance and sensitivity to environmental stress (Su Jin et *al.,* 2023). Community composition and distribution are influenced by coastal morphology, turbidity, hydrodynamics, nutrient inputs and climatic conditions (Traiche et *al*, 2018). The presence of the invasive species *Caulerpa racemosa* var. cylindracea, which tends to colonize disturbed ecosystems, could explain the reduction in native algal flora (Piazzi and Ceccherelli, 2006; Klein and Verlaque, 2008; Klein 2007).

Conclusion

This study aims to assess the diversity of macroalgae on the intertidal area zone of the Algerian west coast. A total of 22 taxa (5 Phaeophyceae, 8 Ulvophyceae and 9 Florideophyceae) are listed in this study. The classification of species according to their frequency makes it possible to better situate

the degree of attachment of species to environmental conditions, in particular to environmental disturbance conditions. Our results showed that the cover rate of each of the 22 species varies from one station to another. The Aïn-Franine, and Kristel stations have a more or less good to acceptable quality environment, without neglecting continuous biomonitoring. The station of Mars El Hadjaj deserves more attention by first making sure to reduce anthropogenic pressure and discharges of domestic and industrial wastewater.

Compliance with Ethical Standards

Conflict of interest: The author(s) declare no actual, potential, or perceived conflict of interest for this article.

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

Data availability: Data will be made available on request from the author(s).

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Ultraviyole radyasyonunun istilacı *Grateloupia turuturu* üzerine fizyolojik etkileri

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ÖZ

İstilacı türler, antropolojik faaliyetler sonucunda bir ekosisteme giren organizmalardır. Bu türler yerleştikleri ortamdaki yerel türlerle rekabete girerek, ekolojik yapı üzerinde tehdit oluşturmakta ve biyoçeşitliliği değiştirebilmektedir. İstilacı bir tür olarak bilinen *Grateloupia turuturu* türü Türkiye'den ilk kez Mayıs 2015 tarihinde Bandırma kıyılarından rapor edilmiş ve günümüzde Erdek ve Mudanya kıyılarında da yayılış gösterdiği belirlenmiştir. Genel olarak istilacı türlerin ekolojik değişkenlere karşı geniş bir toleransa sahip olduğu bilinmektedir. Bu nedenle, bu çalışmada UVR'nin *G. turuturu* türü üzerine fizyolojik etkilerinin belirlenmesi ve *G. turuturu* türünün UVR'ye karşı cevaplarının araştırılması hedeflenmiştir. Bu amaçla *G. turuturu* örnekleri 3 farklı ışık rejiminde (fotosentetik aktif radyasyon, UVA ve UVB) kültüre alınmış ve fizyolojik cevapları araştırılmıştır. Elde edilen veriler UVR'ye maruz kalan örneklerin fotosentetik etkinliğinde ve nitrat redüktaz enzim aktivitelerinde artış olduğunu, buna karşın aksesuar pigment içeriklerinin değişmediğini göstermiştir. Sonuç olarak, *G. turuturu* türünün UVA enerjisini fotosentezde ışık kaynağı olarak kullanabildiği, UVR'ye karşı hassas olmayıp toleranslı olduğu anlaşılmaktadır. Bu nedenle *G. turuturu* türünün yerel türlerle rekabette avantajlı olabileceği düşünülmektedir.

Anahtar Kelimeler: Fotosentez, *Grateloupia*, İstilacı, Ultraviyole radyasyon

ABSTRACT

Physiological effects of ultraviolet radiation on invasive *Grateloupia turuturu*

Invasive species are organisms that enter an ecosystem as a result of anthropological activities. These species compete with native species in the environment where they settle, threatening the ecological structure and changing biodiversity. *Grateloupia turuturu*, known as an invasive species, was reported for the first time in May 2015 from the coast of Bandırma in Turkey and it was determined that it is now distributed in Erdek and Mudanya coasts. In general, invasive species are known to have a wide tolerance to ecological variables. Therefore, the aim of this study was to determine the physiological effects of UVR on *G. turuturu* species and to investigate the responses of *G. turuturu* species to UVR. For this purpose, *G. turuturu* samples were cultured in 3 different light regimes (photosynthetically active radiation, UVA and UVB) and their physiological responses were investigated. The data obtained showed that the photosynthetic activity and nitrate reductase enzyme activities of the samples exposed to UVR increased, whereas the accessory pigment content did not change. In conclusion, *G. turuturu* is able to utilize UVA energy as light sources in photosynthesis and is tolerant but not sensitive to UVR. Therefore, it is thought that *G. turuturu* may be advantageous in competition with local species.

Keywords: Photosynthesis, *Grateloupia*, Invasive, Ultraviolet radiation

Giriş

İstilacı türler, antropolojik faaliyetler sonucunda bir ekosisteme gelen ve yerleştikleri ortama hızla yayılan türleri ifade etmektedir (Sakai ve ark., 2001). Bu türler aynı çevrede yaşayan yerli türleri, çevreyi, insan sağlığını ve ekonomiyi olumsuz yönde etkilemektedir. Deniz ticareti ve taşımacılığının yoğun olduğu alanlara istilacı türlerin girişi ve yayılımı hızlanmaktadır. Türkiye kıyılarına çeşitli yollarla giriş yapan yaklaşık 539 yabancı deniz türü olduğu bilinmektedir (Çınar ve ark., 2021). Bunlar arasında *Galaxaura rugosa*, *Caulerpa taxifolia* gibi deniz yosunları da bulunmaktadır (Turan ve ark., 2011; Taşkın ve ark., 2017).

Kırmızı deniz yosunlarından olan *Grateloupia turuturu* Y. Yamada 1941 (Halimeniaceae) türü Japonya- Hokkaido kıyılarında doğal olarak yayılış göstermektedir (Yamada, 1941). Sonrasında Atlantik adaları, çeşitli Avrupa ülkeleri, Kuzey Amerika, Karayip Adaları, Batı Atlantik, Güney Amerika, Afrika ülkeleri, Avusturalya ve birçok Asya ülkesinden rapor edilmiştir (Guiry ve Guiry, 2024). Ayrıca, Amerika (Villalard-Bohnsack ve Harlin, 1997), Meksika (Miller ve ark., 2011), Portekiz (Barbara ve Cremades, 2004), Brezilya (de Azevedo ve ark., 2015), İspanya (Barbara ve Cremades, 2004), Fransa (Goulletquer ve ark., 2002), İngiltere (Farnham, 1980), İtalya (Tolomio, 1993) ve İsrail (Katsanevakis ve ark., 2014) kıyılarında istilacı tür olarak kayıtlara geçmiştir.

Türkiye'den ilk kez Mayıs 2015 tarihinde Bandırma kıyılarından rapor edilmiştir (Bariche ve ark., 2020). İstilacı bir tür olarak bilinen *G. turuturu* türünün, günümüzde Erdek ve Mudanya kıyılarında da yayılış gösterdiği belirlenmiştir. Marmara Denizi'nin yoğun bir deniz trafiğine sahip olması göz önüne alındığında *G. turuturu* türünün Türkiye'ye balast suyu yoluyla ulaşmış olabileceği düşünülmektedir.

Genel olarak istilacı türlerin ekolojik değişkenlere karşı geniş bir toleransa sahip olduğu bilinmektedir (Bommarito ve ark., 2024). Ekolojik değişkenlerden biri olan ultraviyole radyasyonu (UVR)'nun yerküreye ulaşan miktarı, atmosferik sera gazlarının artışıyla birlikte artış göstermiştir. Bu artış deniz canlılarını da olumsuz yönde etkilemektedir. Artan UVR'nin deniz yosunları üzerine etkilerini araştıran birçok çalışma bulunmaktadır (Wiencke ve ark., 2000; Dobretsov ve ark., 2021; Rothausler ve ark., 2022). Bu çalışmalar UVR'nin deniz yosunlarında fotosentez (Xu ve Gao 2016; Rothausler ve ark., 2022), besin alımı (Vinegla ve ark., 2006), üreme (Dobretsov ve ark., 2020) ve büyüme (Polo ve Chow, 2020; Schneider ve ark., 2022) üzerinde etkileri olduğunu göstermektedir. Ancak yapılan çalışmalar deniz yosunlarının UVR'ye karşı fizyolojik cevabının türler arasında farklı olduğunu göstermektedir. Bu nedenle, bu çalışmada UVR'nin *G. turuturu* türü üzerine fizyolojik etkilerinin belirlenmesi ve *G. turuturu* türünün UVR'ye karşı cevaplarının araştırılması hedeflenmiştir.

Materyal ve Metot

Grateloupia turuturu örnekleri, Ekim – 2022'de Mudanya (Bursa) kıyılarından toplanmıştır. Laboratuvara getirilen örnekler sentetik deniz suyu ile yıkanarak epifitlerinden arındırılmıştır. Temizlenen örnekler, içerisinde filtre edilmiş 23 ppt tuzluluğa sahip deniz suyu bulunan ve provasoli çözeltisi (Provasoli 1968) ile zenginleştirilmiş akvaryumlarda 4 günlük alışma sürecine bırakılmıştır. Alışma süreci boyunca ortam sıcaklığı 22°C, pH:8.0 olarak ayarlanmış ve 60 µmol foton $m^{-2}s^{-1}$ Fotosentetik Aktif Radyasyon ile aydınlatılmıştır. Alışma sürecinden sonra sağlıklı örnekler seçilerek 3 farklı deney grubu oluşturulmuş ve stres çalışmaları yapılmıştır. Bu gruplar;

F: Fotosentetik Aktif Radyasyon (60 µmol foton $m^{-2}s^{-1}$)

FA: Fotosentetik Aktif Radyasyon (60 µmol foton $m^{-2}s^{-1}$) + Ultraviyole-A (2.07 W $m^{-2}s^{-1}$)

FAB: Fotosentetik Aktif Radyasyon (60 µmol foton $m^{-2}s^{-1}$) + Ultraviyole-A (2.07 W $m^{-2}s^{-1}$) + Ultraviyole-B (4.14 W $m^{-2}s^{-1}$)

Deney grupları 10L filtre edilmiş sentetik deniz suyu içeren akvaryumlarda hazırlanmıştır. Hazırlanan kültür ortamında tuzluluk 23ppt, sıcaklık 22°C, aydınlanma periyodu 12A:12K ve pH: 8.0 olarak ayarlanmıştır. Akvaryumlar Provasoli çözeltisi ile zenginleştirilmiştir. F uygulaması için 60 µmol foton $m^{-2}s^{-1}$, FA uygulaması için ilave olarak 2.07 W $m^{-2}s^{-1}$ UVA radyasyonu ve FAB uygulaması için diğer ışıklara ilaveten 4.14 W $m^{-2}s^{-1}$ UVB radyasyonu kullanılmıştır. FAR için Osram Biolux floresan lambası, UV-A için Philips TL-K 40W/10-R UVA floresan lambası, UV-B için Philips TL 20W/01 RS UVB floresan lambası kullanılmıştır. Örnekler yukarıda belirtilen koşullara 1 hafta maruz bırakılmış ve sonrasında aşağıdaki analizler yapılmıştır.

Fotosentetik performans ölçümleri Fotosistem-II'nin (PSII) modülasyonu klorofil floresansının *in-situ* olarak ölçülmesiyle belirlenmiştir. PSII'nin maksimum fotokimyasal kuantum ürününün (F_v/F_m) ölçülmesinde, ölçümü yapılacak tallus parçası cihazın ilgili aparatına konularak karanlık ortam sağlanmıştır. Örnek üzerine uzak-kırmızı ışın verilerek her iki fotosistem merkezinin kapalı konuma gelmesi için, 10 dk karanlıkta bekletilmiş ve F_v/F_m ölçümü alınmıştır. Sonrasında örnekler üzerine, kademeli olarak artan 10 farklı yoğunlukta

aktinik ışık uygulanmıştır. Her 30 saniyede bir 0,8 saniye doygunluk atışı yapılarak, etkili PSII kuantum ürünü ($\Delta F/F_{m'}$) kaydedilmiş ve bir üst aktinik ışık seviyesi verilmeye başlanmıştır. Ölçümlerden elde edilen $\Delta F/F_{m'}$ değeri kullanılarak fotosentetik etkinlik parametreleri olan maksimum göreceli elektron transfer oranı ($rETR_{max}$), fotosentezin doygun olduğu ışık yoğunluğu (I_k) ve alfa değerleri Eilers ve Peeters (1988) tarafından önerilen formül kullanılarak hesaplanmıştır.

Nitrat redüktaz aktivitesi Corzo ve Niell (1991) tarafından önerilen yönteme göre *in situ* olarak yapılmıştır. Azot gazı ilavesiyle anaerobik ortam koşulları oluşturularak 0.1M fosfat tamponu içerisine yaklaşık 0.1g tal parçası konularak 60dk karanlıkta inkube edilmiştir. Süre sonunda tampon çözeltisindeki NO_2^{-2} miktarı belirlenerek enzim aktivitesi hesaplanmıştır.

Protein tayini Bradford (1976) yöntemine göre yapılmıştır. 0.1 g tal parçası, 3ml 0.2M pH; 6.8 sodyum fosfat tamponu ile homojenize edilmiştir. Elde edilen homojenat 3000g'de 5 dk santrifüj edilmiştir. Süpernatant Coomassie Brillant Blue G250 ile boyanarak 595nm'de absorbansı okunmuştur.

Klorofil analizi için sıvı azotla dondurulan örnekler, Inskeep ve Bloom'un (1985) metoduna göre N,N-Dimethylformamide ile karanlık ortamda ekstrakte edilmiştir. Ekstraktın spektrofotometrik olarak ölçülmesinden sonra, aşağıdaki formül yardımı ile klorofil-a miktarları hesaplanmıştır.

Chl-a = 12.70 x A664.5 – 2.79 x A647

Fikoeritrin ve fikosiyanin analizi için sıvı azotla dondurulan örnekler Beer ve Eshel (1985) metoduna göre fosfat tamponu ile ekstrakte edilmiştir. Ekstraktın spektrofotometrik olarak ölçülmesinden sonra, aşağıdaki formüller kullanılarak fikoeritrin ve fikosiyanin miktarları hesaplanmıştır.

Fikoeritrin: [(A564 – A592) – (A455 – A592) x 0.20)] x 0.12

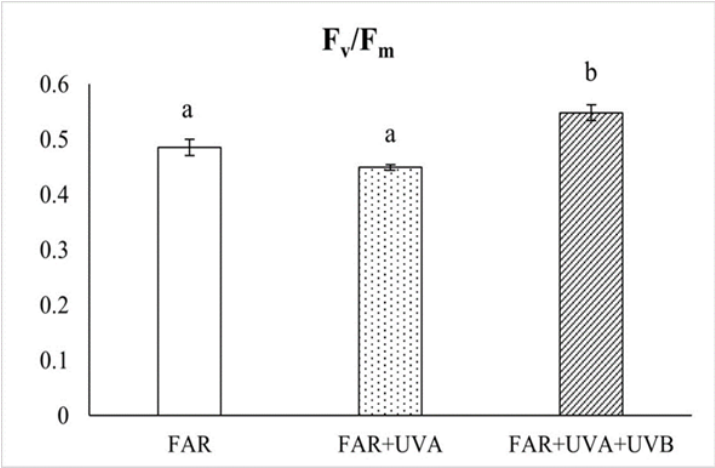
Fikosiyanin: [(A618 – A645) – (A592 – A645) x 0.51)] x 0.15

UV absorbe eden bileşikler (UVACs) absorbans spektrumu ile verilmiştir. Sıvı azot ile dondurulmuş örnekler 25% metanol çözeltisi ile havanda homojenize edildikten sonra, 5dk sonikatorde bekletilmiştir. Sonrasında 45ºC su banyosunda inkube edilmiş ve 5 dk 5000g'de santrifüj edilmiştir. Süpernatantın 280-400nm'de absorbansı alınmış ve örnek ağırlıklarına göre standardize edilmiştir.

Yapılan analizlerin uygulamalar arasındaki farklılığı tek yönlü varyans analizi ile test edilmiştir. Çoklu karşılaştırma testi olarak Tukey HSD testi uygulanmıştır. Varyansın homojenliği ve normalite, Levene testi ve Kolmogorov-Smirnov ile belirlenmiştir. Tüm istatistiksel analizler IBM SPSS 29.0 paket programı ile yapılmıştır.

Bulgular ve Tartışma

Ekofizyolojik çalışmalarda yaygın olarak kullanılan F_v/F_m oranı, optimum kuantum ürününü temsil etmektedir. Farklı ışık rejimlerine maruz kalan *G. turuturu* örneklerinde F_v/F_m oranı farklılık göstermiştir (F:16.78; p=0.001; Şekil-1). En yüksek F_v/F_m oranı FAB uygulamasında 0.548 ± 0.014 olarak bulunmuştur. Çoklu karşılaştırma testleri F ile FA uygulamalarındaki örneklerin F_v/F_m oranlarının birbirine benzer olduğunu, FAB uygulamasının ise diğer uygulamalardan farklı olduğunu göstermiştir.



Şekil 1. Farklı ışık rejimlerine maruz kalan *Grateloupia turuturu* örneklerinin F_v/F_m oranları (ortalama ± standart hata, sütunlar üzerindeki harfler istatistiksel farklılıkları göstermektedir)

Figure 1. Fv/Fm ratios of *Grateloupia turuturu* samples exposed to different light regimes (mean ± standard error, letters on the bars indicate statistical differences)

Alfa değeri düşük ışık yoğunluklarındaki elektron transfer oranını, dolayısıyla da antenna sistemindeki pigmentlerin fotosentetik etkinliğini göstermektedir. Çalışmada elde edilen veriler en yüksek alfa değerinin F uygulamasında olduğunu göstermiştir (Tablo 1). En düşük alfa değeri ise FA uygulamasında bulunmuştur. İstatistiksel olarak F ve FA uygulamalarının birbirinden farklı olduğu (F:4.73; p=0.031), FAB uygulamasının ise diğer iki uygulamayla benzer olduğu gözlenmiştir.

Fotosentezin doygun olduğu ışık yoğunluğunu gösteren I_k değeri 44.7 – 74.85 µmol foton $m^{-2}s^{-1}$ arasında bulunmuş (Tablo 1) ve istatistiksel olarak uygulamalar arasında farklılık olduğu tespit edilmiştir (F:6.23; p=0.011). Çoklu karşılaştırma testleri, alfa değeriyle benzer olarak I_k değerlerinin de F ile

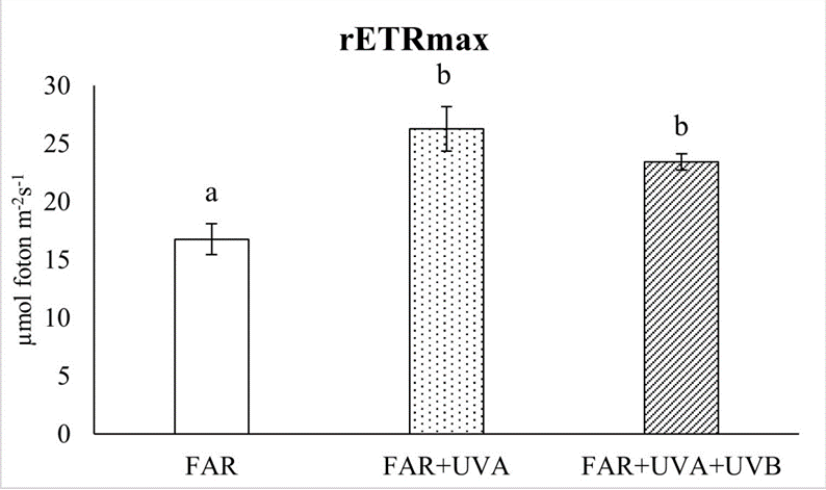
FA uygulamaları arasında benzer olduğunu, FAB uygulamasının ise her iki uygulamayla benzer olduğunu göstermiştir.

En düşük $rETR_{max}$ değeri F uygulamasındaki örneklerde 16.77 ±1.33 µmol foton $m^{-2}s^{-1}$ olarak bulunmuştur (Şekil 2). FA ile FAB uygulamalarına maruz kalan örneklerde ise daha yüksek $rETR_{max}$ değeri kaydedilmiştir. Tek yönlü varyans analizi sonuçları uygulamalar arasında farklılıklar olduğunu (F:11.98; p=0.001) ve FA ile FAB uygulamalarının birbirine benzer olduğunu göstermiştir.

Tablo 1. Farklı ışık rejimlerine maruz kalan *Grateloupia turuturu* örneklerinin alfa, I_k, protein, klorofil-a, fikoeritrin ve fikosiyanin içerikleri (ortalama ±standart hata)

Table 1. Alpha, I_k, protein, chlorophyll-a, phycoerythrin and phycocyanin contents of *Grateloupia turuturu* samples exposed to different light regimes (mean ±standard error)

	FAR (F)	FAR+UVA (FA)	FAR+UVA+UVB (FAB)
Alfa	0.431±0.016	0.339±0.029	0.374±0.016
I_k (µmol foton m^{-2} s^{-1})	44.700±4.517	74.850±7.656	64.933±5.892
Protein (mg/g)	5.949±0.178	6.075±0.054	5.628±0.060
Klorofil-a (mg/g)	0.360±0.017	0.316±0.008	0.385±0.014
Fikoeritrin (mg/g)	1.013±0.058	0.970±0.113	1.002±0.156
Fikosiyanin(mg/g)	0.041±0.004	0.034±0.006	0.043±0.008

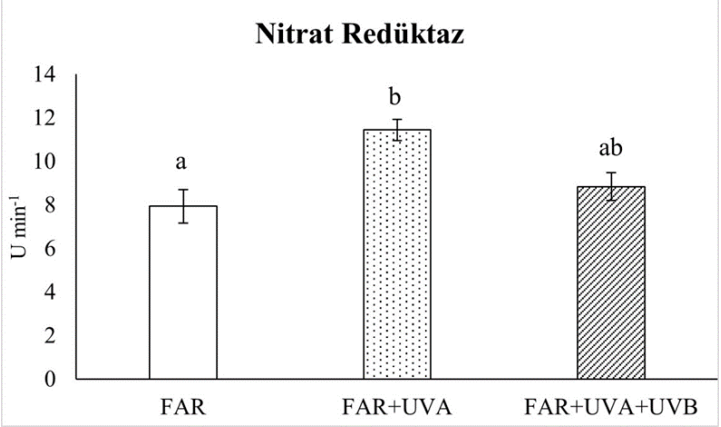


Şekil 2. Farklı ışık rejimlerine maruz kalan *Grateloupia turuturu* örneklerinin $rETR_{max}$ değerleri (ortalama ± standart hata, sütunlar üzerindeki harfler istatistiksel farklılıkları göstermektedir)

Figure 2. $rETR_{max}$ values of *Grateloupia turuturu* samples exposed to different light regimes (mean ± standard error, letters on columns indicate statistical differences)

Tablo-1'de farklı uygulamalardaki örneklerin klorofil-a, fikoeritrin, fikosiyanin ve toplam protein içerikleri verilmiştir. Örneklerin klorofil-a değerleri 0.316 – 0.385 mg/g aralığında bulunmuştur. FA ile FAB uygulamalarına maruz kalan örneklerin klorofil-a içerikleri arasında farklılık olduğu, ancak F uygulamasındaki örneklerin diğer iki uygulamayla benzer klorofil-a içeriğine sahip olduğu tespit edilmiştir. Tüm uygulamalardaki örneklerin fikoeritrin ve fikosiyanin içerikleri ise birbirine benzer değerler göstermiştir (F:0.036; p=0.97; F:0.52; p=0.61; sırasıyla). Uygulamalar arasında en düşük protein içeriği FAB uygulamasına maruz kalan örneklerde bulunmuştur. Ancak istatistiksel olarak FAB uygulaması ile F uygulaması arasında farklılık olmadığı gözlenmiştir. Benzer şekilde FA uygulamasındaki örneklerin protein içerikleri de F uygulaması ile benzer bulunmuştur.

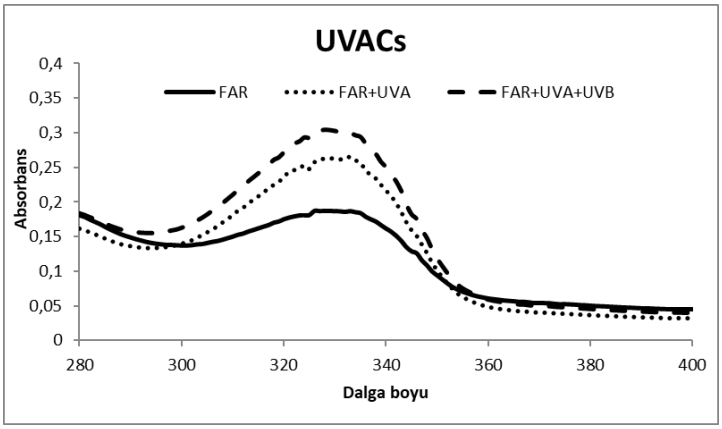
Farklı uygulamalara maruz kalan örneklerin nitrat redüktaz aktiviteleri Şekil 3'de gösterilmektedir. En düşük nitrat redüktaz aktivitesi F uygulamasına maruz kalan örneklerde bulunmuştur. En yüksek enzim aktivitesi ise FA uygulamasındaki örneklerde kaydedilmiştir. FAB uygulamasındaki örnekler ise diğer iki uygulamayla benzer bulunmuştur.



Şekil 3. Farklı ışık rejimlerine maruz kalan *Grateloupia turuturu* örneklerinin nitrat redüktaz aktivitesi (ortalama ± standart hata, sütunlar üzerindeki harfler istatistiksel farklılıkları göstermektedir)

Figure 3. Nitrate reductase activity of *Grateloupia turuturu* samples exposed to different light regimes (mean ± standard error, letters on columns indicate statistical differences)

Bir hafta boyunca farklı ışık uygulamalarına maruz kalan *G. turuturu* örneklerinin UVACs spektrumları Şekil 4'de verilmiştir. Tüm uygulamalarda en yüksek absorbans değerleri 325-330nm dalga boyunda ölçülmüştür. Elde edilen veriler F uygulamasındaki örneklerin diğer uygulamalardan daha düşük absorbans spektrumuna sahip olduğunu göstermiştir. FA ve FAB uygulamalarındaki örneklerin absorbans spektrumları birbirine yakın olmakla birlikte, FAB uygulamasında daha yüksek bulunmuştur.



Şekil 4. Farklı ışık rejimlerine maruz kalan *Grateloupia turuturu* örneklerinin UVACs spektrumları

Figure 4. UVACs spectra of *Grateloupia turuturu* samples exposed to different light regimes

Optimum kuantum ürününü temsil eden F_v/F_m oranı, antenna sistemlerinden PSII reaksiyon merkezine enerji transferinin etkinliğini göstermektedir. Bu oran ekofizyolojik çalışmalarda bireylerin fizyolojik durumu hakkında bilgi vermektedir. F_v/F_m oranı sağlıklı bireylerde genellikle sabit değerler göstermektedir. Ancak bu değer algal divisyolar arasında farklılık göstermektedir. Chlorophyta türlerinde ~0.8 civarında, Phaeophyceae türlerinde ~0.7 civarında ve Rhodophyta türlerinde ise diğer divisyolardan daha düşük olarak ~0.6 civarında olduğu bilinmektedir (Büchel ve Wilhelm, 1993).

Günümüze kadar yapılan birçok çalışmada UVR'nin F_v/F_m oranı üzerine etkileri araştırılmıştır (Chaloub ve ark., 2010; Xu ve ark., 2023; Li ve ark., 2024). Genel kanı UVR'nin deniz yosunlarında F_v/F_m oranını düşürdüğü yönündedir (Figureoa ve ark., 2003). Ancak bazı çalışmalarda ise UVR'ye maruz kalan örneklerin F_v/F_m oranının değişmediği veya arttığı gözlenmiştir (Dring ve ark. 1996; Bischof ve ark., 2000). Bu çalışmalarla benzer olarak FAB uygulamasına maruz kalan *G. turuturu* örneklerinin F_v/F_m oranı diğer uygulamalardan yüksek bulunmuştur. F_v/F_m oranındaki bu artış uygulanan dozdaki UVR'nin *G. turuturu* türünde strese yol açmadığı anlamına gelmektedir.

$rETR_{max}$ değeri fotosentetik etkinlik hakkında bilgi veren en önemli parametrelerdendir. Bu çalışmada elde edilen veriler UVR'ye maruz kalan örneklerin daha yüksek $rETR_{max}$ değerine sahip olduğunu göstermektedir. Bu veri, UVR'nin *G. tu-*

ruturu türünde fotosentetik aktiviteyi artırdığı anlamına gelmektedir. Ancak FA uygulaması ile FAB uygulamasındaki örneklerin $rETR_{max}$ değerlerinin benzer olması, fotosentetik aktivitedeki artışın UVA ışınlarından kaynaklandığını düşündürmektedir. UVA ile birlikte UVB varlığı deniz yosunlarının fizyolojisinde antagonistik, sinerjistik veya nötral etki gösterebilmektedir (Bischof ve ark., 2006; Rex ve Mukherjee 2023). Bu çalışmada FAR ve UVA ışınlarına ilaveten UVB varlığı $rETR_{max}$ değerinde pozitif veya negatif yönde bir değişime neden olmamıştır. Dolayısıyla verilerimiz, *G. turuturu* türünün UVA ışınlarını fotosentez için kullanabildiğini düşündürmektedir. Vinegla ve ark., (2006) tarafından *Fucus spiralis* türünde UVA radyasyonunun fotosentezi stimüle ettiğini belirtmesi de bu düşüncemizi desteklemektedir. İlaveten *Gracilaria lemaneiformis* (Gao ve Xu, 2008) türünün de UVA ışınlarının etkisiyle fotosentetik performansını arttırdığı belirtilmiştir. *Fucus gardneri* türünün ise UVA'ya maruz kaldığında büyüme oranının arttığı rapor edilmiştir (Henry ve Alstyne, 2004). Hatta, Xu ve Gao, (2016)'da UVA enerjisinin fotosentetik karbon fiksasyonunda kullanılmasının bazı kahverengi, kırmızı ve yeşil alglerde olduğunu belirtmesi, bu durumun deniz yosunları arasında yaygın olduğunu göstermektedir.

F uygulaması ile kıyaslandığında, FA ve FAB uygulamalarında kaydedilen daha düşük alfa değerleri ve daha yüksek I_k değerleri de UVR'ye maruz kalan *G. turuturu* örneklerinin daha yüksek fotosentetik etkinliğe sahip olduğunu doğrulamaktadır. Alfa değeri düşük ışık yoğunluklarındaki $rETR_{max}$'ı dolayısı ile de fotosentetik pigmentlerin etkinliğini göstermektedir. Çalışmamızda elde edilen pigment miktarlarının uygulamalar arasında farklılık göstermemiş olması, örneklerin antenna boyutunda değişim olmadığı anlamındadır. Antenna boyutunda bir değişim olmazken, UVR'ye maruz kalan örneklerin daha düşük alfa değerleri göstermesi, antenna pigmentlerinin fotosentezde daha etkin oldukları anlamına gelmektedir. I_k değeri de fotosentezin maksimum doygunluğa ulaşabilmesi için gereken ışık yoğunluğunu temsil etmektedir. I_k değerinin daha yüksek olması, fotosentetik etkinliğin de daha fazla olduğu, bu nedenle de daha fazla ışığa ihtiyaç duyulduğu anlamındadır.

Deniz yosunları fotosentez yoluyla karbon bileşiklerini sentezliyor olsa da diğer besin tuzlarını deniz suyundan temin etmek durumundadırlar. Bu besin tuzları arasında azot, amino asitlerin ve proteinlerin yapısına katılması nedeniyle yaygın olarak araştırılmaktadır. Deniz yosunları inorganic azot formlarından amonyum ve nitratı kolaylıkla kullanabilmektedir (Rosenberg ve Ramus 1984; Hurd ve ark., 2014). Nitrat redüktaz enzimi nitrat asimilasyonunda ilk basamak olan nitratın nitrite indirgenmesini katalizleyen bir enzimdir. Oluşan nitrit, daha sonra amonyuma çevrilmekte ve amonyumda doğrudan amino asit ve proteinlerin sentezlenmesinde kullanılmaktadır. Bu nedenle, nitrat reduktaz enzim aktivitesinin belirlenmesi, deniz yosunlarının azot metabolizması hakkında bilgi vermektedir.

UVB radyasyonunun deniz yosunlarında azot alımını inhibe ettiği bilinmektedir (Sinha ve ark., 1995). Buna karşılık bazı çalışmalarda UVB radyasyonunun azot alımını etkilemediği belirtilmiştir (Braune ve Döhler 1996). Diğer yandan UVB'nin deniz yosunlarında azot alımını ve nitrat reduktaz aktivitesini stimule ettiğini bildiren çalışmalar da bulunmaktadır (Vinegla ve ark., 2006; Flores-Moya ve ark., 1998). Bu çalışmada UVR'ye maruz kalan örneklerde enzim aktivitesinde artış olmuştur. Diğer bir ifadeyle, UVR *G. turuturu* türünün nitrat alımını artırmıştır. Diğer uygulamalardan farklı olarak, FAB uygulamasındaki örneklerde, azot alımındaki artış, örneklerin protein içeriğine yansımamıştır. Hatta FAB uygulamasındaki örneklerin protein içeriklerinin azalma eğiliminde olduğu görülmektedir. Deniz yosunlarının strese maruz kaldıklarında mikosporin benzeri aminoasitler gibi sekonder metabolitlerin sentezini artırdığı bilinmektedir (Vega ve ark., 2021). Yapılan çalışmalar UVR'ye maruz kalan *Sacchorhiza dermatodea* (Roleda ve ark., 2006), *Iridaea tuberculosa* (Jofre ve ark., 2020), *Sargassum horneri* (Xu ve ark., 2022) türlerinde sekonder metabolitlerde artış olduğunu göstermektedir. UVB'ye maruz kalan *G. turuturu* örneklerinde nitrat redüktaz aktivitesindeki artışa rağmen protein miktarının artmaması, hatta azalma eğiliminde olması, örneklerin büyümeden çok UVR ile başa çıkabilmek için sekonder metabolit sentezini artırdığını düşündürmektedir. FA ve FAB uygulamalarındaki örneklerin UVACs absorbanslarının da yüksek olması bu düşüncemizi desteklemiştir.

Sonuç

Sonuç olarak elde edilen veriler, *G. turuturu* türünün UVA enerjisini fotosentezde ışık kaynağı olarak kullanabildiğini, UVR etkisindeyken azot alımını artırdığını göstermektedir. Bu nedenle *G. turuturu* türünün UVA ve UVB radyasyonuna karşı hassas olmadığını, hatta toleranslı olduğunu düşünmekteyiz. Kıyılarımızda yayılış gösteren *G. turuturu* türünün UVR'ye karşı toleranslı olması, istilacı tür karakteristiği ile de uyumlu gözükmektedir. Ancak, UVB'ye maruz kalan örneklerin protein içeriğinin azalma eğilimi göstermesi, daha uzun süreli çalışmalara ihtiyaç olduğunu göstermektedir.

Etik Standart ile Uyumluluk

Çıkar çatışması: Yazarlar bu yazı için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan etmişlerdir.

Etik izin: Çalışma etik izin gerektirmemektedir.

Veri erişilebilirliği: Veriler talep üzerine sağlanacaktır.

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Teşekkür: -

Açıklama: -

Kaynaklar

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Heat inactivation of *Escherichia coli* O157:H7 and *Salmonella* Enteritidis in sous vide-cooked anchovy enriched with ascorbic acid at low temperature

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ABSTRACT

Low-temperature cooking during the sous vide process enhances sensory properties, particularly in heat-sensitive foods. While enhancing efficiency, it also raises the risk of foodborne pathogen persistence. In this study, butterfly anchovy fillets were inoculated with a low dose of *Escherichia coli* O157:H7 or *Salmonella* Enteritidis. To amplify the effect of heat treatment, ascorbic acid (AA) was incorporated into sous vide anchovies before thermal processing at 55°C. Sampling was conducted at 5-minute intervals up to 30 min, followed by longer intervals. The initial *E. coli* load was 4.49 log CFU/g. The addition of AA significantly reduced ($P<0.05$) bacterial counts at and after the 45th min compared to the untreated control (C) group. The lowest count, 1.30 log CFU/g, was observed in the AA group at 120 min of cooking. A tailing effect was noted after 30 min of heating in both groups. On the other hand, *Salmonella* counts gradually declined without statistically significant differences ($P>0.05$) between groups. No colonies (<1.00 log/g) were detected after the 30th and 45th min in the AA and C samples, respectively. *Salmonella* exhibited greater heat sensitivity than *E. coli*. Further research is needed to assess the safety of incorporating AA into low-temperature cooked sous vide seafood.

Keywords: Thermal inactivation, Food safety, Food poisoning, Pathogenic bacteria, Vitamin C

Introduction

Low temperature is being utilized progressively in the food industry and integrated into modern culinary practices (Becker et al., 2016). This approach ensures that the food consistently exhibits a pleasing texture and colour every time it is cooked. Heat-sensitive foods are frequently cooked at low temperatures to create a delicate and tender texture (Misu et al., 2024). Tenderness, juiciness, flavour, and appearance are crucial factors in evaluating the organoleptic quality of food products intended for human consumption (Christensen et al., 2012). Due to seafood's high susceptibility to quality deterioration, preserving its sensory attributes during heat treatment is essential.

Anchovy is the most popular seafood species among Turkish consumers. In 2023, 387,115 tonnes of marine fish were captured from seas in Türkiye. Anchovy accounted for 185,000 tonnes (%48) of this total, making it the most frequently wild-caught species (TUIK, 2024). Anchovy is used in a variety of dishes, including soup, rice dishes, stews, and marinades, and can be prepared using methods such as frying, steaming and grilling (Uran & Gokoglu, 2014). However, elevated cooking temperatures cause changes in myofibrillar proteins that can toughen the texture of food (Becker et al., 2016). Besides, frying results in the loss of EPA and DHA omega-3 fatty acids in fatty fish, which are essential for human health (Ansorena et al., 2010).

Fine dining restaurants have been preparing vacuum-packed, namely, sous vide foods, in temperature-controlled water baths at low temperatures (55 to 60°C) for the past decades (Mortensen et al., 2012). Sous vide is a cooking technique that involves vacuum sealing raw or pre-treated material and pasteurizing it at a precisely controlled temperature (50 to 95°C) for a predetermined time. Then the food can either be served or refrigerated. Rapid chilling prior to cold storage is crucial for ensuring safety (Coşansu et al., 2022). Mild heat treatment during sous vide cooking for an insufficient duration can pose a risk of foodborne illness, as some bacteria may thrive in the "temperature danger zone". The danger zone is the range of temperatures between 5°C and 60°C. Within this temperature range, pathogenic foodborne bacteria may reach hazardous levels, potentially leading to food poisoning (FSIC, 2014; USDA, 2017). Therefore, applying low-temperature heat treatment for an insufficient duration during sous vide cooking may compromise food safety.

Seafood contamination resulting from improper handling and processing before cooking is a major challenge, as mild heat treatments only partially eliminate microorganisms. Although pathogenic bacteria in food do not lead to any observable changes and cannot be detected visually, the consumption of contaminated seafood poses significant health risks (Mol & Coşansu, 2022). *Escherichia coli* O157:H7 and *Salmonella* Enteritidis are foodborne pathogenic bacteria that cause infectious diseases. They are ubiquitous in water and soil environments and are part of the intestinal flora of many animals (Newell et al., 2010). *Escherichia coli* O157:H7 lead to hemorrhagic colitis and hemolytic uremic syndrome, while *Salmonella* Enteritidis typically cause gastroenteritis, characterized by symptoms such as nausea, abdominal cramps, vomiting, and diarrhoea (Coşansu, 2018; Vencia et al., 2015). Therefore, additional hurdles are needed to inhibit unwanted bacteria in food when cooked at low temperatures.

Ascorbic acid (AA) is a natural powerful antioxidant that is found in almost all fruits and vegetables. It is an essential water-soluble micronutrient in human diets. Vitamin C, its biologically active form (L-AA), supports several cellular processes that help the immune system (Davey et al., 2000; Naidu, 2003). The Recommended Dietary Allowance (RDA) is 75 and 90 mg/day for adult women and men, respectively (Institute of Medicine, 2000). AA is extensively utilized in seafood processing to inhibit lipid oxidation, decelerate spoilage, and maintain product quality by preserving colour and texture (Deng et al., 1978; Hambre et al., 2003). Besides its high reducing power, AA appears as an antimicrobial agent in various studies (Giannuzzi & Zaritzky; 1996, Sangcharoen et al., 2017; Przekwas et al., 2020). It has also been utilized in food research to investigate its potential antimicrobial effects by incorporating it into carrot juice (Tajkarimi & Ibrahim, 2011), cheese (Elafify et al., 2022), pork (Ogden et al., 1996) and sole (Zambuchini et al., 2008). In seafood processing, AA functions as an antimicrobial agent, enhancing the safety of seafood products by curbing the proliferation of microorganisms (Sanjúas-Rey et al., 2012).

By lowering the pH and acidifying the environment, AA inhibits bacterial growth through oxidative stress and membrane disruption. Oxidative stress is a disturbance in the balance between reactive oxygen species (ROS) and antioxidants. ROS such as hydroxyl radical, hydrogen peroxide and superoxide radical cause oxidative damage to bacterial proteins, lipids, and nucleic acids, disrupting vital metabolic pathways. As a ROS scavenger, AA leads to the leakage of essential intracellular compounds. Thereby, it destroys the structural stability of the cell and results in bacterial death (Betteridge, 2000; Liu et al., 2021; Ma et al., 2024).

Food safety concerns related to ready-to-eat seafood products cooked at low-temperature can be addressed through the addition of antimicrobial compounds such as organic acids. This study aimed to evaluate the antimicrobial effects of ascorbic acid in sous vide-cooked anchovy, particularly in inhibiting *E. coli* and *S.* Enteritidis that may persist due to insufficient heat treatment.

Materials and Methods

Materials

Iced fresh anchovy was purchased from Karaköy fish market, İstanbul, Türkiye. A total of 3 kg of anchovy was obtained. Fish were brought to the laboratory without breaking the cold chain. Anchovies were beheaded and gutted, and the backbones were removed to obtain butterfly fillets. Cleaned fish (ca. 1.8 kg) were taken to gastronom trays and equally divided into two groups: 1) control (C), and 2) ascorbic acid-added (AA). Food-grade ascorbic acid (Balmumcu Kimya LTD., Türkiye) was used to prepare a 10% stock solution (w/v). The AA group was prepared by adding 0.5% (w/w) ascorbic acid solution directly to the fillets and manually mixed under aseptic conditions. For the control group, the same amount of sterile distilled water was added to the fish. Ten grams of fish were placed into heat-stable polyethylene-polyamide pouches (Apack Ambalaj, Türkiye) appropriate for sous vide cooking. Each group yielded 90 bags, of which at least seventy-five were used. Then, the samples were frozen at -24 °C until use. *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Enteritidis were obtained from the culture collection of the Department of Food Engineering, Sakarya University.

Preparation of Inoculum and Inoculation

Stock cultures of *Escherichia coli* O157:H7 and *Salmonella* Enteritidis were activated in 10 mL Tryptic Soy Broth (TSB) at 35°C for 24 h, separately. A 100 µL of culture from the tube was transferred to TSB again and incubated for another day. Then, the culture solution was centrifuged at 4000 rpm for 10 min. After discarding the supernatant, the pellet was washed twice with 10 mL of 0.1% peptone water (PW) (Üçok Alakavuk et al., 2021). Finally, the precipitation was mixed with 10 mL of PW. This culture solution was serially diluted to obtain 5-6 log CFU/mL inoculum for each bacterium.

Fish samples in pouches were thawed overnight in a refrigerator (4°C). Two hundred microliters of inoculum were inoculated into pouches. The transfer of bacteria to the flesh was allowed for over 20 min. The samples were then vacuum packaged in preparation for heat treatment.

Heat Treatment

Heat treatment trials of *E. coli* and *S.* Enteritidis in sous vide anchovy were performed separately. For sous vide cooking, a water-circulating heater bath (Daihan, WBC 22, S. Korea) was used. Low-temperature cooking was carried out at 55 ±0.5°C. The inner temperature of anchovies, which were not inoculated with bacteria, was monitored with a needle temperature probe. Sampling was performed at 0, 5, 10, 15, 20, 25, 30, 45, 60, 90 and 120 min of cooking. Once the predetermined cooking time was up, each pouch was immediately submerged in an ice water tank to cool down.

Enumeration

Ten grams of fish sample were diluted with 10 mL Maximum Recovery Diluent (MRD) and homogenized in sterile filter bags. By transferring 1 mL, serial dilutions were prepared in 9 mL MRD from each group. A 100 µL of appropriate dilutions were spread plated on Tryptic Soy Agar (TSA) plates in duplicate. For the recovery of the heat-injured bacteria, plates were incubated at 25°C for 2 h. Subsequently, plates were overlaid with a 10-12 mL layer of selective media. For *E. coli*, Sorbitol MacConkey (SMAC) Agar supplemented with Cefixim-Tellurite (CT) solution was used, while xylose lysine tergitol-4 (XLT4) agar supplemented with XLT4 Agar Supplement solution was utilized for *Salmonella*. All plates were incubated at 37°C for 24 h, and the counted colonies were expressed as logarithmic colony forming units per gram (log CFU/g) (Brashears et al., 2001; Lee & Kang, 2009).

Statistical Analysis

Two independent trials were carried out for the predetermined samplings of each bacterium. All data were presented as means ± standard deviation. The differences among the groups were determined by independent samples t-test and performed by using SPSS v21.0 software (IBM Inc., IL). The significance level between the means was considered $P<0.05$.

Results and Discussion

Conventional cooking, which aims for a specific core temperature, involves a higher temperature and leads to a decline in sensory quality due to increased toughness and reduced juiciness (Becker et al., 2016). The Sous vide technique allows cooking at low temperatures without deteriorating the key qualities of sensitive foods such as fish (Misu et al., 2024). On the other hand, inadequate cooking cannot eliminate the risk of pathogenic bacteria that may be present in food. Contamination of food products with *E. coli* and *Salmonella* spp. is associated with numerous cases of foodborne diseases (Interagency Food Safety Analytics Collaboration, 2022).

The incorporation of ascorbic acid into seafood that is cooked at low temperatures, can enhance food safety by inhibiting foodborne pathogenic bacteria. AA is a water-soluble antioxidant that is directly influenced by the cooking conditions. A reduction in the AA content of food is expected depending on the cooking process. Ascorbic acid undergoes rapid oxidation to dehydroascorbic acid, hydrolysis to 2,3-diketogulonic acid, and ultimately polymerization to other non-nutritive components. For instance, boiling red, orange and yellow paprika for 5 min resulted in the loss of AA content due to the leaching into boiling water. On the other hand, AA reductions were not significantly different from those of the raw peppers after microwave heating (Chuah et al 2008). Vacuum packing the anchovy before sous vide cooking, minimized AA loss, resulting in a more nutritious ready-to-eat meal.

Shiga toxin-producing *E. coli* O157 can cause severe infections and even death. Contaminated water and food, direct contact between people, and interaction with animals or their surroundings are the transmission routes to humans. Proper food handling and adequate heat treatment are essential to prevent outbreaks (Heiman et al., 2015). In the current study, cooking at 55°C was not sufficient to destroy all *E. coli* in both sous vide anchovy groups at 120 h. The amount of *E. coli* inoculum transferred to the flesh was 4.49 and 4.43 log CFU/g in control (C) and ascorbic acid added (AA) samples, respectively. No statistically significant difference ($P>0.05$) was observed between C and AA samples until the 45th min of sampling. Thereafter, the *E. coli* counts gradually decreased over time at 45, 60, 90 and 120 min, and significant differences ($P<0.05$) were revealed between the groups. On the other hand, the greatest reduction (%) in bacterial count was achieved at 120 min when the treated group was compared to the control samples (Table 1). The combined effect of lower pH and heat compromised bacterial integrity by leading to cell death and providing an effective bacterial inactivation. The bacterial load decreased progressively over time, showing a downward trend at each sampling point. However, a tailing effect was observed, indicating that the bacteria persisted at low levels during the prolonged heat treatment at 55°C (Figure 1). In a similar study, investigating the thermal inactivation fate of *E. coli* ATCC 25922, holding inoculated sous vide cooked beef steaks at 54 °C resulted in >6 log reductions at various durations. The bacteria counts were 0.51, 0.47, and 1.01 log CFU/g at 64.5, 86, and 107.5 min, indicating a tailing effect (Hunt et al., 2021). Juneja et al. (2009) reported that the time required to eliminate *E. coli* O157:H7 in sous vide minced meat cooked at 55°C by 1 log (D value) was 67.79 ±5.48 min.

Salmonella is a vegetative pathogen frequently detected in raw meat products, which can survive low-temperature heat treatment (Hunt et al., 2023). The inoculum dose of *Salmonella* Enteritidis absorbed by the flesh was 4.45 and 4.41 log CFU/g in the C and AA groups, respectively. The *Salmonella* counts substantially decreased throughout the study period. However, the differences among groups were not statistically significant ($P>0.05$) at any duration of sampling. The *Salmonella* load in the C group was found to be 1.90 log CFU/g at 45 min, while the bacteria was undetectable (<1.00 log CFU/g) in the AA group. After the 45th min of sampling, no colonies (<1.00 log/g) were detected, suggesting the absence of viable bacteria in both groups. The highest reduction percentage was 8.33% observed at the 30th min of cooking when compared to group C (Table 2). Moreover, the comparison of time-dependent-survival curves of *Salmonella* in C and AA groups was presented in Figure 2. By these findings, Juneja (2007) stated that the count of a four-strain mixture of *Salmonella* spp. in chicken breast cooked in the bag at 55°C declined by 1.91 log within 15 min and by 4.0 log after 30 min, which were plated on TSA. Moreover, Hunt et al. (2023) studied the thermal inactivation of 7 log-inoculated *Salmonella enterica* serotypes Typhimurium, Enteritidis, and Heidelberg in sous vide-cooked beef steaks. The minimum time needed for a 5-log reduction at 54.4°C was 64.5 min, and the initial bacterial load was reduced to 0.97 log CFU/g after 107.5 min of cooking. In another study, D values of *Salmonella*, inoculated to sous vide chicken breast, were determined to be 47.65 ±3.68 min and 34.12 ±1.73 min in untreated and acidic teriyaki sauce marinated samples at 55°C. The D value of marinated samples was 28.4% lower than the control samples' values (Karyotis et al., 2017). Bacteria are more susceptible to heat when food is subjected to acid treatment. The acidic environment damages the bacterial cell membrane and alters its internal structure (Dogruyol et al., 2020).

According to the Codex Alimentarius Commission (2024), the maximum use of vitamin C (L-AA) in cooked and/or fried fish and fish products, including molluscs, crustaceans, and echinoderms is permitted under Good Manufacturing Practices (GMP). In this study, 0.5% ascorbic acid was added to anchovies prior to sous vide cooking. This AA concentration is used in various food research avoiding sensory deterioration (Giroux et al., 2001; Ouattara et al., 2002; Elafify et al., 2022). Furthermore, a 1-log reduction (6.53 to 5.70 log CFU/g) in the total bacterial count of medium-fat ground beef patties was reported during the first 4 days of cold storage, when 0.5% AA added (Ouattara et al., 2002). However, there exists a limited number of studies on the effectiveness of AA against *E. coli* and *Salmonella* in food. Elafify et al. (2022) stated that the application of 0.5% AA to artificially contaminated cheese reduced *Salmonella* Enteritidis counts significantly by 0.9 log CFU/g in comparison to untreated samples

during 4 weeks of cold storage. Aligned with the current research, Ouattara et al. (2002) reported that the counts of total coliforms and *Enterobacteriaceae* were not statistically different after the incorporation of AA to beef patties at the beginning of the study. In another study, Chiasson et al. (2004) stated that using AA along with carvacrol reduced the radiosensitivity of *E. coli* and *S.* Typhi significantly in ground beef. The incorporation of 0.03% ellagic acid in combination with 1.71% AA and 1.98% sodium ascorbate extended the shelf life of fresh sole for 2 more days and significantly delayed the growth of total aerobic, psychrotrophic and *Pseudomonas* bacteria (Zambuchini et al., 2008).

In the in vitro studies, Verghese et al. (2017a, 2017b) reported that AA significantly inhibited the growth of *E. coli* in Tryptic Soy Broth and emphasised the possibility of incorporating vitamin C as a safe and effective antimicrobial agent, against climbing antimicrobial resistance. In another study evaluating the effect of vitamin C on the secondary contamination of food, the biofilm formation of pathogenic bacteria was observed. It was reported that the 25.0 mg/mL AA inhibited the biofilm growth of *E. coli*, *L. monocytogenes*, and *S. aureus* by 93.4%, 74.9%, and 40.5%, respectively (Przekwas et al. 2020). Ascorbic acid obtained from Japanese apricot extract also presented antibacterial activity against *E. coli* and the minimum inhibitory concentration (MIC) was determined to be 8.00 mg/mL (Gao et al., 2012). Selim et al. (2012) specified that AA eliminated *E. coli, Salmonella indica* and *Staphylococcus aureus* after 2 hours, and the minimum bactericidal concentrations (MBC) were 32 mg/mL for the above bacteria. Conversely, Sangcharoen et al. (2017) highlighted that AA alone had no inhibitory effect on the growth of *S.* Enteritidis ATCC 13076 in Mueller Hinton broth incubated at 35°C.

AA reduces reactive oxygen species (ROS) levels by improving antioxidant enzyme activity, preventing oxidative damage and controlling radical accumulation. AA exhibits antibacterial activity by inducing intracellular damage, whereas heat treatment exerts antibacterial effects by compromising the cell membrane and triggering protein denaturation (Ma et al., 2024). Consequently, the distinct mechanisms of AA and heat underpin the enhanced reduction in *E. coli* counts observed when they were used in combination. Similarly, Kang et al. (2021) emphasized that hot water and citric acid induced the synergistic generation of ROS or superoxide, resulting in excessive destruction of the cell membrane of *E. coli,* triggering the ROS leakage within the cell.

Figure 1. Comparison of the decrease and tailing effect in the *E. coli* counts over time

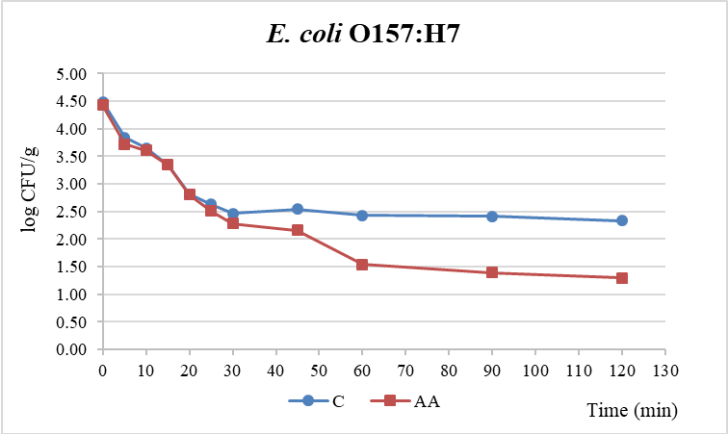

Table 1. Mean *E. coli* O157:H7 counts (log CFU/g) in control (C) and ascorbic acid-added (AA) groups at each sampling time after sous vide cooking and reduction percentage of the bacteria

E. coli O157:H7		C		AA		% Reduction
		Mean	±STD	Mean	±STD	
	Inoculum	6.67	±0.06	6.67	±0.06	
Time	**0**	4.49	±0.01[a]	4.43	±0.03[a]	1.23
	5	3.84	±0.05[a]	3.72	±0.09[a]	3.30
	10	3.65	±0.12[a]	3.61	±0.09[a]	1.34
	15	3.34	±0.02[a]	3.35	±0.01[a]	-0.04
	20	2.81	±0.03[a]	2.81	±0.13[a]	0.22
	25	2.63	±0.06[a]	2.51	±0.03[a]	4.60
	30	2.47	±0.08[a]	2.28	±0.10[a]	7.56
	45	2.55	±0.07[a]	2.16	±0.01[b]	15.27
	60	2.43	±0.09[a]	1.54	±0.09[b]	36.56
	90	2.41	±0.12[a]	1.39	±0.55[b]	42.44
	120	2.34	±0.12[a]	1.30	±0.42[b]	44.34

[a,b]: Letters indicate the statistical difference between groups at each given time point. Percentage (%) reduction was calculated by subtracting the count of AA from the count of C and then dividing by C, with the result multiplied by 100.

Figure 2. Comparison of the decrease and tailing effect in the *Salmonella* counts over time

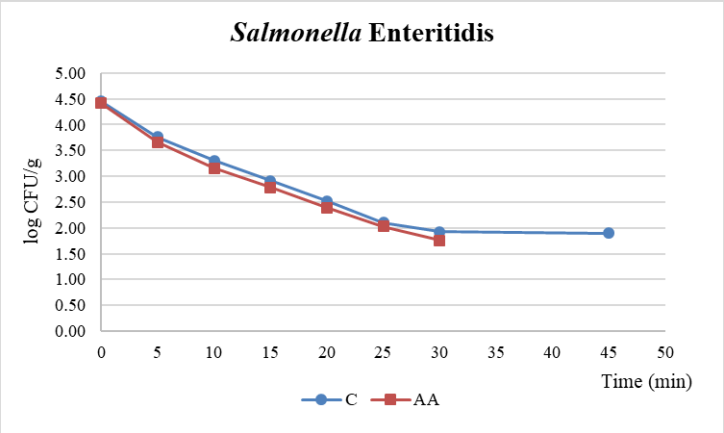

Table 2. Mean *Salmonella* Enteritidis counts (log CFU/g) in control (C) and ascorbic acid-added (AA) groups at each sampling time after sous vide cooking and reduction percentage of the bacteria

Salmonella Enteritidis		C		AA		% Reduction
		Mean	±STD	Mean	±STD	
	Inoculum	5.09	±0.49	5.09	±0.49	
Time	**0**	4.45	±0.17[a]	4.41	±0.06[a]	0.85
	5	3.76	±0.09[a]	3.66	±0.03[a]	2.68
	10	3.31	±0.01[a]	3.16	±0.05[a]	4.52
	15	2.91	±0.34[a]	2.78	±0.00[a]	4.53
	20	2.52	±0.17[a]	2.39	±0.23[a]	5.19
	25	2.10	±0.28[a]	2.03	±0.01[a]	3.32
	30	1.92	±0.38[a]	1.76	±0.09[a]	8.33
	45	1.90	±0.00	<1.00		
	60	<1.00		<1.00		
	90	<1.00		<1.00		
	120	<1.00		<1.00		

[a,b]: Letters indicate the statistical difference between groups at each given time point. Percentage (%) reduction was calculated by subtracting the count of AA from the count of C and then dividing by C, with the result multiplied by 100.

In this study, *S.* Enteritidis was more vulnerable to heat at 55°C than *E. coli* in both C and AA groups, during sous vide cooking. Contrary to the current findings, Patil et al. (2024) reported that *Salmonella* Montevideo exhibited a lower decline in bacteria counts than *E. coli* O157:NM, in sous vide cooked ground beef at 62.5°C at the 5th, 10th and 20th min of samplings. It was also stated that >4 log and 5 log CFU/g reductions were achieved after 20 min and 120 min treatments for both pathogens.

Conclusion

Ascorbic acid incorporated into sous vide anchovies prior to thermal treatment significantly reduced the *E. coli* load at and after the 45th min. However, there were no distinct differences among groups in *Salmonella* counts at any sampling times. While *Salmonella* was undetectable after the 30th min in the AA group and after the 45th min in the control group, *E. coli* was present even at the 120th min. Based on the results, it was suggested that *Salmonella* was more vulnerable to heat treatment compared to *E. coli* in both groups. Due to its advantages, such as being easy to obtain, cost-effective, and capable of reducing contamination levels in food at low concentrations, ascorbic acid holds potential as a natural additive that not only preserves but also enhances the sensory properties of foods. Further research is needed to ensure the food safety of sous vide products processed at low temperatures with the addition of ascorbic acid, particularly for heat-sensitive foods like fish.

Compliance with Ethical Standards

Conflict of interest: The author declares no actual, potential, or perceived conflict of interest for this article.

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

Data availability: Data will be made available on request from the author.

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Disclosure: -

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Occurrences of the large male smoothhound, *Mustelus mustelus* (Linnaeus, 1758) in the Sea of Marmara

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ABSTRACT

On 18 December 2023 and 17 August 2024, two male specimens of *Mustelus mustelus* (1321 mm and 1425 mm TL) were caught during scientific bottom trawling in the northern Sea of Marmara, the first off, the coast of Prince Islands and the second off Ambarlı. The results of the present study showed that male *M. mustelus* can grow extremely large (at least 292 mm) than previously published total length (TL) data for specimens caught in Turkish waters. The smaller size of male smoothhounds caught in Turkish waters in recent years is, of course, causing a phenomenon well known to the new generation of shark researchers in Türkiye, known as the "shifting baseline syndrome". Currently, *M. mustelus* is not a protected shark species in Turkish waters but is also one of the most sought-after sharks by commercial fishermen. In conclusion, to avoid capturing mega-spawning specimens of *M. mustelus* in commercial fisheries, an upper size limit can be implemented as a first step towards effectively conserving the species.

Keywords: *Mustelus mustelus*, Megaspawner, Shifting baseline, Conservation

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Introduction

The smoothhound, *Mustelus mustelus* (Linnaeus, 1758), is a relatively large shark species in the family Triakidae of the order Carcharhiniformes (Ebert et al., 2021). In the eastern Atlantic, the distribution of *M. mustelus* extends from the waters around the British Isles to Morocco, the Canary Islands, the Azores and Madeira in the north, and from Angola to South Africa in the south (Ebert et al., 2021). It is one of the well-known members of the Mediterranean shark fauna (Barone et al., 2022), which also occurs in Turkish waters over a wide range from the eastern Mediterranean to the Sea of Marmara (Bilecenoğlu, 2024).

Although the size (total length, TL) of *M. mustelus* is reported to be 2000 mm in several references (e.g. Ninni, 1923; Reiner, 1996), much of the contemporary general ichthyological or shark-specific literature reports the TL of the smoothhound to be ≤1750 mm (e.g. Bauchot, 1987; De Maddalena et al., 2001; Ebert et al., 2021). Furthermore, the largest sizes (TL ranging from 1650 to 1750 mm) for *M. mustelus* have been attributed exclusively to females, and despite the largest males are commonly reported to be around 1100 mm (De Maddalena et al., 2001; Ebert et al., 2021), males can grow up to 1445 mm in the Mediterranean Sea (Saïdi et al., 2008) or 1450 mm TL in South African waters (Smale & Compagno, 1997).

In the present paper, the authors report on capturing very large male smoothhounds in the Sea of Marmara and provide detailed morphometric measurements of the specimens examined. They also review the available literature on the maximum size of *M. Mustelus*'s from the perspective of the "shifting baseline syndrome" (SBS) (Pauly, 1995) and "let the mega spawners live" (LML) concepts (Froese, 2004).

Materials and Methods

The male smoothhounds examined in this study were caught using bottom-trawl gear (codend mesh size 14 mm and maximum mesh size 22 mm) and towed according to MEDITS standards (Anonymous, 2017). Bottom-trawl hauls were conducted aboard the *R/V Yunus-S*, a 510 hp stern trawler operated by Istanbul University, and the towing duration was 30 minutes. The bottom trawl stations where the studied smoothhounds were caught are shown in Figure 1. In order to keep the captured smoothhounds alive, they were gently removed from the codend and not gaffed to avoid injury, following the best practice procedure for shark handling (FAO & ACCOBAMS, 2018). As Ellis et al. (2017) suggested, a survival tank was equipped with a large volume container and a seawater hose with an adjustable nozzle, and captured specimens were held in the tank before examination and released alive following videography and measurements. Species identification was followed by Ebert & Stehmann (2013), Ebert et al. (2021) and Barone et al. (2022). 65 morphometric distances were measured either with a tape measure to the nearest 0.5 mm (for distances> 10 cm) or with a vernier calliper to the nearest 0.05 mm (for distances ≤ 10 cm) following the methodology outlined in Ebert et al. (2021). Total length (TL) is the distance between the tip of the snout and the tip of the upper lobe of the caudal fin lying in the natural position (Ebert et al., 2021), and TL measurements were documented by videography. Video evidence of the specimens examined is available on request from the first author.

Results and Discussion

On 18 December 2023 a male smoothhound (specimen 1, 1321 mm TL) was caught off the southwestern sector of Prince Islands over a muddy-sandy bottom at the depths of 24-28 m between the following coordinates: start of the towing: 40º56'33" N 29º1'48" E; end of the towing: 40º55'56" N 29º1'15" E. On 17 August 2024 the second male (specimen 2, 1425 mm TL) was caught off Ambarlı coast also over a muddy-sandy bottom at the depths of 126-132 m between the following coordinates: start of the towing: 40º55'861" N 28º25'595" E; end of the towing: 40º55'337" N 28º27'364" E. Total weights of specimens 1 and 2 were 6850 g and 8000 g, respectively. Morphometric measurements of the specimens are presented in Table 1. The following description is based on the examined smoothhounds: Specimens 1 and 2 were large sharks with relatively slender bodies and short heads, in which the mean prepectoral length is 18.8% in TL; eyes large, in which the mean length of eye is 2% in TL, and mean interorbital space is 6.5% in TL; a moderately long snout, in which the mean preoral snout is 5.4% in TL; mean length of anterior margins of the moderately large pectoral fins constitute 13% of TL and mean length of posterior margins constitute 12.6% of TL. Molar-like low-crowned teeth are arranged on the upper and lower jaws, and the joint of the Meckelian cartilages of the lower jaw constitutes a prominent

angle. Posterior edges of the first and second dorsal fins are not fringed. Dorsal colouration of the examined smoothhounds was uniformly brownish grey, without white or dark spots, and ventral surfaces whitish; narrow dark grey bands are visible on the posterior edges of dorsal and caudal fins; pale grey colouration is visible posteriorly on the ventral surfaces of pectoral and pelvic fins. Photographs of the examined smoothhounds are presented in Figure 2. Detailed morphometric measurements of the examined specimens are presented in Table 1. The above description is consistent with the descriptions given in Ebert & Stehmann (2013), Ebert et al. (2021) and Barone et al. (2022), thus the examined males were positively identified as *Mustelus mustelus*.

Despite the slight differences, most of the morphometric measurements of the examined smoothhounds (Table 1) fell within the ranges given in Ebert & Stehmann (2013) for *M. mustelus*. Nevertheless, two of the morphometric measurements given in the present study, which are mean eye length (EYL) to TL and interorbital space (INO) to TL, were found to be quite different from the published ratios (Ebert & Stehmann, 2013). Contrary to Ebert & Stehmann (2013) stating that mean EYL is 2.3 to 4 % of TL, the same ratio is 2% of TL in the examined specimens. Furthermore, despite Ebert & Stehmann (2013) reporting that INO is 3.7-4.8% of TL, the same ratio is 6.5% of TL in the examined smoothhounds. Allopatric or geographically distant populations of the same fish species may tend to show morphometric distances at the opposite margins of the well-accepted proportions ranges, as Cailliet et al. (1986) proposed. Therefore, the differences seen in the present proportions of EYL-TL and INO-TL can be considered admissible by the allopatry.

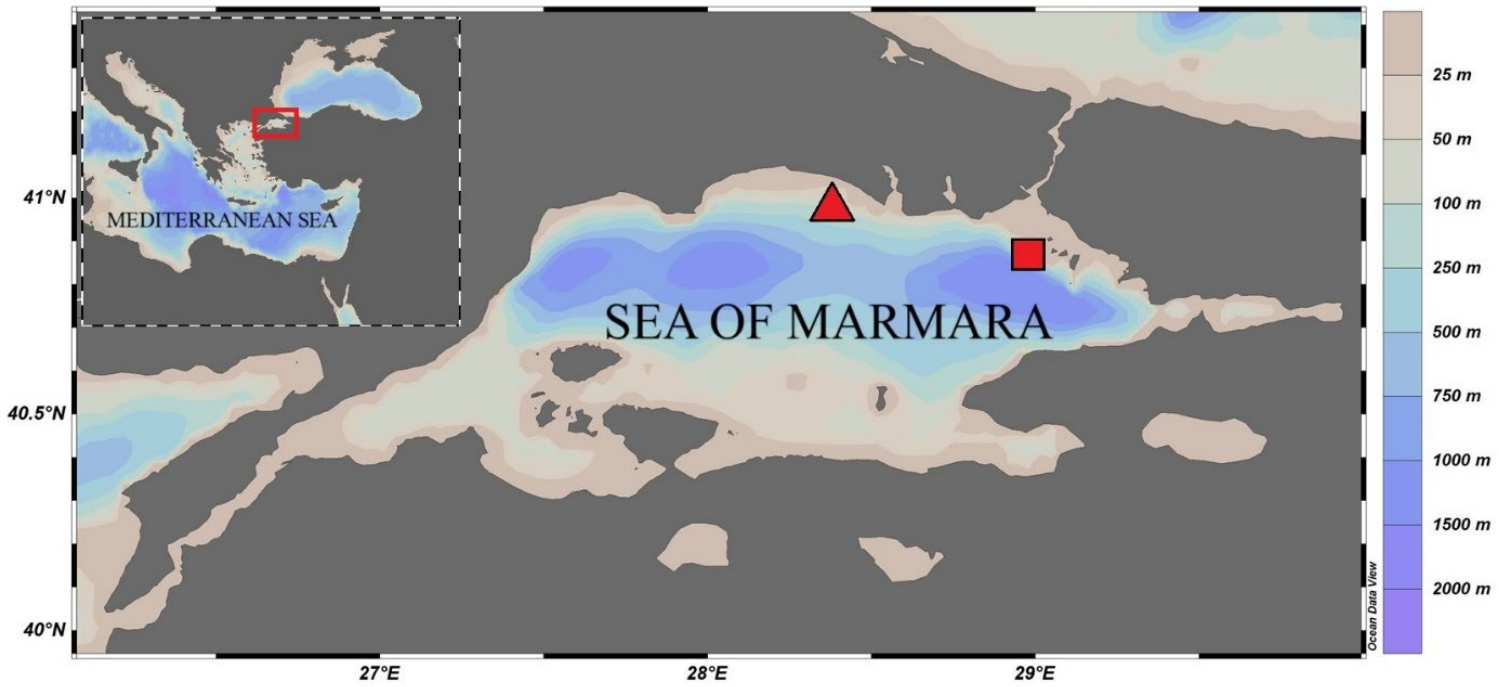

Figure 1. Map showing the approximate localities where the examined male smoothhounds were caught (▲, specimen 1 and ■, specimen 2). Red rectangle in the small map showing the geographical position of the Sea of Marmara in the Mediterranean ecosystem

Figure 2. The largest male specimens of *Mustelus mustelus* were caught and released in the Sea of Marmara. Side views of (a) specimen 1, 1321 mm TL and (b) specimen 2, 1425 mm TL, and (c) ventral view of specimen 2. Arrows denote the claspers, which were remarkably more extended than the pelvic fins and hardened

The largest sizes of *M. mustelus* published in various references from 1923 to 2021 are reviewed in Table 2. Regardless of sex, the TL of *M. mustelus* specimens varied between 1500 mm and 2000 mm, with the largest sizes mainly attributed to female smoothhounds. Despite reports of large smoothhounds of 2000 mm TL (Ninni, 1923; Reiner, 1996), the TL of the most significant confirmed female of *M. mustelus* was reported to be 1732 mm, caught in South African waters (Goosen & Smale, 1997). This female was considered the species' most significant (record size) specimen ever recorded in the 20th century (De Maddalena et al., 2001). On the other hand, based on the literature (Compagno, 1984; Smale & Compagno, 1997; Goosen & Smale, 1997; Ebert et al., 2021), the maximum TL of males is smaller than that of females. *M. mustelus* is a viviparous shark. Therefore, females require more volume in the abdominal cavity to maintain developing embryos during pregnancy (Ebert et al., 2021), resulting in larger sizes than males. Furthermore, Smale & Compagno (1997) reported that the TL of the most prominent male was 1450 mm in South African waters, while Saïdi et al. (2008) reported a male of 1445 mm TL from the Gulf of Gabès (southern Tunisia, south-central Mediterranean Sea). Therefore, based on the available data in the scientific literature, one of the present male smoothhounds, which was 1425 mm TL, is probably one of the largest males of *M. mustelus* ever recorded in Turkish waters. However, the present study's authors do not exclude the possibility of unpublished males being captured that may be longer than the male smoothhounds examined.

Table 1. Morphometric measurements and percent TL of mean of respective distances of examined males. Distances in bold represent the morphometric percentages used to describe *Mustelus mustelus* in Ebert & Stehmann (2013).

Measurement (mm)	Specimen 1	Specimen 2	Mean	% TL of mean	Published descriptive % of TL[§]
Total Length (TL)	1321	1425	1373	100	
Precaudal-Fin Length (PCL)	1050	1132.7	1091.33	79.5	N/R
Pre-Second Dorsal Fin Length (PD2)	804	867.3	835.65	60.9	N/R
Pre-First Dorsal Fin Length (PD1)	350	377.6	363.78	26.5	N/R
Prepectoral-Fin Length (PP1)	**249**	**268.6**	**258.80**	**18.8**	**17-21**
Prepelvic-Fin Length (PP2)	569	613.8	591.40	43.1	N/R
Snout-Vent Length (SVL)	631	680.7	655.84	47.8	N/R
Preanal-Fin Length (PAL)	876	945.0	910.48	66.3	N/R
Interdorsal Space (IDS)	**302**	**325.8**	**313.89**	**22.9**	**18-25**
Dorsal Caudal-Fin Space (DCS)	138	148.9	143.43	10.4	N/R
Pectoral-Fin Pelvic-Fin Space (PPS)	270	291.3	280.63	20.4	N/R
Pelvic-Fin Anal-Fin Space (PAS)	251	270.8	260.88	19	N/R
Anal-Fin Caudal-Fin Space (ACS)	**108**	**116.5**	**112.25**	**8.2**	**6.3-8.8**
Head Length (HDL)	229	247.0	238.01	17.3	N/R
Prebranchial Length (PG1)	196	211.4	203.72	14.8	N/R
Prespiracular Length (PSP)	116	125.1	120.57	8.8	N/R
Preorbital Length (POB)	**82**	**88.5**	**85.23**	**6.2**	**5.9-8**
Prenarial Length (PRN)	43	46.4	44.69	3.3	N/R
Preoral Length (POR)	**71.5**	**77.1**	**74.31**	**5.4**	**5.3-7.4**
Eye Length (EYL)*	**26.5**	**28.6**	**27.54**	**2**	**2.3-4**
Eye Height (EYH)	13.5	14.6	14.03	1	N/R
Interorbital Space (INO)*	**85.8**	**92.6**	**89.18**	**6.5**	**3.7-4.8**
Spiracle Length (SPL)	7.9	8.5	8.21	0.6	N/R
Eye-Spiracle Space (ESL)	12.5	13.5	12.99	0.9	N/R
Nostril Width (NOW)	18.9	20.4	19.64	1.4	N/R
Internarial Space (INW)	**29.3**	**31.6**	**30.45**	**2.2**	N/R
Mouth Width (MOW)	72.5	78.2	75.35	5.5	N/R
Mouth Length (MOL)	**42.3**	**45.6**	**43.97**	**3.2**	N/R
Intergill Length (ING)	64.1	69.1	66.62	4.9	N/R
First Gill Slit Height (GS1)	25.9	27.9	26.92	2	N/R
Second Gill Slit Height (GS2)	27.1	29.2	28.17	2.1	N/R
Third Gill Slit Height (GS3)	28.9	31.2	30.04	2.2	N/R
Fourth Gill Slit Height (GS4)	37.5	40.5	38.98	2.8	N/R
Fifth Gill Slit Height (GS5)	16.9	18.2	17.57	1.3	N/R
Pectoral-Fin Anterior Margin (P1A)	**172**	**185.5**	**178.77**	**13**	**13-17**
Pectoral-Fin Base (P1B)	53.9	58.1	56.02	4.1	N/R
Pectoral-Fin Inner Margin (P1I)	91.3	98.5	94.89	6.9	N/R
Pectoral-Fin Posterior Margin (P1P)	**166**	**179.1**	**172.53**	**12.6**	**8.2-14**
Pelvic-Fin Anterior Margin (P2A)	**93.9**	**101.3**	**97.60**	**7.1**	**6.5-9.9**
Pelvic-Fin Length (P2L)	121.6	131.2	126.39	9.2	N/R
Pelvic-Fin Base (P2B)	69.8	75.3	72.55	5.3	N/R
Pelvic-Fin Inner Margin (P2I)	61	65.8	63.40	4.6	N/R
Anal-Fin Anterior Margin (ANA)	82.8	89.3	86.06	6.3	N/R
Anal-Fin Length (ANL)	101.9	109.9	105.91	7.7	N/R
Anal-Fin Base (ANB)	67	72.3	69.64	5.1	N/R
Anal-Fin Inner Margin (ANI)	31.7	34.2	32.95	2.4	N/R
First Dorsal-Fin Length (D1L)	190	205.0	197.48	14.4	N/R
First Dorsal-Fin Anterior Margin (D1A)	140	151.0	145.51	10.6	N/R
First Dorsal-Fin Height (D1H)	113.45	122.4	117.92	8.6	N/R
First Dorsal-Fin Base (D1B)	130.8	141.1	135.95	9.9	N/R

First Dorsal-Fin Inner Margin (D1I)	63.1	68.1	65.58	4.8	N/R
First Dorsal-Fin Posterior Margin (D1P)	139	149.9	144.47	10.5	N/R
Second Dorsal-Fin Length (D2L)	139.5	150.5	144.99	10.6	N/R
Second Dorsal-Fin Anterior Margin (D2A)	109	117.6	113.29	8.3	N/R
Second Dorsal-Fin Height (D2H)	106.26	114.6	110.44	8	N/R
Second Dorsal-Fin Base (D2B)	101.3	109.3	105.29	7.7	N/R
Second Dorsal-Fin Inner Margin (D2I)	39.1	42.2	40.64	3	N/R
Second Dorsal-Fin Posterior Margin (D2P)	98	105.7	101.86	7.4	N/R
Dorsal Caudal-Fin Margin (CDM)	262	282.6	272.31	19.8	N/R
Preventral Caudal-Fin Margin (CPV)	97	104.6	100.82	7.3	N/R
Subterminal Caudal-Fin Margin (CST)	44.1	47.6	45.84	3.3	N/R
Terminal Caudal-Fin Lobe (CTL)	95.7	103.2	99.47	7.2	N/R
Clasper Outer Length (CLO)	119.2	128.6	123.89	9	N/R
Clasper Inner Length (CLI)	157	169.4	163.18	11.9	N/R
Clasper Base Width (CLB)	14.9	16.1	15.49	1.1	N/R

§Ebert & Stehmann (2013); *morphometric percentages slightly differing than those given in Ebert & Stehmann (2013); N/R, not reported in Ebert & Stehmann (2013)

Table 2. A review of reported sizes of *Mustelus mustelus* was published in several references in chronological order. N/R, not reported

Reference	Maximum TL (mm)	Maximum TL of males	Region
Ninni (1923)	2000	N/R	Sea of Marmara
Compagno (1984)	1640	1100	Worldwide
Branstetter (1984)	1500	N/R	Northeastern Atlantic Ocean and Mediterranean Sea
Bauchot (1987)	1600	N/R	Mediterranean Sea
Akşıray (1987)	1500	N/R	Turkish seas
Reiner (1996)	2000	N/R	Eastern-central Atlantic Ocean
Smale & Compagno (1997)	1650	1450	South African waters
Goosen & Smale (1997)	1732	1280	South African waters
De Maddalena et al. (2001)	1650	N/R	Adriatic Sea
Saïdi et al. (2008)	1650	1445	Mediterranean Sea
Ebert & Stehmann (2013)	1500	N/R	North Atlantic Ocean
Ebert et al. (2021)	1750	1100	Worldwide
Present study	---	1321	Sea of Marmara
Present study	---	1425	Sea of Marmara

Contrary to previous literature where the maximum size of *M. mustelus* was reported to be 2000 mm TL (Ninni, 1923; Reiner, 1996), contemporary literature reports the maximum size of the species in its global range to be 1750 mm TL (Ebert et al., 2021), and even smaller for Turkish waters (975 mm TL in Filiz & Mater, 2002; Filiz, 2009; 1133 mm TL in Eronat & Özaydın, 2014). Furthermore, the contemporary maximum TL information for males caught in Turkish waters (577 mm TL in Filiz & Mater, 2002; 852 mm TL in Filiz, 2009; 915 mm TL in Eronat & Özaydın, 2014) was remarkably smaller than the TLs reported globally (1100 mm, Ebert et al., 2021), for Tunisian waters (1445 mm, Saïdi et al., 2008) or South African waters (1450 mm, Smale & Compagno, 1997). The apparent smaller size of male smoothhounds caught in Turkish waters in recent years naturally raises a well-known phenomenon among the new generation of shark researchers in Türkiye, known as the "Shifting Baseline Syndrome" (SBS), first proposed by Pauly (1995). In short, SBS is a phenomenon that occurs when past information or historical experience is missing or forgotten and causes members of the new generation of researchers to accept current data, such as TL measurements, stock size or species composition, as usual (Pauly, 1995). From this perspective, the contemporary size data reported for males of *M. mustelus* caught in Turkish waters is a typical example of SBS. The maximum TL of *M. mustelus* reported in Eronat & Özaydın (2014) is 79.5% of the TL (1425 mm TL) of specimen 2 caught in the Sea of Marmara. Therefore, the present results indicate that

male *M. mustelus* can grow at least 292 mm larger than previously published TL data for specimens caught in Turkish waters.

According to Serena (2005), males of *M. mustelus* mature between 700 mm and 960 mm TL in the Mediterranean. Considering the TLs (1321 mm and 1425 mm) and the very long and hard claspers of the present males (Figure 2), it is obvious that they are mature males. Furthermore, as the TLs of specimen 1 and specimen 2 were 132.6% and 148.4% longer than the maximum TL (960 mm; Serena, 2005) for mature males, respectively, the present male smoothhounds can also be described as "mega spawners", defined as specimens of any fish species that are larger than the length at first maturity (Froese, 2004). Based on the definition of a mega spawner fish (Froese, 2004), e.g. optimum length (the size of the fish slightly larger than the length at first maturity) plus 10%, based on the above-mentioned percentage differences (132.6% and 148.4%) of the present male smoothhounds, they can also be described as very large mega spawners of male *M. mustelus* in the Mediterranean Sea. In line with the concept of "let the mega-spawners live" (LML) proposed by Froese (2004), the present male smoothhounds were kept in a survival tank supplied with fresh seawater prior to the measurements, and they were handled carefully throughout the process and released alive.

Conclusion

The present study shows that males of *Mustelus mustelus* can grow extremely large compared to those reported in the available literature. According to Froese (2004), the goal of the current management regime should be to implement a fishing strategy that results in 0% (none) of the megaspawners being caught in fisheries or being injured or killed in case of unintentional capture. Currently, *M. mustelus* is not a protected shark species in Turkish waters and one of the most sought-after sharks by commercial fishermen. However, *M. mustelus* is listed in Appendix III of the Barcelona Convention and therefore the exploitation of this species needs to be regulated throughout its Mediterranean range (GFCM, 2018). Combining this regulatory requirement proposed in the Barcelona Convention with the above-mentioned LML concept, monitoring the spatial and temporal distribution of mega-spawning males and females of *M. mustelus* throughout the Turkish seas and implementing a rational strategy for managing this shark in commercial fisheries are clear and unavoidable requirements. Therefore, in order to avoid the killing of mega-spawning specimens of *M. mustelus* in commercial fisheries, an upper size limit can be implemented as a first step towards the effective conservation of the species.

Compliance with Ethical Standards

Conflict of interest: The author(s) declare no actual, potential, or perceived conflict of interest for this article.

Ethics committee approval: Since the captured sharks are not included in the list of protected species and were released alive, ethics committee approval is not required.

Data availability: This work has been supported by the "Integrated Marine Pollution Monitoring (DEN-İZ) 2023-2025 Programme" carried out by the Ministry of Environment, Urbanization and Climate Change / General Directorate of Environmental Impact Assessment, Permit and Inspection and coordinated by TUBITAK-Marmara Research Center.

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Disclosure: -

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AQUATIC RESEARCH
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Keywords (indexing terms), usually 3-6 items

Introduction

Material and Methods

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Conclusion

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1. No data was used for the research described in the article.
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Table 1. Limitations for each manuscript type

Type of manuscript	Page	Abstract word limit	Reference limit
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Review Article	no limits	200	60
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Tables should be included in the main document and presented after the reference list, and they should be numbered consecutively in the order they are referred to within the main text. A descriptive title must be placed above the tables. Abbreviations in the tables should be defined below them by footnotes (even if they are defined within the main text). Tables should be created using the "insert table" command of the word processing software and arranged clearly to provide easy reading. Data presented in the tables should not be a repetition of the data presented within the main text but should support the main text.

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

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

References

The citation style and methods that comply with the scientific standards that should be used in the "Aquatic Research" journal for the sources used by the authors in their works are given below.

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....(Bhujel, 2014).

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A book in print

Bhujel, R.C. (2014). A manual for tilapia business. CABI Nosworthy Way Wallingford Oxfordshire OX10 8DE UK, 199 p. ISBN 978-1-78064-136-2. https://doi.org/10.1079/9781780641362.0000
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