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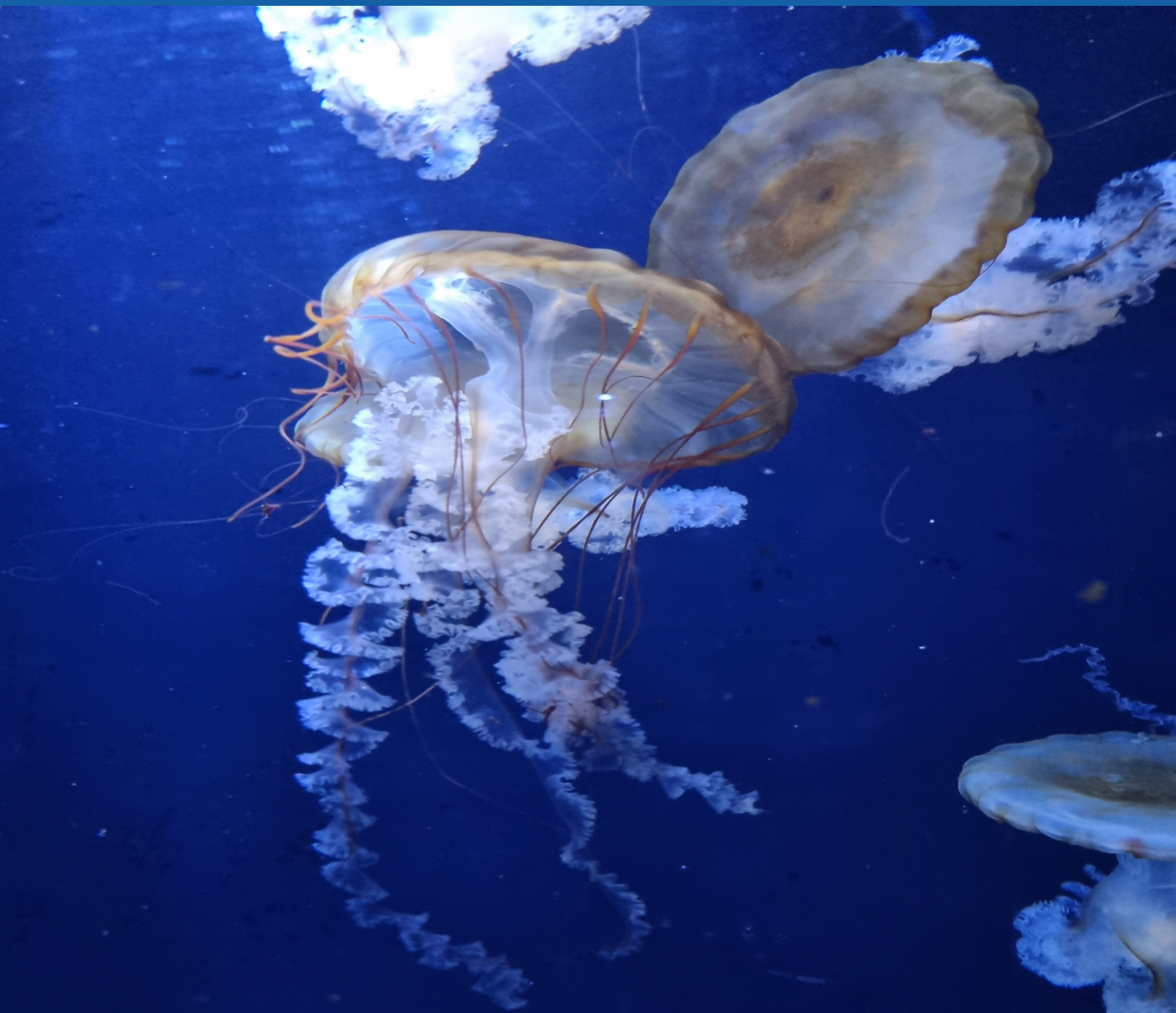

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Aquatic Sciences and Engineering aims to contribute to the literature by publishing manuscripts at the highest scientific level on all fields of aquatic sciences. The journal publishes original research and review articles that are prepared in accordance with the ethical guidelines.

The scope of the journal includes but not limited to; aquaculture science, aquaculture diseases, feeds, and genetics, ecological interactions, sustainable systems, fisheries development, fisheries science, fishery hydrography, aquatic ecosystem, fisheries management, fishery biology, wild fisheries, ocean fisheries, biology, taxonomy, stock identification, functional morphology freshwater, brackish and marine environment, marine biology, water conservation and sustainability, inland waters protection and management, seafood technology and safety.

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Research Article

Determination of the Antibacterial Activity of Microalgae Isolated from Giresun Streams

Sibel Altürk Karaca[1], Elif Neyran Soylu[1]

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ABSTRACT

The rapid emergence of antibiotic resistance has become a global crisis, threatening public health, food security, and agriculture. Particularly, the control of zoonotic diseases and the assurance of microbial safety in animal products necessitate the development of new and sustainable solutions. In this context, research on antimicrobial agents derived from natural sources has been gaining significant importance. Microalgae, with their ability to synthesize bioactive compounds, represent a promising natural resource in this regard. Studies on the antibacterial properties of freshwater microalgae in Türkiye remain limited. However, the rich biodiversity of these ecosystems provides valuable opportunities for the discovery of novel antimicrobial agents. This study investigates the antibacterial activity of *Chlorococcum hypnosporum*, *Stichococcus bacillaris*, *Chlorella vulgaris*, *Chlorolilaea pamvotia*, and *Desmodesmus opoliensis* isolated from the Aksu, Batlama, and Büyük Güre streams in Giresun, Türkiye. Bioactive compounds were extracted using acetone, ethanol, and methanol, and their antibacterial effects were tested against five bacterial strains via the agar well diffusion method. Notably, the acetone extract of *Chlorella vulgaris* exhibited significant activity against *Bacillus subtilis* at 40 µl/petri, and also showed high antibacterial activity against *Escherichia coli*. Additionally, the ethanol extract of *Chlorococcum hypnosporum* exhibited antibacterial activity against both *Salmonella Typhimurium* and *Staphylococcus aureus*. Other microalgae species also demonstrated significant antibacterial properties against the tested bacterial strains. These findings enhance our understanding of the antibacterial potential of Türkiye's freshwater microalgae and highlight their potential as sustainable antimicrobial agents for ensuring microbial safety in animal products. This study further emphasizes the importance of microalgae as natural and environmentally friendly alternatives in combating antibiotic resistance and preventing agricultural microbial contamination.

Keywords: Microalgae, freshwater, molecular characterisation, antibacterial activity

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INTRODUCTION

The discovery of antibiotics marked a revolutionary step in medical science, enabling the effective treatment of various infectious diseases. However, prolonged and widespread use of antibiotics has led to the development of resistance by microorganisms. This resistance, driven by the evolutionary survival mechanisms of bacteria, progressively reduces the effectiveness of antibiotics (Salam et al., 2023). Antibiotic resistance has emerged as one of the most critical issues on the global agenda in recent years, not only due to its significant impact on public health but also because of the economic burden it imposes. The rapidly increasing rates of antibiotic resistance worldwide have profound implications for health, sustainable development, the economy, trade, and the stability of nations. It is anticipated that this issue will have far-reaching consequences in the years to come (Demyanyuk et al., 2023; Vanegas-Múnera

and Jiménez-Quiceno, 2020; Frieri et al., 2017; Lee Ventola, 2015).

The increasing threat of antibiotic resistance underscores the importance of developing alternative therapeutic strategies and discovering new antibiotics (Wright, 2014). In this context, the investigation of biologically active compounds derived from microorganisms in natural sources has become a significant area of research. Algae produce secondary metabolites in response to various environmental stress factors, such as high UV radiation, salinity, biofilm formation, thallus damage, and competition with other aquatic organisms (Kolácková et al., 2023). These metabolites play a critical role in the algae's survival and adaptation to stress conditions, and exhibit biological activities such as antibacterial, antifungal, and antioxidant properties (Little et al., 2021; Ördög et al., 2004). Among these metabolites, long-chain unsaturated fatty acids are particularly associated with the antibacterial activity of algae. These fatty acids target bacterial cell membranes, causing membrane damage, disrupting cellular respiration, and allowing intracellular contents to leak out (Saritha et al., 2018; Amaro et al., 2011; Borowitzka, 1995). The antibacterial potential of algae has gained prominence in recent years as a promising alternative in the fight against antibiotic-resistant bacteria. Studies have demonstrated that secondary metabolites derived from algae exhibit potent antibacterial effects against pathogenic bacteria (Bhowmick et al., 2020; Besednova et al., 2020). In this context, the biotechnological applications of algae have generated considerable interest, not only as antibacterial agents but also as potential biological sources for the pharmaceutical and cosmetic industries (Adhithya et al., 2022). Studies on the antibacterial potential of algae have demonstrated these organisms as promising alternatives in the fight against antibiotic-resistant bacteria. In this context, the biochemical properties of various algae species, particularly their antibacterial activities, have gained significant attention. This study investigates the antibacterial effectiveness of microalgae species such as *Chlorococcum hypnosporum*, *Stichococcus bacillaris*, *Chlorella vulgaris*, *Chlorolilaea pamvotia*, and *Desmodesmus opoliensis*.

Chlorococcum hypnosporum is a unicellular green microalga belonging to the Chlorophyceae family. The cell size of this species ranges from 21 to 20 microns, and the cell wall thickness is less than 0.50 microns. Cultured cells are always green (Lee, 1970). It is known that *Chlorococcum* produces astaxanthin, adonixanthin, cantaxanthin, β-carotene, lutein, and ketocarotenoids (Yuan et al., 2002; Zhang and Lee, 1999). Astaxanthin is a natural carotenoid that possess antibacterial activity (Karpinski et al., 2021; Shanmugapriya et al., 2018). *Stichococcus bacillaris* is a green soil microalga characterized by rod-shaped or cube-shaped cells and containing more than 14 strains (Neustupa et al., 2007). A study by Harder and Opperman (1953) reported that *Stichococcus bacillaris* exhibits antibiotic activity due to the fatty acids it contains. Nucleic acids, vitamins, minerals, amino acids, essential fatty acids, carotenoids, and enzymes are powerful sources of bioactive compounds that can be produced by *C. vulgaris*, which has great potential in various biological applications related to human health (Sarkar et al., 2021; Maadane et al., 2017; Rao et al., 2010). *Chlorolilaea pamvotia*, first isolated in Türkiye by Altürk Karaca and Soylu (2025), is recognized for its chloroplasts, which vary from cup-like to reticulate shapes, and its ability to accumulate lipid droplets and starch. Lortou and Gkelis (2023) investigated the antibacterial properties of *Chlorolilaea pamvotia* in their study and reported that this species exhibits significant antibacterial activity. *Desmodesmus opoliensis* consists of tapered, fusiform cells arranged in groups of 2, 4, or 8. The cell dimensions are 15 x 6 micrometers (Médard et al., 2018). *Desmodesmus* has significant potential for producing algal biomass rich in carbohydrates, vitamins, proteins, as well as micro and macro elements (Hosseini et al., 2020). Previous studies have reported the antibacterial properties of species such as *Chlorella vulgaris* and *Stichococcus bacillaris*, and the secondary metabolites produced by *Chlorococcum* and *Desmodesmus* have also been shown to exhibit antibacterial effects in response to environmental stress (Rinaldi et al., 2024; Hussein et al., 2018; Sivasubramanian, 2011).

The streams in Giresun are vital ecosystems in the Black Sea Region, providing rich biodiversity and ideal conditions for microalgae growth. Their nutrient-rich waters make them excellent sources for isolating microalgae with potential antibacterial and biotechnological applications. Studying these streams not only enhances our understanding of regional biodiversity but also supports sustainable utilization of their biological resources.

This study aims to evaluate the antibacterial activities of *Desmodesmus opoliensis*, *Chlorella vulgaris*, *Chlorococcum hypnosporum*, *Stichococcus bacillaris*, and *Chlorolilaea pamvotia*, isolated from water samples collected from the Batlama, Aksu, and Büyük Güre Streams in Giresun, against pathogenic bacteria, including *Escherichia coli*, *Staphylococcus aureus*, *Salmonella Typhimurium*, and *Enterococcus faecalis*. By investigating the antimicrobial potential of these microalgal species, the study seeks to provide valuable insights into their biological activities and contribute to the development of alternative strategies for managing bacterial infections. Additionally, the research aims to address global health challenges such as the decreasing efficacy of antibiotics and the growing threat of antibiotic resistance by identifying new biologically active compounds. Exploring the antibacterial properties of these microalgae could play a significant role in the discovery of novel therapeutic agents targeting both bacterial infections and resistant strains. The findings may support the development of effective and sustainable treatment strategies, aligning with global efforts to reduce reliance on antibiotics.

MATERIALS AND METHODS

Isolation of Algal Species

Water samples were collected from the benthic and pelagic zones of the Aksu, Batlama, and Büyük Güre Streams within the central district of Giresun Province using 1 L plastic bottles and transported to the laboratory. The microalgae samples collected are provided in Table 1.One milliliter of each water sample was inoculated into BG11 and Allen media solidified with 1% agar (Allen, 1984; Allen and Stanier, 1968). The culture plates were incubated at 26°C in a SANYO MLR 351 incubator under a light intensity of approximately 155 µmol/m²/s with a 12:12 light (L) pho-

toperiod. After one month, all distinct colonies formed on the agar plates were transferred using an inoculating loop to fresh solid media. This process was repeated until single-species isolates were obtained (Demiriz, 2008). The isolated species were then transferred to liquid media and left to grow under controlled incubation conditions.

Table 1. Streams of Microalgae Sample Collection.

Sample	Stream	Coordinates
A1	Aksu	40.561389 38.216111
A2	Aksu	40.561389 38.216111
A3	Aksu	40.561389 38.216111
B3	Batlama	40.909003 38.355779
BG2	Büyük Güre	40.915570 38.334224

Morphological Identification and Molecular Characterization of Algal Species

Samples were taken aseptically from the cultures grown in liquid media. Light microscopy and inverted microscopy were employed for species identification, and measurements were conducted using a micrometric eyepiece. The identification process utilized references such as *Freshwater Algae of North America* and the AlgaeBase database (Wehr and Sheath, 2003; Guiry and Guiry, 2023).

Light microscopy, although widely used for the identification of microalgae, makes it difficult to distinguish morphologically similar species, particularly those that are small or challenging to identify. Due to these limitations, molecular methods provide a more accurate and rapid means of identifying algal species. Ribosomal RNA gene sequencing is an effective technique that facilitates the phylogenetic identification of microalgae, especially for species that cannot be cultured. This method is widely used to assess microbial population diversity and provides more precise results compared to microscopic identification and culturing techniques. In this study, ribosomal RNA gene sequencing was employed for the molecular characterization of microalgal species. The primary goal was to accurately identify species that are difficult to define with light microscopy and cannot be cultured. The 18S rRNA gene sequencing served as an important tool in determining the phylogenetic relationships of microalgae, leading to a more accurate classification of the species. Molecular methods enable the reliable identification of species that may be overlooked in traditional microscopic examinations (Fawley et al., 2004; Fawley et al., 2005; Lewis and Lewis, 2005; Hoshina, 2014). DNA isolation and molecular identification of the algal species were conducted by BM Software Consulting and Laboratory Limited Company. DNA isolation was performed using the EURx GeneMATRIX Isolation Kit (EURx, Gdańsk, Poland) following the manufacturer's protocol.After DNA isolation, the quantity and purity of the obtained DNA were assessed through spectrophotometric measurements using a Thermo Scientific Nanodrop 2000 (USA) device. In the PCR procedure, target gene regions were amplified using the universal primers EukA and EukB for species identification. The primer sequences and PCR conditions are as follows:

EUK A (5′-AACCTGGTTGATCCTGCCAGT-3′),

EUK B (5′-GATCCTTCTGCAGGTTCACCTAC-3′).

The PCR procedure was carried out.First, an initial denaturation was performed at 95°C for 5 minutes. Then, 30 cycles were conducted with the following conditions for each cycle: denaturation at 95°C for 45 seconds, annealing at 57°C for 45 seconds, and extension at 72°C for 90 seconds. After the cycles, a final extension was performed at 72°C for 5 minutes, and the reaction was completed by cooling to 4°C. The amplification results obtained by PCR (kyratec thermocycler) were run on a 1.5% agarose gel prepared with 1x TAE buffer at 100 volts for 90 minutes, and the gel was stained with ethidium bromide and visualized under UV light. A single-step PCR was performed to amplify the approximately 1800 base pair region. PCR reactions were carried out with Solis Biodyne (Estonia) FIREPol® DNA Polymerase Taq polymerase enzyme.Results were evaluated using the NCBI-BLAST program.

Preparation of Algal Extracts

The species *Desmodesmus opoliensis* (P.G. 16 Richter) E. Hegewald, *Chlorella vulgaris* Beijerinck, *Chlorococcum hypnosporum* R.C. Starr 1955, *Stichococcus bacillaris* Nägeli, and *Chlorolilaea pamvotia* (Lortou & Gkelis) Lortou & Gkelis were subjected to centrifugation at 8000 rpm for 5 minutes to concentrate the algal cells. The resulting algal pellets were then dried in an oven at 65°C for 24 hours to remove excess moisture.

One gram of dried algal biomass was extracted with 10 mL of ethanol, methanol, and acetone to evaluate their efficiency in extracting bioactive compounds. The extraction was carried out in a water bath at 50°C for 48 hours to ensure optimal conditions for the release of bioactive compounds (Vehapi et al., 2018; Kumar et al., 2023). Following extraction, the samples were filtered using Whatman No. 1 filter paper to remove any solid residues. Subsequently, the solvents were evaporated in an oven for 2 hours under dark conditions to isolate the extracted bioactive compounds, making the samples suitable for further analysis (Bennour et al., 2020; Foerster et al.,2023). This procedure ensured that the extracts were free of solvents and prepared for subsequent testing.

Determination of Antibacterial Activity of Algal Extracts

The bacterial strains used in this study included *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6051), *Escherichia coli* (ATCC 25922), *Enterococcus faecalis* (ATCC 29212), and *Salmonella enterica Serovar Typhimurium* (ATCC 14028). The antibacterial activity of the algal extracts was assessed using the agar well diffusion method (Perez et al., 1990). Bacteria were pre-incubated on Nutrient Agar (NA) at 37°C overnight. A single colony from each bacterial strain was inoculated into 5 mL of Nutrient Broth and incubated at 37°C for 18 hours. These bacterial cultures were spread onto NA plates using sterile cotton swabs. Wells with a diameter of approximately 6 mm were created on

the NA plates, and 20 µL or 40 µL of algal extracts were added to each well. Plates were left at +4°C overnight to allow diffusion. Negative controls included acetone, methanol, and ethanol. After 24 hours of incubation at 37°C, the inhibition zones around the wells were measured using a millimetric ruler. The study was conducted under aseptic conditions and repeated three times.

RESULTS AND DISCUSSION

Morphological and Molecular Characterization of Algae

In this study, the morphological and molecular characterization of five isolated algal species was conducted (Table 1). The molecular characterization of the isolated microalgae species revealed that *Chlorococcum hypnosporum* (A1) was identified with 100% similarity. Additionally, *Chlorella vulgaris* (A3) showed 99.90% similarity, *Chlorolilaea pamvotia* (B3) showed 97.39% similarity, and *Desmodesmus opoliensis* (BG2) showed 99.55% similarity based on 18S rRNA analysis. Owing to the unsuccessful PCR amplification, the characterization of *Stichococcus bacillaris* (A2) was performed only morphologically (Table 2).

Determination of Antibacterial Activity Using the Agar Well Diffusion Method

The antibacterial effects of *Chlorococcum hypnosporum* (A1), *Stichococcus bacillaris* (A2), *Chlorella vulgaris* (A3), *Chlorolilaea pamvotia* (B3), and *Desmodesmus opoliensis* (BG2) against *Bacillus subtilis*, *Enterococcus faecalis*, *Salmonella Typhimurium*, *Staphylococcus aureus*, and *Escherichia coli* were investigated. In this study, solvents based on acetone, ethanol, and methanol were used to prepare the algal extracts. The extracts were tested on NA medium at concentrations of 20 µL/petri and 40 µL/petri.

Table 3 presents the antibacterial activity of acetone, ethanol, and methanol extracts from *Chlorococcum hypnosporum* at concentrations of 20 µL/petri and 40 µL/petri against the test bacteria.In our study, the ethanol extract obtained from *Chlorococcum hypnosporum* at a dosage of 40 µL/petri dish exhibited the highest antibacterial activity against *Bacillus subtilis* ATCC 6051. Both ethanol and methanol extracts at 40 µL/petri dish demonstrated antibacterial activity against *Escherichia coli* ATCC 25922, while acetone, ethanol, and methanol extracts at 20 µL/petri dish did not show any antibacterial activity against the same bacterium. Additionally, Staphylococcus aureus ATCC 25923 exhibited resistance to the methanol extract, whereas it was inhibited by both the acetone and ethanol extracts. In the study conducted by Elshobary et al. (2020), which investigated the antibacterial activity of *Chlorococcum minutum*, it was found that the acetone extracts exhibited the highest antibacterial activity, inhibiting the most sensitive pathogens, while methanol and ethanol extracts showed lower activity. In contrast, in our study, the ethanol extract of *Chlorococcum hypnosporum* demonstrated high antibacterial activity against *Bacillus subtilis* ATCC 6051. While acetone extracts generally exhibited higher activity in the study by Elshobary and colleagues (2020), our study observed that the ethanol extract was more effective. These differences may be attributed to variations in the biochemical composition of the microalgae species used, the diversity in extraction methods. Elshobary et al. employed sequential extraction techniques, while our study utilized a single solvent extraction method. This could be a significant factor influencing antibacterial activity. Furthermore, the varying susceptibilities of bacterial strains and the polarity of solvents may also contribute to these differences.

Table 4 shows the antibacterial effects of 20 µL/petri and 40 µL/petri acetone, ethanol, and methanol extracts obtained from *Stichococcus bacillaris* against the test bacteria.It was determined that the *Salmonella Typhimurium* ATCC 14028 bacterial strain was resistant

Table 2. Sample Code Sequencing Data Results and BLAST Results.

Sample Code	Sequencing Data Results	BLAST Results
A1	CCATGCATGTCTAAGTATAGTCCCTTATACTGCGAAACTGCGAATGGCTCATTA- AACAGTTATAATTTATTTGATGGTACTTACTACTCGGATAACCGTAGTAATTCTA- GAGCTAATACGTGCGCAAATCCCGACTTCTGGAAGGGACGTATTTATTAGATA- AAAGGCCAGCCGGGCTTTGCCCGACCTGCGTTGATTCATAATAACCATAC- GAATCGCATGGCCTTGTGCCGGCGATGTTTCAAATAAATATCTGCCCTAT- CAACTTTCGATGGTAGGATAGAGGCCTACCATGGTGGTAACGGGTGACG- GAGGATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCA- CATCCAAGGAAGGCAGCAGGCGCGCAAATTGCCCAATCCCGATTCGGGGAG- GCAGTGACAATAAATAACAATACCGGGCATTCAATGTCTGGTAATTGGAAT- GAGTACAATCTAAATCTCTTAACGAGGATCCATTGGAGGGCAAGTCTGGT- GCCAGCAGCCGCGGTAATTCCAGCTCCAAAAGCGTATATTTAAGTTGTTG- CAGTTAAAAGGCTCGTAGTCGAAACTCGGGTTCGGTCCAGCGGTCCG- CCTCTGGTGTGCACTGCTGGTACTGTTCCTTTCTGTCGGGGACGGGCTCCT- GGGCTTCATTGTCTGGGACCCGGGCTCGGCGAGGTTACTTTGAGTAAATT- AGAGTGTTCAAAGCAGGCGATCGCCCTGAATACATTAGCATGGAATAGCAC- GATAGGACTCTGGCCTATCTTGCTGGTCTGTAGGACCGGAGTAATGATTAA- GAGGGACAGTCGGGGGCATTGGTATTTCCGGGTCAGAGGTGAAATTCTTG- GATTCCGGAAAGACCATCCACTGCGAAAGCATTTGCCAAGGATGTTTTCATT- GATCAAGAACGAAAGTTGGGGGCTCGAAGACGATTAGATACCGTCGTAGTCT- CAACCATAAACGATGCCGACCAGGGATTGGCGGGCGTTCTTTTGATGAC	*Chlorococcum hypnosporum* 100 %
A2	Cannot be evaluated according to the sequencing analysis	

Table 2. Continued.

Sample Code	Sequencing Data Results	BLAST Results
A3	GTCTAAGTATAAACTGCTTTATACTGTGAAACTGCGAATGGCTCATTAAAT- CAGTTATAGTTTATTTGATGGTACTTACTACTCGGATACCCGTAGTAAATCTA- GAGCTAATACGTGCGTAAATCCCGACTTCTGGAAGGGACGTATTTATTAGATA- AAAGGCCGACCGGGCTTCTGCCCGACTCGCGGTGAATCATGATAACTTCAC- GAATCGCATGGCCTTGTGCCGGCGATGTTTCATTCAAATTTCTGCCCTAT- CAACTTTTGATGGTAGGATAGAGGCCTACCATGGTGGTAACGGGTGACG- GAGGATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCA- CATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTGACACAGG- GAGGTAGTGACAATAAATAACAATACTGGGCCTTTTCAGGTCTGGTAATTG- GAATGAGTACAATCTAAACCCCTTAACGAGGATCAATTGGAGGGCAAGTCT- GGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTTAAGTTGCT- GCAGTTAAAAAGCTCGTAGTTGGATTTCGGGTGGGATCTGCCGGTCCGC- CGTTTCGGTGTGCACTGGCAGGGCTCACCTTGTTGCCGGGGACGGGCTCCT- GGGCTTCACTGTCCGGGACTCGGAGTCGGCGCTGTTACTTTGAGTAAATT- AGAGTGTTCAAAGCAGGCCTACGCTCTGAATACATTAGCATGGAATAACAC- GATAGGACTCTGGCCTATCCTGTTGGTCTGTAGGACCGGAGTAATGATTA- AGAGGGACAGTCGGGGGCATTCGTATTTCATTGTCAGAGGTGAAATTCTTG- GATTTATGAAAGACGAACTACTGCGAAAGCATTTGCCAAGGATGTTTTCATTA- ATCAAGAACGAAAGTTGGGGGCTCGAAGACGATTAGATACCGTCCTAGTCT- CAACCATAAACGATGCCGACTAGGGATCGGCGGATGTTTCTTCGATGACTCC	*Chlorella vulgaris* 99.90 %
B3	TCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAGG- CAGCAGGCGCGC AAATTACCCAATCCCGACACGGGGAGGTAGTGACAATAAATAACGATACTG- GGCATTTTTGTCTGGTAATCGGAATGAGCACAACGTAAATACCTTAACGAG- TATCCATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTC- CAATAGCGTATATTTAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTC- GGGCGGCGCGTCGCGGTCTGCCCTTTGGGTACGTACTGCGCCCGCGTC- GCCTTGCTGCCGGGGACGCGCTCCTGGGCTTCACTGTCCGGGACGCG- GAGTCGGCGAGGTGACCTTGAGCAAACAGGAGTGTTCATAGCAAGCGCAC- GCTCTGAACTATTTAGCATGGAATCACGCGATAGGACTCTGGCCTATCCCG- GTGGTCTGTAAGACCGGAGTAATGATTAAGAGGGACAGTCGGGGGCAT- TCGTATTTCATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGACGAACTTCT- GCGAAAGCATTTGCCAGGGATGTTCTCATTGATCAAGAACGAAAGTTGGG- GGCTCGAAGACGATTAGATACCGTCGTAGTCTCACCCATAAACGATGCCG- ACTAGGGATCGGCGGGTGTTCCATTGAT	*Chlorolilaea pamvotia* 97.39 %
BG2	TCTAAGTATAAACTGCTTATACTGTGAAACTGCGAATGGCTCATTAAATCAGT- TATAGTTTATTTGGTGGTACCTTCTTACTCGGAATAACCGTAAGAAAATTA- GAGCTAATACGTGCGTAAATCCCGACTTCTGGAAGGGACGTATATATTAGATA- AAAGGCCGACCGGGCTCTGCCCGACCCGCGGTGAATCATGATATCTTCAC- GAAGCGCATGGCCTTGTGCCGGCGCTGTTCCATTCAAATTTCTGCCCTAT- CAACTTTCGATGGTAGGATAGAGGCCTACCATGGTGGTAACGGGTGACG- GAGGATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCA- CATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTGATACGGG- GAGGTAGTGACAATAAATAACAATACCGGGCATTTCATGTCTGGTAATTG- GAATGAGTACAATCTAAATCCCTTAACGAGGATCCATTGGAGGGCAAGTCTG- GTGAACCAAGCAACGCAATGCTGTTGACGCCAGAGATAGTAGGGCAGTTG- CACTCTAGTGTACCTGTTACGCCTGCTAGTCGAGCAATCATTCAATTGAACGG- GCTGATTGCCGGCAAGACGACCTGGTACGGGGAAGGCCTTCAAGATCCACT- GGCTAATCCCGTGGCGAGCTTGCATAGGGTGACCTTTGTAAAGCCGTCGTAAC- GCACGCAAAGGCGTCGGCTGACTCACTGAAGTTGGCTTAAGGGACGTGCTA- ACCCCATCCGATGATAAAGGATGCTTGAAGCAATAGCACCCGTTCTGCAAAG- GCTTCAAGGGGCAATAGTGTGCTGAGGAGATGCTTCACACTGCTCGGTAT- TCAAGCATTGGAAACTCAATCTGTGGCGACTAGGCAAGCGTGACGACAATTA- AGCTCGCAAATCGCGATTCTTGGAATTGACGTCGTCGCTGCCTCGTACAGATC- GAATCCAGTGCGAAAGGGGCCAGCAGCCGCGGTAATTCCAGCTCCAATAG	*Desmodesmus opoliensis* 99.55 %

to the 40 µL/petri acetone extract obtained from *Stichococcus bacillaris*. Additionally, *Staphylococcus aureus* ATCC 25923 was found to be resistant to the 40 µL/petri methanol extract of *Stichococcus bacillaris*. No antibacterial activity was observed for the 20 µL/petri and 40 µL/petri acetone, ethanol, and methanol extracts of *Stichococcus bacillaris* against *Bacillus subtilis* ATCC 6051, *Enterococcus faecalis* ATCC 29212, and *Escherichia coli* ATCC 25922. Sivakumar et al. (2014) reported that the *Stichococcus bacillaris* Sia2011 strain produced high levels of lipids, suggesting its potential use in the pharmaceutical industry. Bozkurt (2019), in their thesis study, reported that the ethanol and methanol extracts of *Stichococcus bacillaris* demonstrated significant antimicrobial activity against *S. aureus*, while the methanol extract exhibited weak antibacterial activity against *E. coli*. In contrast, the ethanol extract showed no antibacterial activity against *E. coli*. These findings align with our study, which revealed resistance of *S. aureus* to the methanol extract and the lack of activity of the ethanol extract against *E. coli* for the same species.

Table 5 exhibits the antibacterial effects of 20 µL/petri and 40 µL/petri acetone, ethanol, and methanol extracts obtained from *Chlorella vulgaris* against the test bacteria.It was determined that the 20 µL/petri and 40 µL/petri acetone extracts obtained from *Chlorella vulgaris* exhibited antibacterial activity against *Bacillus subtilis* ATCC 6051. Additionally, only the 40 µL/petri acetone, ethanol, and methanol extracts of *Chlorella vulgaris* demonstrated antibacterial activity against *Enterococcus faecalis* ATCC 29212, while the 20 µL/petri acetone and methanol extracts showed no activity against this strain. *Typhimurium* ATCC 14028 exhibited resistance to the 40 µL/petri acetone extract of *Chlorella vulgaris*, with an inhibition zone diameter of 8 mm. Moreover, *Staphylococcus aureus* ATCC 25923 was resistant to the 40 µL/petri ethanol extract, forming a 6 mm inhibition zone, while the 20 µL/petri methanol extract had no antibacterial effect on this microorganism. The 40 µL/petri acetone extract of *Chlorella vulgaris* showed sensitivity to *Escherichia coli* ATCC 25922, producing a 14 mm inhibition zone. Syed et al. (2015), in their study investigating the antibacterial effects of ethanol, methanol, and chloroform extracts of *Chlorella vulgaris* against *Klebsiella* sp., *E. coli*, and *Bacillus* sp., reported that the ethanol extract exhibited high antibacterial activity against *E. coli*, while the methanol extract produced a lower inhibition zone. Similarly, Sukhikh et al. (2022) noted that the fatty acid content of *Chlorella vulgaris* contributed to its antibacterial effect against *Bacillus subtilis*.

Table 3. Antibacterial activity of 20 µL/petri and 40 µL/petri acetone, ethanol, and methanol extracts of *Chlorococcum hypnosporum* microalgae against test bacteria.

Extract	Microalgae doses	*B. subtilis*	*E. faecalis*	*S.Typhimurium*	*S. aureus*	*E. coli*
Acetone	20 µL/petri	4 mm	4 mm	-	5 mm	-
	40 µL/petri	7 mm	5 mm	-	3 mm	-
Ethanol	20 µL/petri	-	4 mm	3 mm	4 mm	-
	40 µL/petri	8 mm	6 mm	10 mm	10 mm	8 mm
Methanol	20 µL/petri	2 mm	-	-	-	-
	40 µL/petri	3 mm	2 mm	-	-	7 mm

Table 4. Antibacterial activity of 20 µL/petri and 40 µL/petri acetone, ethanol, and methanol extracts of *Stichococcus bacillaris* microalgae against test bacteria.

Extract	Microalgae doses	*B. subtilis*	*E. faecalis*	*S. Typhimurium*	*S. aureus*	*E. coli*
Acetone	20 µL/petri	-	-	-	-	-
	40 µL/petri	-	-	6 mm	-	-
Ethanol	20 µL/petri	-	-	-	-	-
	40 µL/petri	-	-	-	-	-
Methanol	20 µL/petri	-	-	-	-	-
	40 µL/petri	-	-	-	4 mm	-

Table 5. Antibacterial activity of 20 µL/petri and 40 µL/petri acetone, ethanol, and methanol extracts of *Chlorella vulgaris* microalgae against test bacteria.

Extract	Microalgae doses	*B. subtilis*	*E. faecalis*	*S. Typhimurium*	*S. aureus*	*E. coli*
Acetone	20 µL/petri	2 mm	-	-	2 mm	2 mm
	40 µL/petri	12 mm	4 mm	8 mm	3 mm	14 mm
Ethanol	20 µL/petri	4 mm	-	2 mm	3 mm	-
	40 µL/petri	6 mm	4 mm	3 mm	6 mm	5 mm
Methanol	20 µL/petri	3 mm	-	-	-	-
	40 µL/petri	12 mm	2 mm	2 mm	2 mm	7 mm

Table 6 shows the antibacterial effects of 20 µL/petri and 40 µL/petri acetone, ethanol, and methanol extracts obtained from *Chlorolilaea pamvotia* against the test bacteria. It was determined that the 40 µL/petri ethanol and methanol extracts obtained from *Chlorolilaea pamvotia* exhibited antibacterial activity against *Bacillus subtilis* ATCC 6051. The 20 µL/petri and 40 µL/petri acetone extracts of *Chlorolilaea pamvotia* showed no antibacterial activity against *Enterococcus faecalis* ATCC 29212, while both the 20 µL/petri and 40 µL/petri ethanol extracts demonstrated antibacterial activity against this microorganism. Specifically, the 40 µL/petri ethanol extract produced a 9 mm inhibition zone against *E. faecalis* ATCC 29212, indicating sensitivity to this extract. On the other hand, no extracts of *Chlorolilaea pamvotia* displayed antibacterial activity against Salmonella *Typhimurium* ATCC 14028 and *Escherichia coli* ATCC 25922. In the study conducted by Lortou and Gkelis (2023), it was reported that *Chlorolilaea pamvotia* exhibited antibacterial activity against *S. Typhimurium*. However, in our study, no antibacterial activity of this species against the same microorganism was observed. This discrepancy could be attributed to methodological differences. Specifically, factors such as the extraction method, the type of solvent used, genetic or phenotypic variations in the microbial strains tested, and experimental conditions (e.g., incubation time, pH, or temperature) may have influenced the results.Moreover, variations in the chemical composition of extracts obtained from *C. pamvotia* could result in differences in the quantity and diversity of active compounds, thereby affecting antibacterial activity. This highlights the importance of standardizing methods in such studies and underscores the significant influence of environmental and experimental conditions on the biological activities of microalgae.

Table 7 illustrates the antibacterial activity of acetone, ethanol, and methanol extracts (20 µL/petri and 40 µL/petri) derived from *Desmodesmus opoliensis* against the test bacteria The 40 µL/petri acetone, ethanol, and methanol extracts of the microalgae *Desmodesmus opoliensis* exhibited antibacterial activity against *Bacillus subtilis* ATCC 6051 and *Enterococcus faecalis* ATCC 2921. The 40 µL/petri acetone extract demonstrated antibacterial activity against *Salmonella Typhimurium* ATCC 14028. Additionally, the 40 µL/petri ethanol extract exhibited antibacterial activity against *Staphylococcus aureus* ATCC 25923. In the 20 µL/petri ethanol extract, antibacterial activity was observed only against *Staphylococcus aureus*, whereas in the 40 µL/petri ethanol extract, activity was recorded against *Bacillus subtilis*, *Enterococcus faecalis*, *S. aureus*, and *E. coli*. Hosseini et al. (2020) demonstrated in their study that *Desmodesmus* microalgae exhibit notable antibacterial activity, particularly when cultured under stress conditions.

The antibacterial effects of the solvents themselves were evaluated, and no inhibition zones were observed with acetone, ethanol, or methanol against *Bacillus subtilis* ATCC 6051, *Enterococcus faecalis* ATCC 29212, *Salmonella Typhimurium* ATCC 14028, *Staphylococcus aureus* ATCC 25923, and *Escherichia coli* ATCC 25922. Similarly, in the study conducted by Demiriz (2008), the antibacterial effects of methanol, ethanol, n-butanol, acetone, hexane, and 0.5M Tris-HCl solvents on test bacteria were evaluated, and it was concluded that none of these solvents exhibited antibacterial activity against *Salmonella enteritidis* ATCC 13076, *Escherichia coli* O157:H7, *Staphylococcus aureus* ATCC 19213, and *Bacillus subtilis* ATCC 6633.Both studies highlight the ineffectiveness of solvents and algal extracts at low doses against the tested bacteria. The findings suggest the need for a more comprehensive investigation of antibacterial activity involving various solvent types and microalgal metabolites.

Consequently, the variation in the inhibitory activity of each isolated algal species against Gram-positive and Gram-negative bacteria is believed to be related to the antibacterial com-

Table 6. Antibacterial activity of 20 µL/petri and 40 µL/petri acetone, ethanol, and methanol extracts of *Chlorolilaea pamvotia* microalgae against test bacteria.

Extract	Microalgae doses	*B. subtilis*	*E. faecalis*	*S. Typhimurium*	*S. aureus*	*E. coli*
Acetone	20 µL/petri	-	-	-	-	-
	40 µL/petri	-	-	-	-	-
Ethanol	20 µL/petri	-	2 mm	-	-	-
	40 µL/petri	3 mm	9 mm	-	2 mm	-
Methanol	20 µL/petri	-	-	-	-	-
	40 µL/petri	2 mm	5 mm	-	2 mm	-

Table 7. Antibacterial activity of 20 µL/petri and 40 µL/petri acetone, ethanol, and methanol extracts of *Desmodesmus opoliensis* microalgae against test bacteria.

Extract	Microalgae doses	*B. subtilis*	*E. faecalis*	*S. Typhimurium*	*S. aureus*	*E. coli*
Acetone	20 µL/petri	2 mm	-	-	-	-
	40 µL/petri	3 mm	2 mm	2 mm	2 mm	-
Ethanol	20 µL/petri	-	-	-	2 mm	-
	40 µL/petri	4 mm	4 mm	-	2 mm	4 mm
Methanol	20 µL/petri	4 mm	-	-	-	-
	40 µL/petri	4 mm	2 mm	-	2 mm	-

pounds, specifically secondary metabolites, which are present in varying concentrations in each algal species and influence the mechanism of action against the bacteria. In this study, it was concluded that as the doses of the extracts prepared for the tested bacteria increased, the antimicrobial activity also increased. Vehapi (2016) reported that with the increase in concentration of all microalgae extracts, there was an observed increase in the inhibition rates against Gram positive; *Mycobacterium smegmatis* RUT and Gram negative; *Morganella morganii*, *Proteus mirabilis* BC 6624, *Aeromonas hydrophila* ATCC 7965.

CONCLUSION

This study highlights the antibacterial potential of green microalgae and emphasizes their biotechnological significance as natural alternatives to antibiotics. Specifically, the antibacterial activity exhibited by *Chlorococcum hypnosporum*, *Stichococcus bacillaris*, *Chlorella vulgaris*, *Chlorolilaea pamvotia*, and *Desmodesmus opoliensis* species against various pathogenic bacteria demonstrates their capacity to produce bioactive compounds with antibacterial properties. This finding reveals the potential of these microalgae as antimicrobial agents. For instance, the ethanol and methanol extracts of *Chlorococcum hypnosporum* and *Chlorella vulgaris* exhibit significant antibacterial activity against *Bacillus subtilis* and *Escherichia coli*, underscoring their potential as new therapeutic options in health-related applications.

Furthermore, this study points to the importance of microalgae as a biotechnological resource for combating antibiotic-resistant bacterial strains. In the face of rising concerns over antibiotic resistance, microalgae present a promising natural alternative. Species such as *Chlorococcum hypnosporum*, *Stichococcus bacillaris*, *Chlorella vulgaris*, *Chlorolilaea pamvotia*, and *Desmodesmus opoliensis* offer considerable promise for the development of alternative therapeutic agents and natural preservatives.

Species like *Chlorella vulgaris*, *Desmodesmus opoliensis*, and *Stichococcus bacillaris* are well-adapted to diverse environments, from freshwater to marine habitats, making them ideal for biotechnological applications. Their genetic diversity and environmental adaptability enhance their suitability for large-scale industrial production. For example, the antibacterial properties of *Chlorella vulgaris* and its ability to thrive in various conditions make it an excellent candidate for use in the pharmaceutical and food safety sectors. Similarly, the antibacterial activities and environmental resilience of *Desmodesmus opoliensis* and *Stichococcus bacillaris* support their potential in sustainable biotechnological processes.

In conclusion, this study demonstrates that green microalgae possess significant antibacterial potential and could be used as natural alternatives to antibiotics in preventing zoonotic diseases and ensuring the microbial safety of animal products. Given their ability to produce bioactive compounds, microalgae warrant further research for the discovery of new antimicrobial agents and expanded biotechnological applications. The results of this study strongly support the notion that microalgae represent a promising resource for the development of alternative antimicrobial solutions.

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Ethics committee approval: Ethics committee approval is not required. Both authors declare that this study does not include any experiments with human or animal subjects.

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Research Article

Recent Plant Traits of *Caulerpa taxifolia* var. *distichophylla* in the Turkish Aegean Sea

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ABSTRACT

A research cruise was conducted along the Turkish coast of the Aegean Sea between May and August 2024 to assess the distribution and plant characteristics of *Caulerpa taxifolia* var. *distichophylla*. During the survey, samples of *Caulerpa taxifolia* var. *distichophylla* were identified at one station in August 2024 in Tatlısu Gulf, Altınova Bay, Balıkesir among 321 stations. Specimens were collected from one station located at a depth of 20 m. The density of shoots was estimated to be 344 m-2. Morphological analyses revealed that the specimens were characterized by a light green, delicate, feathery thallus with thin prostrate stolons and erect fronds with pinnae. The erect fronds ranged in height from 2.10 mm to 105 mm, with a mean of 46.51 ± 1.4 mm, and in width from 1.30 mm to 4.10 mm, with a mean of 2.68 ± 0.003 mm. A significant relationship was found between the number of pinnae and frond length, with a slope of approximately 2.15. The relationship between frond length and width followed a curved-linear (logarithmic) model, with a slope of approximately 0.515. This study provides the latest comprehensive biometric data on *C. taxifolia* var. *distichophylla*, which has spread to the north of the Turkish Aegean Sea after its in the Gulf of Izmir in 2011, and contributes to the understanding of its invasive potential and ecological impacts in the region.

Keywords: *Caulerpa taxifolia* var. *distichophylla*, biometry, invasive species, Turkish Aegean Sea

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INTRODUCTION

The Mediterranean basin, especially the eastern basin and seas such as the Levant and the Aegean, has become a hotspot for the introduction and invasion of exotic species (Zenetos & Galanidi, 2020; Çinar et al., 2021). Invasions of organisms can threaten the status of species found in natural communities (Ceccherelli & Cinelli, 1998). Fish, benthic fauna and macroinvertebrates represent the most intentionally and accidentally introduced species in the eastern Mediterranean. Most of the invaders are species of Indo-Pacific origin and temperate and tropical specimens (Çinar et al., 2021). The invaders affect the already established Mediterranean ecosystem and change the ecosystem in time and space. Their density and population dynamics of seaweeds guide to understand the interaction with strengths of variation in their life history between the isolated and mixed populations (Schemske et al., 1994). Clonal vegetative growth is common and induces highly populated aggregations, foraging and movement to suitable adjacent space, followed by rapid rates of expansion and a reduced risk of mortality (Wright, 2005).

Ninety-eight species of marine algae have been introduced into the Mediterranean Sea. Nine species were invasive and had ecological and economic impacts (Siguan & Ribera, 2002). These nine species were *Caulerpa taxifolia*, *Caulerpa racemosa*, *Sargassum muticum*, *Lam-*

inaria japonica, *Asparagopsis armata*, *Undaria pinnatifida*, *Womersleyella setacea*, *Acrothamnion preissii* and *Lophocladia lallemandii*. Fifty species have increased in number in the last 20 years. In the western Mediterranean, sixty-seven species were non-indigenous species with locality of Japanese or Pacific waters (Siguan & Ribera, 2002). Twenty-nine non-indigenous species (Red Sea or Indian Ocean origin) were found in the eastern basin (Siguan & Ribera, 2002). Zenetos & Galanidi (2020) updated the non-indigenous seagrass species found in the Mediterranean. Cinar et al. (2021) revised the phytobenthos in the Turkish waters of the eastern Mediterranean. Out of a total of 253 alien species compiled by Çinar et al. (2021) for Turkish Aegean waters, 28 belonged to phytobenthos.

The genus *Caulerpa* has some species having invasive feature in the Mediterranean and other seas. The presence of the efficient mechanisms of plant tissue regeneration makes the genus *Caulerpa* highly invasive and adaptive to dispersal and establishment of new population (Welling et al., 2009). There is no information on overall potential grazers of *Caulerpa taxifolia* var. *distichophylla* in the Mediterranean, but a gastropod, *Bittium reticulatum* has been observed on *C. taxifolia* var. *distichophylla*, presumably feeding on it. Besides, Mutlu et al. (2022) observed a large-sized non-indigenous invasive gastropod, *Conomurex persicus* grazing on fronds of *C. t.* var. *distichophylla* in Antalya Gulf, Turkey.

The Chlorophyta *Caulerpa taxifolia* var. *distichophyla* is a taxonomically recognized variant of *Caulerpa taxifolia* native to southwestern Australia that has recently emerged in the Mediterranean region (Tsirintanis et al., 2022). This variety was first documented in Syria in 2003 (Bitar et al., 2017) and later in Turkey in 2006 (Cevik et al., 2007, misidentified as *C. taxifolia*). Later, the species was identified in Sicily in 2007, where significant drift biomass was observed along the coastline (Cormaci & Furnari, 2009; Meinesz et al., 2010; Jongma et al., 2013). The invasive Chlorophyta rapidly expanded its range in the central and eastern Mediterranean (Musco et al., 2014; Aplikioti et al., 2016; Picciotto et al., 2016; Ellul et al., 2019a). The species was colonized on the bottom of dead *Posidonia oceanica* matte in Sicily (Musco et al., 2014). Maritime traffic and global warming are likely to facilitate the further spread of *C. taxifolia* var. *distichophylla* in the Mediterranean (Mannino et al., 2019).

The distribution history of the species in Mediterranean and Turkish waters is as follows: *Caulerpa taxifolia* var. *distichophylla* was first reported from Syria in 2003, followed by Turkey in 2007 (Cevik et al., 2007). The species was then chronologically observed from waters of Sicily (Cormaci & Furnari, 2009; Meinesz, Chancollon & Cottalorda, 2010; Jongma et al., 2013; Musco et al., 2014), Cyprus (Çicek et al., 2013; Tsiamis et al., 2014; Aplikioti et al., 2016), Malta (Schembri et al., 2015), Rhodes (Aplikioti et al., 2016), Libya (Shakman et al., 2017), Lebanon (Bitar et al., 2017) and Tunisia (Chartosia et al., 2018). In Turkish marine waters the species occurred in Iskenderun Bay (Cevik et al., 2007), in Izmir Gulf, Aegean Sea (Turan et al., 2011), Antalya Bay (Mutlu et al., 2022), Alanya Bay, Sea of Marmara (Taşkın et al., 2023). Mutlu et al. (2022) documented the species' seasonal and depth-related plant characteristics and competition with *C. prolifera*. Some alien macrophytes including *C. taxifolia* var. *distichophylla* occurred interestingly in some hydrothermal systems of Aeolian Islands, Tyrrhenian Sea, Italy where occurrence of *Caulerpa cylindracea* Sonder, 1845, *Caulerpa taxifolia* and *Halophila stipulacea* (Forsskal) Ascherson, 1867 as well was reported by Gaglioti & Gambi (2018).

Understanding of invasion with mechanisms and succession of marine organisms can help researchers to regulate and conserve marine organism population by knowing biotic and abiotic characteristics of ecosystem, disturbance regimes and life history traits of invaders (Sol et al., 2012). Therefore, the present study was aimed to provide initial assessment of the invasion of *C. taxifolia* var. *distichophylla* with an emphasis on the morphometric characteristic from the Turkish Aegean Sea water.

MATERIAL AND METHODS

Specimens and environmental data acquisition

A research cruise conducted from late May to late August 2024 (Figure 1b) aimed to assess the distribution and species composition of submerged vegetation along the Turkish coast of the Aegean Sea. Some seagrasses (*Posidonia oceanica*, *Cymodocea nodosa*, *Zostera* spp, and *Halophila stipulacea*) and many species of seaweeds were recorded and their distribution will be detailed in a later paper in preparation. A total of 321 survey stations were established during daylight hours (Figure 1). In addition, a detailed transect in Altınova Bay extended from the shoreline to deeper waters and included four different bottom depths of 10, 15, 20, and 30 m to check the occurrence of the species in the vicinity, as specimens of *Caulerpa taxifolia* var. *distichophylla* were identified at one station in Tatlısu Gulf, Altınova Bay, Balıkesir Province, Turkey.

Specimens of *C. taxifolia* var. *distichophylla* were collected from a station (designated as A20) situated at a depth of 20 meters (Figure 1). SCUBA divers meticulously gathered running stolons from the specimens within a defined 0.4 x 0.4 m quadrant. This sampling procedure was applied for all 321 stations located between 7 m and 30 m by SCUBA diving conducted at 10, 15, 20 ad 30, and sometimes at 5 m where the bottom was suitable for the ship security.

The material examined comprises unpreserved specimens, including 15 stolons and 55 fronds. Of these, 54 fronds were used for the purpose of counting the pinnae of the fronds. The specimens were collected at locations with the coordinates 39.21621 N and 26.70309 E, at a depth of 20 m by SCUBA diving. The collection was made at 17:29 by Yaşar Özvarol and Barış Akçalı on 9 August 2024.

On board the R/V "Akdeniz Su", after sorting the materials obtained by SCUBA divers the tangled fronds, stolons, and rhizoids of the species were untangled in preparation for biometric measurements (see Figure 5). Measurements were performed on fresh, unpreserved specimens.

During the shipboard sampling, physicochemical (temperature, salinity, pH, oxygen, and total suspended solids) and optical parameters (Secchi disk depth, photosynthetic active radiation, PAR)

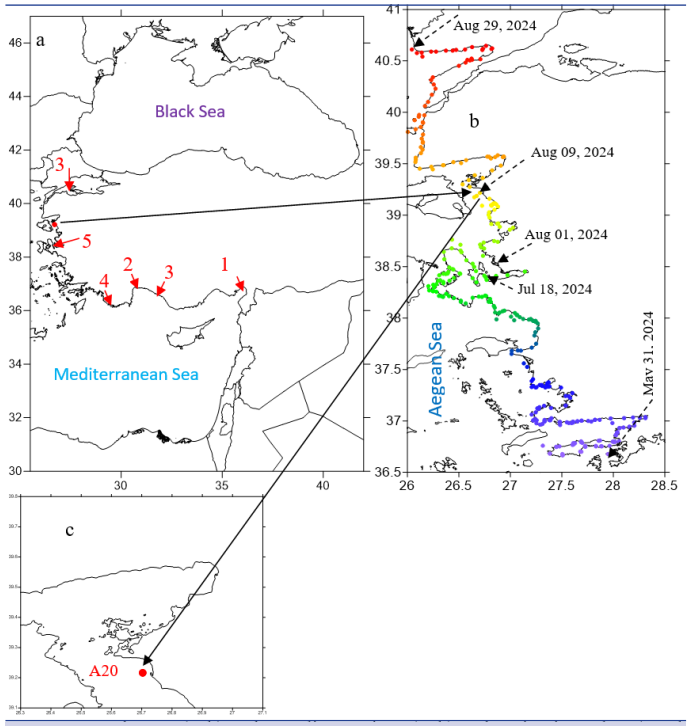

Figure 1. Study area (a, b) and sampling stations (o, b) and station in Tatlısu (Freshwater) Gulf, Altınova, Balıkesir, Turkey where *Caulerpa taxifolia* var. *distichophylla* occurred (o, c). Arrow (a) shows arrival time during the present study (b) and locations and citation number of previous records of the species along the Turkish waters: 1: Cevik et al. 2007, 2: Mutlu et al. (2022), 3: Taşkın et al. (2023), 4: Jongma et al. (2013) and 5: Turan et al. (2011). Colored stations (b) were purposed to show location in T-S diagram (see Figures. 2, 3).

were measured from surface and near-bottom waters. Water samples were collected on board using a 5-l Niskin bottle at surface and near-bottom waters. The physicochemical parameters were measured using multiparameter probes (AZ Combo, model 84051). PAR was measured using an ampoule (Spherical SPQA-4671 model, Li-Cor Inc.) and a multiparameter recorder (LI-1400 model, Li-Cor Inc). PAR ampoule was casted from the surface (on air and then 20-30 cm below surface) to the near-bottom depth down to max 50 m which was the length of the PAR cable. The profiled PAR values were then converted to percent values for each water depth in referring sea surface value as 100% at each station. The light extinction rate (Kd) was then calculated. These all environmental measurements were deployed at all 321 stations.

Biometrical measurements

The biometry was characterized by morphometry (frond length: FL, frond widest width: FW, bud length: BL, and bud widest width: BW) of the samples (see Figure 5 for details of the measurements). The morphometric parameters (length and width) were measured using a micrometer caliper. Population parameters were the density (number of shoots/m^2 and per quadrant; TS and for buds, BNo), number of fronds per stolon (FNo), number of stolons (#S), number of paired pinnae and ratio of number of pinnae per rachis and per 1 cm of rachis (#F).

A total of 15 runners were measured. The number of pinnae per frond was counted from a total of 36 fronds. The number of buds

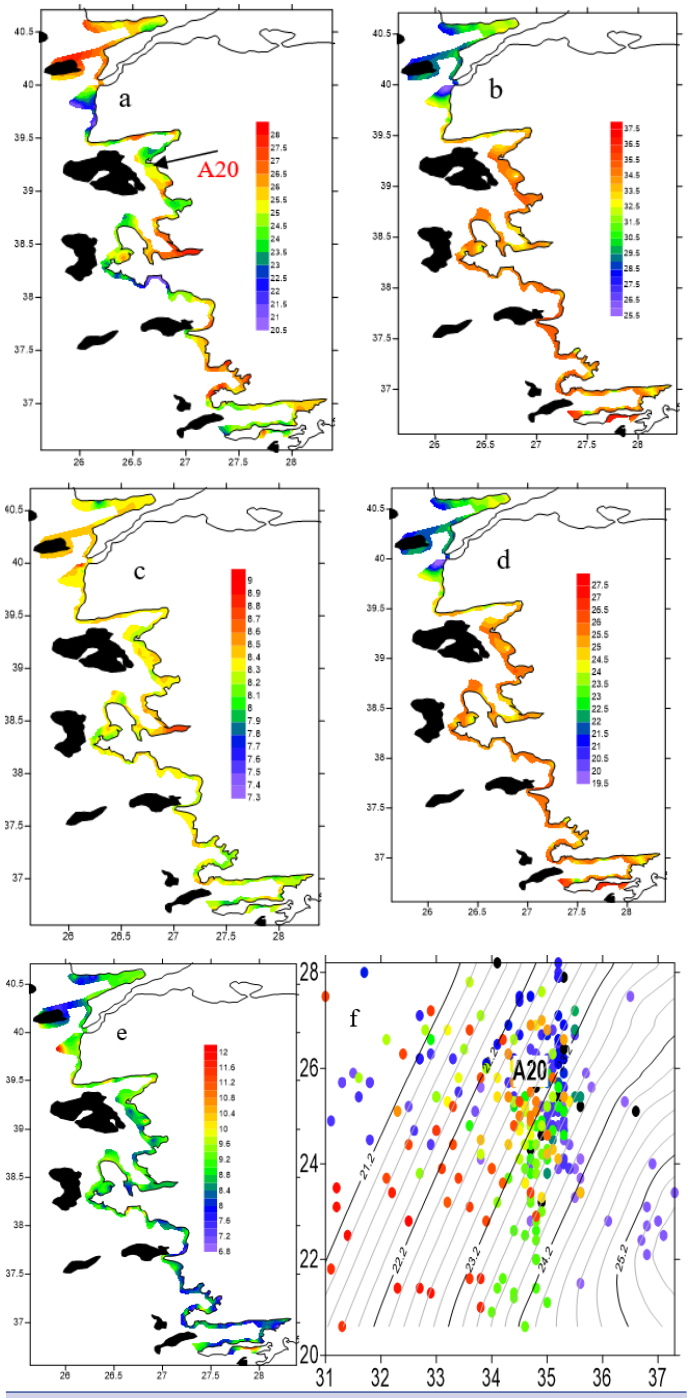

Figure 2. Sea surface water temperature in oC (a), salinity in ppt (b), pH (c), dissolved oxygen in mg/l (d), total suspended matter in mg/l (e) and T-S diagram with isoclines of density, σt at the stations colored with geographical latitudes (f) (see Figure 1b for the station locations).

on the frond was counted by eyes and measured for biometric parameters using a micrometer caliper. Individual weight could not be measured on board since onboard balance which is not affected from the rock and movement of the ship was required. This would be purposed for later laboratorial work at land.

Pearson correlation was used to measure the degree of relationship between number of pinnae and frond length, and between FW (widest width) and FL. These relationships were regressed linearly and logarithmically, respectively. The Student's t-test (Ho: r=0) was used to test the significance of the correlation coefficients at $p < 0.05$.

RESULTS AND DISCUSSION

Study environment

During the survey, sea surface temperature varied between 20.5 and 28.5 °C in the whole study area, while near-bottom waters had a range of water temperature from 18 to 28 °C. Salinity tended to have a decreasing gradient from south to north in the study area. This was more pronounced in the near-bottom waters. Oxygen content and pH increased slightly from south to north in the study area, in contrast to total suspended solids (Figures 2-3).

Cold water of the Black Sea through the Sea of Marmara occurred in the Dardanelles Strait exit in the Aegean Sea, but warmer water occurred in northern part of the Aegean Sea. In this specific region with the effect of river Meriç located in the northernmost study area, the less saline water occurred as compared to the southern part of the study area (Figures 2, 3).

At station A20, sea surface temperature was about 25.5 °C and sea surface salinity was 34.6 (Table 1, Figures 2-3). Sea surface pH was 8.26 and near bottom water pH was 8.31 at A20. Dissolved oxygen was high in both waters, where total suspended solids were measured at around 25.5 mg/l (Table 1).

Regarding the sea surface water, the TS plot showed that the stations in Altınova Bay had similar temperatures, but A20 (σt=23.0) was different from the other stations in the bay. Like the sea surface values, the near-bottom water density at A20 was estimated to be σt=23.4 (Figures 1-3).

The Secchi disk read 18 m at A20, which measured PAR at 17:29 on August 9, 2024. PAR values were measured in units of µmol photons/cm^2/s. However, the percentage of light reaching the near-bottom waters was similarly estimated to be ~ 20% of the surface PAR (Figure 4). Kd was estimated to be 0.069 (22.83%) per unit depth (Figure 4b).

Species traits

Description: The *Caulerpa* specimens from the Turkish Aegean were identified as *Caulerpa taxifolia* var. *distichophylla* (Sonder) Verlaque, Huisman & Procaccini, 2013 based on their morphological characteristics. The specimens were characterized by a light green thallus, feathery and delicate, with narrow prostrate stolons and erect fronds bearing pinnules (Figure 5). The stolons were slender with short rhizoidal stems (Figure 5). The erect fronds were simple, ranging from 2.10 to 105 mm with an average length of 46.51±1.4 mm. The erect fronds were observed to be terete at the base and compressed towards the apex, bearing distichous (rarely tristichous) and closely arranged (but never overlapping) pinnules in one plane. The pinnules themselves were noted to be compressed (Figures 5, A1). There was a significant correlation between the number of pinnules and the length

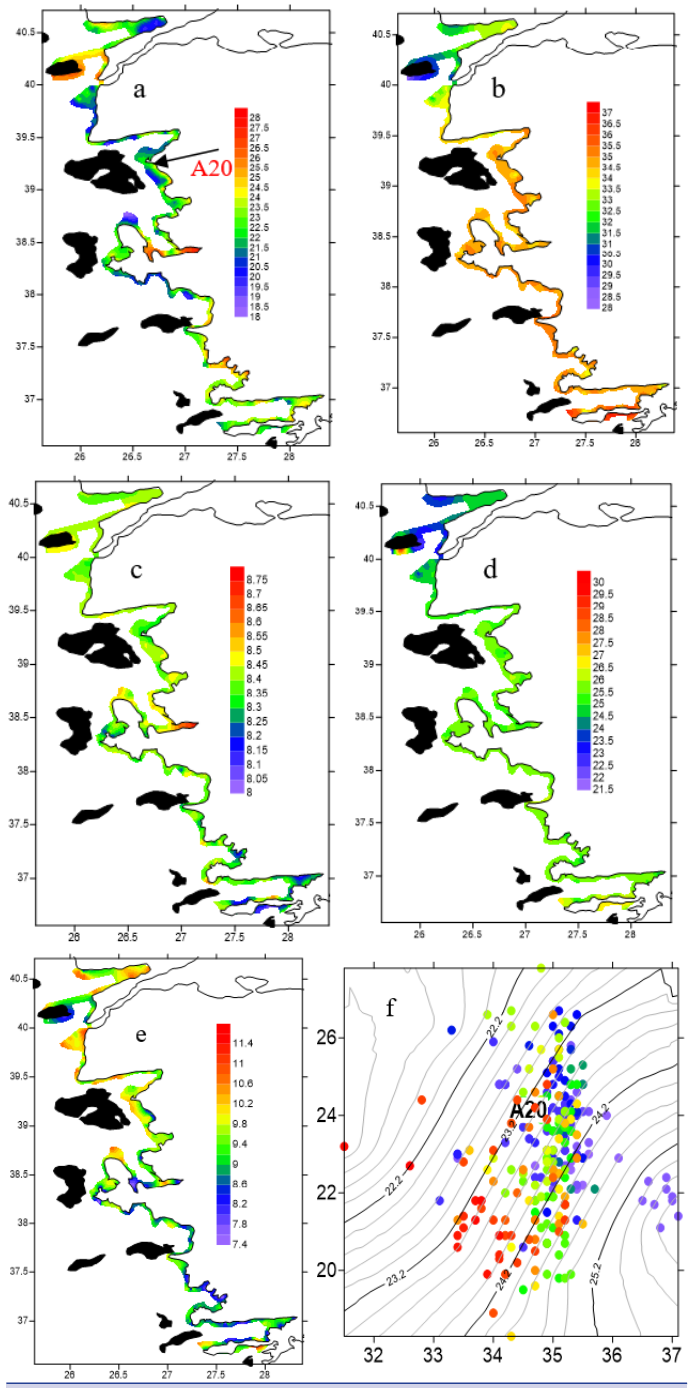

Figure 3. Near-bottom water temperature in oC (a), salinity in ppt (b), pH (c), dissolved oxygen in mg/l (d), total suspended matter in mg/l (e) and T-S diagram with isoline of density, σt at the stations colored with geographical latitudes (see Figure 1b for the station locations).

of the frond. The slope of the relationship was approximately 2.15. The number of pinnae showed considerable variation with a mean of 106. The number of lateral branchlets (pinnules and ramuli) per 1 cm frond length showed considerable variation, with values ranging from 1.24 to 7.14 branchlets for the main frond and from 0.57 to 5.20 for the buds (Figure 7). However, the mean values for both frond types were similar. The relationship between frond length and width was modeled using a curved-linear (logarithmic) function (Figure 8). The slope of the relationship was estimated to be approximately 0.515.

Remarks: *C. taxifolia* var *distichophyla* has a similar identical structure to *Caulerpa mexicana* and both *C. mexicana* and *C. taxifolia* have the type of distichous frond but the *C. mexicana* has a type between clavate; club-shaped (wider than tip parts of ramuli) falcate ramuli and *C. taxifolia* has variant of falcate; sickle-shaped ramuli. The basal part of ramuli of both species was contracted, but it is not contracted for the *C. taxifolia* var *distichophyla*. For *C. taxifolia*, the maximum frond length ranged from 16.6 to 18.1 cm and width from 13.3 to 18.5 mm. The number of branchlets varied between 6-18 and 156-210 with an average of 76 to 94 and 10-12 per 1 cm frond length. For *C. mexicana*, maximum frond length ranged from 10 to 12.5 cm and width from 7 to 9 mm. The number of branchlets varied from 10-28 to 120-194 with an average of 12-15 to 20-27 with 16-18 per 1 cm frond length and the slope of the relationship between frond length and the number of branchlets was estimated to be less (0.81-0.97) than 1 for *C. taxifolia* and more (1.49-1.73) than 1 for *C. mexicana* (Fig. A2) (Mutlu et al., 2024). The number of branchlets of *C. taxifolia* varied between 6-18 and 156-210 with an average of 76 to 94 and 10-12 per 1 cm frond length. The slope of the relationship between frond length and number of branchlets was estimated to be less (0.81-0.97) than 1 for *C. taxifolia* and more (1.49-1.73) than 1 for *C. mexicana* (Fig. A2) (Mutlu et al., 2024).

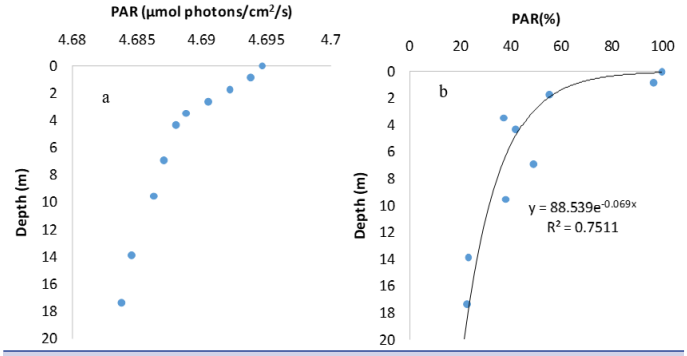

Figure 4. PAR profile in µmol photon/cm2/s (a) and percent based on sea surface PAR (b) along the water depth from the surface to bottom at 20 m.

Distribution: In addition to the previous record in the Sea of Marmara, Iskenderun Bay, İzmir Bay and Antalya Bay in the Levant Sea of the Turkish coasts (Figure 1), one site was inhabited by specimens of *Caulerpa taxifolia* var. *distichophylla* in shallow and coastal waters of the Aegean Sea (Altınova Bay, Turkey). The distribution occurred at 20 m.

Biometry

Specimens of *Caulerpa taxifolia* var. *distichophylla* at the station were found in runner stolon organization attached to the bottom substrates (Figure 5). Such occurrence was identical to the species on the bottom, unlike *Caulerpa mexicana* and *Caulerpa taxifolia* (Mutlu et al., 2024) with highly elongated stolon throughout the bottom. The biometric parameters of the species were recognized as density and morphometric variables to characterize the recent measurements made from the live specimens in the Turkish waters of the Aegean Sea (Table 2).

Frond length (FL) varied between 2.10 and 105 mm with a mean of 46.51±1.4 mm and frond width between 1.30 and 4.10 with a mean of 2.68±0.003 mm (Table 2). Turan et al. (2011) characterized biometry of *C. taxifolia* collected from the Izmir Gulf, Aegean Sea as follows: The mean stolon diameter, width of fronds, maximal length of pinnules and width of pinnules of a total of 50 *C. taxifolia* samples were 1.6±0.5 mm, 9.9±2.3 mm, 5.4 ±1.3 mm and 1.1 ±0.1 mm, respectively.

Table 3. Basic statistics of number of pinnae (ramuli) per 1-cm frond length for the main fronds, and for the buds (BP#/BL).

	FP#/FL	BP#/BL
Min	1.24	0.57
Max	7.14	5.20
Mean	2.39	2.27
SD	0.77	0.96

Table 1. Physicochemical properties of the sea surface and near-bottom waters at station A20.

Depth (m)	Secchi	T (°C)	S (ppt)	pH	TSM (mg/l)	DO (mg/l)	σ_t
0.5		25.5	34.7	8.26	25.6	9.4	22.9636
20	18	23.7	34.6	8.31	25.5	9.5	23.4286

Table 2. Depth-wise distribution of morphometrical and density variables of *Caulerpa taxifolia* var. *distichophylla* (mean±SD). FL: frond length in mm, FW: frond width in mm, BL: bud length in mm, BW: bud width in mm, LA: leaf area in m^2 per quadrant, TS: shoot density in shoots/m^2, and LAI: Leaf area index (m^2/m^2).

FL	FW	BL	BW	LA	TS	LAI
46.5±1.4	2.68±0.003	29.6±1.4	2.29±0.68	$1.36*10^{-4}$±$5.2*10^{-6}$	344±0	0.165±0

The length of the budding frond ranged from 4 mm to 266 mm and the width ranged from 0.8 mm to 4.0 mm (Table 2).

Shoot density was calculated as 344 shoots/m^2 and leaf area index as 0.165 at A20 (Table 2).

Budding

The stolons of the species appeared in a continuous line formed by the bud (Figure 5). Of the total specimens examined in the present study area, the number of fronds per stolon varied between 1 and 7 fronds, with a maximum of about 6 fronds. The maximum number of buds was also about 6 buds per frond (Figure 6). 18 out of 54 fronds were budded with a percentage of 33%. Multiple budding occurred rarely on one main shoot of the specimens (Figure 6).

Interbiometry relationship

The number of lateral branchlets (pinnalus, ramuli) per 1-cm frond length varied between 1.24 and 7.14 branchlets for the main frond, and for the buds, less than that of the main frond, varying between 0.57 and 5.20, but the average values of both frond types were similar to each other (Table 1).

Figure 5. *Caulerpa taxifolia* var. *distichophylla* appearance of entire specimens (a and b) and close-up view of fronds (c and d). F: frond, St: stolon, Rh: rhizoid, FL: frond length, FW: frond width, P: pinnae, R1: rachis 1, and R2: rachis 2. B is bud. Dashed line arrow is remark.

The number of pinnae-frond length relationships was significantly established for the species with a correlation of r=0.9353 (p= 9.0305*10^{-25}). The slope of the relationship was approximately 2.15. The number of pinnae varied between 10 and 260, with an average of 106 (Figure 7).

The relationship between frond length and width was fitted in a curved-linear (logarithmic) model (r= 0.5842, p= 2.163*10^{-5}) (Figure 8). The slope of the relationship was estimated to be approximately 0.515.

The Aegean Sea, particularly the Turkish waters were influenced at south by the Mediterranean Sea and at north by Black Sea through the straits system (Özsoy & Latif, 1996). This peculiarity induced a variety of marine environments in the Aegean Sea and a wide range measurement in the physicochemical and optic pa-

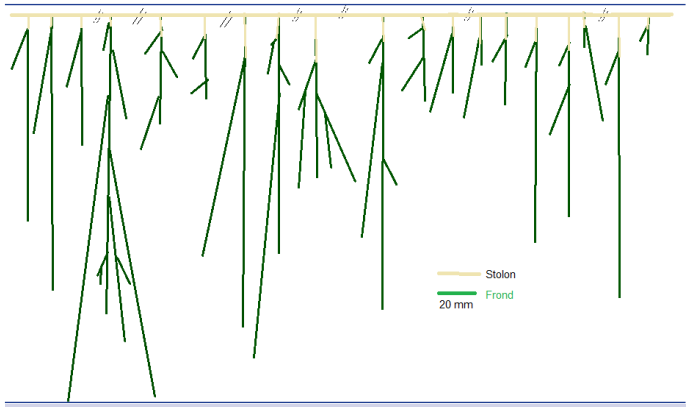

Figure 6. Schematized frond budding (see Fig. 5 for real appearance of budding reticulated with branches) of *Caulerpa taxifolia* var. *distichophylla* found at 20 m. Light green is frond (scale: 2 cm), beige is stolon unscaled. Diagonal break line denotes fronds belonging to each stolon.

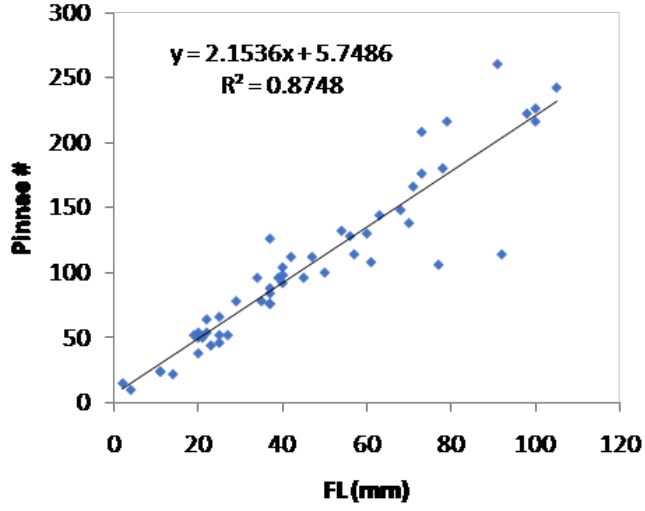

Figure 7. Relationships between frond length and number of pinnae (#P).

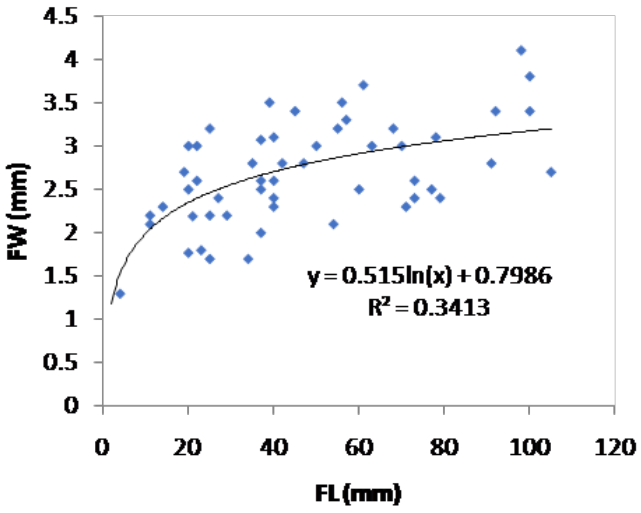

Figure 8. Relationships between frond length and number of pinnae (#P).

rameters (Uçkaç, 2005). Besides, the present entire study area was anthropogenically impacted by the highly populated cities along the coast through the rivers (Çulha et al., 2022). For instance, Izmir Gulf, Yelekçi et al. (2021) measured extreme nutrients, chl-*a* and Çinar et al. (2012) high total carbon content in the sediments. Mutlu (2021) reported hypoxia in the water of the Izmir Gulf.

In line with Musco et al. (2014), we hypothesize that *C. taxifolia* var. *distichophylla* must be considered potentially invasive and that the *Caulerpa taxifolia* species cluster in general appear to possess ecological traits (Andreakis & Schaffelke, 2012) that make the species particularly adapted for rapid colonization of the shallow-water Mediterranean ecosystem.

After Turan et al. (2011) recorded the *Caulerpa taxifolia* var. *distichophylla* for the first time in the Aegean Sea, the present study on second occurrence of the species expanding to the north in the Turkish Aegean Sea provided significant insights into the morphological and biometric characteristics of this invasive species. The findings indicated that this variant exhibited a delicate, feather-like thallus structure, characterized by narrow prostrate stolons and erect fronds with distinct pinnules. The research highlighted the species' adaptability and potential for rapid colonization in the Mediterranean ecosystem, underscoring its invasive nature. The biometric data collected, including frond length, width, and the number of pinnae, established a foundational understanding of the species' growth patterns and density in its new habitat.

Caulerpa taxifolia var. *distichophylla* was found at one station at 20 m in Altınova Bay among 321 stations visited during the survey (Figure 1). During the survey *Caulerpa mexicana* and *Caulerpa taxifolia* (Figures A1, A2) were found at two stations (10 m and 15 m) in Izmir Gulf and four stations (10, 15, 20 and 30 m) in Dikili Bay, respectively (Mutlu et al., 2024). In Turkish waters, *Caulerpa taxifolia* var. *distichophylla* has been reported for its occurrence in Iskenderun Bay, Kaş, Antalya Bay in the Levant Sea, and the Sea of Marmara (Figure 1a) (Mutlu et al., 2022; Jongma et al., 2013; Taşkın et al., 2023). The present occurrence of the species filled the gap for the records between eastern Mediterranean Sea and Sea of Marmara. In Cyprus, *C. taxifolia* var. *distichophylla* was found both in very shallow waters and at 42 m and 18 m depth on the island of Rhodes (Table 4).

In particular, the relationship between frond length and frond width and the relationship between frond length and the number of lateral branchlets showed a difference in the relationships among the congeneric species. In the present study, three congeneric species were found, and they showed a significant difference in the relationships among the species (Figure A2). Regarding the relationship between frond length and number of lateral branchlets, *Caulerpa taxifolia* var. *distichophylla* had a slope greater than 2, *C. mexicana* greater than 1 but less than 2, and *C. taxifolia* less than 1 (Figure A2).

The frond length of *C. taxifolia* var. *distichophylla* was higher than *C. mexicana* and *C. taxifolia* at maxima and on average (Fig. A2, Mutlu et al., 2024). Frond width was narrower than that of the other two species. Frond length was measured around the maximum, like other Mediterranean sites (Table 4). Frond width was narrower in the Levantine basin than in the western Mediterranean (Table 4).

The shoot density of *C. mexicana* varied between 469 shoots/m^2 (at 15 m) and 630 shoots/m^2 (at 10 m), and *C. taxifolia* between 343 shoots/m^2 estimated at 15 m and 1397 shoots/m^2 at 30 m. The average shoot density of *C. taxifolia* var. *distichophylla* was lower than that of the other two species (Mutlu et al., 2024). The shoot density estimated for the present study was found to be lower than the range of the results obtained from the other sites (Table 4). However, the biometric measurements had similar range as compared to that in the other Mediterranean location, but not in Sea of Marmara (Table 4). This density and morphometric difference could be due to newly establishment of the species in the Turkish Aegean Sea and Sea of Marmara.

However, exclusive biometrics and plant characteristics were not supported to the knowledge of the species from the previous publications from the Turkish waters, including the occurrence in the Turkish Aegean Sea. Although the specimens were found in only one station, this biometric information could still be useful set. Further future studies will help to understand the dynamics of *C. taxifolia* var. *distichophylla* in the Aegean Sea between the Mediterranean Sea and the Sea of Marmara.

The significant correlation between frond length and the number of pinnae, and frond width along with the observed relationships between frond dimensions, contributed to the existing body of knowledge regarding the non-broad-scaled ecological dynamics of invasive marine species and helped to identify of three congeneric species of *Caulerpa*. Furthermore, the study emphasized the importance of ongoing monitoring and research to assess the ecological impacts of *C. taxifolia* var. *distichophylla* on native marine communities.

Table 4. Plant traits (TS: shoot density in shoots/m^2, LAI: leaf area index in m^2/m^2, FNo: number of lateral branchlets per 1-cm frond length, RL: rachis length in mm, FL: frond length in mm, FW: frond width in mm, RhL: rhizoid length in mm, Cov.: surface coverage on ground in m^2, D: bottom depth in m and bottom and substrate type) and distribution of *C. taxifolia* var. *distichophylla* in seas of the Mediterranean basin.

TS	LAI	FNo	RL	FL	FW	RhL	Cov.	D	Loc.	C#
		1.3		25–100	1.5–4.2	1–4		42*	Cyprus	1
		1.3		25–100	1.5–4.2*	1–4		18*	Rhodes	1
				< 60-70		16/18			Sicily	2
				< 60-70		16/18			Sardine	2
				80-100				sandy	Sicily	3
				150				shaded	Sicily	3
				35*, 20				shaded by *P. oceanica*	Sicily	3
				200 vs. 80,					Sicily	3
				140-160				sheltered from the waves, "matte"	Tunisia	4
276.3 (± 51.21							125	4–6	Malta	5
				5.90–170.33	3.60–1.25	0.63–3.48	0.37	14–15	Malta	5
91–972				12.3–41.8*				40*	Malta	6
972 N/m*										6
				100				3m,snady, rocky	Sicily	7
				<100				2, rocky	Sicily	7
				< 50				Out shipwreck	Sicily	7
				100–150				In shipwreck	Sicily	7
				<100				1-2, rocky	Sicily	7
				50-100				4-5m, rocky, edge of *Posidonia*	Sicily	7
				50-100				9-10, border *C. nodosa*	Sicily	7
				30–40	2–4	3			Sicily	8
<100-600	0.0010-0.0084*		5.8-8.1, 16*	24.7-48.4, 40	1.03-1.74*			5-30	Antalya, TR	9
				40	2.0-3.0	4-5		4	Sea of Marmara	10
344	0.165	1.24-7.14, 2.39 0.57-5.20*, 2.27*		2.1-105, 46.5	1.3-4.1, 2.6*	3-80, 20,12 1.3-30*, 9.4*		20	Aegean Sea, TR	PS
*max	*mean	*for bud frond	*max	*RL+FL	*mean	*for bud frond		*max depth		

C#: 1: Aplikioti et al., 2016, 2: Di Martino, Stancanelli & Cantasano, 2018, 3: Musco et al., 2014, 4: Chartosia et al., 2018, 5: Schembri et al., 2015, 6: Ellul et al., 2019b, 7: Mannino et al., 2019, 8: Picciotto et al., 2016, 9: Mutlu et al., 2022, 10: Taşkın et al., 2023.

CONCLUSION

The species has been spreading toward the north where the eutrophic regions are available, which can threat Sea of Marmara and Black Sea when invaded by the specimens in the future. In the central Mediterranean the species had higher length, width and shoot density of the frond as compared Levantine and Ae-

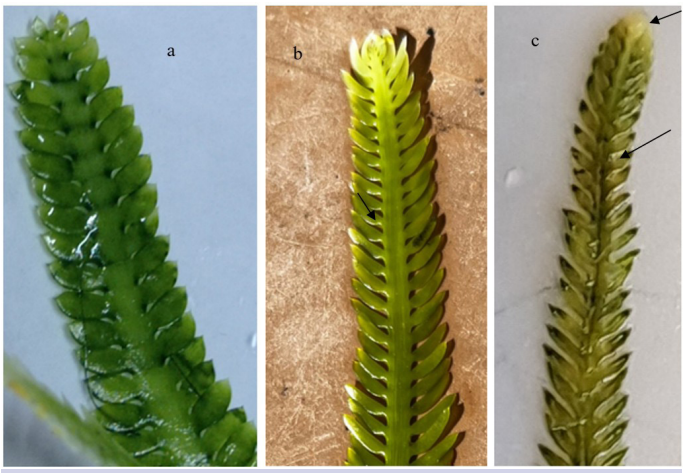

Figure A1. Fronds, rachises and pinnae of *Caulerpa mexicana* (a), *C. taxifolia* (b) and *C. taxifolia* var. *distichophylla* (c) from the Turkish Aegean waters obtained during the present study (Mutlu et al., 2024).

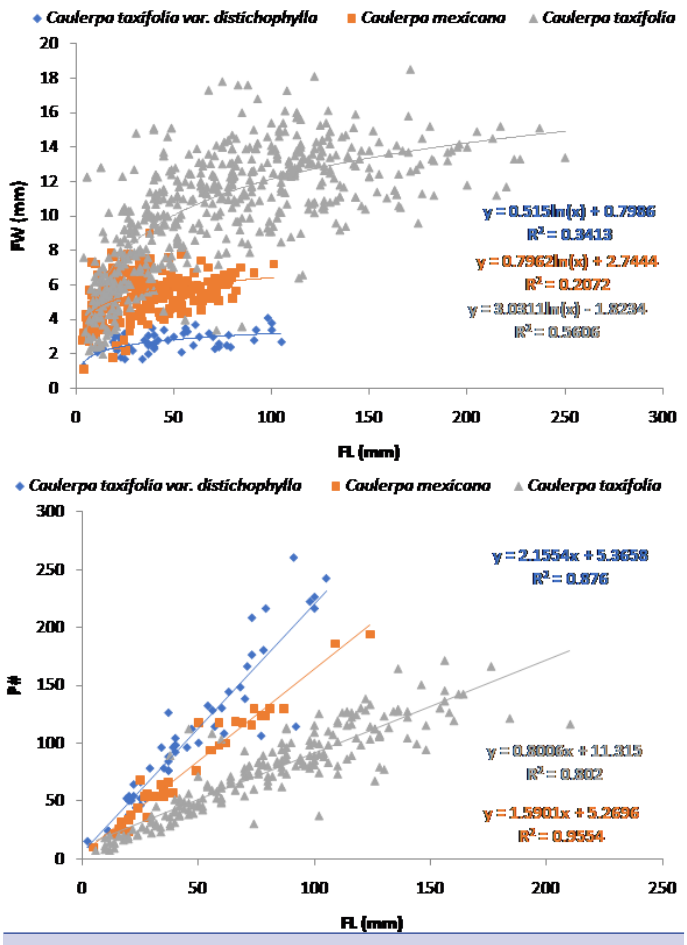

Figure A2. Frond length (FL)-width (FW) and frond length (FL)-number of pinnae (P#) of *Caulerpa mexicana*, *Caulerpa taxifolia* and *C. taxifolia* var. *distichophylla* specimens collected during the present study in the Turkish coast of the Aegean Sea (Mutlu et al., 2024).

gean specimens. Given the potential threats posed by invasive species to local biodiversity, this research served as a crucial step in understanding the implications of biological invasions in the Mediterranean region, particularly considering changing environmental conditions and human activities that may facilitate further spread. Overall, the findings underscored the need for effective management strategies to mitigate the impacts of invasive marine species on fragile ecosystems. Further studies are needed to study spreading of the species and population dynamic of species with compressive environmental parameters such as contents of water nutrients and sedimentary variables.

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Compliance with Ethical Standard: The authors declare that all applicable guidelines for sampling, care, and experimental use of animals in the study have been followed.

Conflict of interest: The authors have no conflicts of interest to declare that are relevant to the content of this article.

Ethical approval: The authors declare that all applicable guidelines for sampling, care, and experimental use of animals in the study have been followed.

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Research Article

Investigating the Suitability of Remineralized Aquaponics Sludge for Microalgae Culture: Biomass Production and Nutritional Composition

Murat Yeşiltaş[1], Mehmet Ali Turan Koçer[1], Faruk Pak[1], Özgür Aktaş[1], Edis Koru[2]

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ABSTRACT

Microalgae are promising resources for valuable products, and cultivating them requires a suitable culture medium to optimize growth and desired biochemical content. Aquaponic sludge, a byproduct of aquaponics systems, offers a sustainable and cost-effective alternative to conventional media by recycling waste and reducing environmental impact. This study aimed to compare the performance of standard BG-11 (Blue-Green 11) medium with remineralized sludge-water (RSW) and RSW supplemented with micronutrient solution (RSW+Mn) for cultivating *Chlorella minutissima, Botryococcus braunii,* and *Haematococcus pluvialis*. The highest specific growth rate (μ) of 0.097±0.011 was observed for *C. minutissima* in BG-11 medium, nearly 28% higher than in RSW medium. However, the highest dry biomass productivity (P_b) of 0.012±0.011 was achieved by *H. pluvialis* in RSW+Mn medium, significantly 94% higher than in RSW medium. Additionally, the volumetric productivity of biomass (Q_x) for *H. pluvialis* in RSW medium was 0.045±0.017, nearly 50% higher than in BG-11 medium. The best doubling time (td) of 8.83±0.93 days was observed for *H. pluvialis* in RSW medium. Notably, *C. minutissima* cultured in RSW medium yielded the highest crude protein (55.77±1.81%) and total lipid (4.69±0.88%) contents. These results demonstrate that RSW medium can be tailored to achieve desired outcomes, such as optimizing growth rate or lipid content. This study highlights the potential of remineralized aquaponic sludge as a sustainable culture medium for microalgae, contributing to waste recycling and resource efficiency in aquaponics systems. Future studies should focus on optimizing RSW medium for large-scale cultivation of target microalgae species with specific biochemical profiles.

Keywords: Microalgae, aquaculture, wastewater, recirculating aquaculture system, sustainability, waste recycling

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INTRODUCTION

The integration of aquaponics and microalgae production using wastewater represents a significant advancement in sustainable aquaculture. Medium-scale integrations offer valuable data for aquapreneurs, enabling them to develop new initiatives.

In aquaponics, fish and plants are grown together in a closed recirculating system (Goddek and Keesman, 2020). The system is managed by mimicking a natural aquatic environment with fish, plants, and nitrification bacteria consortia in a limited area (Tunçelli and Memiş, 2024). The recirculation characteristics of aquaponics make it a valuable system that contributes to reducing natural freshwater consumption by up to 90% (Danish et al., 2021). As compared to conventional irrigation systems, recirculating aquaculture systems like aquaponic systems use less irrigation water for agricultural activities and prevent soil salinization (Colt et al., 2022). However, recirculating aquaculture systems produce sludge that contains a variety of nutrients, including nitrogenous compounds,

phosphorus, and other dissolved organic carbons that could negatively affect the environment when their concentration is higher than usual (Jasmin et al., 2020). Furthermore, the water in aquaponics is typically warm, which is ideal for microalgae growth. Due to these kinds of opportunities, aquaponics byproduct, sludge-water, has the potential to be a sustainable alternative as it can be collected easily and remineralized for microalgae cultivation (Boedijn et al., 2021; Addy et al., 2017).

Three primary methods are commonly used for integrated vegetable and fish production in aquaponics systems. Nutrient film technique (NFT), media bed, and deep water culture methods are frequently used methods for establishing aquaponics (Li et al., 2019). NFT aquaponics utilizes thin water surfaces and air simultaneously to provide optimal conditions for plant growth (Su et al., 2020). With its wide air surface area, NFT aquaponics is considered one of the most advantageous aquaponic systems for converting nitrogenous ions to nitrates through aerobic oxidation bacteria (Thakur et al., 2023). Moreover, aquaponic systems can be integrated with microalgae cultivation to enhance ecosystem-friendly aquaculture (Ansari et al., 2021). However, this sludge-water can be treated through remineralization and repurposed as a nutrient-rich medium for microalgae cultivation.

Studies have shown that microalgae grown in different wastewaters have similar growth rates and biomass yields as microalgae grown in traditional culture mediums. Studies have shown that microalgae can effectively utilize various wastewaters for growth, including urban wastewater (Robles et al., 2020), textile wastewater (Wu et al., 2020), pig farm wastewater (Nagarajan et al., 2019), brewery effluent (Ferreira et al., 2019), shrimp wastewater (Krasaesueb et al., 2019), and even palm oil wastewater (Ahmad et al., 2019). Because microalgae culture medium is one of the most expensive entries of the microalgae production after labor, illumination conditions, agitation, and photosynthetic efficiency of the system (Mtaki et al., 2023). As a result, using cheaper raw materials like aquaponics sludge-water, optimizing nutrient composition in culture medium, recycling wastewaters, cost-effective production techniques, and exploring alternative microalgae culture mediums can be done to reduce the cost of the culture medium. Microalgae have the ability to produce highly valuable bioactive compounds such as vitamins, minerals, essential amino acids, fatty acids, carotenoids, and enzymes (Zhou et al., 2022). Therefore microalgae that are cultured in aquaponics sludge-water are very good candidates to get biomass for bioactive compounds. Nitrification bioreactors can recover nitrogen from sludge-water via remineralization (Wongkiew et al., 2021), and the resulting culture medium can be used for microalgae cultivation.

Ammonium-oxidizing bacteria like *Nitrosomonas* sp., *Nitrosococcus* sp., and *Nitrosospira* sp. are the leading microorganisms of the nitrification process (Al-Ajeel et al., 2022). These bacteria are commonly used in wastewater treatment facilities. Aerobic-activated sludge application is one of the widely used processes for municipal and industrial wastewaters to get rid of their hazardous compounds such as ammonium, nitrite, and nitrate ions (Singh and Dey, 2024). Because these pollutants can cause eutrophication with precipitation in narrow bays, closed-water basins or lakes (Zhang et al., 2020). Eutrophication is usually characterized by algal blooms, low water quality, and mass fish mortality (Kapsalis and Kalavrouziotis, 2021). Compounds like ammonium and nitrite in sludge-water that pose a danger to aquatic and terrestrial animals make it possible for microalgae to grow effectively (Chamoli et al., 2024).

First studies of using sludge extracts to produce microalgae date back to the 1970s. Using sludge extracts, Wong et al. (1977) attempted to increase the production of *Chlorella pyrenoidosa* in the Kuhl Medium. In a different study, sludge extracts were discovered to be more beneficial than other common microalgae cultivation mediums (Wong, 1977). Aquaponics sludge-water is a waste product that would otherwise need to be disposed of, so using it as a culture medium can help to reduce the amount of waste produced by the aquaponics system and potentially reduce the cost of microalgae cultivation. Additionally, using aquaponics sludge water can help to improve the efficiency of the aquaponics system by recycling the nutrients that are present in the water.

However, depending on the species of fish and plants in the aquaponics system, the sludge-water might be contaminated with some pathogens, heavy metals, or other pollutants, which might impact negatively on microalgae growth. There are some important physicochemical parameters such as temperature (Elisabeth et al., 2021), pH (Fernandes et al., 2022), culture medium (de Medeiros et al., 2020), carbon dioxide (Li et al., 2023), and conductivity (Barahoei et al., 2021) that have effects on the concentration and quality of lipids and proteins in microalgae. Therefore, it's important to evaluate the quality of the sludge water before using it as a culture medium and to consider other factors such as pH, temperature, and light conditions to optimize the growth of the microalgae. Plants and protists require nitrogenous nutrient salts such as ammonia, nitrite, and nitrate as well as algae to take advantage of these compounds, as well (Ribeiro et al., 2020; Kyriacou et al., 2019).

Chlorella sp. is one of the most common microorganisms used in the production of biomass from industrial, municipal, and even aquaponics wastewater (Fimbres-Acedo et al., 2020; Chen et al., 2019; Wang et al., 2019; Addy et al. 2017; Fang et al., 2017). On the other hand, *B. braunii* and *H. pluvialis* are other microalgae species that have a growing trend of experimental and commercial cultivation. *B. braunii* is known for its rich lipid content and hydrocarbon production ability (Nazloo et al., 2024), *H. pluvialis* is known for its natural astaxanthin production capability (Mularczyk et al., 2020). Bioethanol wastewater streams (Nishshanka et al., 2022), primary treated wastewater (Pan et al., 2021), synthetic brewery wastewater (Yap et al., 2022), and domestic secondary effluent (Sirotiya et al., 2023) were used as culture mediums for the production of *H. pluvialis* biomass. *B. braunii* were used as a wastewater treatment organism for piggery wastewater (Mkpuma et al., 2023), sewage wastewater rich in ammonium nitrogen (Miura et al., 2022), aerated swine lagoon wastewater (Li et al., 2022). According to these studies, a sustainable ecosystem approach to biomass production was made by using the help of different microalgae.

This study's hypothesis focuses on a novel and potentially sustainable approach to microalgae culture medium using aqua-

ponic byproducts. Traditionally, microalgae are cultivated using well-defined but expensive medium like BG-11. This study investigates a potentially lower-cost option by exploring remineralized sludgewater from aquaponics as a culture medium. There's a growing interest in using wastewater as a culture medium, and this study specifically focuses on remineralized sludgewater due to its potentially enhanced nutrient profile, making it even more suitable for microalgae growth.

The purpose of the study was to determine the performance of microalgae biomass production by utilizing sludge-water obtained from aquaponics. A comparison was made among the performance of microalgae grown in RSW medium, RSW medium including micronutrient solution (RSW+Mn), and commonly used BG-11 medium. For the cultivation of microalgae species, the BG-11 medium is one of the most commonly used nutrient mediums, and it can be compared with other microalgae culture mediums easily. Microalgae cell numbers, doubling times, biomass productivities, specific growth rates, and biochemical contents were determined and evaluated in the RSW and RSW+Mn culture mediums.

MATERIALS AND METHODS

Aquaponic system setup

This study was conducted at the Mediterranean Fisheries Research Production and Training Institute's (MEDFRI) Nutrient Film Technique (NFT) aquaponics research facility in Antalya Province for 42 days, from September to October. The recirculating aquaculture system (RAS) consisted of a 2.5 m^3 fiber aquaculture tank, two 80-liter radial flow separators, two 150-liter biofilter tanks (each containing 20 liters of media), 12 PVC rafts, and a 150-liter sump/pump tank. A 0.25 kW submersible water pump (Pedrollo, Tamworth UK) was installed in the sump/pump tank to circulate water. Daily, 10 liters of sludge-water were collected from the aquaculture tank discharge pipe and radial flow separators. This untreated discharge poses a risk of eutrophication in nearby inland waters. The collected sludge-water underwent a one-day remineralization process in 10-liter tanks. This process employed an air pump (2.5 L/min) to convert hazardous nutrients into bioavailable forms. A 0.45-micron cellulose ester membrane filter removed solid waste. To prevent bacterial interference, the remineralized sludge-water (RSW) was autoclaved daily at 121 °C and 1 atm for 25 minutes. The combined RSW was used as a microalgae culture medium and compared to the BG-11 medium. Dead fish were promptly removed from the aquaculture tank to minimize bacterial contamination. Figure 1 illustrates the catfish-lettuce aquaponics system utilized in this study.

Fish

A total of 348 African catfish (*Clarias gariepinus*) were used with an average weight of 28.83±11.68 g were used in the study. The total biomass of the fish was 10 kg (4 kg/m^3 initial density). The fish were fed twice daily (09:00 and 16:00) at a rate of 2% of their total biomass per feeding (4% total daily ration) for six weeks using commercial carp feed. The diet contained 50% raw protein, 8% total fat, 3.7% cellulose, 9.7% ash, 1.5% calcium, 0.94% phosphorus, 0.42% sodium, 8000 $IUkg^{-1}$ vitamin A, 3000 $IUkg^{-1}$ vitamin D3, 350 $mgkg^{-1}$ vitamin E, 30 $mgkg^{-1}$ manganese oxide, 60 $mgkg^{-1}$ zinc oxide, 20 $mgkg^{-1}$ iron chelate of glycine hydrate, 2 $mgkg^{-1}$ calcium iodide, 6 $mgkg^{-1}$ copper sulfate pentahydrate, 0.2 $mgkg^{-1}$ sodium selenite. The following fish performance parameters were assessed during the study: feed conversion ratio (FCR), specific growth rate (SGR) (μmax), survival rate (SR), relative growth rate (RGR), and weight gain (WG). Calculation methods for each parameter are described as follows:

FCR=Dry feed weight (g) / Weight gain (g)

SGR= ((ln Last Weight – ln Initial Weight) / Duration of experiment) × 100

SR= (Last number of fish in fish tank / Initial number of fish in fish tank) × 100

RGR= ((Last weight of fish – Initial weight of fish) / Initial weight of fish) × 100

WG= (Last weight – Initial weight)

Figure 1. Nutrient film technique aquaponics 1: Fish tank, 2: Separation tanks, 3: Bio-filter, 4: Hydroponic unit, 5: Sump/Pump, 6: Sludge-water discharge pipe

Plant

Three-week-old lettuce (*Lactuca sativa*) seedlings, with an average initial weight of 1.69±0.42 g, were planted in a hydroponics unit at a density of 12 × 12 cm, using fiber as a substrate. The total of 120 lettuce plants were used in the experiment to ensure statistical reliability. The total wet weights of all plants were meticulously measured after separating them from the fiber substrates. The lettuce shoots were separated from the roots by cutting them just above the root collar (the point where the stem meets the roots). The following parameters were calculated:

Total harvested plant weight (g): Measured for each plant.

Leaf number: Counted for each plant.

Yield (gm^{-2}): Calculated as Biomass/Area, where the area of the hydroponics unit was 2.88 m^2.

Survival rate (SR): Determined as (Final number of plants/Initial number of plants) × 100.

Shoot/root ratio (s/r): Calculated as plant shoot length divided by plant root length.

Microalgae strains and culture system setup

Chlorella minutissima, *Haematococcus pluvialis*, and *Botryococcus braunii* were selected for cultivation using standard BG-11, RSW, and RSW+Mn mediums. Firstly, all stock microalgae species used in the present study were pre-cultured in standard BG-11 medium ($NaNO_3$ (1500 mgL^{-1}), K_2HPO_4 $3H_2O$ (40 mgL^{-1}), $MgSO_4$ $7H_2O$ (75 mgL^{-1}), $CaCl_2$ $2H_2O$ (36 mgL^{-1}), $C_6H_8O_7$ (6 mgL^{-1}), $C_6H_8O_7$.xFeH_3N (6 mgL^{-1}), Na-EDTA (1 mgL^{-1}), Na_2CO_3 (20 mgL^{-1}) and micronutrient solution: H_3BO_3 (2.86 μgL^{-1}), $Co(NO_3)_2$ $6H_2O$ (0.494 μgL^{-1}), $MnCl_2$ $4H_2O$ (1.81 μgL^{-1}), $ZnSO_4$ $7H_2O$ (2.22 μgL^{-1}), Na_2MoO_4 $2H_2O$ (3.9 μgL^{-1}), $CuSO_4$ $5H_2O$ (0.79 μgL^{-1})) in 1 L erlenmeyer flasks. The microalgae were then cultivated in three different media: standard BG-11, RSW, and RSW+Mn in the study. All microalgae species were cultivated in 10 L low-density polyethylene (PE) plastic bags. Each species was cultured in triplicate 10-liter low-density polyethylene (PE) plastic bags within a microalgae culture platform (Figure 2). A 16:8 light/dark photoperiod was used as one of the most important parameters for biomass production (Ratomski et al., 2021). The cultures were illuminated with 36-watt cool-white fluorescent lights. It was attempted to maintain the temperature at 25 °C by using the central heating system of the Aquarium Unit at MEDFRI. The light adjustment at the surface of the microalgae culture plastic bags was realized by using a light meter, TES 1332A (TES Electrical Electronic Corp., Taipei, China). Microalgae cultures were continuously aerated using filtered air at a constant flow rate of 2.5 $Lmin^{-1}$ for each plastic bag. Microalgae were grown in the live feed laboratory of the Mediterranean Fisheries Research Production and Training Institute for 27 days. Microalgae cell numbers were monitored every day using a light microscope (Leica Microsystems, Wetzlar, Germany) and a haemocytometer (depth 0.1mm; five replicates were averaged). At the end of the cultivation process, batch cultures were harvested and then freeze-dried by a high-vacuum freeze dryer (Telstar Cryodos-50, Terrassa, Spain) for 2 days at 0.023 mBar and -55±2 °C. All biochemical analyses were conducted on freeze-dried samples obtained from batchwise cultures, and they were stored at -18 °C.

Physicochemical parameters and ion concentration of microalgae culture mediums

Evaluating the physicochemical parameters and nutrient salts of the microalgae culture medium is an important step in optimizing microalgae growth. This influences the biochemical composition of the obtained microalgae, such as crude protein, total carbohydrates, and total lipids. By doing this, microalgae researchers can minimize culture medium costs while maintaining optimal growth conditions using alternative techniques and resources.

BG-11 medium is commonly used for green microalgae and cyanobacteria species (Rippka et al., 1979; Ozturk et al., 2019). The RSW culture medium, obtained daily from the aquaponics system, underwent a 42 day oxidation and sterilization process before being pooled (or "combined"/"formulated") in PE plastic bags. After remineralization, the ion concentration of RSW was analyzed using a Dionex ICS 3000 ion chromatography system consisting of a dual gradient pump unit (Dionex, Sunnyvale, CA, USA). Ion concentrations of BG-11, RSW, and RSW+Mn culture mediums are shown in Table 4.

Figure 2. Microalgae batch culture platform 1: Polyethylene plastic bags with microalgae, 2: 36 Watt fluorescent lamps, 3: 2.5 L/min aeration

A handheld multiparameter unit, the Yellow Spring Instrument Pro-DSS (YSI, Xylem Inc., Yellow Springs, OH, USA), was used to measure physicochemical parameters of microalgae culture medium, including temperature (°C), conductivity (μScm^{-1}), salinity (ppt), and pH every day. At the end of the remineralization process, the daily collected and remineralized sludge-waters were combined and analyzed by ion chromatography. Daily collected RSW culture medium samples (3 mL) were filtered using 0.22 μm pore size polytetrafluoroethylene (PTFE) filters before analysis with two Dionex ICS 3000 ion chromatographs (Thermo Scientific, Waltham, Massachusetts, USA) equipped with IonPac AS9-HC and IonPac AS11-HC columns according to the manufacturer instructions.

Microalgae cell number and biomass

Maintaining optimal nutrient levels and growth conditions for microalgae leads to a higher biomass yield. By understanding how culture medium and conditions affect cell production, they can manipulate the conditions to favor the desired product. By doing this, it can be formulated cost-effective and optimized culture medium for large-scale production.

Three different mediums (BG-11, RSW, and RSW+Mn) were compared to determine microalgae growth (cell numbers). Microalgae cultures were counted daily using a binocular microscope (Leica DM2000, Leica Microsystems Canada, Richmond Hill, Ontario). The microalgae cells were counted daily after homogenization using a Neubauer haemocytometer and results were expressed as cellsmL^{-1}. For determining dry biomass, 10 mL algal suspension was filtered daily using glass microfiber filters (Whatman GF/F, 47 mm, nominal pore size 1.6 μm) and all microalgae dried at 105 °C for 4 hours. Pre-weighed glass microfiber filters were used for algae culture filtration. After filtration, the filters containing the algae were dried at 105 °C for 4 hours. The filters were then cooled down in a vacuum desiccator and weighed again. The difference in weight represents the dry biomass of the algae culture (mgL^{-1}) (Simonazzi et al., 2021; APHA, 1997). Vacuum pressure during filtration was maintained at 45 mm Hg. All microalgae groups were filtered in triplicate.

According to Vonshak (1986), the below formulation was used to calculate specific growth rate (μ).

$\mu = \ln(X2 - X1) / t$

$X1$= Initial biomass concentration

$X2$= Final biomass concentration

t= Time

Productivity (Q*x*) was calculated using the formula below (Liu ve ark., 2013).

Qx (gLday^{-1})= $(X2-X1)/t$

$X1$= Initial biomass concentration

$X2$= Final biomass concentration

t= Time

Doubling time (t*d*) was determined using the following formula (Yoshimura ve ark., 2013).

t*d* (day)= $\ln 2/\mu = 0.693/\mu$

t*d*: Doubling time or regeneration time

Dry biomass productivity calculated was applied using the following formula (Liu et al., 2013).

Biomass productivity (P*b*) (gm^{-2} day^{-1})= $(DWL_n \times DWL_0)/n$

DWL_n= Dry weight of day n

DWL_0= Dry weight of day 0

n= cultivation days

Determination of biochemical content of microalgae

Determining the biochemical content of obtained microalgae biomass is a key aspect for evaluating target products like crude protein, total carbohydrates, and total lipids. Crude protein in the biomass is suitable primarily for human consumption and animal feed. Total carbohydrates from the algae can be used for bioethanol and other biofuel applications. Total lipids are valuable for mainly supplements and biodiesel production.

Triplicate analyses were conducted on lyophilized microalgae to determine their total lipids, crude protein, raw ash, carbohydrates, and moisture content. The total lipid concentrations were determined gravimetrically after extraction using the modified Bligh and Dyer (1959) method by Kates (1972). Using the Dumas combustion method, the crude protein content of microalgae was determined (Chiacchierini et al., 2003). In order to determine the moisture content in freeze-dried microalgae biomass, approximately 1 g of the sample was oven dried at 105°C for 1 hour (Lee et al., 2013). Microalgae raw ash content was determined using the protocol of the AOAC method 942.05 (Helrich, K., 1990). Total carbohydrates (including crude fiber) were calculated as 100% minus the sum of the moisture, protein, fat, and ash contents obtained using proximate analysis as previously explained (Eyeson & Ankrah, 1975).

Statistical analysis

The statistical analyses were performed using JMP 13 software (SAS Institute Inc., Cary, N. C.). Following a Shapiro-Wilk homogeneity test, analysis of variance (One-way and two-way ANOVA) was conducted to compare results for culture mediums (BG-11, RSW, and RSW+Mn) within each algal species. Tukey's HSD tests were used to determine significant differences (P <0.05) among all culture mediums and algal species. The effects of temperature, pH, conductivity, and salinity were assessed using a repeated measures ANOVA. Data are presented as means±standard deviations in the tables.

RESULTS AND DISCUSSION

Growth performance of fish

The growth performance of African catfish was evaluated using the following criteria: fish feed conversion ratio (FCR), specific growth rate (SGR), relative growth rate (RGR), and survival ratio

(SR). At the conclusion of the 42 day of the aquaponics study, 45.14 kg of fish were harvested. However, accumulation of organic matter in the fish and separation tank, leading to declining dissolved oxygen (DO) levels throughout the study period and the cannibalistic nature of African catfish resulted in the mortality of 47 out of 348 fish. The final average weight of the fish was 149.97±70.38 g. FCR, SGR, RGR, and SR were calculated to be 0.98, 3.59% day-*1*, 351.1%, and 86.49%, respectively. The overall health of the African catfish was assessed as good. The absence of supplemental aeration in the fish tank is a potential factor in the 13.5% fish mortality observed by the end of the study. FCR of African catfish were found as 1.18-1.33 in spinach and mustard green integrated aquaponics (Endut et al., 2016), 1.02-1.09 in floating raft hydroponics integrated with cucumber (Baßmann et al., 2017), and 1.03-1.14 in co-cultivated basil integrated aquaponics (Baßmann et al., 2018). African catfish fed with fish diets containing different proportions of potassium diformate and potassium chloride exhibited specific growth rates (SGR) ranging from 1.25±0.09% day^{-1} to 1.52±0.12% day^{-1} (Siqwepu et al., 2020) which is lower than this study's results. In another study, African catfish grown in an aquaponic system with basil (*Ocimum basilicum*) at moderate and high densities had lower SGR values (0.71% day^{-1} and 0.80% day^{-1}, respectively) compared to this study (Baßmann et al., 2018). Hagar et al. (2019) reported an RGR value of 97.28±0.03 for African catfish grown in recirculating aquaculture systems (RAS). In comparison, studies have shown higher survival rates for African catfish integrated with aquaponics (e.g., 94.25±2.12% with pumpkin cultivation) compared to recirculating systems (80.60±1.20%) and static systems (59.24±1.91%) (Oladimeji et al., 2020). African catfish are found to be an appropriate candidate for aquaponics systems. Studies have shown promising results, with survival rates reaching 100% when integrated with lettuce cultivation using microwave pyrolysis biochar (Su et al., 2020). Additionally, research by Suhl et al. (2018) reported total weight gain ranging from 10.0 kg $tank^{-1}$ to 267.5 kg $tank^{-1}$ in a tomato-African catfish aquaponics system with an innovative suction filter that reduces nitrogen loss. However, cannibalism can be a major cause of mortality in African catfish.

Growth performance of plant

Lettuce was harvested from the hydroponics unit after 42 days of the experiment. Average plant weight, average leaf number, average leaf area, average stem diameter, fresh weight (yield), survival rate, and average shoot/root ratio were calculated as 91.85±35.36 g, 36.79±7.33, 48.26±32.20 cm^2, 2.16±0.39 cm, 164.06±54.09 gm^{-2}, 100%, and 4.83±1.21, respectively. Tunçelli and Memiş (2024) Palm et al. (2014), Calone et al. (2019), and Byrd et al. (2022) reported similar results for hydroponic lettuce production in aquaponics. However, the fresh weight was lower compared to the findings of Maucieri et al. (2019). Badrey et al. (2024) found that lettuce (*Lactuca sativa* L.) grown in a polyculture aquaponic system using polyculture effluent (ASTAF-Pro) achieved a significantly higher average weight (450±70 g) compared to those grown in monoculture (360±45 g). Matysiak et al. (2023) reported a romaine lettuce yield of 86 g per plant within 21 days in a vertical aquaponic farm. This translates to a yield of 3.4 kg m^{-2} at a planting density of 40 plants m^{-2}. In a study of lettuce (*Lactuca sativa*) production in northern latitudes using aquaponics, Abbey and Anderson (2019) observed significant differences in fresh weight based on fish species. Lettuce grown in a deep water culture (DWC) system with perch had the lowest mean fresh weight (65.6 g), while those grown with tilapia achieved the highest (172.3 g).

Leafy vegetables like lettuce are the most preferred plant species in aquaponics due to their easy integration. However, in this study, the lettuce showed a relatively lower growth performance compared to other studies. This might be attributed to the specific ion concentration in the effluent (circulating water) of the aquaponic system, which may not have been optimal for lettuce growth.

Ion concentrations of the microalgae culture mediums

As expected, the BG-11 medium had the highest NO_3-N concentration at 247.06 mgL^{-1}. In contrast, RSW and RSW+Mn media had higher concentrations of NH_4-N, NO_2-N, SO_4, K, Mg, Ca, and Cl compared to standard BG-11 (Table 1). Statistically significant differences ($p<0.05$) were found in nutrient concentrations between RSW and RSW+Mn media compared to BG-11, suggesting they might be more suitable for *Chlorella* sp. growth. Based on the combined daily aquaponic sludge-water, ammonia levels were relatively low (1.06±1.18 mgL^{-1}) compared to nitrite (3.97±6.63 mgL^{-1}) and nitrate (4.32±7.9 mgL-*1*) levels. Phosphate levels were also moderate (1.52±1.65 mgL^{-1}). Green microalgae like *Chlorella* sp. require adequate nitrogen (particularly NO_3-N) and phosphorus (PO_4-P) for maximum biomass production (Chakraborty et al., 2016). These values are all considered relatively low compared to other studies (e.g., Gao et al., 2016; Tanikawa et al., 2018). In another study, for an axenic cultivation, nitrate and phosphate concentrations of aquaculture wastewater were found as 17.6 mgL^{-1} and 16.9 mgL^{-1}, respectively. Indigenous microalgae consortia consisting of *Chlorella* sp. (95.2%), *Chlamydomonas* sp. (3.1%), *Stichococcus* sp. (1.1%), *Chlorella* sp., and *Scenedesmus quadricauda* were used to produce microalgal biomass with aquaculture wastewater, successfully (Halfhide et al., 2014).

Physicochemical parameters of *Chlorella minutissima*culture medium

The trends in physicochemical parameters (e.g., temperature, pH, salinity, EC) for *H. pluvialis* cultured in BG-11, RSW, and RSW+Mn media are shown in Figure 3. Average values of these parameters for *C. minutissima* culture are presented in Table 2. The maximum temperature (26.8±0.10 °C) was observed in the BG-11 medium on day 7, while the minimum temperature (22.53±0.05 °C) was measured in the RSW+Mn medium on day 21. Average temperatures were calculated as 24.59±1.23 °C in BG-11, 24.32±1.26 °C in RSW, and 24.42±1.26 °C in RSW+Mn media. While temperature showed no significant differences among groups ($F(2,81) = 0.3420$, $P>0.05$), pH ($F(2,81) = 9.711$), salinity ($F(2,81) = 109.3$), and EC ($F(2,81) = 140.5$) exhibited statistically significant differences ($P < 0.05$). The highest EC was measured in the BG-11 medium on the 27th day (1599.33±57.41 μScm^{-1}), while the lowest conductivity was found in the same medium on day 1 (700.33±1.70 μScm^{-1}). The average EC was calculated as 1499±227.69 μScm^{-1} in BG-11, 992.35±31.86 μScm^{-1} in RSW, and 976.45±15.99 μScm^{-1} in RSW+Mn mediums. BG-11 ex-

Table 1. The ion concentrations of BG-11, RSW, and RSW+Mn mediums

Descriptions	BG-11	RSW	RSW+Mn
NO_2-N (mg/L)	-	3.97±6.83	3.97±6.83
NO_3-N (mg/L)	247.06	4.32±7.9	4.32±7.9
PO_4-P (mg/L)	9.25	1.52±1.65	1.52±1.65
SO_4 (mg/L)	29.33	109.42±43.41	109.42±43.41
NH_4-N (mg/L)	0.32	1.06±1.18	1.06±1.18
K (mg/L)	5.67	8.68±5.15	8.68±5.15
Mg (mg/L)	7.40	33.36±7.88	33.36±7.88
Ca (mg/L)	9.81	155.5±29.36	155.5±29.36
Na (mg/L)	410.29	55.65±58.14	55.65±58.14
Cl (mg/L)	9.00	89.87±132.74	89.87±132.74
Co (mg/L)	0.0091	-	0.0091
Mo (mg/L)	0.1546	-	0.1546
Mn (mg/L)	0.5025	-	0.5025
Zn (mg/L)	0.0500	-	0.0500
Cu (mg/L)	0.0204	-	0.0204

BG-11: Blue-green microalgae culture medium, RSW: Remineralized sludge-water, RSW+Tr: Remineralized sludge-water + BG-11 microalgae culture medium trace element solution

hibited a statistically significant difference (P <0.05) in conductivity compared to the other culture media. No significant differences were found in salinity among all mediums (P >0.05). Figure 3 illustrates the observed trends in pH, with a maximum value of 8.78±0.02 on day 16 and a minimum of 7.42±0.01 at the beginning of the experiment. Despite the difference in nutrient salt concentration between BG-11 and RSW, the RSW medium yielded better results in terms of biomass production compared to BG-11. The average physicochemical parameters of *Chlorella minutissima* culture are presented in Table 2 below. Temperature of the culture mediums of *C. minutissima* showed no significant changes. However, RSW and RSW+Mn mediums differed from BG-11 in terms of pH, salinity and conductivity, potentially indicating a difference in nutrient composition.

Temperature of the culture mediums of *C. minutissima* showed no significant changes. However, RSW and RSW+Mn mediums differed from BG-11 in terms of pH, salinity and conductivity, potentially indicating a difference in nutrient composition.

For comparison, a study by Ribeiro et al. (2020) found an optimal temperature of 28°C for *Chlorella sorokiniana* production using a combination nitrogen medium (urea, ammonia, and nitrate). Microalgae growth also depends on pH response and reaction (Berge et al., 2012). Chiu (2015) reported that agricultural and livestock breeding wastewater offered a good potential for *Chlorella* sp. cultivation due to higher nutrient concentrations. These

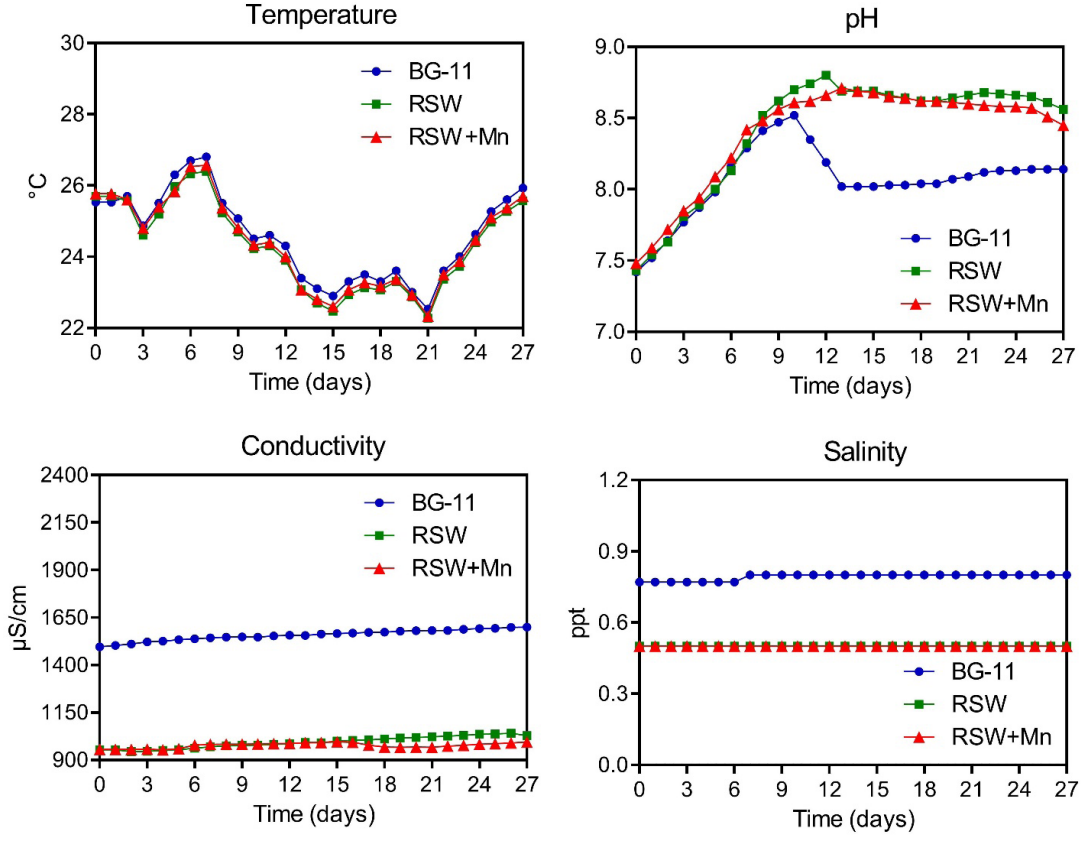

Figure 3. Time-dependent change of physicochemical parameters of BG-11, RSW, and RSW+Mn for *Chlorella minutissima*

Table 2. *C. minutissima* culture mediums average physicochemical parameters

Parameter/ Culture medium	BG-11	RSW	RSW+Mn
Temperature (°C)	24.59±1.23^{A}	24.32±1.26^{A}	24.42±1.26^{A}
pH	8.06±0.25^{A}	8.42±0.41^{B}	8.41±0.36^{B}
Salinity (ppt)	0.76±0.13^{A}	0.50±0.00^{B}	0.50±0.00^{B}
Conductivity (µScm^{-1})	1499.01±227.69^{A}	992.35±31.86^{B}	976.45±15.99^{B}

Letters A-B indicates significant differences between samples (P <0.05)

Table 3. Growth parameters of *C. minutissima*

Abbreviations	BG-11	RSW	RSW+Mn
µ	0.097±0.011^{A}	0.070±0.010^{A}	0.068±0.016^{A}
Q_x	0.0046±0.0006^{A}	0.0034±0.0001^{A}	0.0041±0.0005^{A}
t_d	7.05±0.98^{A}	10.18±1.67^{A}	10.96±3.04^{A}
P_b	0.000054±0.000024^{A}	0.000064±0.000021^{A}	0.000117±0.000047^{A}

µ= specific growth rate, Q_x= productivity, t_d= doubling time, P_b= biomass productivity

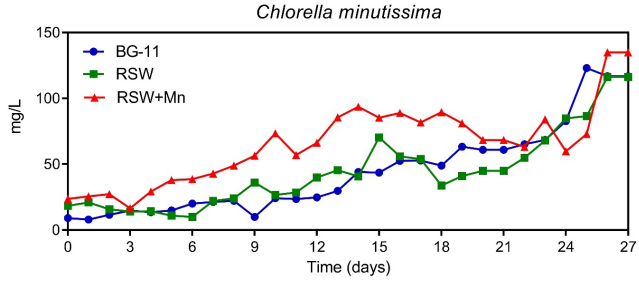

Figure 4. Time-dependent change of *C. minutissima* dry biomass

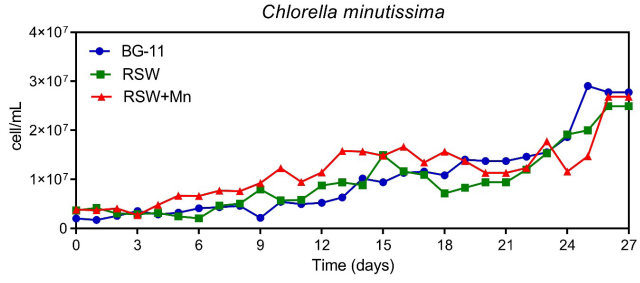

Figure 5. Time-dependent change of C. minutissima cell number

wastewaters contained total nitrogen ranging from 185-3213 mgL^{-1} and total phosphorus around 30-987 mgL^{-1}. In contrast, domestic secondary effluent had a relatively low concentration of both total nitrogen (15-90 mgL^{-1}) and total phosphorus (5-20 mgL^{-1}). *Chlorella* sp. demonstrates adaptability to various wastewater sources. For instance, Wang et al. (2010) cultivated *Chlorella* sp. in municipal wastewater, reporting effluent from an aeration tank to contain nitrite (0.074±0.003 mgL^{-1}) and nitrate (16.95±0.07 mgL^{-1}). In contrast, Yu et al. (2019) used anaerobic digestion effluent containing high ammonium (40 mgL^{-1}) for *Chlorella vulgaris* and *Chlorella protothecoides*. A pH of 5.7 to 6.5 was sufficient for optimal growth of *Chlorella pyrenoidosa* grown in anaerobically digested activated sludge. However, when pH levels increased above 9.1 to 9.6 it was unable to grow in the wastewater (Tan et al., 2016). *Chlorella vulgaris* was used for removal of toxic chemicals from tannery wastewater, as well (Das et al., 2017). Temperature, pH, electrical conductivity, ammonium-nitrogen, and phosphate of the diluted tannery wastewater were found to be 15-20 °C, 7.78±0.20, 2.19±0.16 mScm^{-1}, 8.12±0.60 mgL^{-1} and 10.68±1.63 mgL^{-1}, respectively (Subashini and Rajiv, 2018).

Growth trend of *Chlorella minutissima*

As shown in Figures 4 and 5, *C. minutissima* exhibited higher growth rates or cell densities in RSW and RSW+Mn media compared to BG-11. A distinct separation in algal dry biomass concentrations between cultures was observed from day 3 to day 22, when comparing all media used for *C. minutissima* cultivation. Figure 5 illustrates a time-dependent increase in cell number of *C. minutissima*, with all culture media exhibiting either exponential or linear growth patterns. The maximum cell concentration of *C. minutissima* was observed as (2.70±1.17)×10^{7} cellsmL^{-1} in the RSW+Mn culture medium on the 27th day of the study. No statistically significant differences (P >0.05) were found in *C. minutissima* cell concentrations among all culture media. The average biomass of all culture mediums was measured 51.62±38.40 mgL^{-1} in BG-11, 42.06±27.53 mgL-*1* in RSW, and 65.61±29.49 mgL^{-1} in RSW+Mn.

Dry weight of biomass is one of the most important parameters for assessing biomass yield in microalgae culture (Chioccioli et al., 2014). Interestingly, Mutanda et al. (2011) reported no statistically significant difference in growth parameters between *Chlorella* spp. cultured in BG-11 and post-chlorinated wastewater, despite observing a higher biomass yield (116.3 mgL^{-1}) in the post-chlorinated medium compared to BG-11 (69.9 mgL^{-1}). *Chlorella* sp. was used as a phytoremediation species and it can produce biomass in highly concentrated municipal wastewater. *Chlorella* sp. exhibited a high biomass concentration of 86 mgL^{-1} (Li et al., 2011). In another study, Cabanales et al. (2013) investigated the use of five distinct stages of domestic wastewater depuration for eliminating nutrient salts while producing *Chlorella vulgaris* biomass. They reported biomass yields ranging from 39 to 195 mgL^{-1} dry weight per day (mgL^{-1} dW day^{-1}), similar to the values observed in our study. A study reported biomass yields of 0.1 gL^{-1} dW day^{-1} for *C. vulgaris*, 0.4 gL^{-1} dW day^{-1} for *Scenedesmus obliquus*, and 0.9 gL^{-1} dW day^{-1} for a consortium of Chlorella, Chaetophora, Scenedesmus, and Navicula when cultivated using urban wastewater in a photobioreactor (Gouveia et al., 2016). *C. minutissima* has gained attention for its ability to serve

two purposes: remediating wastewater by removing nutrient salts and producing microalgae biomass, even when cultivated in saline aquaculture water. A study reported that the cell density of microalgae increased almost fivefold during wastewater treatment, reaching a peak five times higher than the initial concentration after 10 days (Hawrot-Paw et al., 2020). In another study, *Scenedesmus* sp., *C. variabilis*, and *C. sorokiniana* were applied to tannery wastewater to produce biomass, which could then be used for biofuel production. *Scenedesmus* sp., *C. variabilis*, and *C. sorokiniana* cultivated in different tannery wastewater concentrations exhibited substantial growth, as evidenced by increased cell density, chlorophyll content, and sugar content, compared to the control group. *C. sorokiniana* displayed impressive growth in a short period, achieving a threefold increase in biomass compared to the BG-11 control group within just 16 days (Nagi et al., 2020). There were no statistically significant differences among the groups when it comes to microalgal growth parameters as seen in Table 3 [F(2,9) = 0.1272], (*P*=0.8821). Researchers found that the specific growth rate ranged from 0.289 to 0.408 day^{-1} after adhering to photoheterotrophic fermentation and adding glycerin to the culture medium (Yang et al., 2011). BG-11 medium supported faster growth, higher productivity, and potentially greater overall biomass production compared to RSW and RSW+Mn mediums. Doubling time is inversely related to growth rate, the higher μ value in BG-11 suggests a potentially shorter doubling time compared to RSW and RSW+Mn mediums.

Physicochemical parameters of *Botryococcus braunii* culture medium

The average physicochemical parameters of the *B. braunii* cultures in BG-11, RSW, and RSW+Mn media are presented in Table 2, while Figure 6 illustrates the trends observed in these parameters throughout the experiment. Temperature remained consistent across all culture media (BG-11, RSW, and RSW+Mn) throughout the experiment, with no statistically significant differences observed [F=(2, 81)= 0,2701] (*P* >0.05). For conductivity and salinity, BG-11 was found statistically important compared to other culture mediums [F(2, 81)= 96.79], (*P* <0.05). When it comes to pH, among all groups were found statistically significant differences (*P* <0.05). The maximum pH level was determined as 8.87±0.07 at RSW medium on the last day of experiment while minimum pH level was measured as 7.66±0.04 on the 5th day of experiment. A statistically significant difference was found between BG-11 and other mediums in terms of pH [F(2.81)= 2795] (*P* <0.05). Figure 6 shows physicochemical parameters of *B. braunii* in different culture mediums over time. The average physicochemical parameters of *B. braunii* culture can be seen in Table 4 below.

Repeated measures ANOVA revealed a statistically significant effect of treatment on temperature [F(1.049, 28.33)= 23.25], (*P* <0.0001), pH [F(2.142, 57.83)= 59.31], (*P* <0.0001), conductivity [F(1.148, 31.00)= 831.4], (*P* <0.0001), and salinity [1.086, 29.32)= 32.47], (*P* <0.0001) over time.

All three mediums had similar average temperatures with some small variations. BG-11 medium had a slightly lower pH compared to RSW and RSW+Mn. While *B. braunii* tolerates a wide pH range, it generally prefers slightly acidic conditions (pH 6-6.5) to produce hydrocarbons (Nugroho et al., 2020). BG-11 had slightly higher salinity (0.66 ppt) compared to RSW (0.59 ppt) and RSW+Mn (0.58 ppt), but the differences are minor. BG-11 had the highest conductivity (around 1325 μScm^{-1}), followed by RSW (around 1180 μScm^{-1}) and RSW+Mn (around 1153 μScm^{-1}). This difference in nutrient composition might influence *B. braunii* growth and other parameters. A culture medium temperature of 23 °C was determined to be the optimum temperature for growing *B. braunii* (Qin and Li, 2006). Yoshimura et al. (2013) found the growing temperature of *B. braunii* strain SHOWA between 5 to 35 °C and optimum growth temperature was determined as 30 °C. Tarhan et al. (2021) tried to grow *C. minutissima* and *B. braunii* using different dilutions (50x, 100x 200x, and 400x) of orange and olive pomace aqueous phases. At low dilution rates they found shorter generation times and higher growth rates for microalgae. In another study, *B. braunii* strain CHN 357 was cultured at different temperatures among 20 to 30 °C and the optimum temperature was found as 23 °C (Qin and Li, 2006). The highest EC was measured as 1385.33±46.58 μScm^{-1} at the end of the study in BG-11 medium. Similarly, Órpez et al. (2009) found the EC as 978 μScm^{-1} in secondarily treated sewage wastewater in the study of production performance of *B. braunii*. The growth performance of *B. braunii* strain BOT-22 was also evaluated in soybean curd wastewater (SCW). SCW is diluted as 1%, 2%, 5%, and 10% and compared to control AF-6 medium's microalgae biomass performance. SCW medium ion concentration was reported as 3 mgL^{-1} ammonium, 100 mgL^{-1} phosphate, 92 mgL^{-1} sulfate, 35 mgL^{-1} magnesium, 1280 mgL^{-1} potassium, 366 mgL^{-1} calcium, and 41 mgL^{-1} sodium. Compared to AF6 microalgae culture medium like the one used in that study, SCW yielded better biomass results with its nutrient variables (Yonezawa et al., 2012). In another study, secondarily treated sewage (STS) was used as *B. braunii* culture medium in the batch culture system. STS derivatives were found to have better concentrations of nitrite, ammonium, conductivity, and total phosphorus compared to CHU 13 microalgae culture medium (Sawayama et al., 1992). Aerated swine lagoon wastewater without sterilization and pH adjustment was also tested as an alternative *B. braunii* culture medium for open microalgae production systems. When aerated swine lagoon wastewater (ASLW) is compared with swine lagoon wastewater (SLW), it was observed that there are significant differences in terms of pH, conductivity, dissolved oxygen, nitrate-nitrogen, ammonium-nitrogen, total nitrogen, and total phosphorus. Similarly to the present study, *B. braunii* cultivation was made at 25 °C temperature and light intensity of 120 μmol photons $m^{-2}s^{-1}$ (Liu et al.,

Table 4. *B. braunii* culture mediums average physicochemical parameters

Parameter/ Culture medium	BG-11	RSW	RSW+Mn
Temperature (°C)	26.75±1.68^{A}	26.52±1.56^{A}	26.81±1.55^{A}
pH	8.03±0.34^{A}	8.64±0.19^{B}	8.51±0.15^{B}
Salinity (ppt)	0.66±0.05^{A}	0.59±0.05^{B}	0.58±0.04^{B}
Conductivity (μScm^{-1})	1325.16±44.00^{A}	1180.00±60.91^{B}	1153.75±43.43^{B}

Letters A-B indicates significant differences between samples (*P* <0.05)

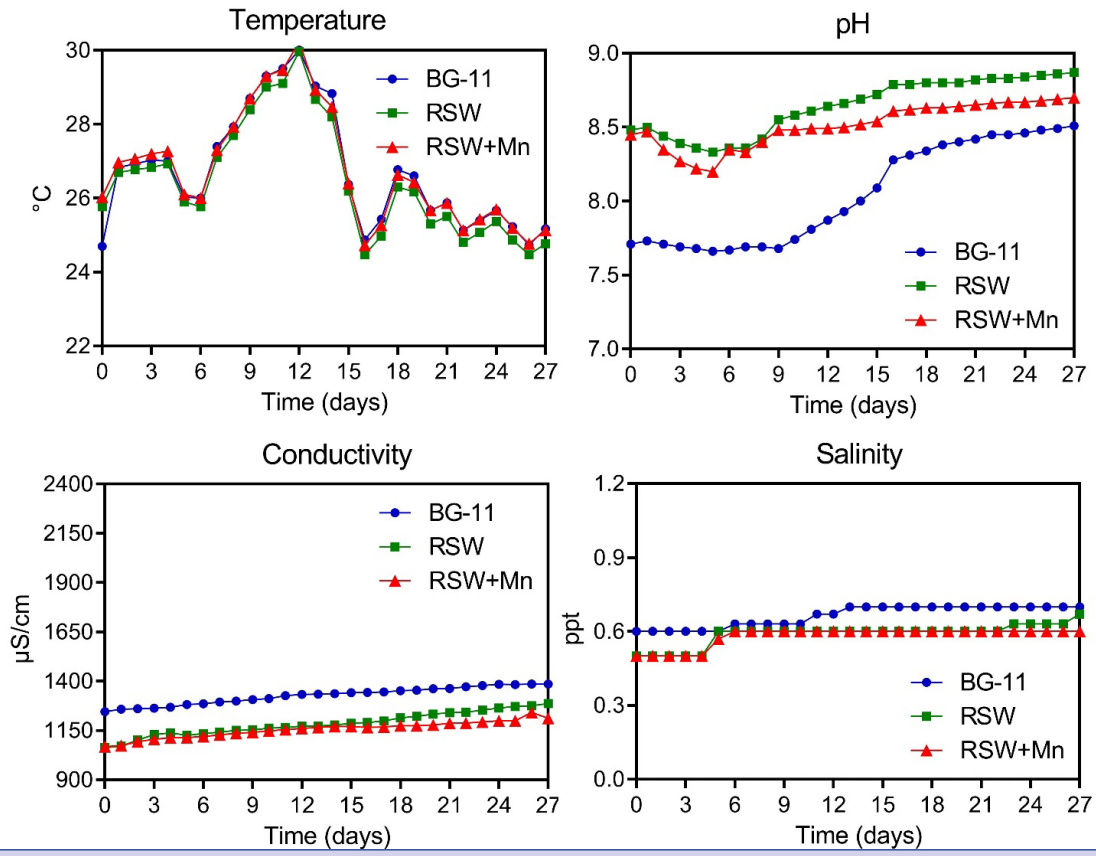

Figure 6. Time-dependent change of physicochemical parameters of BG-11, RSW, and RSW+Mn for *Botryococcus braunii*

2013). A pretreated or untreated wastewater resource can be an effective microalgae medium, since it can be used for microalgae cultivation at a low cost.

Growth trend of *Botryococcus braunii*

Dry biomass and cell concentration changes of *B. braunii* grown in different culture mediums are given in Figure 7 and Figure 8, respectively. Mean dry biomass of *B. braunii* obtained from BG-11, RSW, and RSW+Mn were calculated as 379.40±200.12 mgL^{-1}, 385.19±305.01 mgL^{-1}, 130.20±138.63 mgL^{-1}, respectively. As can be seen in Figure 7, *B. braunii* grew better in the RSW medium compared to others. The 21st day of the experiment revealed the highest cell concentration of *B. braunii* in RSW+Mn medium at 3.6×105±5.3×104 $cellsmL^{-1}$. 21 days of *B. braunii* production in the RSW culture medium can be considered sufficient.

After the 18th day of the experiment, the biomass yield of the *B. braunii* cultured in RSW medium, skyrocketed. There were no statistically significant differences among the groups when it comes to microalgal growth parameters as seen in Table 5 [$F(2,9) = 0.5292$], ($P = 0.6063$).

RSW medium had the highest specific growth rate compared to BG-11 and RSW+Mn. This suggests *B. braunii* grew faster in RSW. Similar to specific growth rate, RSW medium had the highest productivity compared to BG-11 and RSW+Mn mediums. RSW medium (8.04±0.47) has the shortest doubling time, followed by BG-11 (11.78±2.59) and RSW+Mn (13.31±0.80). This aligns with the trend observed in specific growth rate. Biomass productivity of the BG-11 medium was found as the highest when compared to RSW and RSW+Mn.

In a study investigating the biomass production performance of secondarily treated piggery wastewater, the removal of nitrogen-phosphorus and produce *B. braunii* biomass was found advantageous and sustainable. In that study, biomass production was found as between 1 gL^{-1} and 7.5 gL^{-1} in different nitrogen concentrations (102 $mgNL^{-1}$, 204 $mgNL^{-1}$, 510 $mgNL^{-1}$, +1020 $mgNL^{-1}$) in batch culture (An et al. 2003). Nitrogen concentrations of secondarily treated wastewaters were found much higher compared to this study and low values of biomass may be caused by the lack of nitrogenous compounds in the culture mediums. Órpez et al. (2009) grew *B. braunii* in secondarily treated sewage wastewater at

Table 5. Growth parameters of *B. braunii*

Abbreviations	BG-11	RSW	RSW+Mn
µ	0.061±0.012^{A}	0.086±0.0052^{A}	0.052±0.0031^{A}
Q_x	0.033±0.023^{A}	0.044±0.0062^{A}	0.013±0.0015^{A}
t_d	11.78±2.59^{A}	8.04±0.47^{A}	13.31±0.80^{A}
P_b	0.0086±0.009^{A}	0.0062±0.0008^{A}	0.0021±0.00019^{A}

µ= specific growth rate, Q_x= productivity, t_d= doubling time, P_b= biomass productivity

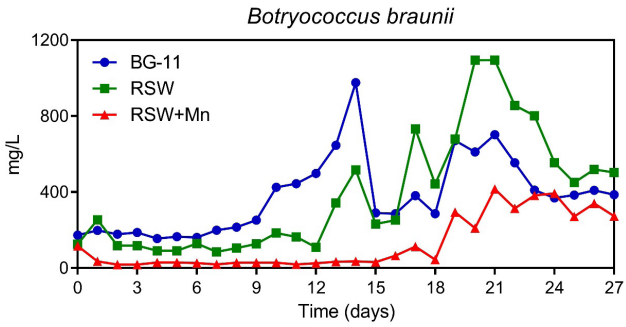

Figure 7. Time-dependent change of *B. braunii* dry biomass

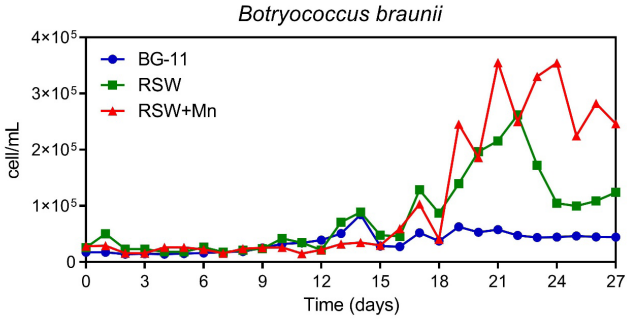

Figure 8. Time-dependent change of *B. braunii* cell number

pH 8 and maximum specific growth rate was found as 0.21 gL^{-1} day^{-1}. Secondarily treated sewage wastewater showed similar findings to this study with its nutrient concentrations like ammonium (15 mgL^{-1}), nitrates (0.9 mgL^{-1}), nitrites (0.14 mgL^{-1}), phosphates (11.5 mgL^{-1}). In a research that aims carotenoid production from *B. braunii* at different light intensities (100 and 500 µmol photons $m^{-2}s^{-1}$), and in various culture mediums (modified CHU 13 medium, modified CHU 13 medium without nitrogen, and modified CHU 13 without N+2Fe), the highest biomass yield was determined as 0.6 gL^{-1} on day 16 at the highest light intensity (Indrayani et al., 2022). When compared to this study, similar results were found with the biomass of *B. braunii* cultured in BG-11 medium. Qin and Li (2006) reported specific growth rates between 0.061±0.003 - 0.095±0.003 which was very similar to this study.

Physicochemical parameters of *H. pluvialis* culture medium

The trend differences among physicochemical parameters of BG-11, RSW, and RSW+Mn culture mediums for culturing *H. pluvialis* were shown in Figure 9. The average physicochemical parameters of the study were shown in Table 3. While the maximum temperature was determined as 26.83±0.05 °C at RSW+Mn culture medium on the 3rd day, the minimum temperature was observed as 22.13±0.05 °C at BG-11 medium on the 17th day of the experiment. In this study, temperature was held at appropriate levels. The maximum pH level was determined as 8.57±0.07 at RSW medium on the 8th day, and the minimum pH level was measured as 7.41±0.03 at RSW+Mn in the beginning of the experiment. In RSW+Mn medium, the highest conductivity was measured as 2316.33±30.07 µScm^{-1} at the end of the study, while the lowest conductivity was measured as 1070.33±3.40 µScm^{-1} on the first day of the experiment. The average physicochemical parameters of *H. pluvialis* culture can be seen in Table 6 below.

Table 6. *Haematococcus pluvialis* culture mediums physicochemical parameters

Parameter/ Culture medium	BG-11	RSW	RSW+Mn
Temperature (°C)	24.26±1.27^{A}	24.35±1.25^{A}	24.54±1.26^{A}
pH	7.83±0.15^{A}	8.27±0.35^{B}	8.22±0.32^{B}
Salinity (ppt)	0.79±0.05^{A}	0.58±0.04^{B}	1.24±0.70^{C}
Conductivity (µS/cm)	1597.91±70.93^{A}	1146.29±35.63^{B}	2207.35±54.02^{C}

Letters A-C indicates significant differences between samples (P <0.05)

BG-11, RSW, and RSW+Mn had very similar average temperatures with some variations. Temperature wasn't a differentiating factor for *H. pluvialis* growth in the experiment. BG-11 medium had a slightly lower pH compared to RSW and RSW+Mn. *H. pluvialis* can tolerate a wide range of pH (6-8.5) (Do et al., 2021). The statistically significant differences suggest that the pH levels in the BG-11 medium are different from both RSW and RSW+Mn. RSW+Mn had a significantly higher salinity (1.24 ppt) compared to both BG-11 and RSW mediums. The statistically significant differences suggest variations in the amount and type of dissolved nutrients between the mediums. BG-11 and RSW+Mn likely have higher concentrations of dissolved salts compared to RSW medium.

Optimum growth temperature of *H. pluvialis* was found between 25-28 °C in a study that aims to find optimal temperature and irradiance for *H. pluvialis* (Fan et al., 1994). 35 °C had detrimental effects on *H. pluvialis* cells according to Borowitzka et al. (1991). The beginning pH level of the culture medium for *H. pluvialis* was determined as 7.5, according to Choi et al. (2017).

Growth trend of *H. pluvialis*

The maximum *H. pluvialis* cell concentration was elicited as 8.9×10^{4}±3.2×10^{4} in RSW+Mn medium on the 20th day of the study. The dry weight of biomass obtained from BG-11, RSW, and RSW+Mn mediums were calculated as 70.62±20.29 mgL^{-1}, 169.42±84.21 mgL^{-1}, 631.52±336.90 mgL^{-1}, respectively (Figure 10 and Figure 11). As can be seen in Figure 10, the highest dry biomass increase was found at the RSW+Mn medium at the 20th day of the experiment for *H. pluvialis*. Some green cells transitioned to aplanospore stage but more than 90% of the cells never transitioned into the red (astaxanthin production) stage. There were no statistically significant differences among the groups when it comes to microalgal growth parameters as seen in Table 7 [$F(2,9) = 0.6714$], (P =0.5348).

There was no statistically significant difference between RSW (0.079±0.0081) and RSW+Mn (0.080±0.015) medium, which have the higher specific growth rates. This suggests *H. pluvialis* grew slower in BG-11 medium. Similar to growth rate, RSW+Mn medium (0.045±0.017) has the highest productivity, followed by RSW (0.010±0.0057) and BG-11 (0.0030±0.0001). This indicates that RSW+Mn supported the highest rate of biomass production. A lower doubling time signifies faster

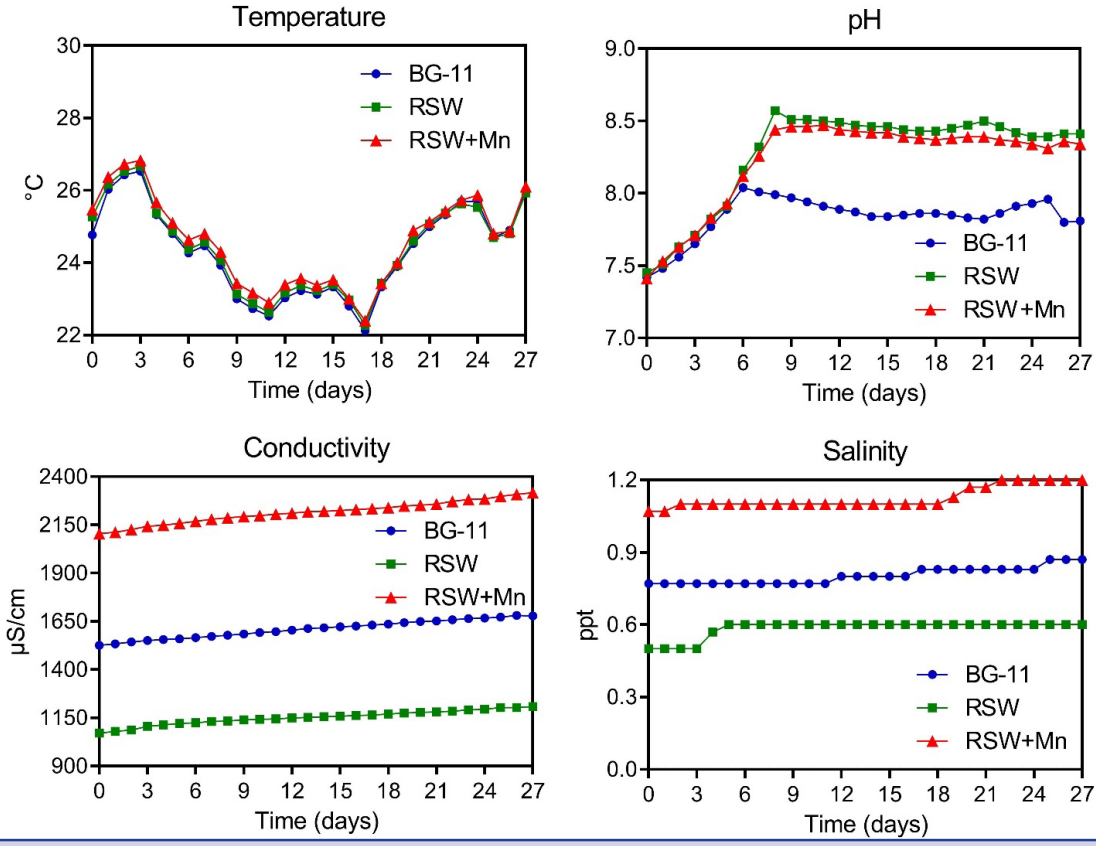

Figure 9. Time-dependent change of physicochemical parameters of BG-11, RSW, and RSW+Mn for *H. pluvialis*

Table 7. Growth parameters of *H. pluvialis*

Abbreviations	BG-11	RSW	RSW+Mn
μ	0.044±0.012^{A}	0.079±0.0081^{A}	0.080±0.015^{A}
Q_x	0.0030±0.0001^{A}	0.010±0.0057^{A}	0.045±0.017^{A}
t_d	17.40±5.69^{A}	8.83±0.93^{A}	8.92±1.44^{A}
P_b	0.00020±0.00013^{A}	0.00074±0.00083^{A}	0.012±0.011^{A}

μ= specific growth rate, Q_x= productivity, t_d= doubling time, P_b= biomass productivity

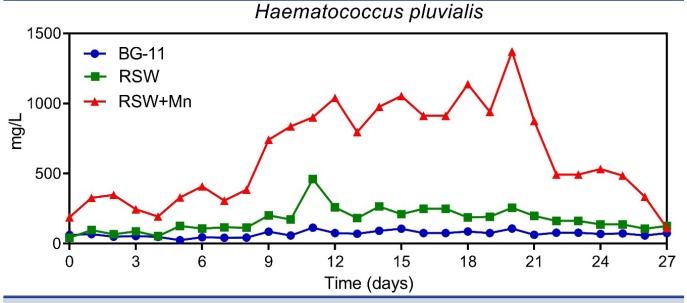

Figure 10. Time-dependent change of *H. pluvialis* dry biomass.

population growth. RSW medium (8.83±0.93) has the shortest doubling time, followed by RSW+Mn (8.92±1.44) and BG-11 (17.40±5.69). This aligns with the trend observed in specific growth rate. RSW+Mn medium showed the highest biomass productivity (0.012±0.011).

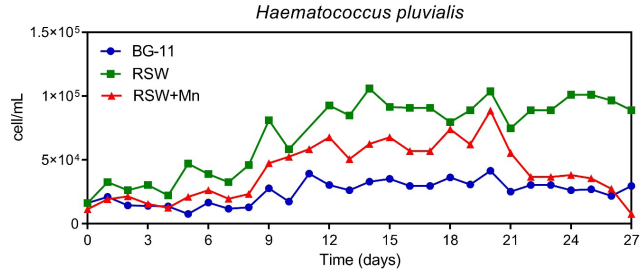

Figure 11. Time-dependent change of *H. pluvialis* cell number.

In general two-step production policy followed for commercial cultivation. In the first stage of production, the vegetative process is maximized. At the second stage, stress conditions are tried to be provided for astaxanthin production (Shah et al., 2019). Only vegetative processes were studied in this study. The results of a study which was related to 32 days of cultivation of *H. pluvialis* using standard laboratory prepared wastewater under different light spectrums has a slightly better performance in terms of cell concentration (51×10^4 cellsmL^{-1}) than this study (Mourya et al., 2023). The maximum biomass concentration was found as 27.8 mgL^{-1} day^{-1} cultured in a domestic secondary treated effluent for *H. pluvialis* (Wu et al. 2013). In another study, cas-

sava wastewater obtained from three different cassava producers diluted to different concentrations (2.5%, 5%, and 10%) to produce microalgae. The highest biomass was obtained from 2.5% diluted CW medium as 9.31 cells mL^{-1} (Rodrigues et al., 2021).

Statistical analyzes revealed significant differences in cell densities among the tested microalgae species (*C. minutissima*, *B. braunii*, and *H. pluvialis*) across varying culture media (BG-11, RSW, and RSW+Mn) $F(27, 504)= 17.09$, ($P <0.0001$). As expected, *C. minutissima* exhibited higher cell densities compared to both *B. braunii* and *H. pluvialis*. Furthermore, the RSW+Mn medium negatively impacted *C. minutissima* growth, resulting in significantly reduced cell densities compared to BG-11 and RSW media $F(8, 504)= 299.3$, ($P <0.0001$). This effect was specific to *C. minutissima*, as the other species did not exhibit the same sensitivity to the RSW+Mn medium.

Two-way ANOVA and Tukey's HSD post-hoc test revealed significant effects of all microalgae species (*C. minutissima, B. braunii,* and *H. pluvialis*) and culture mediums (BG-11, RSW, and RSW+Mn) on biomass productivity $F(27, 504)= 5.182$, ($P <0.0001$). *C. minutissima* exhibited significantly greater biomass productivity in BG-11 compared to *B. braunii* ($P <0.0001$). *H. pluvialis*, however, achieved maximal biomass productivity in RSW+Mn, significantly outperforming all other species-medium combinations $F(8, 504)= 68.84$, ($P <0.0001$). The significant interaction effect between species and medium ($P <0.0001$) highlights the differential responses of these microalgae to varying culture conditions.

Nutritional value of microalgae in aquaponics remineralized sludge-water

Nutritional value of *C. minutissima, B. braunii* and *H. pluvialis* were determined and discussed below following headings. In Table 8, 9, and 10 are shown comparison of the biochemical contents of *C. minutissima*, *B. braunii*, and *H. pluvialis*, respectively. Microalgae's biochemical composition can be affected by multiple factors, such as nutrient concentration, composition, light intensity, and temperature.

Crude protein

C. minutissima crude protein concentrations were determined for BG-11, RSW, and RSW+Mn groups as 51.88±0.32%, 55.77±1.81%, and 21.53±0.70%, respectively. *C. minutissima* cultured in RSW+Mn showed a statistically significant difference from other cultures in terms of crude protein levels. RSW medium was found to have the best protein concentration compared to other groups for *C. minutissima*. *B. braunii* crude protein contents were determined for BG-11, RSW, and RSW+Mn groups as 49.77±0.62%, 38.50±0.78%, and 37.39±0.61%, respectively. Comparatively, *B. braunii* cultured in BG-11 medium exhibited statistically significant difference ($P <0.05$). *H. pluvialis* crude protein contents in BG-11, RSW, and RSW+Mn were calculated as 40.61±4.06%, 33.86±2.75%, and 30.25±0.84%, respectively. BG-11 culture medium had statistically significant difference compared to other RSW culture mediums for *H. pluvialis* ($P <0.05$).

The lowest protein contents in this study were obtained from *H. pluvialis*. Nitrogen to protein conversion factor (ki) have determined as 4.78 by Lourenço et al. (2004) and the factor mostly used in microalgae studies. In this study, results were also calculated using a 4.78 nitrogen to protein conversion factor. The presence of trace elements in the culture medium can result in a high ash content in the biomass and a reduction in protein concentration (Schüler et al., 2020). And low nitrogen concentration in the culture medium causes low crude protein in the biomass (Ördög et al., 2012). Freeze-dried Chlorella 71105 strain grown in standard culture medium was found to contain 55.5% crude protein (Lubitz, 1963). This percentage of protein concentration is similar to this study's conclusion. Protein concentrations of different culture groups of *B. braunii* were shown to be similar and relatively high results when compared to other studies (Cabanelas et al., 2015; Ashokkumar and Rengasamy, 2012). Sydney et al., (2011) investigated *C. vulgaris* and *B. braunii* microalgae species biodiesel production potential using domestic wastewater and they found the maximum protein content as 40.4% of biomass. It is possible that macronutrients and micronutrients obtained from RSW can influence the nutritional value of microalgae. All microalgae species cultured in an RSW medium can be evaluated as having the potential to be a raw material in different sectors with their high protein concentrations.

Total lipids

C. minutissima cultured in BG-11, RSW, and RSW+Mn the total lipid contents were determined as 4.18±0.33%, 4.69±0.88%, and 1.35±0.48%, respectively. Comparing RSW+Mn with other groups, a statistically significant difference was found ($P <0.05$). *B. braunii* is known for the production of hydrocarbons and high levels of lipids. However, cultured *B. braunii* in BG-11, RSW, and RSW+Mn mediums the total lipid contents were found as 0.62±0.11%, 0.48±0.11%, and 0.53±0.31%, respectively. Finally, *H. pluvialis* BG-11, RSW, and RSW+Mn culture groups' total lipid contents were found as 0.57±0.30%, 0.70±0.07%, and 0.11±0.02%, respectively. No statistically significant difference was found among all culture mediums regarding total lipids for *H. pluvialis* and *B. braunii* ($P >0.05$).

Lipid contents of the all microalgae species were found relatively low when compared to other studies (Sonkar et al., 2023; Liang et al., 2015; Jackson et al., 2020). Microalgae cultures under controlled conditions that promote biomass multiplication resulted in a low total lipid concentration (Mularczyk et al., 2020). Different Chlorella strains which have 40% crude protein, 20-25% carbohydrates, and 20-26% lipids were reported grown at seawater based F2 medium in outdoor cultivation (Guccione et al. 2014). *C. vulgaris* which is grown at modified Fitzgerald medium (Hughes et al. 1958) showed 40-55% lipid composition with nitrogen depletion in medium (Widjaja, 2009). In another study, *C. vulgaris* cultured in artificial wastewater medium had 42% average lipid content and the lipid productivity was 147 $mgL^{-1}day^{-1}$ (Feng et al., 2011). Giraldo et al. (2021) reported that high bicarbonate dosages increased biomass and lipid productivity in *B. braunii*. In another study, total lipid concentration of *B. braunii* was found higher than 40% (Cheng et al., 2013). Damiani et al. (2010) investigated the impact of continuous high light intensity with nitrogen-sufficient medium and high light intensity with nitrogen-deprivation medium on the total lipid content (dry weight) and they found 34.85% and 32.99%, respectively. Similar to other studies, the high light intensity and nitrogen deprivation have the ability to change the lipid production (46.71%-

56.92%) of *H. pluvialis*, dramatically (Liang et al., 2015). All microalgae species used in this study had low total lipid concentrations compared to above studies.

Total carbohydrates

Total carbohydrate concentrations of *C. minutissima* were determined in BG-11, RSW, and RSW+Mn as 18.66±3.68%, 16.90±0.99%, and 20.20±5.47%, respectively. *B. braunii* total carbohydrate contents were calculated in BG-11, RSW, and RSW+Mn as 23.67±1.89%, 39.14±2.43%, and 31.32±2.35%, respectively. *H. pluvialis* total carbohydrate content was found in BG-11, RSW, and RSW+Mn as 34.94±5.04%, 27.94±6.75%, and 28.52±0.80%, respectively. In comparing all culture mediums for *C. minutissima* and *B. braunii*, there was no statistically significant difference (P >0.05). However, BG-11 exhibited a statistically significant difference for *H. pluvialis* (P <0.05).

The dominant energy storage products in Chlorophytic microalgae are carbohydrates and oils (Subramanian et al., 2013). The low lipid content in dry microalgal biomass can be explained by the high carbohydrate produced within the microalgae cells. It has been suggested by Freitas et al. (2017) that arabinose and xylose can be used as carbon sources for microalgal cultures to increase the amount of carbohydrates in biomass (53.8%). In another study, Andreeva et al. (2021) reached 47.9% carbohydrate content in *Chlorella vulgaris* biomass using carbohydrate additives (a mixture of glucose, fructose, sucrose, and maltose). The total carbohydrate concentration was determined between 20 to 76% in 16 different *B. braunii* strains (Gouveia et al., 2017). The maximum sugar content of *B. braunii* was found to be 28.96% in treated domestic sewage wastewater, which was lower than the carbohydrate content of this study (Sydney et al., 2011). It has been understood that the content of microalgae culture medium directly affects the carbohydrate content of microalgae.

Moisture

C. minutissima, cultured in BG-11, RSW, and RSW+Mn culture mediums, moisture content was determined as 11.83±0.18%, 11.09±1.01%, respectively. *B. braunii* moisture content was calculated in BG-11, RSW, and RSW+Mn as 16.76±2.67%, 15.43±0.17%, and 21.59±0.51%, respectively. *H. pluvialis* moisture content was found in BG-11, RSW, and RSW+Mn as 10.76±1.04%, 17.22±0.90%, and 30.10±1.61%, respectively. No statistically significant difference among all culture mediums for *C. minutissima* and *B. braunii* (P >0.05), but BG-11 showed statistically significant difference for *H. pluvialis* (P <0.05).

Fresh algal cells constitutes around 70-95% water after centrifugation (Da Silva et al., 2008). Hosseinizand et al. (2017) stated that the moisture content of Chlorella should be decreased from 35-75% to 10% due to preservation of the biochemical properties. Chlorella species moisture content were decreased using hot air drying from 70.38±2.90% to 0.88±0.05% and using freeze drying from 70.38±2.90% to 3.58±0.19% (Stramarkou et al., 2017).

Ash content

As shown in these tables below, *C. minutissima* ash contents in BG-11, RSW, and RSW+Mn were determined as 11.84±0.19%, 11.09±1.01%, and 55.94±4.57%, respectively. *B. braunii* ash content was calculated in BG-11, RSW, and RSW+Mn groups as 16.76±2.67%, 15.43±0.17%, and 21.59±0.51%, respectively. *H. pluvialis* ash content was found in BG-11, RSW, and RSW+Mn as 10.76±1.04%, 17.22±0.90%, and 30.10±1.61%, respectively. There was no statistically significant difference among all culture mediums for *C. minutissima* and *H. pluvialis* (P >0.05). However, it was determined that there was a statistically significant difference in the RSW and RSW+Mn groups used in the production of *B. braunii* species when compared to BG-11 (P <0.05).

The highest ash content was found in RSW+Mn medium similar to another study which used landfill leachate based mediums (dos Santos et al., 2022). With this study, it is understood that high conductivity and salinity concentrations in the culture mediums caused an increase of the ash content of harvested spe-

Table 8. *C. minutissima* nutrition facts in different culture mediums

Descriptions	BG-11 (%)	RSW (%)	RSW+Mn (%)
Crude protein	51.88±0.32^{A}	55.77±1.81^{A}	21.53±0.70^{B}
Total lipid	4.18±0.33^{A}	4.69±0.88^{A}	1.35±0.48^{B}
Total carbohydrate	18.66±3.68^{A}	16.90±0.99^{A}	20.20±5.47^{A}
Moisture	10.60±0.33^{A}	10.84±0.88^{A}	10.90±0.48^{A}
Ash	11.84±0.19^{A}	11.09±1.01^{A}	55.94±4.57^{A}

Letters A-B indicates significant differences between samples (P <0.05)

Table 9. *B. braunii* nutrition facts in different culture mediums

Descriptions	BG-11 (%)	RSW (%)	RSW+Mn (%)
Crude protein	49.77±0.62^{A}	38.50±0.78^{A}	37.39±0.61^{A}
Total lipids	0.62±0.11^{A}	0.48±0.11^{A}	0.53±0.31^{A}
Total carbohydrates	23.67±1.89^{A}	39.14±2.43^{A}	31.32±2.35^{A}
Moisture	11.41±0.11^{A}	7.20±0.11^{A}	11.49±0.31^{A}
Ash	16.76±2.67^{AB}	15.43±0.17^{A}	21.59±0.05^{B}

Letters A-B indicates significant differences between samples (P <0.05)

Table 10. *H. pluvialis* nutrition facts in different culture mediums

Descriptions	BG-11 (%)	RSW (%)	RSW+Mn (%)
Crude protein	40.61±4.06^{A}	33.86±2.75^{B}	30.25±0.84^{B}
Total lipid	0.57±0.30^{A}	0.70±0.07^{A}	0.11±0.02^{A}
Total carbohydrate	34.94±5.04^{A}	27.94±6.75^{AB}	28.52±0.80^{B}
Moisture	13.11±0.30^{A}	10.28±0.07^{B}	11.03±0.02^{AB}
Ash	10.76±1.04^{A}	17.22±0.90^{A}	30.10±1.60^{A}

Letters A-B indicates significant differences between samples (P <0.05)

cies. It may be possible to use leachate and effluent-based wastes as microalgae culture mediums, however the biomass would have a higher ash content than commercially produced microalgae.

CONCLUSION

The comparative study of using BG-11, RSW and RSW+Mn mediums to culture *C. minutissima*, *B. braunii*, and *H. pluvialis* has shown sufficient concentrations and value-added compounds. Removal of nitrogen and phosphorus from remineralized sludge-water of aquaponics to microalgal biomass was successfully achieved. RSW obtained from aquaponics had an advantage to BG-11 microalgae culture medium due to its low cost, easy for obtaining and more environmentally friendliness. Protein composition of *C. minutissima* in RSW had the highest scores compared to other culture mediums (BG-11 and RSW+Mn). While RSW medium was found as an advantageous for *C. minutissima* cultivation due to its high protein content, but *B. braunii* and *H. pluvialis* were characterized by low lipid contents. Because of this reason RSW medium is not recommended for cultivation of *B. braunii* and *H. pluvialis*. *B. braunii* culture was found to have the highest carbohydrate content in the RSW medium. *B. braunii* is known for its low growth rate and long regeneration time. The RSW medium, however, can be an advantageous sustainable resource with its fertile properties for the production of biomass. Crude protein levels of the microalgae species were suitable when compared to other culture mediums in the literature. However, total lipid contents of the microalgae species were found very low, between 0.11±0.02-4.69±0.88%, due to the nitrogen rich culture mediums. RSW culture medium might be suggested as an alternative culture medium for green microalgae production for batchwise systems. When it comes to culture period, it may be suggested that *B. braunii* and *H. pluvialis* be cultured for 20 days using RSW based microalgae mediums, however, *C. minutissima* may require more than 27 days for cultivation batchwise. Additional research is required to find the optimum physico-chemical conditions, remineralization process of RSW and techno-economic analysis of *C. minutissima*, *B. braunii*, and *H. pluvialis* cultured in RSW.

This study partially fulfills the expected objective. *C. minutissima, B. braunii*, and *H. pluvialis* grew well in remineralized sludge water (RSW) medium obtained from nutrient film technique (NFT) aquaponics. All microalgae species effectively removed nutrients form RSW, achieving bioremediation. *C. minutissima* exhibited the highest crude protein content in RSW medium compared to the other cultures. RSW proved to be a low-cost, readily available, and environmentally friendly alternative to the standard BG-11 for *C. minutissima* cultivation. However, all microalgae species had very low lipid content due to the nitrogen-rich RSW medium. Consequently, RSW was not suitable for maximizing lipid production in *B. braunii* and *H. pluvialis*.

Given its characteristically high total oil content and hydrocarbon production, *B. braunii* is a promising candidate for further studies on maximizing oil yield. Future research can investigate the effects of manipulating nutrients, light, carbon source, and stress factors (including high salinity and various trace elements) on oil production for this species in remineralized aquaponics sludge water. *H. pluvialis*, valued for its high concentration of important antioxidant substances like astaxanthin, canthaxanthin, and lutein, is a promising candidate to investigate the effects of remineralized aquaponics wastewater on production of these antioxidants.

In conclusion, microalgae species exhibited differential growth responses and biomass productivities depending on the culture medium. While *C. minutissima* thrived in BG-11 and RSW, *H. pluvialis* achieved superior biomass productivity in the RSW+Mn medium, underscoring the importance of optimizing culture conditions for each species to maximize yields. Finally, the effects of different concentrations of aquaponic wastewater and stress factors on the biomass and biochemical composition of *C. minutissima* can be studied, as well.

Ethics committee approval: This study was ethically reviewed by the Local Ethics Committee for Animal Experiments of the General Directorate of Agricultural Research and Policies, Mediterranean Fisheries Research, Production, and Training Institute. It was approved in compliance with the principles of the Local Ethics Committee Directive for Animal Experiments on January 28, 2019, under decision number 2019/01.

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Research Article

Length-Weight and Length-Length Relationships of *Etrumeus golanii* DiBattista, Randall & Bowen, 2012 (Clupeiformes: Dussumieriidae) in Antalya Bay (Mediterranean Sea of Türkiye)

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ABSTRACT

Some population biology characteristics of *Etrumeus golanii* DiBattista, Randall & Bowen, 2012 were estimated for 438 individuals captured using gillnets in Antalya Bay (Mediterranean Sea of Turkey) between November 1997 and May 1998. Fish size in TL (total length) ranged from 8 cm to 26.4 cm and the length-weight relationships (LWRs) were calculated for males, females and all individuals as $W=0.0042L^{3.2309}$, $W=0.0086L^{2.999}$ and $W=0.0059L^{3.1218}$ respectively. The results additionally showed that length-length relationships (LLRs) were significantly correlated ($r^2>0.985$, $P<0.001$). This study aims to contribute to the Bayesian hierarchical approach used in LWR calculations in the FishBase database. Additionally, 11 LWRs were gathered from 11 studies carried out between 1984 and 2021 in different marine waters. The present study is an evaluation of previous scientific studies from different areas. The value of the b slope for *E. golanii* varies between 2.626 and 3.443. The mean value of b was 3.0750 and the median value of b was 3.0435, and the Log10 a and b plot was used to detect outliers.

Keywords: *Etrumeus golanii,* length-weight relationships (LWRs), lessepsian fish, Mediterranean Sea, ORMEF

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INTRODUCTION

At the beginning of the 20th century, there has been a steady increase in non-native fish species on the Levant coast (Galil, 2000). The colonisation of Indian-Pacific and Red Sea species in the Mediterranean, known as Lessepsian migration, began with the construction of the Suez Canal in 1869 (Por, 1978; Golani, 1998). Approximately 63 % of all non-native fish species in the Mediterranean enter the sea through the Suez Canal (Bella, 2000; Galil & Zenetos, 2002). The Suez Canal has become the principal entry point for non-native fish species into the Mediterranean, resulting in vast, self-sustaining populations (Bariche et al., 2003).

The ORMEF database presents information on 188 fish taxa that are thus divided: 106 species entered through the Suez Canal; 25 species introduced by human activities; 57 Atlantic species through the strait of Gibraltar (Azzuro et al., 2022) since the first description of the lessepsian migratory fish species in the Mediterranean (Ben-Tuvia, 1953). The ORMEF database is a Mediterranean database of exotic fish records, containing 4015 geo-referenced data from 20 Mediterranean countries and compiled from 670 scientific papers (Azzuro et al., 2022). There is also new evidence that some fish species have recently formed populations in the Mediterranean basin (Bilecenoğlu et al., 2002; Azzurro & Andaloro, 2004). It would not be incorrect to argue that Türkiye is one of the coun-

tries with the most intense migration and population formation of lessepsian species among the Mediterranean countries. Although this quick and consistent migration has an impact on the entire Mediterranean environment, it is clear that further knowledge on the biological properties of these species is needed. Calculations of the length-weight (LWRs) and length-length (LLRs) relationships are used for basic purposes in the evaluation of fish stocks and populations (Ricker, 1968; Ricker, 1979).

LWRs are one of the most valuable tools for assessing various aspects of fish species, including their overall health, reproductive history, life cycle, and physical condition (Nikolsky, 1963; Wootton, 1992; Pauly, 1993; Edelist, 2012). It is crucial to use standardised measurements across all groups to increase the reliability of comparisons between different populations. This is important in terms of developing information on the LWRs exhibited by species under many environmental conditions. The LLRs are of great importance, especially in comparative growth studies (Moutopoulos & Stergiou, 2002). The length of fish may typically be assessed more rapidly and simply than the weight of fish in fisheries research (Kara & Bayhan, 2008). Calculating the LWR makes determining the mass easier when only the length is known. The tail may be damaged and cut off during hunting activities or in case of tail parasites. Such situations make it difficult to accurately measure TL. However, knowing the SL (standard length) will help to calculate the TL.

This study aims to estimate the LWRs and LLRs of females, males and all individuals of the *E. golanii* species caught in Antalya Bay in 1997 and 1998 and to offer the opportunity to compare research materials with single-length measurement data of the species.

MATERIALS AND METHODS

The fish samples were captured monthly using gillnets during the commercial fishing season conducted between 1997 and 1998 in Antalya Bay (Figure 1).

Fish were transported from the commercial boats to the laboratory via a cold chain and measured to the nearest cm TL (total length), FL (fork length) and SL (standard length) and weighed to the nearest g TW (total weight). The correlation between TL and

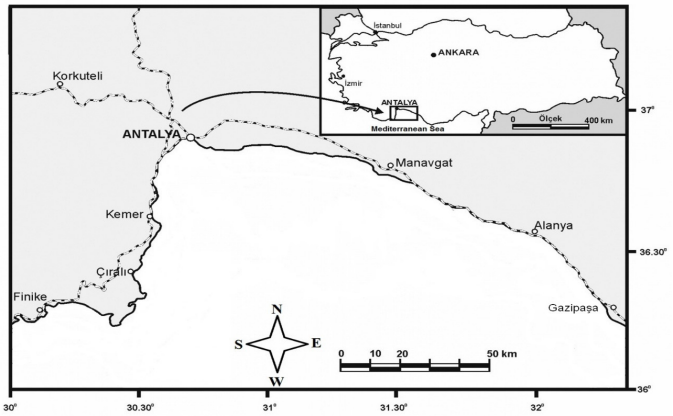

Figure 1. Sampling area.

TW, $W = a \times L^b$, length-weight data can, however, be fitted with a (linear) regression if logarithms are taken of both sides, resulting in $\log_{10} W = a + b \log_{10} L$ (Pauly, 1984). Least-squares regression was used to obtain the parameters a and b, as well as the coefficient of determination (r^2). *A t-test* at the P = 0.05 significance level was used to determine if the b value for each species differed substantially from 3. Furthermore, linear regressions were used for calculating the connections (1) TL vs FL; (2) FL vs SL; and (3) SL vs. TL. To test for potentially significant differences in both the slope and intercept, covariance analysis was performed. All statistical analyses were appraised at $P<0.05$ significance level.

RESULTS AND DISCUSSION

Until a few years ago, the species was frequently used in the literature with the synonym name "*Etrumeus teres*". However, the naming made by Mitchill (1814) was accepted as the real name of the species (Froese and Pauly, 2024). In this study, studies carried out under the name "*Etrumeus teres*" were accepted as *E. golanii* and evaluated for comparison. Keramidas et al. (2023) summarised the invasive history of *E. golanii* in the Mediterranean;

The first record in the Mediterranean was reported by Gruvel, (1931) as *Klupea kowal* (Rüppell, 1837) from Lebanon. It continued to spread westwards with the first record from Lampedusa in the central Mediterranean, and by then the species was recorded under the name *Etrumeus teres* (DeKay 1842). In 2012, DiBattista et al. (2012) reported that the specimens identified as the Lessepsian immigrant *E. teres* in the Mediterranean Sea were actually a completely new species, namely *E. golanii*. For this reason, the *E. teres* species given in the length-weight relationship in the Mediterranean in Table 3 was accepted as *E. golanii*.

Some population biology characteristics of *E. golanii* were estimated for 438 individuals captured using gillnets in Antalya Bay (Mediterranean Sea of Türkiye) between November 1997 and May 1998. Fish size in TL ranged from 8 cm in April to 26.4 cm in December. It was determined that 55.7% of the samples were females (n=244) and 44.3% males (n=194).

The LWRs were calculated for males, females and all individuals as $W=0.0042L^{3.2309}$, $W=0.0086L^{2.999}$ and $W=0.0059L^{3.1218}$ respectively (Table 1). Analysis of covariance revealed significant differences between sexes for the slopes (b) of the regression lines ($P<0.001$). The LWRs of *E. golanii* presented in Table 1 show that the calculated allometric coefficients vary between 3.134 (January) and 3.244 (November) in males and between 2.987 (April) and 3.364 (Nowember) in females.

All LLRs shown in Table 2 were highly significant ($P<0.001$), with all coefficients of determination values greater than 0.985. The LWRs can be obtained from the body length and body weight measurements acquired from the same fish throughout their lifetimes or from a sample of fish caught at some point in time (Wootton, 1990).

All the allometric coefficients (b) estimated in this study were within the expected range between 2.9 and 3.3. Additionallay Bagenal & Tesch (1978); Koutrakis & Tsikliras (2003); Yılmaz & Hoşsucu (2003), allometric coefficients (b) may range from 2 to 4.

Table 1. Estimated parameters of LWRs for both sexes of *E. golanii* in Antalya Bay.

Months	Sex	N	Lenght characteristics		Weight characteristics		Relationship parameters			
			TL Range (cm)	Mean TL (±SD)	W Range (g)	Mean W (±SD)	a	b	SE of b	r^2
November	F	38	15.2-24.6	18.66±3.20	26.8-139.7	61.15±36.69	0.0029	3.364	5.269	0.997
	M	48	14.5-24.7	17.55±3.33	24.2-128.3	50.21±35.46	0.0040	3.244	4.194	0.996
December	F	71	14.4-26.4	21.14±3.35	22.9-168.6	89.27±41.71	0.0036	3.290	7.476	0.995
	M	39	14.8-24.6	19.71±2.52	24.5-137.9	68.90±29.31	0.0033	3.315	6.739	0.984
January	F	24	12.1-20.8	16.60±2.46	14.6-80.2	40.28±19.00	0.0059	3.117	3.820	0.989
	M	32	12.5-24.6	16.85±3.04	15.5-132	43.52±27.41	0.0056	3.134	6.610	0.994
February	F	18	16.1-22.3	19.69±1.56	33.6-94.8	69.94±16.53	0.0050	3.196	3.678	0.955
	M	22	16.1-23.0	19.07±1.77	34.4-109.8	61.79±18.90	0.0039	3.266	3.449	0.983
March	F	42	15.1-22.3	19.51±1.80	30.3-96.4	63.54±17.37	0.0073	3.043	4.083	0.965
	M	5	16.9-21.0	19.34±1.75	36.1-75.0	58.76±16.42	0.0033	3.292	1.293	0.994
April	F	42	8-24.1	20.28±2.52	49.2-128.2	77.74±19.49	0.0092	2.987	4.213	0.970
	M	18	18.1-22.0	20.14±1.08	51.0-92.9	68.09±11.75	0.0051	3.158	2.721	0.960
May	F	9	15.8-22.4	19.07±2.37	33.2-102.6	62.03±23.35	0.0074	3.050	4.882	0.985
	M	30	14.6-24.2	18.24±2.48	23.9-116.9	53.79±23.06	0.0047	3.199	2.864	0.994
Overall	F	244	8-26.4	19.70±3.03	14.6-168.6	71.23±33.45	0.0086	2.999	8.135	0.968
	M	194	12.5-24.7	18.44±2.86	15.5-137.9	56.61±28.61	0.0042	3.231	5.437	0.998
	A	438	8-26.4	19.14±3.02	14.6-168.6	64.76±32.20	0.0059	3.122	7.126	0.982

F: Females, M: Males, A: All Inviduals, N: number of individuals, TL: Total Length, SD: Standard Deviation, W: Weight, a: intercept, b: slope, SE: Standard Error, r^2: coefficient of determination

Table 2. LLRs between the total length, fork length and standard length of *Etrumeus golanii* in Antalya Bay.

Sex	Equation	N	a	b	r^2
Female	TL=a+bFL	244	-0.179	1.124	0.991
	FL=a+bSL		0.468	1.019	0.997
	SL=a+bTL		-0.134	0.865	0.992
Male	TL=a+bFL	194	0.228	1.098	0.992
	FL=a+bSL		0.467	1.020	0.985
	SL=a+bTL		-0.389	0.878	0.990
All	TL=a+bFL	438	-0.013	1.113	0.992
	FL=a+bSL		0.483	1.018	0.992
	SL=a+bTL		-0.255	0.871	0.992

TL: Total Length, FL: Fork Length; SL: Standard Length, N: number of individuals, a: intercept, b: slope, r2: coefficient of determination

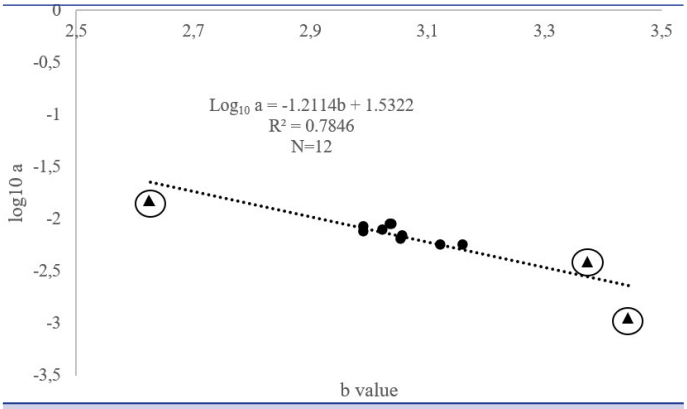

Figure 2. *Log a* and *b* value plot for all available length-weight relationships of *E. golanii* species. The 3 outliers of the type are marked with circular lines in the graph.

Other research on the LWR and LLR connections of *E. golanii* have been conducted in the Turkish Seas and other locations. Table 3 shows the b values reported in this research. Although the majority of the research on the species has been undertaken in the Mediterranean, a comparison of the LWR parameters, particularly the b values, in 11 separate studies conducted in various areas of the Mediterranean has been made. Figure 2 depicts these results.

CONCLUSION

E. golanii can easily be misidentified by confusion with similar pelagic species of high economic importance such as *Engraulis encrasicolus* (European anchovy) and the *Sardina pilchardus* (European pilchard)(Keramidas et al., 2023). *E. golanii* is known as one of the most fished species among the small pelagic fish species

Table 3. LWRs of "*Etrumeus golanii*" from different localities.

Area	N	Sex	Length range (cm)	Length	a	b	Author(s)
Gulf of Suez		All	-	TL	0.0059	3.158	Sanders et al., 1984
Gulf of Suez, Red Sea, Egypt		All	12.0 – 27.0	SL	0.0011	3.443*	El-Sayed, 1996
Gulf of Suez, Red Sea, Egypt	600	All	11.0 – 24.8	TL	0.0091	3.0356	Mehanna & El-Gammal, 2005
Lebanese coast, Medi. Sea		All	Up to 25	TL	0.0039	3.375	Bariche et al., 2006
Eastern Alex. Medi. Sea, Egypt		All	11.0 -.25.0	TL	0.0071	3.055	Akel, 2009
Iskenderun Bay	61	All	10.0-16.70	-	0.0078	2.989	Ergüden et al., 2009
Beymelek Lagoon, SW Turkey	10	All	8.2-10.6	TL	0.015	2.626	Sümer, 2012
Israeli Coasts	16	All	8.0-24.0	TL	0.0088	2.989*	Edelist, 2014
Egyptian Mediterranean waters	656	All	9.0 – 25.0	TL	0.0066	3.051	Farrag et al., 2014
Antalya Bay	68	All	14.6 – 24.1	SL	0.0081	3.021	Türker et al., 2020
Eastern Mediterranean, Egypt	630	All	9-11.7	TL	0.0091	3.036	Mehanna1 & Farouk, 2021
Antalya Bay	438	All	8.0 – 26.4	TL	0.0059	3.1218	This study

N: number of individuals, TL: Total Length, FL: Fork Length, SL: Standard Length, a: intercept, b: slope, r^2: coefficient of determination

belonging to the family Dussumieriidae, which is economically valuable in the Türkiye coasts of the Eastern Mediterranean basin. *E. golanii* originated from the Red Sea and was first recorded in 1961 in Ashdod, Israel, when a few individuals were caught from a trawl at a depth of 100 m. Until today, it has been detected at different depths throughout the Mediterranean basin. After this first record in the Mediterranean, it was found that it was limited to form populations in some parts of the Eastern Mediterranean for years and was several decades late in its geographical distribution, and it started to spread more rapidly from the Central Mediterranean to the Western Mediterranean (Azzuro et al., 2016; Galil et al., 2019; Dikou, 2024). Although *E. golanii* is an economically important species, it is an alien species for the Mediterranean basin and should be monitored in terms of fisheries biology and population dynamics.

Season, nutrition, habitat, gonad development, sex, health, stomach fullness, and preservation procedures all have an impact on the parameters of the fish, LWRs (Tesch, 1971; Bagenal & Tesch 1978; Hossain et al., 2006).

All the allometric coefficients (b) estimated in this study were within the expected range between 2.9 and 3.3. Additionallay Bagenal & Tesch (1978); Koutrakis & Tsikliras (2003); Yılmaz & Hoşsucu (2003), allometric coefficients (b) may range from 2 to 4. There has not been much research on growth parameters such as LLRs and LWRs for *E. golanii*, a Lessepsian species. However, as stated in Table 3, the results of the current study are similar to those of other studies.

While the LWR parameter values compiled from these studies are presented in Table 3, a comparison of the parameters is made in Figure 2. According to data obtained from previous studies, the value of the b slope for *E. golanii* varies between 2.626 and 3.443. The mean value of b was 3.0750, the median value of b was calculated to be 3.0435, and the Log_{10} a and b plot was used to detect outliers (Figure 2).

FaroesE (2000) suggested that plotting log a and b for all known LWRs of a species results in a linear relationship and that this relationship can be used to identify outliers (Stergiou & Moutopoulos, 2001). We applied this method to the LWR 12 data (Figure 2). This method resulted in the detection of more than one point with data for the species deviating from the linear graph. Points deviating from the regression line are shown in circles in Figure 2, and the data to which this data belongs is marked as suspicious in Table 3.

Figure 2 is thought that compiling these findings contributes to the Bayesian hierarchical approach used in LWR calculations in the Froese and Pauly, 2024.

Conflict of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics Committee Approval: No need for ethical approval for this study.

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Research Article

Ectoparasitic Helminths During the Annual Cycle of the European Chub (*Squalius cii*, Richardson, 1857) in the Susurluk Basin, Türkiye: Their Infestation Levels, Identification, and Effect of Host Factors on Infection Levels*

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ABSTRACT

The ectoparasitic helminth in the European chub (*Squalius cii*, Richardson, 1857) was collected from the Susurluk basin from the Northwest region of Türkiye in the period from spring 2020 (April) to winter 2021 (February) once in every three months and also summarised. Only two species of Monogenea were found on the gills of the host and were identified as *Dactylogyrus vistulae* Prost, 1967 (Monogenea: Dactylogyridae) and *Paradiplozoon homoion* (Bychowsky & Nagibina, 1959) (Monogenea: Diplozoidae). *D. vistulae* was the most common and the highest number species. Other factors such as season, host length and sex were investigated to determine their effect on the infection values of ectoparasitic helminth infection, which were calculated from information collected from 79 host fishes. However, according to our current literature, there is no study in which *S. cii* is considered as a valid species and investigated in terms of ichtyohelminthological in Türkiye. This is the first ichtyohelminthological survey of *S. cii* in Türkiye and therefore new host and distribution records for all helminth species. Moreover, with such studies, scientists will have the opportunity to evaluate the infection success of parasite species belonging to this group, depending on the host, environmental conditions and enemies, and their infection success depending on the seasons and host factors. And data will be obtained about the complex life histories of the species belonging to this group and will contribute to the determination of the causes of death that will occur in both cultured and natural fish populations in the future.

Keywords: *Squalius cii*, *Dactylogyrus vistulae, Paradiplozoon homoion*, season, host size, sex

* This paper is a part of Doctorate Thesis named "Determination of Helminth Parasite Diversity of Some Endemic Fish Species in Susurluk Basin.

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INTRODUCTION

The genus *Squalius* of the family Cyprinidae has a wide geographical distribution in Europe and Asia (Özulug & Freyhof 2011).According to the current literature, this genus includes about 47 species (Zardoya & Doadrio 1999) and 21 of 47 from Central and Western Anatolia (Çiçek et al., 2020; Özuluğ& Freyhof 2011). Despite these studies, the taxonomy of these genus members in Türkiye at the species level has not yet been resolved. Indeed, in one of these studies, Özuluğ & Freyhof (2011) revised the species belonging to the genus *Squalius* in Anatolia and defined ten species. Four of them were recognised as new species and the other six as valid species. One of these valid species is*Squalius cii*, which occurs in northwest Anatolia in Türkiye and is present in all branches of the stream in the Susurluk basin (Öztürk &Küçük 2017). In addition, the taxonomic status and distribution of the species of the genus *Squalius* were also studied by Stoumboudi et al. (2006). They has been reported this species from the island of Lesbos (Greece). The studies of these researchers added a locality outside of Türkiye to the distribution area of the species. However, the taxonomic and the current status of the species

of this genus still remain complicated. In fact, while this species was considered as endemic to Türkiye by Aksu et al.(2016), Çiçek et al. (2020) were accepted as a natural species. As for the status of *Squalius cii* in the IUCN, although this species is narrowly distributed and consumed by local people in a small number of locations, it is still Least Concern (LC) in the IUCN.

Although there have been many studies investigating ichthyoparasitological properties of freshwater fishes in Türkiye in recent years, there are a few studies on freshwater fishes belonging to *Squalius* (Aydogdu et al. 2015; Elbay & Öztürk 2021; Soylu et al. 2017; Unal et al. 2017)and none on the European chub (*S. cii)*. However, according to our current literature, there is no study in which *S. cii* is considered as a valid species and investigated in terms of ichthyoparasitological. Therefore; this study is the first ichtyohelminthological survey on *S. cii* in Türkiye. *S. cii* was investigated for ectoparasitic helminth diversity and determined the effect of seasons, host fish age and sex on their infection levels.

MATERIALS AND METHODS

Between Spring 2020 to Winter 2021 (one every three months), specimens of *Squalius cii* were collected seasonally from the locality (Susurluk basin) (Fig. 1). The fish was collected by electrofishing, attempting to collect an equal number of small and medium-sized fish. A total of 79 *S. cii* were sampled and submitted for ichtyohelminthological investigation in the laboratory in within polythene tanks aerated and containing ice to minimize any possible damage from the locality and examined within as soon as possible after collection. The fish specimens were killed by vertebral separation and their length recorded. Specimens were grouped according to length. During dissection, first fins and skin were first observed with a Nikon binocular stereoscopic microscope, and thereafter, the gills of freshly killed fish were dissected into Petri dishes for careful observation for the presence of ectoparasitic helminth. The sex of individual fish were determined after dissection. The monogenea specimens were made into permanent preparations according to the methods described in Malberg 1957; Ergens, 1969. Infection parameters for each parasite species were recorded on the basis of seasons, host sex, and length. The monogenea specimens were identified according to the identification keys of Markevic (1951);Bychowkaya –Palovskaya (1962);Gussev (1985); Khotenovsky (1985). For calculations of the levels of their infection parameters values were calculated using Bush et al. (1997). Infection parameters for each monogenean species were calculated according to Bush et al. (1997). The SPSS V.23 software was applied to determine the significance between the prevalence and intensity of parasite infestation on the basis of host sex, length and seasons.Subsequently, to confirm the morphological identification of the diplozoid species, the molecular analysis was performed as reported by Aydogdu et al. (2020a, b). The BLAST sequence analysis have available in GenBank under accession number MW724525 as *Paradiplozoon homoion*.

Figure 1. Sampling locality of *Squalius cii* in the Susurluk Basin Map.

RESULTS AND DISCUSSION

General infection

Two different ectoparasitic helminths were identified on the gills of 79 individuals of *Squalius cii* examined. Based on the presence of the following morphometric characteristics: the hard parts of haptor and reproductive organs, they were identified: *Dactylogyrus vistulae* (Prost, 1967), (Fig.2a,b,c) was identified from 18 individuals and *Paradiplozoon homoion*(Bychowsky & Nagibina, 1959), (Fig. 3) from 9 individuals of *S. cii*. In total, 62 ectoparasitic helminth specimens were recovered. Among these, *D. vistulae* was the most common and the highest number species (Fig. 4). Figure 4 graphically shows the calculated infection values for two ectoparasitic helminth species.

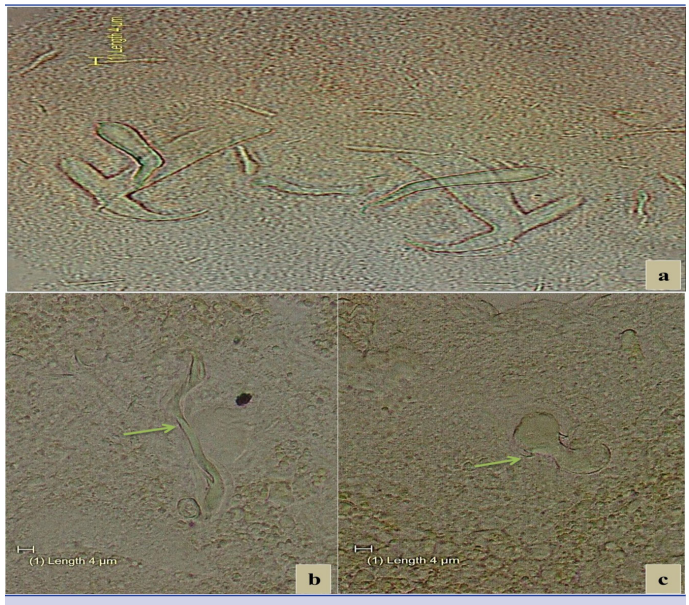

Figure 2. *Dactylogyrus vistulae* a) haptor (scale bar = 4µm) b) copulatory organ(scale bar = 4µm). c) vaginal tube (scale bar = 8µm).

Figure 3. Clamps of the *Paradiplozoon homoion* from Squalius cii (scale bar = 8µm).

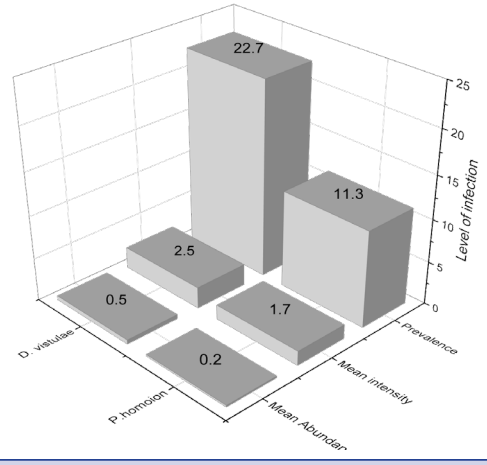

Figure 4. Distribution of the infection value of ectohelminth parasites in *Squalius cii* from the Susurluk Basin, Balıkesir.

The distribution of European chub ectoparasitic helminth with respect to season

The study was conducted seasonally from spring 2020 (April) to winter 2021 (February). *D.vistulae* was recorded in spring and summer, while *P.homoion* was observed throughout all seasons, with the exception of spring. The highest prevalence value of *D. vistulae* was recorded in spring and summer. In *P. homoion*, it was highest in winter (10%) (Fig 5).As far as seasonally intensity is concerned; *D. vistulae* had the highest mean intensity values in spring and summer, while the highest mean intensity for *P. homoion* was observed in summer. As a result of the analysis, it was determined that the parasite infection rates of *D.vistulae* changed significantly according to the seasons (p=0,000), while *P.homoion* was not found to be significant (p=0,262).

The distribution of European chub ectoparasitic helminth with respect to the host length classes

The fishes were measured in term of length with measuring scale and divided into two groups according to their length. The highest prevalence of *D. vistulae* was recorded in length classes II (11.6-16.6 cm), whereas the highest prevalence of *P homoion* was observed in fish between 6.5-11.5cm (length classes I) (Fig.7, 8). For the mean parasite intensity in the present study, the mean intensity of the ectoparasitic helminth also showed differences in the two length classes. In *D. vistulae* and *P.homoion*, the mean parasite intensity was highest in larger fish.

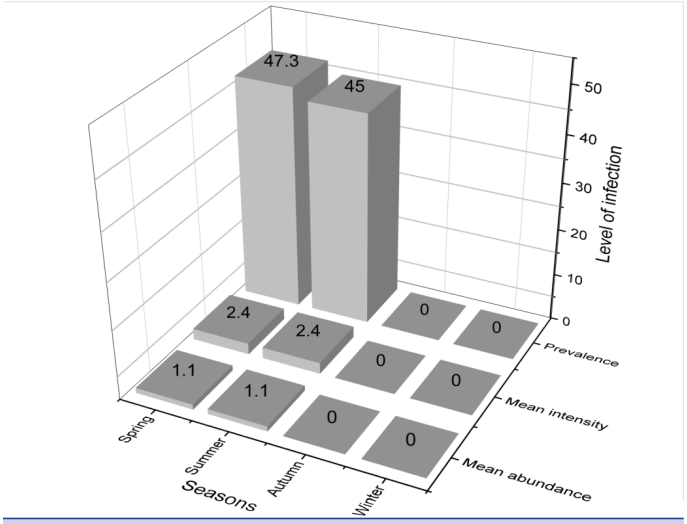

Figure 5. Presence of *Dactylogyrus vistulae* in *Squalius cii* in different seasons.

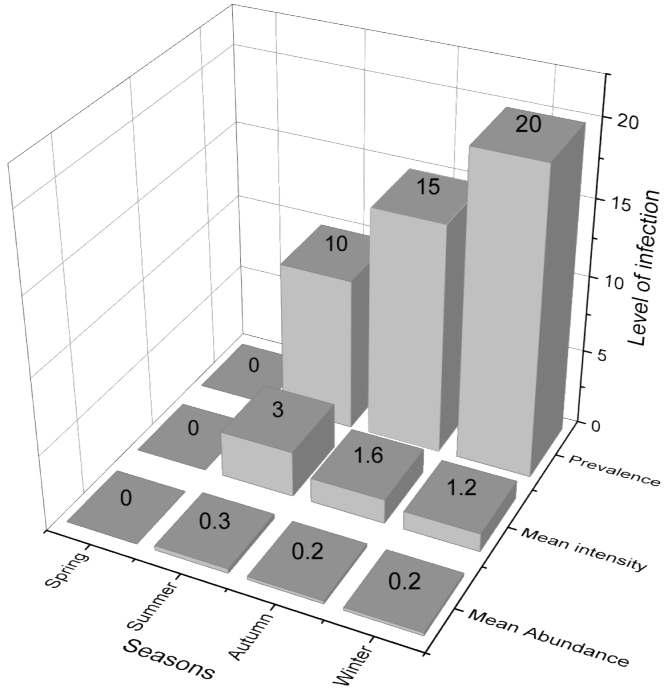

Figure 6. Presenceofthe *Paradiplozoon homoion* in *Squalius cii* indifferent seasons.

In addition, the mean abundance of ectoparasitic helminth specimens was almost equal in length classes (Fig.7, 8). The statistical analyses showed no significant difference in the number of *D. vistulae* and *P homoion* between host length classes (p=0.053 and 0.901, respectively).

The distribution of European chub ectoparasitic helminth with respect to the host fish sex

Of the 79 host *S. cii* individuals, 65.8% were females and 34.13 % were males. The highest prevalence of *D. vistulae* was in males, and the highest mean intensity level was in females (Fig. 9, 10). With respect to *P homoion* infection, the prevalence and mean intensity were both higher in females than in males. However, the difference was not significant based on the statistical analyses (p=0.307 and 0.383, respectively).

This present work provides new host records and a new locality was added for the distribution of two ectoparasitic helminth specimens. The infection values of the ectoparasitic helminth specimens varied between species. *Dactylogyrus vistulae* Prost 1967, (Fig.2a, b, c) had the highest prevalence, intensity and abundance (Fig.4). *D. vistulae* (a monogenean) is very common in fish belonging to the genera *Squalius* and *Chondrostoma* (Gussev 1995) and has been previously reported from five fish species living in different habitats from Türkiye: *Squalius cephalus*, S. *anatolicus, S. recurvirostris, Leuciscus cephalus* and *Chondrostoma regium* collected from Serban Dam Lake, Susurluk Creek, Dogancı Dam Lake, Bursa, Örenler Dam Lake, Yeşilırmak River, Almus Dam Lake and Akçay stream with prevalence and mean intensity varying from 12.04 % and 4.5 to 63.6 % and 8.5%, respectively. (Aydogdu et al. 2001; Açıkel & Öztürk 2012; Turgut et al. 2012; Öztürk 2014; Aydogdu et al. 2015; Elbay & Öztürk 2021). Thus, it can be seen that the prevalence and mean intensity of infection with *D. vistulae* differs according to the host species and locality. As a result, this study and the studies of the researchers mentioned above showed that the prevalence and mean intensity values of *D. vistulae* varied according to the host fish species and locality. The changes in the prevalence and mean intensity values of *Dactylogyrus* spp. infection in freshwater fishes might be influenced by various factors: such as host specificity, host hormonal status, host immunological response, host migration …etc. (Ramadan &Shakweer 1981; Hanzelova & Zitnan 1985; Tombi et al. 2004; Simkova et al. 2005; Açıkel& Öztürk 2012;Koyunet al. 2015).However, the influence of these factors is difficult to distinguish because they are likely interrelated and affect each other.

In the present study, a total of 16 specimens of *Paradiplozoon homoion* (Bychowsky & Nagibina 1959), (Fig. 3) were recovered from 9 of 79*S. cii* examined with prevalence and mean intensity of 11.3% and 1.7 %, respectively. It is also the most recorded species of *Paradiplozoon* in Türkiye. *P. homoion* was previously reported 11 times from various freshwater fish species (Özer, 2021). Aydogdu et al.(2020 a) reported a prevalence of 11.7% in The European bitterling (*Rhodeus amarus*), which occurred in the same locality as this study and agrees with the prevalence of *P. homoion in* our study.

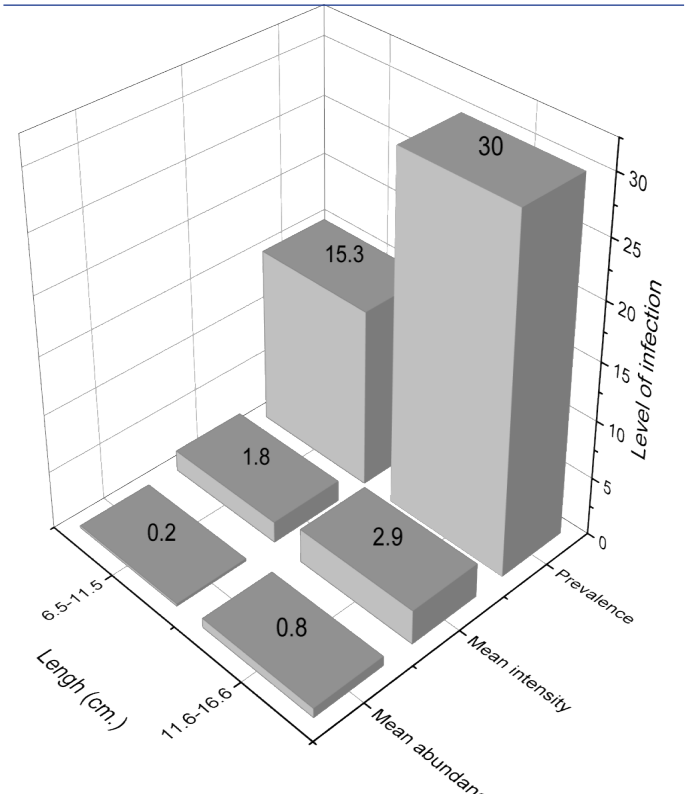

Figure 7. Distribution of the infection value of *Dactylogyrus vistulae* in *Squalius cii* from the Susurluk Basin, Balıkesir, according to the host length.

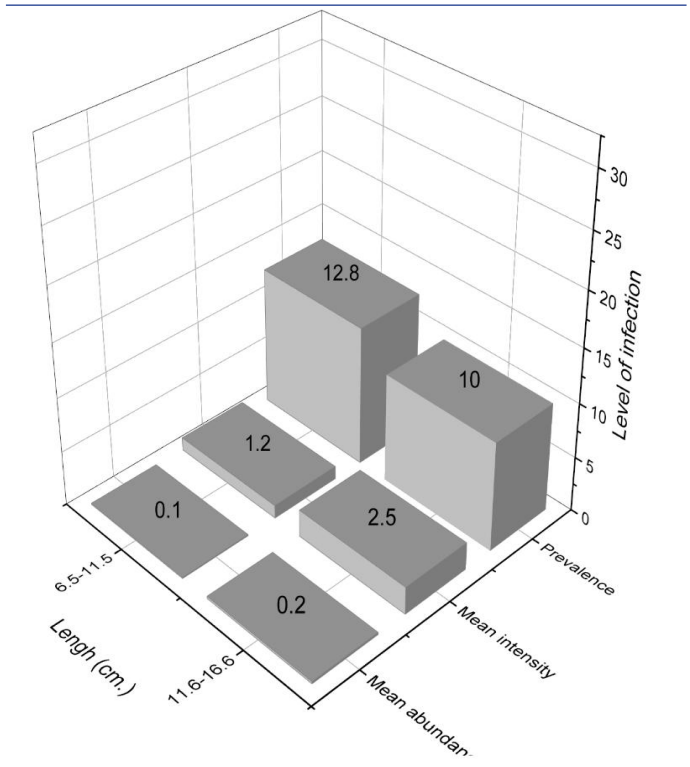

Figure 8. Distribution of the infection value of the *Paradiplozoon homoion* in *Squalius cii* from the Susurluk Basin, Balıkesir, according to the host length.

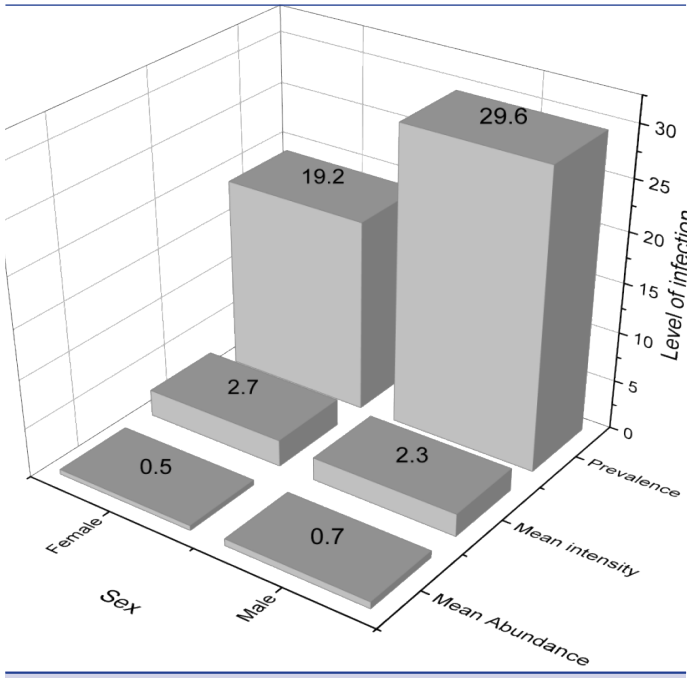

Figure 9. Presence of *Dactylogyrus vistulae* in *Squalius cii* in different sex groups.

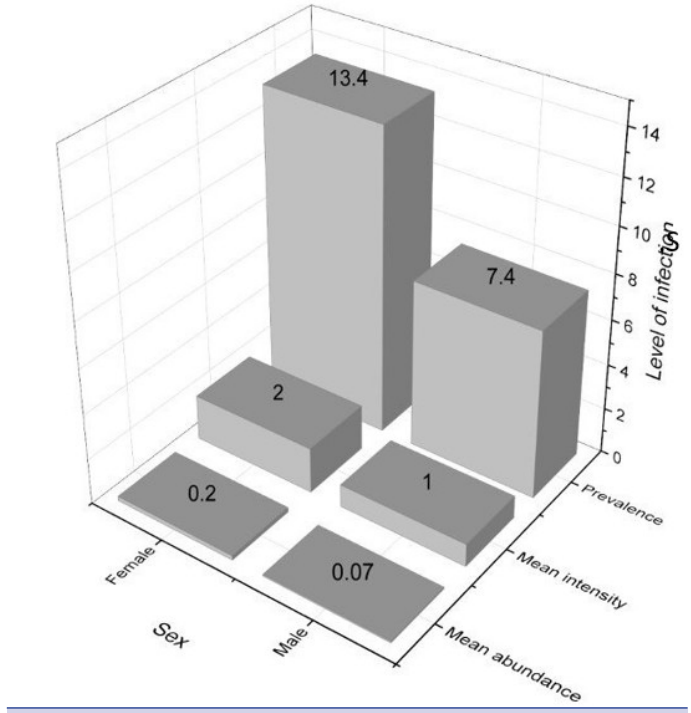

Figure 10. Presence of the *Paradiplozoon homoion* in *Squalius cii* in different sex groups.

With respect to the seasonality, the infection values of two ectoparasites helminths in this study varied, *D. vistulae*, the mean intensity was recorded the same in spring and summer while the prevalence reached high in spring. And this species was not detected in the autumn and winter samples. The finding in this study of *D. vistulae* was similar to that of Öztürk (2014); Aydogdu et al. (2015) and Elbay & Öztürk (2021) for S. *cephalus*, *S. anatolicus* and *S. recurvirostris*, respectively. In addition to these, *D. vistulae* has been identified by various authors many times in Türkiye from various freshwater fish species (Aydogdu et.al. 2001; Kurupınar & Öztürk 2009; Açıkel & Öztürk 2012).All of these authors noted the highest prevalence of *D. vistulae* infection in the spring season, similar to the value of our study, in the above-mentioned studies.

Regarding *P. homoion*, the highest prevalence of infection was recorded in winter. The parasite's infection intensity reached its peak in the summer season (3 parasite/ fish)(Fig. 6).

Many other researchers from Türkiye have investigated the seasonal variation of the infection rates of *P. homoion* in different fish species (*e.g.*Koyun2001; Öztürk 2005; Soylu 2007; Aydogdu et al.2020a,b).Aydogdu et al.(2020a,b) observed the highest infection prevalence value of *P. homoion* in the winter season from Manyas spirlin, *Alburnoides maynasensis*, from the Susurluk stream. Similarly, Soylu (2007) recorded the winter season to have the highest infection prevalence of this parasite species from flower fish, *Pseudophoxinus antalyae*. Contrary to these findings, Koyun (2001) did not encounter this species from the bleak *Alburnus alburnus* in the winter season. Similarly, Öztürk (2005) found *P. homoion* in all seasons (except winter) from Roach (*Rutilus rutilus*), and in the same study, the researcher only found this parasite in the summer season from Danube bleak (*Chalcalburnus chalcoides*). These authors pointed as due to different rates of parasite development in variations in temperature of different fish habitats.

The findings of the present study exhibit consistency with what recorded by Soylu (2007) and Aydogdu (2020a) and support this suggestion.

Generally, as the fish increased in size in this study for *D. vistulae*, the prevalence of infection values changed, the highest prevalence values of *D. vistulae* was recorded in the length classes II (11.6-16.6 cm), while the highest prevalence of *P. homoion* was observed in the length classes I (6.5-11.5 cm) et al.

In the present work, the highest prevalence and mean intensity values of *D. vistulae* recorded in the fish length 11.6-16.6 cm long (Fig. 7). The variation in the infection rates of *D.vistulae* and host fish length has been investigated by several researchers (Kurupınar & Öztürk2009; Açıkel & Öztürk 2012; Turgut et al. 2012; Öztürk2014; Aydogdu et al. 2015). Some of these authors (Kurupınar & Öztürk 2009; Öztürk 2014; Aydogdu et al 2015) found a positive relationship between the infection levels of *D.vistulae* and the length of the host fish. They found higher prevalence and mean intensity levels in medium and large fish. On the other hand, there are studies (Açıkel & Öztürk 2012) which found results the opposite, detecting infection prevalence and mean intensity values of *D. vistulae* the highest in young fish. Furthermore, Turgut et al. (2012) demonstrated no relationship between the mean intensity level of *D.vistulae* and host length. The present results agreed with Kurupınar & Öztürk (2009); Öztürk (2014) and Aydogdu et al.(2015) with a positive correlation between the abundance of parasites and the length of their host.

In case of *P homoion*, the prevalence of infection was highest in smaller fish 6.5-11.5 cm long, while the mean intensity was the highest in fish 11.6 – 16.6 cm long (Fig. 8).To the best of our knowledge, the relationships between *P. homoion i*nfection levels and host fish length in Türkiye have been studied by Koyun 2001; Soylu 2007; Öztürk, 2011 and Aydogdu et al. 2020a. Of these authors, only Aydogdu et al. (2020a) recorded its highest prevalence and mean intensity levels in large fish. In contrast to the findings of Aydogdu et.al.(2020a),others (Koyun2001; Soylu2007 and Öztürk 2011) found no host size-related effects on the infection of *P. homoion* in the increasing size classes of hosts.

As we know, in general, a positive relationship exists between the total number of monogenean parasite species per host and host length. This has been illustrated by several researches (e.g. Fisher & Kelso 1990; Bu and Song 1997; Aydogdu et al. 2003; Simkova et. al.2006; Açıkel & Öztürk, 2012) who found that the infection of *Dactylogyrus* increases with the length of the host fish. They also stated that in general, the number, prevalence and intensity of *Dactylogyrus* species increased with the length of the host fishes. They are of the opinion that larger sized host are probably present a larger gill surface for infection which the volume water that passes through the gills of larger sized fish which this conveys more oncomiracidium, larger sized fish had more surface are to accumulate parasites than smaller sized fish. Being a monogenean, one would expect *D.vistulae* to follow a general trend in the present study. The results of this study strongly confirmed the ideas mentioned above. Exactly as expected, *D. vistulae* preferred or accumulated on larger-sized fish with larger gill surface areas in the present study. Since larger-sized groups of *S. cii* were infested with *D.vistulae*, it could be concluded that larger sized host are large colonised surfaces area to parasites and more time to accumulate parasites than smaller-sized ones. To arrive at this conclusion, it should be noted that a sample size of 42 specimens per survey could possibly be sufficient to illustrate preferences for host size (Fig. 7).

In the light of the above information, a similar trend that infection of parasites increases with the length of the host fish is also expected from *P. homoion,* whereas the results of the present study indicated that this parasite had no preference for larger sized hosts. Therefore, we can conclude that *P. homoion* does not prefer or accumulate larger fish with larger gill surface areas in this study.

In the present study, the highest prevalence of infection of *D. vistulae* with respect to sex was highest in males, whereas it's the highest mean intensity level was observed in females. This agrees well with Özturk (2014) who found a higher prevalence of *D. vistulae* in male individuals of the chub. In the opposite of these findings, Elbay & Öztürk(2021)found that *D. vistulae* in male individuals of *Squalius recurvirostris* were heavily infected.

CONCLUSION

From the current study, two ectoparasitic helminth specimens were identified: the monogeneans, *Dactylogyrus vistulae*(Prost, 1967), and *Paradiplozoon homoion*(Bychowsky & Nagibina, 1959), were found on the gills. D. *vistulae* was the most common species in the host fishes and also found to be the maximum in number than *P. homoion*. In addition to these, other factors such as season, host length and sex were calculated to determine their effect on the prevalence, mean intensity and abundance of ectoparasitic helminth infection in the present study. In addition to these study findings, to the best of our knowledge, the two ectoparasitic helminth specimens recorded in this study were also recorded in studies in which different fish species were investigated in terms of ichtyohelminthological in Türkiye. However, since this study is the first ichtyohelminthological survey of *S. cii* in Türkiye, the host fish represents a new host record for two helminth specimens and thus, new knowledge has contributed to the geographical distribution and host range of these helminth species.

S. cii is a species consumed by local people in a narrow range with a small number of locations, but still has Least Concern (LC) status in the IUCN. While investigating the reasons for the decrease in this fish population in the future (massdeaths, overfishing), Monogenean group parasite epidemics, which are among the fish parasites and are especially lethal in aquaculture and rarely, in fish in the natural environment, should be evaluated and investigated as one of the causes of death. The necessity of investigating the species belonging to this group is because they have complex life history strategies adopted to ensure survival in unpredictable and opposing environments, such as strategies such as multiple reproductive mechanisms. With such studies, scientists will have the opportunity to evaluate the infection success of parasite species belonging to this group, depending on the host, environmental conditions and enemies, and their infection success depending on the seasons and host factors. Furthermore, data will be obtained about the complex life histories of the species belonging to this group and willcontribute to the determination of the causes of death that will occur in both cultured and natural fish populations in the future.

Conflicts of Interest: The authors declare no conflicts of interest.

Ethics Committee Approval: The authors affirm that ethical approval is unnecessary for this study.

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Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Research Article

Fermented Rainbow Trout Production Using *Lactobacillus sakei* and *Saccharomyces cerevisiae*: Effects on Microbiological, Biochemical, and Sensory Quality

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ABSTRACT

The aim of this study was to evaluate the microbiological, biochemical and sensory properties of fermented rainbow trout (*Oncorhynchus mykiss*) using *Lactobacillus sakei* ATCC® 15521™ (L), *Saccharomyces cerevisiae* ATCC® 9080™ (S) and their combination (M) as starter cultures and without starter culture as spontaneous fermentation (F). Rainbow trout slices (94% w/w) were mixed with curing ingredients—sucrose (2% w/w), salt (3% w/w), garlic powder (0.3% w/w), ginger powder (0.3% w/w), and sweet red pepper powder (0.3% w/w)—and kept at 4 °C for 2 days. After the cured trout were dried at 60 °C for 3 hours, starter cultures were evenly injected into the trout slices at a rate of 1% (1 mL starter culture: 100 g cured fish). The fermentation process was conducted at 24 °C for 14 days. During fermentation of the trout, the recommended 6 log CFU/g value for LAB, which is beneficial for gastrointestinal health, was reached on different days depending on the group: on day 12 in the F group (6.07 ± 0.25 log cfu/g), on day 4 in the S group (6.25 ± 0.11 log cfu/g), and on day 2 in the M group (6.52 ± 0.09 log cfu/g). Additionally, the highest value in the L group was 7.21±0.11 log cfu/g on day 2, indicating rapid fermentation. *Enterobacteriaceae* counts remained low across all groups, with values dropping to <1 log cfu/g by the end of fermentation. The results showed that the pH of fresh fish, initially 6.70, decreased significantly to 4.01, 4.18, and 4.09 in L, S, and M groups, respectively, by the end of fermentation. Nutritional analysis revealed higher protein content and reduced lipid oxidation levels in the M group, which also exhibited the lowest levels of total volatile basic nitrogen and thiobarbituric acid, indicating improved freshness and oxidative stability. Sensory evaluation identified the M group as the most preferred due to its balanced acidity, texture, and flavor. These findings suggest the potential of combining *L. sakei* and *S. cerevisiae* to produce high-quality fermented trout with enhanced safety, nutritional value, and consumer acceptance. This study represents the first effort to produce the fermentation process specifically for rainbow trout, marking a contribution to the field of fermented fish production.

Keywords: Fermentation, Starter culture, Lactic acid bacteria, Yeast, Microbial safety, Biochemical properties, Sensory characteristics, Fermented fish

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INTRODUCTION

Rainbow trout (*Oncorhynchus mykiss*) is one of the inexpensive, abundant, and widely consumed freshwater fish species due to its high nutritional value, mild flavor, and versatility in processing (Wilburn & Ryan, 2017; Escamilla-Rosales et al., 2024). However, freshwater fish, unlike marine fish, are marketed primarily for fresh consumption (Zang et al., 2020; Tavares et al., 2021). Traditional fish processing methods such as smoking, salting, and drying of freshwater fish often involve high salt concentrations, which may also limit their nutrition-

al benefits upon consumption (Belleggia & Osimani, 2023). The use of fermentation in processing these fish species appears to be a promising approach. Fermenting fish for human consumption has several benefits (Gelman et al., 2000; Paludan-Muller et al., 2002). It is a low-cost method to develop new fermented fish products that reduce fishy odors, increase the nutritional value and organoleptic qualities of fish, and increase consumption by providing long-term preservation of rainbow trout (Kılınç & Çaklı, 2021; Cai et al., 2024).

The use of LAB and yeasts together in fish fermentation contributes to the microbial safety (barrier technology) and sensory quality of fermented fish products. Therefore, significant research is still needed to better understand the optimal conditions for fish fermentation and the interactions between microorganisms (Wang et al., 2020; Chan et al., 2023). LAB ensure product safety by reducing pH through sugar fermentation, while yeasts affect aroma and texture properties (Glover et al., 2009; Carvalho et al., 2020). The proteolytic and lipolytic activities of these microorganisms contribute to the degradation of proteins and lipids, forming flavor compounds that increase the overall acceptability of the final product (Zang et al., 2018; Laranjo et al., 2019). However, little is known about the microbiological and biochemical changes that occur in freshwater fish fermentation inoculated with the combination of *Lactobacillus sakei* and *Saccharomyces cerevisiae*.

Some fermented fish products are very popular in eastern countries and also in some parts of western countries. In recent years, the use of pure or mixed bacterial cultures to produce fermented products has become increasingly popular (Siddiqui et al., 2023). Previous research has demonstrated the potential of using starter cultures in fermentation in fish processing and highlighted its impact on food safety, sensory properties and overall quality improvement. Bernbom et al. (2009) studied the fermentation of trout (*Salmo trutta*) with salt and boiled rice, showing that only the addition of at least 6% garlic combined with *L. plantarum* 509 effectively inhibited *Salmonella*, improving food safety. Zhu et al. (2019) investigated the fermentation of sturgeon (*Acipenser sinensis*) with *S. cerevisiae*, finding that it enhanced flavor, aroma, texture, and color, while increasing free amino acids, fatty acids, and peptides. Li & Xu (2021) examined the role of *L. plantarum* in fish paste fermentation, reporting that it inhibited the lipolysis and flavor formation caused by *S. cerevisiae*, affecting the biochemical properties of the product. An et al. (2022) evaluated the impact of *L. plantarum*, *Pediococcus acidilactici*, and *P. pentosaceus* on Zhayu, a traditional Chinese fermented fish, demonstrating that lactic acid bacteria improved food safety and quality by lowering pH and TVB-N levels. Zeng et al. (2013) analyzed the effects of autochthonous starter cultures (*L. plantarum*, *Staphylococcus xylosus*, and *S. cerevisiae*) on the microbiological and physicochemical properties of Suan yu, a low-salt fermented fish made from common carp (*Cyprinus carpio*). Zang et al. (2018) investigated the microbial changes during the fermentation of Suan yu, showing that a 1:1:1 mixture of *L. plantarum* 120, *S. xylosus* 135, and *S. cerevisiae* 31, inoculated at 1% concentration and fermented at 25°C, influenced the microbial flora throughout the process. Despite the increasing interest in fish fermentation, research on the production and quality of fermented rainbow trout using specific starter cultures has not been encountered. The aim of this study was to produce fermented trout with *L. sakei* ATCC® 15521™ (L), *S.cerevisiae* ATCC® 9080™ (S), their combination (M), and a spontaneously fermented control group (F) and to monitor their quality throughout fermentation.

MATERIALS AND METHODS

Preparation of the starter cultures

Twice-subcultured starter cultures, *Lactobacillus sakei* ATCC® 15521™ was incubated in Man Rogosa and Sharpe (MRS, Merck, Germany) broth, and *Saccharomyces cerevisiae* ATCC® 9080™ was incubated in Yeasts and Molds (YM, DifcoTM, France) broth at 37°C for 24 hours for use in trout fermentation. After incubation, cell pellets were harvested by centrifugation at 10,000 x g for 10 minutes at 4°C and washed twice with sterile saline (0.9% NaCl). The pellets were resuspended in the same saline solution, and the cell concentrations were adjusted to 8-9 log cfu/g for *L. sakei* and 5-6 log cfu/g for *S. cerevisiae* by measuring the optical density (OD) at 600 nm. A mixed culture of *L. sakei* and *S. cerevisiae* in a 1:1 (v/v) ratio was used for inoculation. The starter cultures were stored at -80°C with 30% sterile glycerol solution.

Preparation of fermented rainbow trout

The fermented trout was prepared according to the suan-yu fermented carp fish production technique described by Zeng et al. (2013), with modifications based on preliminary studies of the fermentation process on sliced trout. The Black Sea rainbow trout used in the study were sourced fresh from Isparta Fish Market (July 2024), cleaned, and aseptically sliced into pieces weighing 100 ± 10 g at the Food Processing Laboratory of the Eğirdir Fisheries Faculty. A total of 8 fish, each weighing 1.5 ± 0.5 kg, were used, resulting in approximately 12 kg of fish. Rainbow trout slices (94% w/w) and curing ingredients—sucrose (2% w/w), salt (3% w/w), garlic powder (0.3% w/w), ginger powder (0.3% w/w), and sweet red pepper powder (0.3% w/w)—were kept at 4 °C for 2 days. Then cured samples were dried in a digital oven (Crystal, CKD 044 E, Germany) at 60 °C for 3 hours. After drying for fermentation, 1% (1 mL starter culture: 100 g cured fish) of the inoculation solution was homogeneously applied to the trout slices (Sürengil, 2024). The trout slices were divided into four groups, each containing 25 slices. These slices were tightly wrapped in stretch film and packed in polystyrene containers for fermentation, with two slices per package. The containers were sealed to prevent contamination and moisture loss during the fermentation process. Fermentation was conducted in an incubator (NÜVE ES110, Türkiye) at 24 ± 2°C for 14 days. Analyses were performed on all groups at day 0 (before inoculation), after curing, and on days 2, 4, 6, 8, 10, 12, and 14. Additionally, analyses were performed on fresh fish (T), cured fish (C), and fermented trout groups: without bacteria (F); with *L. sakei* starter culture (L); with *S. cerevisiae* starter culture (S); and with a combination of *L. sakei* and *S. cerevisiae* starter cultures (M).

Microbiological analyses

Microbiological analyses were performed in the Food and Processing Laboratory of Isparta University of Applied Sciences, Eğirdir Fisheries Faculty (AOAC 2000). In these analyses, 10 g of fish sample was transferred to a sterile bag under aseptic condi-

tions and homogenized with 90 ml of sterile peptone water (Maximum recovery diluent, MRD, Merk, Germany) solution in Stomacher (Seward 400, London, UK) for 1 minute. The microbial counts were determined using the pour plate method: LAB on De Man, Rogosa and Sharpe (MRS, Merk, Germany) agar medium at 96 hours at 30°C; Yeast-mold (YM) on Yeast Extract Glucose Chloramphenicol (YGC, Merk, Germany) agar medium at 24 hours at 30 °C; and Enterobacteriaceae (ENT) on Violet Red Bile Dextrose (VRBD, Merk, Germany) Agar medium at 24 hours for 30 °C. The analysis results were expressed as colony-forming units per gram (log CFU/g).

Biocochemical and proximal analyses

Moisture determination was performed by placing approximately 0.5 g of sample in a moisture measuring device (AND MX-50, Japan). The moisture content was calculated as a percentage of the weight loss. The total ash content (%) was determined by burning 2 g of the sample in a muffle furnace (Ash Furnace Nüve TS 500) at approximately 500°C for 4 hours. Fat content determination; a 5 g sample was first dried in an oven (WiseVen Witeg, Germany) at 105°C, and then crude fat was extracted using the Soxhlet method with diethyl ether. The amount of fat (%) in each the sample was calculated. Protein content determination; the Velp UD-20 protein pre-combustion unit was used according to the Kjeldahl method (Nx6.25) with the Velp UDK 142 protein distillation unit. As the exact fermentation time was not determined, the proximate analyses were performed on the 2nd day, when the first bacteria were inoculated. pH analysis; 10 g of the samples were weighed and 10 ml of distilled water was added and homogened in the homogenizer. The pH meter (Hanna HI221) probe was immersed in the prepared sample, and the reading was recorded (AOAC, 1990). Lactic acid value; a 10 g sample was taken, homogenized with 100 ml of pure water, and then titrated with 0.1 N NaOH to pH 8. The % lactic acid was determined based on the amount of NaOH consumed (Acton and Keller, 1974).

Determination of thiobarbituric acid (TBA) and total volatile basic nitrogen (TVB-N)

TBA analysis was performed according to the method by Gassem (2019). Ten grams of the sample were homogenized with 50 mL of distilled water. The pH was adjusted to 1.5 by adding 2.5 mL of 4 N HCl. Five milliliters of the distillate solution were boiled with 5 mL of TBA reagent (0.2883 g thiobarbituric acid in 100 mL of 90% acetic acid) for 35 minutes. The absorbance was measured at 535 nm using a spectrophotometer (PG INSTRUMENTS- T80+ UV/VIS), and MDA concentration was calculated as mg MDA per kg by multiplying the absorbance by 7.8.

TVB-N analysis was performed according to the method by Antonocoupoulos and Vyncke (1989). The sample was homogenized by adding 1-2 g of MgO to 10 g of the sample in Kjeldahl tubes, and the solution was distilled with 3% boric acid using the Velp UDK 142 protein distillation unit. The final distillate was titrated with 0.1 N HCl and the consumption was calculated based on the amount of acid used.

Sensory evaluation

Fermented fish produced was submitted for sensory evaluation to analyze whether the control sample (F) and the samples with mixed starter cultures (L, S and M) differed. A panel of 20 individuals, consisting of faculty members and graduate students with experience in seafood consumption at Isparta University of Applied Sciences, was selected, and the panelists were informed about the product. Fermented products at room temperature were coded with three-digit random numbers; they were evaluated for appearance, color, odor, taste, saltiness, texture and overall taste. A nine-point hedonic scale for evaluation; in which 1 point equals very dislike, 5 points equals moderate neither like nor dislike, and 9 points equals extremely liked. The sensory evaluation results were calculated by taking the mean and standard deviation of the panelists' scores (Zeng et al., 2014).

Statistical analysis

All analyses were conducted in three parallel groups in fermented fish groups, and one-way analysis of variance (ANOVA) was performed with the SPSS (version 22.0, IBM, USA) statistical package program, and the differences were determined with the Duncan Multiple Comparison Test. Values are expressed as "mean ± standard deviation".

RESULTS AND DISCUSSION

Microbiological results

Fermentation significantly influenced LAB development in different groups. While LAB was not detected in the naturally fermented F and L groups on the first day after heat treatment, the S group (inoculated with S. cerevisiae) had 5.78 ± 0.01 log CFU/g, and the M group had 4.82 ± 0.11 log CFU/g ($P<0.05$). LAB counts were performed before inoculation to ensure consistency and observe fermentation-related changes. The LAB presence in the S group is attributed to the natural microbiota of the fish and spontaneous fermentation, though its increase remained lower than in the inoculated groups. A synergistic effect between LAB and yeast was observed, but dominant species exhibited more pronounced growth, highlighting the role of starter cultures.

LAB counts in fermented fish with mixed cultures increased significantly throughout fermentation ($P<0.05$, Table 1). The initial LAB count was 2.39 ± 0.20 log CFU/g in fresh fish, 3.85 ± 0.27 log CFU/g in cured trout, and 3.16 ± 0.53 log CFU/g on day 0 after heat treatment. These values were obtained from preliminary analyses conducted prior to the fermentation process. LAB growth in naturally fermented trout was slower in the early stages than in starter culture-inoculated products ($P<0.05$), emphasizing the impact of controlled fermentation on microbial dynamics. The 6 log value, which is recommended for beneficial effects on gastrointestinal health (Hemarajata and Versalovic, 2013), was reached on different days depending on the group. Notably, in the L group, the highest value of 7.21 ± 0.11 log CFU/g was reached on the 2nd day, while in the M group, the highest value of 6.52 ± 0.09 log CFU/g was also observed on the 2nd day, indicating rapid growth at early stages of fermentation. Zeng et al. (2013) reported that the LAB count in carp suan-yu fermented fish inoculated with different mixed starter cultures was significantly different from the control group and gradually increased over 14 days, reaching values between 8.59 and 8.73 log CFU/g. However, the control group reached 8.31 log CFU/g with a slower increase by the 21st day, and the LAB count remained stable during

Table 1. Microbiological results in the fermented trout during fermentation (Log cfu/g).

DAY		0	2	4	6	8	10	12	14
LAB	F	3.16±0.53^{aA}	4.17±0.21^{aAB}	4.33±0.42^{aB}	5.01±0.12^{aB}	5.21±0.25^{aB}	5.34±0.15^{adB}	6.07±0.25^{aB}	6.01±0.21^{aB}
	L	3.16±0.53^{aA}	7.21±0.11^{dB}	8.56±0.04^{dC}	8.87±0.02^{cC}	9.12±0.35^{cdC}	9.34±0.66^{cC}	9.35±0.75^{cC}	9.37±0.05^{cC}
	S	3.16±0.53^{aA}	5.14±0.12^{bB}	6.25±0.11^{bC}	6.67±0.52^{bC}	7.11±0.24^{bCD}	7.87±0.01^{bD}	7.79±0.25^{bD}	7.61±0.02^{bC}
	M	3.16±0.53^{aA}	6.52±0.09^{bB}	7.75±0.17^{cC}	8.99±0.13^{cCD}	9.24±0.08^{cE}	9.27±0.04^{cE}	9.12±0.42^{cE}	9.07±0.11^{cE}
YM	F	<1	3.60±0.42^{aA}	4.73±0.02^{bB}	4.86±0.17^{bB}	5.11±0.13^{bB}	5.10±0.05^{bB}	5.15±0.42^{bB}	5.03±0.07^{bB}
	L	<1	3.67±0.12^{aA}	3.85±0.31^{aA}	3.72±0.03^{aA}	3.88±0.17^{aA}	4.19±0.13^{aA}	4.12±0.42^{aA}	4.21±0.06^{aA}
	S	<1	6.45±0.18^{cA}	6.58±0.15^{cA}	6.92±0.14^{cA}	6.98±0.32^{cA}	6.93±0.15^{cA}	6.91±0.31^{cA}	6.87±0.07^{cA}
	M	<1	5.85±0.37^{bA}	5.90±0.11^{bcA}	6.15±0.15^{cA}	6.82±0.01^{cA}	6.86±0.11^{cA}	6.85±0.21^{cA}	6.52±0.33^{cA}
ENT	F	2.29±0.05^{aA}	2.51±0.04^{aA}	2.42±0.3^{aA}	2.31±0.22^{aA}	2.30±0.2^{aA}	2.28±0.17^{aA}	<1	<1
	L	2.29±0.05^{aA}	2.17±0.03^{aA}	<1	<1	<1	<1	<1	<1
	S	2.29±0.05^{aA}	2.13±0.03^{aA}	2.12±0.2^{aA}	<1	<1	<1	<1	<1
	M	2.29±0.05^{aA}	<1	<1	<1	<1	<1	<1	<1

F: naturally fermented trout, L: *L. sakei* starter culture added, S: *S. cerevisiae* starter culture added, M: *L. sakei* and *S. cerevisiae* starter cultures added
Values with different uppercase letters in the same row indicate significant differences between days (P<0.05).
Values with different lowercase letters in the same column indicate significant differences between groups (P<0.05).

subsequent ripening. Similar to the current study, the LAB counts in samples inoculated with starter cultures were significantly different from those in the control groups (P < 0.05). This indicates that L. sakei adapted well to the fish during fermentation and became the dominant flora.

Yeast and mold (YM) counts varied significantly among the fermentation groups (P < 0.05, Table 1). While YM was detected in the F and L groups 3.60±0.42 and 3.67±0.12 on the 2th days, it was 6.45±0.18 log CFU/g in the S group, which received *S. cerevisiae*, and 5.85±0.37 log CFU/g in the M group. In the naturally fermented F group, a gradual increase was observed, reaching 5.15 ± 0.42 log CFU/g by day 12. The L group exhibited the lowest YM counts throughout fermentation, with values remaining below 4.21 ± 0.06 log CFU/g. In contrast, the S and M groups, which contained *S. cerevisiae*, showed significantly higher yeast counts from the early stages. The S group reached its highest value (6.98 ± 0.32 log CFU/g) on day 8, while the M group followed a similar trend, peaking at 6.86 ± 0.11 log CFU/g on day 10. These results indicate that the addition of *S. cerevisiae* significantly influenced yeast proliferation, whereas the *L. sakei*-inoculated L group exhibited lower YM growth, likely due to LAB-driven competition or inhibitory effects. These results showed that LAB were the dominant microorganisms during early fermentation due to the intense fermentation activity in fish meat. *L. sakei* exhibited strong competitive power, growing rapidly during trout fermentation. In rainbow trout fermented with mixed cultures, *S. cerevisiae* and *L. sakei* demonstrated strong competitiveness, inhibiting the growth of other microorganisms, including yeast, through the production of organic acids and bacteriocins (P > 0.05). Similarly, Liu et al. (2021) observed that YM did not grow in the initial stages of fermentation in suan-yu products prepared without the addition of *L. plantarum* B7 and LAB. After 7 days, the pH of the fermentation stabilized, and acid-resistant yeasts reached a maximum value of 4 log CFU/g. Kılınç and Sürengil (2016) found that LAB was the dominant flora compared to YM during fermented pastrami production in whiting. LAB counts increased throughout the storage period, suppressing *Enterobacteriaceae* counts at the beginning of storage.

The *Enterobacteriaceae* (ENT) values are shown in Table 1. After heat treatment, the ENT count was 2.29 ± 0.05 log CFU/g on the first day. There was no significant difference between the groups on the first day (P > 0.05). The ENT counts were significantly lower in starter culture-inoculated groups during fermentation compared to the naturally fermented group (P < 0.05). During fermentation, ENT counts exhibited a significant decrease in starter culture-inoculated groups compared to the naturally fermented F group (P < 0.05). In the F group, ENT counts gradually declined, becoming undetectable (<1 log CFU/g) by day 12. In contrast, the L group showed a rapid reduction, with ENT dropping below detectable levels by day 4. The S group reached undetectable levels by day 6, and the M group showed a rapid decrease, becoming undetectable as early as day 2, as indicated in the table 1. The observed reductions in ENT levels align with previous findings on the antimicrobial effects of LAB. The acidification caused by LAB activity likely inhibited pathogenic and spoilage microorganisms, creating unfavorable conditions for ENT survival. Adab et al. (2018) emphasized the role of LAB in inhibiting enterobacteria through organic acid production, a mechanism that contributes to the stability of fermented meat and fish products. Likewise, Anihouvi et al. (2007) reported significant decreases in ENT counts in fermented fish inoculated with mixed starter cultures, reinforcing the competitive advantage of LAB over enterobacteria. Additionally, Yang et al. (2012) and Darbandi et al. (2022) highlighted the role of LAB-produced bacteriocins in suppressing ENT and other foodborne pathogens. The combination of *L. sakei* and *S. cerevisiae* (M group) demonstrated the most rapid suppression of ENT, suggesting a synergistic effect between these cultures in improving microbial safety. The results of this study confirm that controlled fermentation using starter cultures enhances food safety by effectively suppressing ENT growth in fermented fish products.

Chemical results

The pH and titratable acidity (TA) values of the fresh rainbow trout used in the study on the first day were determined as 6.70 ± 0.06 and 0.17 ± 0.01, respectively, while the values for the cured trout were 7.10 ± 0.08 and 0.21 ± 0.05. In the fermented products,

the pH values of the natural and starter culture-applied samples were observed to be lower than those detected in the control group during fermentation, as shown in Figure 1. While the pH of fresh fish meat was 6.70, the pH values of the L, S, and M groups decreased to 4.01, 4.18, and 4.09, respectively, within 14 days. However, the pH of the control group remained at values between 5.37 and 5.08 until the 10th day of fermentation, eventually decreasing to 4.52. These shows that the pH values are significantly different ($P < 0.05$) compared to products prepared with mixed starter cultures.

In the fermented products, the TA values of the natural and starter culture-applied samples were observed to be higher than those detected in the control group during fermentation, as shown in Figure 2. While the TA of fermented products increased significantly ($P < 0.05$), the F, L, S, and M values increased from 0.17 to 1.05, 3.07, 2.18, and 3.13, respectively, during 14 days of fermentation and storage. The TA values increased in parallel with the LAB count throughout the fermentation periods ($P < 0.05$) but remained almost unchanged at the end of storage, i.e., during ripening ($P > 0.05$). Although there was no statistically significant change in the F group samples, gradual and slow increases were observed throughout storage ($P > 0.05$). However, the TA of the control group was lower than that of the products prepared with starter cultures ($P < 0.05$). Significant differences were observed in product M compared with other groups ($P < 0.05$), although no significant change occurred after the 4th day ($P > 0.05$). Similarly, no significant changes were observed in the L group from day 4 or in the S group from the 8th day ($P > 0.05$). In previous studies conducted on fermented fish, a similar result was obtained in Suanyu products, and it was reported that the pH value in fermented fish using starter culture decreased below 5 after 1 week (Zang et al., 2018; Lv et al., 2022). The decrease in pH is attributed to the increase in organic acids in meat due to LAB development during fermentation (Hwanhlem et al., 2011). It has also been stated that the rapid decrease in pH to 4.5 within the first 48 hours of fermentation is essential for preventing the development of pathogens and harmful bacteria, ensuring the safety, texture, and flavor development of fermented products (Kılınç, 2004; Panda et al., 2011; Benkerroum, 2013). Acid changes and the density and increase of various organic acids (mostly lactic acids) are crucial for the fermentation of fish (Kuley et al., 2020). The gradual decrease in pH in all products during fish fermentation, particularly the rapid decrease in pH and the increase in acid value in the groups where *L. sakei* was applied as a starter culture, indicate that trout meat adapted well to the lactic acid environment. The significantly higher lactic acid concentrations in the starter culture-inoculated groups further support this finding.

Nutritional component analyses of natural and starter culture-applied samples of fermented products are shown in Table 2. The nutrient compositions of fresh rainbow trout and fermented products differed statistically significantly in terms of protein, moisture, fat and ash contents ($P < 0.05$). The presence of high protein content and low moisture in all fermented fish indicates that fermented fish can be consumed as a nutritious and safe product. The results reveal that all fermented fish products, whether naturally fermented or inoculated with starter cultures, showed notable changes compared to fresh trout, indicating the significant influence of fermentation processes on the nutritional profile of the fish ($P < 0.05$). The addition of starter cultures further enhanced protein content, with the L group and M group showing higher protein levels (25.03 ± 0.45 g/100 g and 26.18 ± 0.48 g/100 g, respectively) compared to the naturally fermented group (F), which had a protein content of 20.08 ± 0.51 g/100 g. The synergistic effect of the combined LAB and yeast cultures in the M group may have accelerated the breakdown of proteins, leading to more efficient protein degradation and an overall increase in protein content. The L group (*L. sakei*) also showed a higher fat content (3.00 ± 0.05 g/100 g) compared to the naturally fermented group (2.90 ± 0.03 g/100 g), though it

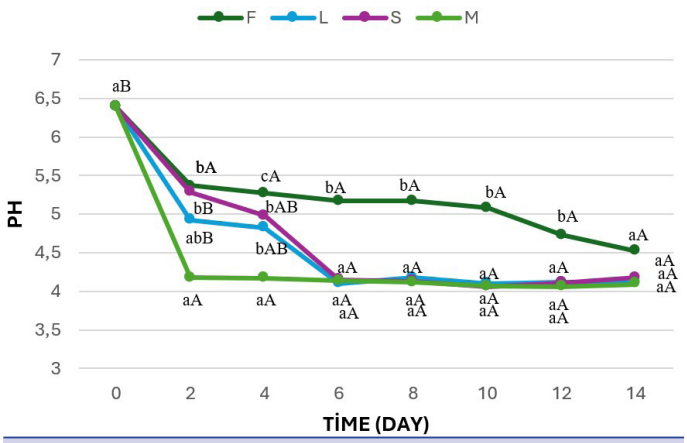

Figure 1. The pH in fermented trout during fermentation.
F: naturally fermented trout, L: with *L. sakei* starter culture added, S: with *S. cerevisiae* starter culture added, M: with *L. sakei* and *S. cerevisiae* starter cultures added
Values with different uppercase letters in the same row indicate significant differences between days (P<0.05).
Values with different lowercase letters in the same column indicate significant differences between groups (P<0.05).

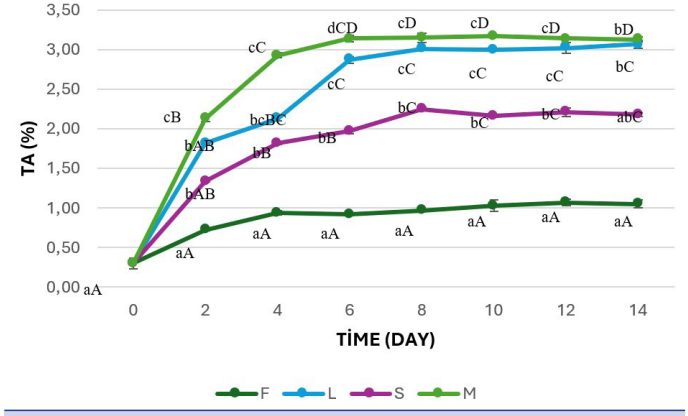

Figure 2. The titratable acidity in fermented trout during fermentation.
F: naturally fermented trout, L: with L. sakei starter culture added, S: with S. cerevisiae starter culture added, M: with L. sakei and S. cerevisiae starter cultures added
Values with different uppercase letters in the same row indicate significant differences between days (P<0.05).
Values with different lowercase letters in the same column indicate significant differences between groups (P<0.05).
Values with different lowercase letters in the same column indicate significant differences between groups (P<0.05).

Table 2. Nutritional component of fermented trout (g/100 g).

	Protein	Fat	Moisture	Ash
T	19.39 ± 0.35^{a}	2.05 ± 0.04^{a}	76.36 ± 0.15^{a}	2.63 ± 0.01^{a}
F	20.08 ± 0.51^{b}	2.90 ± 0.03^{bc}	69.0 ± 0.11^{b}	3.09 ± 0.04^{b}
L	25.03 ± 0.45^{cd}	3.00 ± 0.05^{c}	68.6 ± 0.15^{b}	3.10 ± 0.08^{b}
S	24.68 ± 0.76^{c}	2.47 ± 0.12^{b}	68.9 ± 0.17^{b}	3.01 ± 0.02^{b}
M	26.18 ± 0.48^{d}	3.96 ± 0.07^{d}	68.7 ± 0.21^{b}	3.11 ± 0.01^{b}

T: fresh rainbow trout, F: naturally fermented trout, L: *L. sakei* starter culture added, S: *S. cerevisiae* starter culture added, M: *L. sakei* and *S. cerevisiae* starter cultures added
Values with different uppercase letters in the same row indicate significant differences between days (P<0.05).
Values with different lowercase letters in the same column indicate significant differences between groups (P<0.05).

was slightly lower than the M group. The S group (*S. cerevisiae*) showed a moderate increase in fat content (2.47 ± 0.12 g/100 g), which could indicate that yeast alone has a less pronounced effect on lipid metabolism in fermented fish. The L, S, and M groups showed similar moisture levels (68.6 ± 0.15 g/100 g to 69.0 ± 0.11 g/100 g), suggesting that the addition of starter cultures (whether LAB or yeast) had similar effects on moisture retention during fermentation. This is consistent with the idea that fermentation not only reduces moisture but also modifies the texture and enhances the overall flavor profile of the fish. Ash content, which is an indicator of the mineral content, was highest in the M group (3.11 ± 0.01 g/100 g) and lowest in the T group (2.63 ± 0.01 g/100 g). The increase in ash content during fermentation is expected, as minerals from the fish tissue become more concentrated due to the loss of moisture. The addition of starter cultures, particularly the combination of LAB and yeast in the M group, may have contributed to enhanced mineral retention by affecting the solubility of mineral compounds during fermentation. This is in line with findings from other studies, where the fermentation process improved the mineral content of fermented products by reducing water content and concentrating mineral elements. Xu et al. (2010) reported an increase in fish meat protein values in fermented silver carp sausage using *Pediococcus pentosaceus* during a 48-hour fermentation period. The gradual increase in protein is attributed to the degradation of salt-soluble and water-soluble proteins as pH decreases. Wang et al. (2017) found that adding LAB could increase acid activity in fish meat, thereby promoting a decrease in moisture and a parallel increase in lipid content and protein degradation. Thus, fermentation of fish meat with starter cultures causes significant changes in nutritional content due to the rapid proliferation of LAB and a rapid decrease in pH ($P < 0.05$).

In the fermented products, total volatile basic nitrogen (TVB-N) values of natural and starter culture-applied samples were observed to be higher in the control group compared to the starter culture-added groups during fermentation, as shown in Figure 3. In all starter culture-inoculated groups, TVB-N increased to 21.93, 19.91, and 17.13 mg/100 g in the L, S, and M groups, respectively, at the end of storage. According to the quality classification based on chemical criteria, samples with TVB-N values below 25 mg/100 g are considered very good, those with values around 30 mg/100 g are classified as good, samples with values between 30–35 mg/100 g are considered marketable, while those exceeding 35 mg/100 g are regarded as spoiled (Huss, 1988; Varlık et al., 1993). The TVB-N of naturally fermented trout increased from

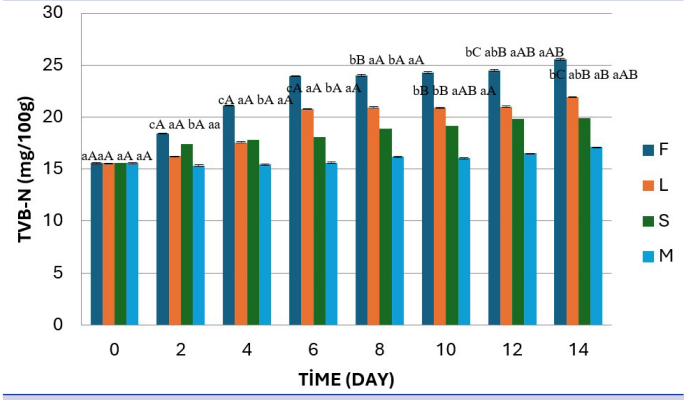

Figure 3. The TVB-N (mg/100g) in fermented trout during fermentation.
F: naturally fermented trout, L: with L. sakei starter culture added, S: with S. cerevisiae starter culture added, M: with L. sakei and S. cerevisiae starter cultures added
Values with different uppercase letters in the same row indicate significant differences between days (P<0.05).
Values with different lowercase letters in the same column indicate significant differences between groups (P<0.05).

15.58 ± 0.06 mg/100 g to 25.57 mg/100 g, remaining within the acceptable freshness range. Although it reached the freshness standard (25 mg/100 g–30 mg/100 g), this TVB-N remained below the national standard (35 mg/100 g). TVB-N is a commonly used index for evaluating fish freshness (Oyelese et al., 2013), and it increases steadily during fermentation due to the production of alkaline, nitrogen-containing substances by enzymatic and microbial decomposition of fish (Weiner et al., 2015). These compounds also contribute to the characteristic flavor of fermented fish (Debevere and Boskou, 1996). Preventing the increase in TVB-N and improving the safety of fermented fish can be achieved using mixed cultures that facilitate carbohydrate breakdown. TVB-N contents in group L and S samples increased gradually during fermentation, but the TVB-N content of naturally fermented trout was significantly higher than that of products fermented with starter culture ($P < 0.05$). Hu et al. (2008) reported similar findings, noting that LAB and *S. cerevisiae* applications slowed TVB-N accumulation by producing lactic acid and bacteriocins, consistent with our study.

In the fermented products, thiobarbituric acid (TBA) values of the natural and starter culture-applied samples were observed to be higher in the control group compared to the starter culture-add-

ed groups during fermentation, as shown in Figure 4. TBA values are often used as an indicator of lipid oxidation in foods (Wang et al., 2016). According to Sikorski et al. (1990), the TBA value of fish products should be <5 mg/kg. In this study, all products remained below this limit. The growth rate of TBA content in naturally fermented trout was higher than that in trout fermented with starter culture ($P < 0.05$). The TBA content of naturally fermented trout was significantly higher ($P < 0.05$) than that of trout inoculated with starter culture. Bao et al. (2018) reported initial TBA contents of natural, L, W, and LW samples ranging from 0.03 to 0.72 mg MDA/kg. By the end of a 5-day fermentation period, the highest TBA value reached 1.93 mg MDA/kg in the natural group, while the LAB group exhibited the lowest TBA value at 1.21 mg MDA/kg. LAB has been reported to have antioxidant effects on unsaturated fatty acids, suppressing the increase in TBARS in fermented fish (Zeng et al., 2013) and dry sausages (Sun et al., 2016). Consistent with these findings, starter cultures in this study effectively prevented oxidation of unsaturated fatty acids, demonstrating strong antioxidant activity (Zeng et al., 2014).

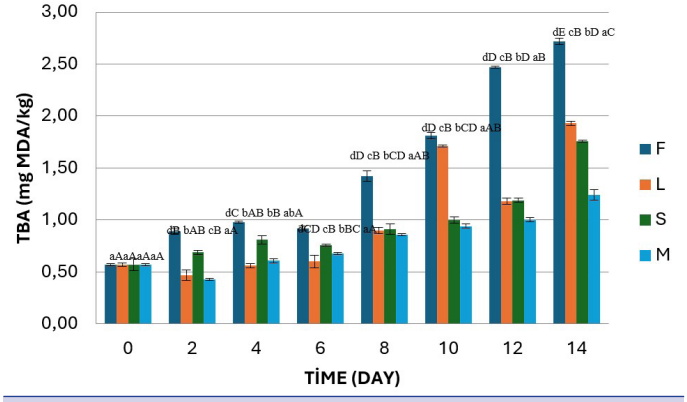

Figure 4. The TBA (mg MDA/kg) in fermented trout during fermentation.

F: naturally fermented trout, L: with L. sakei starter culture added, S: with S. cerevisiae starter culture added, M: with L. sakei and S. cerevisiae starter cultures added
Values with different uppercase letters in the same row indicate significant differences between days ($P<0.05$).
Values with different lowercase letters in the same column indicate significant differences between groups ($P<0.05$).

Sensory evaluation

The sensory properties of fermented trout are shown in Figure 5. It was determined that the appearance and color sensory properties of the L and F groups were similar to each other ($P>0.05$), while the S group was the most liked in terms of these criteria ($P<0.05$). The sensory properties of the M and S groups were statistically similar ($P>0.05$), indicating positive sensory effects in the presence of S. cerevisiae in the product. Controlled humidity adjustment as a result of controlled heat treatment before fermentation created a similar sensory salinity effect for all groups; therefore, no significant difference was observed between them ($P>0.05$).

The most liked group in terms of taste, texture, and overall appreciation, which was significantly different from the other groups, was the product with mixed starter culture ($P<0.05$). These findings are consistent with the expected results that using starter culture in fermented products can enhance sensory quality (Abdel-Naeem et al., 2021; Christ-Ribeiro et al., 2021). This improvement is likely due to the faster pH decrease, which may accelerate product ripening and improve taste and appearance. Liu et al. (2021) reported that the high-salt (15-20%) Suan-yu products they produced contained high-quality proteins and had an intense acidic taste without a fishy smell or taste, further supporting the benefits of this method of production.

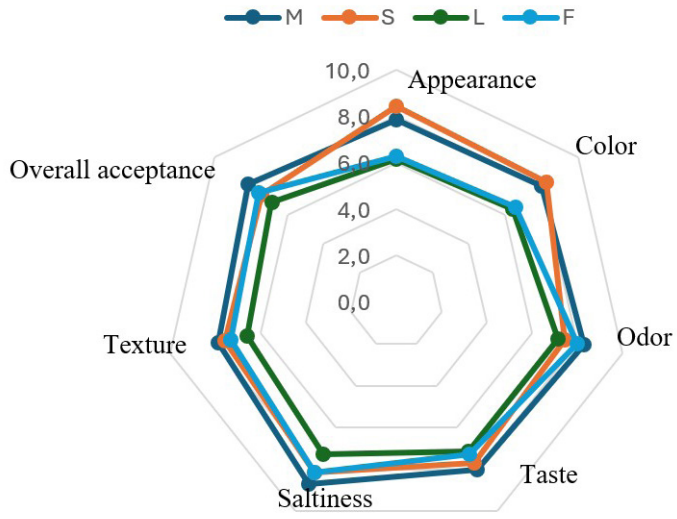

Figure 5. Sensory properties of fermented trout.

F: naturally fermented trout, L: with L. sakei starter culture added, S: with S. cerevisiae starter culture added, M: with L. sakei and S. cerevisiae starter cultures added
Values with different uppercase letters in the same row indicate significant differences between days ($P<0.05$).
Values with different lowercase letters in the same column indicate significant differences between groups ($P<0.05$).

CONCLUSION

This study effectively demonstrated the synergistic contributions of *L. sakei* and *S. cerevisiae* in enhancing the sensory, nutritional, and microbiological qualities of fermented trout. The findings underline their roles in providing appropriate acidity, salinity, and stability at low temperatures, while also contributing to higher fat and protein content, as well as enhanced antioxidative and antimicrobial effects. The achievement of the recommended 6 log value for probiotics critical for gastrointestinal health at different stages of fermentation highlights the efficiency of the starter cultures, particularly in groups L and M, which achieved this threshold as early as the second day. They was achieved on the 12th day in the F group (6.07 ± 0.25 log cfu/g), on the 4th day in the S group (6.25 ± 0.11 log cfu/g), on the 2nd day in the M group (6.52 ± 0.09 log cfu/g), and on the 2nd day in the L group (7.21 ± 0.11 log cfu/g).

The study also confirmed the ability of starter cultures to mitigate lipid oxidation (TBA) and total volatile base nitrogen (TVB-N) levels by producing lactic acid and bacteriocins. These properties emphasize the potential of using mixed starter cultures to ensure the safety, quality, and shelf life of fermented fish products.

The conclusion that fermented trout can be successfully prepared and consumed under low-salinity and room-temperature conditions is significant for broadening the scope of fish fermentation techniques and consumer accessibility. Fermented trout can be successfully products prepared and consumed by slicing 1.5–2 kg trout into 100–150 g portions when stored at low salinity and room temperature, based on their technological and sensory properties.

Conflict of Interests: The authors declare that there is no potential conflict of interest.

Ethics Committee Approval: Ethics approval was not required for this study.

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Research Article

Effect of Polypropylene Microplastic and Florfenicol Antibiotic on Some Hormonal and Haematological Biomarkers in Yellowfin Seabream (*Acanthopagrus latus*)

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ABSTRACT

Microplastics (MPs) and antibiotics, such as florfenicol (FFC), are emerging pollutants affecting aquatic ecosystems. This research examined the separate and joint impacts of MPs and FFC on hematological and hormonal indicators in yellowfin seabream *(Acanthopagrus latus)*. The fish were categorized into four groups according to their dietary treatments: a control group with a standard diet and three experimental groups receiving diets containing 15 mg/kg of FFC, 100 mg/kg of MPs, or both for 10-day. The ELISA method was used to measure hormones in plasma. Blood collection occurred on the first, fourth, seventh, and fourteenth days after feeding stopped to evaluate FFC concentration in the plasma and various hematological and hormonal parameters. Exposure to FFC and MPs, alone or together, considerably decreased red blood cell, hemoglobin levels, hematocrit values, mean corpuscular hemoglobin, mean corpuscular hemoglobin, white blood cell, lymphocyte, and erythropoietin concentrations. Neutrophil and cortisol levels increased. Mean corpuscular volume was elevated only in the group receiving both FFC and MPs. After a 14-day recovery period, all measured parameters returned to baseline levels in the FFC-only group. Co-exposed group showed the highest concentration of FFC in the plasma on the first day. The groups administered MPs, individually or collectively, exhibited a reduction in thyroid hormones. These findings indicate that both MPs and FFC induce anemia and stress in yellowfin seabream, with co-exposure exacerbating these effects. Although the toxic effects of FFC were temporary, the lasting presence of MPs indicates potential long-term risks.

Keywords: Yellowfin seabream, *Acanthopagrus latus*, Florfenicol, Haematological/ Hormonal indices, Microplastics

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INTRODUCTION

Microplastics (MPs), artificial polymers smaller than 5mm in size, are predominantly found in the pharmaceutical and cosmetics industry (Athira et al., 2024). They enter marine environments through wastewater treatment plant leaks and surface water runoff (Patil et al., 2024). MPs are classified into various types based on the compounds used, with polypropylene and polyethylene and their by-products being commonly found in marine environments (Maghsodian et al., 2021). At present, MPs are a significant pollution risk in coastal ecosystems, leading to growing public concern (Athira et al., 2024). MPs can be transferred through the food chain when aquatic organisms consume them, resulting in their accumulation in different fish species. This accumulation in the essential tissues of fish can cause harmful effects on their hematological indices, immune system function, oxidative stress, and DNA integrity (Li et

al., 2025; Sankar et al., 2025). Aquatic environments are heavily influenced by anthropogenic, resulting in the presence of multiple types of pollutants, such as antibiotics, cyclic aromatic hydrocarbons, heavy metals, MPs, and pesticides. MPs have the capacity to absorb various pollutants, particularly antibiotics, which enhances their bioavailability and intensifies the toxic effects on aquatic organisms (Banaee et al., 2023).

Antibiotics such as florfenicol (FFC) are designed for application in veterinary medicine and aquaculture and enter ecosystems through wastewater from animal and aquatic farming (Bardhan et al., 2022). Antibiotics can easily penetrate the cell membrane due to their high lipophilic properties and generate reactive oxygen species that may harm cells (Zheng et al., 2025). Previous researchers have observed a toxic effect of FFC on blood indices and hematopoietic tissues of Nile tilapia (*Oreochromis niloticus)* (Bardhan et al., 2024).

Although there is growing awareness of the individual hazards posed by MPs and antibiotics (Akbari & Jaafari, 2025; Yi et al., 2025), there remains a considerable lack of understanding regarding their combined effects on aquatic. Most research has concentrated on the impact of single pollutants; however, in natural settings, organisms frequently encounter multiple stressors at once. Therefore, additional studies are essential to enhance our comprehension of how various pollutants interact and their effects on marine ecosystems. This understanding could facilitate the creation of effective strategies to tackle this issue.

The yellowfin seabream (*Acanthopagrus latus*) is a marine species known for its resilience to challenging environmental conditions. It is widely fished and farmed, especially in Iran, underscoring its importance for both ecological and economic research (Shirmohammadi et al., 2017). Changes in hematological and hormonal parameters, which serve as biological indicators, are often influenced by physiological and environmental and hormonal factors. Therefore, when fish are exposed to stressors, it is expected that certain hematological and hormonal parameters will undergo changes. In fact, the hematological and hormonal characteristics of fishes are crucial evidence of their physiological stages and reflect the relationship between the aquatic ecosystem and their health (Harikrishnan et al., 2024; Azam, 2025). The enzyme-linked immunosorbent assay (ELISA) is a highly sensitive, specific, and reproducible method for measuring hormones (Azam et al., 2025), making it the preferred technique for this study. The aim of this study was to assess the hypothesis that simultaneous exposure to MPs and FFC enhances hematological and hormonal stress responses in yellowfin seabream more than exposure to either substance individually. Also, in this study, thyroid hormones (T3 and T4) were evaluated as critical biomarkers to assess endocrine disruption potential of MPs and FFC in fish.

MATERIALS AND METHODS

Chemicals

The Jampilen Petrochemical Company in Iran supplied the polypropylene powder (less than 44 µm). The Rooyan Darou Company in Iran provided the FFC. Kits for Hemoglobin (Hb; Code: THB-Z-100) are sourced from ZiestChem in Iran, while erythropoietin (EPO; Code: RK02771) is obtained from ZellBio in Germany. Additionally, triiodothyronine (T3; Code: E-T3), thyroxine (T4; Code: T4-192-10), and cortisol (Code: E-S-2601) kits are supplied by IDEAL in Iran. All additional chemical substances needed for the analyses were purchased from Merck in Germany. Florfenicol ($C_{12}H_{14}Cl_2FNO_4S$; MW 358.2 g/mol) is a broad-spectrum bacteriostatic antibiotic that inhibits protein synthesis by binding to the 50S ribosomal subunit. It exhibits moderate lipophilicity (log Kow = 3.2), water solubility of 1.1 g/L at 25°C, and pKa of 8.7. Polypropylene microplastics are hydrophobic polymers (water contact angle ~100°) with low density (0.85-0.92 g/cm³) with a typical melting point of 160–170°C.

Fish maintenance

A total of 144 young yellowfin seabream that were healthy had an average weight of 41.12 ± 10.2 g. These fish were obtained from a private company located in Khuzestan province, Iran. For a period of 10 days, the fish were adjusted to their new environment in twelve 300 L tanks. During this adaptation phase, they were fed a commercial pelleted diet provided by Beyza 21 Manufacturing Company in Iran. The water conditions and the nutritional content of their basic diet followed the standards outlined by Shirmohammadi et al. (2024) for yellowfin seabream. The fish received two feedings daily, which totaled 2% of their body weight. To maintain good water quality, approximately 80% of the water in the tanks was replaced each day to remove waste. Throughout the study, the water's physicochemical properties were regularly checked and kept at ideal levels.

Preparation of trial diet

Initially, the primary diets were pulverized. Subsequently, the FFC and MPs were incorporated into the mixture in the preferred ratios, along with water to create a thick paste. A meat grinder was then used to shape this paste into noodles. After that, the noodles were dried in the air and cut into suitable lengths. They were then frozen at -20 °C until ready for use (Banaee et al., 2023).

Experimental design and sampling

After the adjustment period, the fit fish were randomly split into four groups, with three replications in each group, totaling 12 fish per group. The fish involved in the experiment were grouped as follows:

Control: received a standard commercial diet without any additives.

FFC: was given a diet containing 15 mg/kg of FFC.

MPs: had a diet including 100 mg/kg of polypropylene.

FFC+MPs: consumed a diet that included 15 mg/kg of FFC and 100 mg/kg of polypropylene.

Throughout the 10-day trial, the fish were provided with 2% of their body weight in food two times a day. They were served the experimental diet in the morning and the standard diet in the afternoon (Del Piano et al., 2023). The amounts of polypropylene utilized in this study were determined based on earlier toxicity studies conducted on fish (Jeyavani et al., 2023; Yedier et al., 2023). The chosen therapeutic dose of FFC aimed to assess any potential harmful effects of the treatment on yellowfin seabream.

This evaluation aimed to determine the safety and tolerability of the administered dose concerning aquatic health (Shirmohammadi et al., 2024). Any observable changes in fish behavior were monitored daily.

On the 1st, 4th, 7th, and 14th days after stopping the feeding, three fish from each tank were randomly selected and sedated with a solution of 200 µl/L of 2phenoxyethanol. Blood was collected from the tail vein using a syringe treated with heparin and was split into two parts. One part was used for analyzing hematology parameters, while the other part was spun in a centrifuge at 6000 rpm for 10 min to assess hormone levels and FFC build-up. The plasma obtained was stored at -80°C (Shirmohammadi et al., 2017). The tests were performed following the regulations of the National Ethical Committee for Animal Research in Iran.

FFC accumulation assay

Plasma FFC levels were determined using High-Performance Liquid Chromatography (HPLC) equipped with a UV detector (Model K2500, KNAUER, Germany), following Jangaran Nejad et al. (2017). In summary, 1 mL of plasma was combined with 4 mL of ethyl acetate. The supernatant was then evaporated with nitrogen at 40°C for 45 minutes, and the remaining substances were dissolved again in 1 mL of mobile phase and 0. 5 mL of hexane. After the centrifugation process (16,000 rpm for 20 min), the aqueous phase was filtered (0.45 µm nylon) and analyzed (20 µL injections). Results were expressed as µg/mL.

Haematological assay

Heparinized blood was mixed with NattHerrick's solution at a ratio of 1:30 to measure the white blood cell (WBC; $10^3/mm^3$) and red blood cell (RBC; $10^6/mm^3$) counts using a hemocytometer. Giemsa stained smears were employed for the differential leukocyte counts. A microhematocrit centrifuge (Hettich, Germany) was utilized to find the hematocrit (Ht, %), and Hb (g/dL) was assessed colorimetrically using the cyanmethemoglobin method. RBC indices including the average corpuscular volume (MCV; fl/cell), average corpuscular hemoglobin (MCH; pg/cell), and average corpuscular hemoglobin concentration (MCHC; g/dL) were also calculated (Shirmohammadi et al., 2018).

Hormonal assay

Plasma cortisol, EPO, T3, and T4 levels were quantified via ELISA using a DYNEX DS2 plate reader (USA), following kit protocols. Results were reported as ng/mL (cortisol), mU/mL (EPO), and ng/mL (T3, T4) (Barry et al., 1993; Lai et al., 2006; Azam et al., 2025). Initially, 50 µL of each plasma sample and standard was added to the wells of a 96-well microplate. Next, 100 µL of enzyme-conjugated antibody was dispensed into all wells (except the blank control wells), followed by incubation at room temperature (25°C) for 2 hours in the dark. After incubation, the microplates were washed five times with a wash buffer (saline containing surfactant and the preservative ciprofloxacin hydrochloride). Subsequently, 100 µL of 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added to each well, and the plates were incubated for 35 minutes at 25°C. The reaction was then stopped by adding 1 N sulfuric acid. After 10 minutes, the absorbance was measured at 450 nm (with a reference wavelength of 630 nm) using a microplate reader. The concentration of the samples was determined using the standard curve and the corresponding line equation derived from it.

Statistical analysis

Data were expressed as means ± standard deviation (SD; n=9). Normality was assessed with the Shapiro-Wilk test. One-way ANOVA with Duncan's post hoc test (IBM-SPSS v26.0, Chicago, USA) evaluated inter-group differences across sampling times, with significance set at $p < 0.05$

RESULTS AND DISCUSSION

The results indicated that the group exposed to both FFC and MPs showed the highest concentration of FFC in plasma on the first day after exposure ($p < 0.05$; Figure 1). This suggests that MPs could act as a carrier for adsorbing antibiotic residues, facilitating their uptake into aquatic tissues (Shirmohammadi et al., 2024). The study observed a decreasing trend ($p < 0.05$) over the 14-day recovery period in plasma levels of FFC, likely attributed to its metabolism. This finding aligns with Kverme et al. (2019), who reported similar results in lumpfish (*Cyclopterus lumpus*) treated with FFC for the same duration.

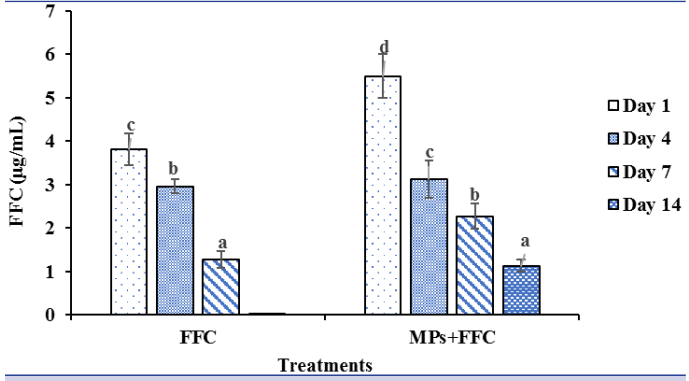

Figure 1. FFC accumulation in plasma at different times (Mean ± SD; n=9). The fish that were tested on various days within the same groups showed notable differences, which were marked by different letters ($p < 0.05$).

The results of this study showed that exposure to FFC and MPs, whether separately or together, led to a notable decrease in RBC, Hb, Ht, and WBC ($p < 0.05$; Figures 2a, b, c and 3a). The observed hematological reductions suggest a potential risk of anemia and immune dysfunction, likely attributable to impaired erythropoiesis in hematopoietic tissues, oxidative damage to cellular membranes, and possible internal hemorrhaging (Kondera et al., 2020; Harikrishnan et al., 2024). Comparable hematological disturbances were noted in rainbow trout (*Oncorhynchus mykiss*) subjected to FFC (Shiry et al., 2020) and in zebrafish (*Danio rerio*) exposed to MPs (Ammar et al., 2023).

The research discovered that being exposed to FFC and MPs, whether separately or in combination, led to a notable reduction in MCH and MCHC levels. Conversely, the presence of both substances was linked to an increase in MCV values ($p < 0.05$; Figure

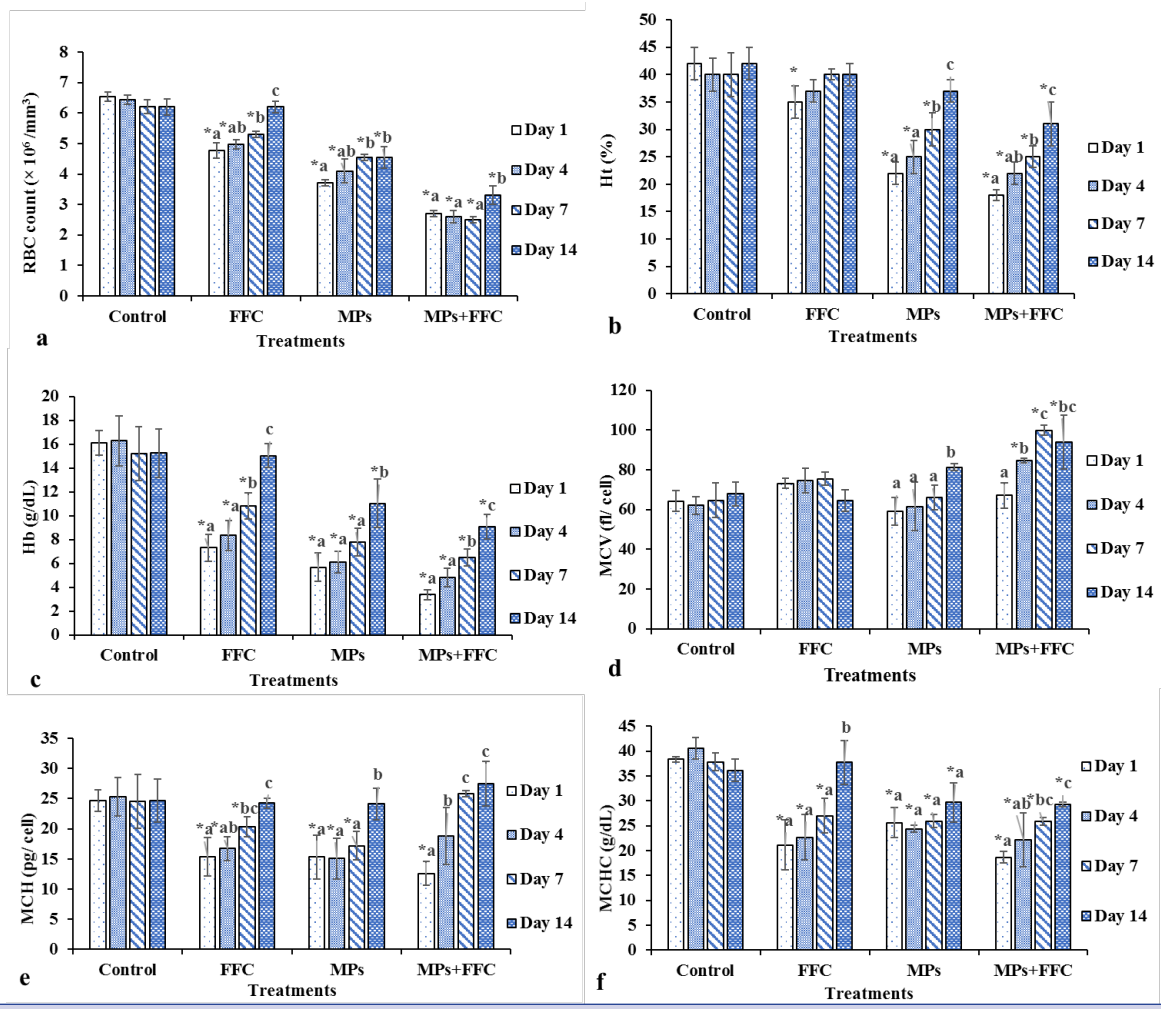

Figure 2. The impact of feeding yellowfin seabream with FFC and MPs, either separately or together, on hematological factors (a) red blood cell (RBC), (b) hematocrit (Ht), (c) Hemoglobin (Hb), (d) Mean corpuscular volume (MCV), (e) Mean corpuscular hemoglobin (MCH) and (f) Mean corpuscular hemoglobin concentration (MCHC) (Mean ± SD; n=9). The fish that were tested on various days within the same groups showed notable differences, which were marked by different letters. An asterisk (*) indicated a meaningful difference between the treated group and the control group ($p < 0.05$).

2d, e, f). The alterations in these indicators are typically associated with variations in the quantity and dimension of RBCs, as well as the synthesis of Hb in fish exposed to pollutants (Ammar et al., 2023). Elevated MCV and decreased MCHC indicate macrocytic and hypochromic anemia, resulting from impaired erythropoiesis and immature RBCs in circulation (Clauss et al., 2008). Similarly, exposure to chloramphenicol led to decreased levels of MCH and MCHC in African catfish (*Clarias gariepinus*; Nwani et al., 2014). In a related finding, exposure to polypropylene resulted in an increase in MCV while simultaneously lowering MCH and MCHC in Nile tilapia (Nair & Perumal, 2024), suggesting disruptions in hematological parameters.

In this research, every treatment (FCC, MPs and FFC+MPs) led to a noticeable rise in the neutrophil count ($p < 0.05$; Figure 3b), probably as a result of heightened phagocytic activity in reaction to cellular damage (Shirmohammadi et al., 2018). In contrast, there was a significant decline in lymphocyte counts ($p < 0.05$; Figure 3c), suggesting possible immunosuppression in fish (Shirmohammadi et al., 2017). Also, the substances had no effect on eosinophils and monocytes (Figure 3d, e). The findings are consistent with earlier research on rainbow trout exposed to FFC, as noted by Shiry et al. (2020). Additionally, Harikrishnan et al. (2024) found that after 20 days of exposure to MPs, zebrafish (*Danio albolineatus*) exhibited a significant decrease in lymphocyte counts in both male and female fish, accompanied by an increase in neutrophils and monocytes.

The study found that after one day of exposure, cortisol levels peaked in all treatment groups ($p < 0.05$; Figure 4a and Figure 5a). While cortisol levels decreased over time in the treated groups, they did not return to control levels, except for the FFC-treated group, which normalized after 14 days. Increased cortisol levels during stress lead to higher energy demands, which can weaken the immune response (Lemos et al., 2023). The continued presence of high cortisol levels in the MPs and FF-

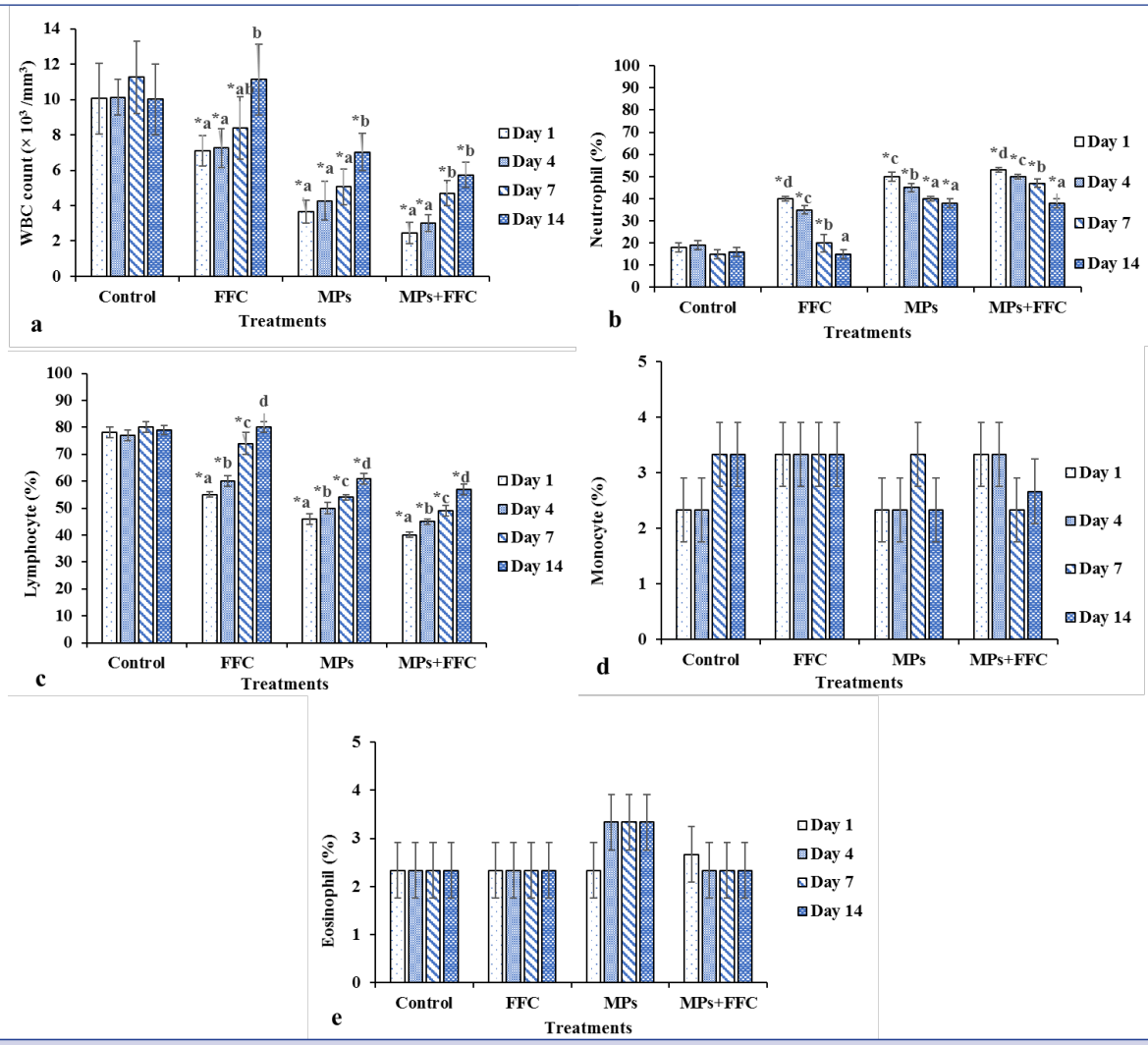

Figure 3. The impact of feeding yellowfin seabream with FFC and MPs, either separately or together, on total and differential white blood cell count (a) white blood cell (WBC), (b) neutrophil, (c) lymphocyte, (d) monocyte and (e) eosinophil (Mean ± SD; n=9). The fish that were tested on various days within the same groups showed notable differences, which were marked by different letters. An asterisk (*) indicated a meaningful difference between the treated group and the control group ($p < 0.05$).

C+MPs groups indicates that MPs might disrupt the hypothalamic-pituitary-interrenal axis, which plays a crucial role in managing stress responses. Such disruption could extend the stress response, persisting even after the stressor has been eliminated (Ding et al., 2025). The findings align with previous research by Harikrishnan et al. (2024) on zebrafish and Hoseini and Yousefi (2019) on rainbow trout, both of which noted increased cortisol levels in response to exposure to MPs and medicinal compounds like oxytetracycline, respectively.

The study found that EPO levels fell in all groups that received treatment on the initial sampling day but slowly rose over the course of the study, though they still remained much lower compared to those in the control ($p < 0.05$; Figure 4b and Figure 5b). Stressors may have damaged the fish's kidney tissue, which is responsible for producing EPO, leading to decreased hormone levels due to impaired hormone secretion (Sadeghi et al., 2015). Our results are consistent with previous research on stressed rohu (*Labeo rohita*; Datta et al., 2022). The FFC group was the only one to show a full recovery of EPO levels after 14 days, further emphasizing the transient nature of the toxicity caused by FFC.

During the sampling period, the concentration of T3 and T4 hormones exhibited an increasing trend in the groups that received MPs either separately or in combination and were not normal after 14 days. In contrast, FFC did not have a significant impact on either of the thyroid hormones ($p > 0.05$; Figure 4c, d and Figure 5c, d). These alterations can be linked to the presence of MPs in the tissue, leading to increased damage to the thyroid. This theory is supported by previous studies conducted by Wang et al. (2022), which investigated the impact of MPs and biphenyls on juvenile Japanese flounder (*Paralichthys olivaceus*).

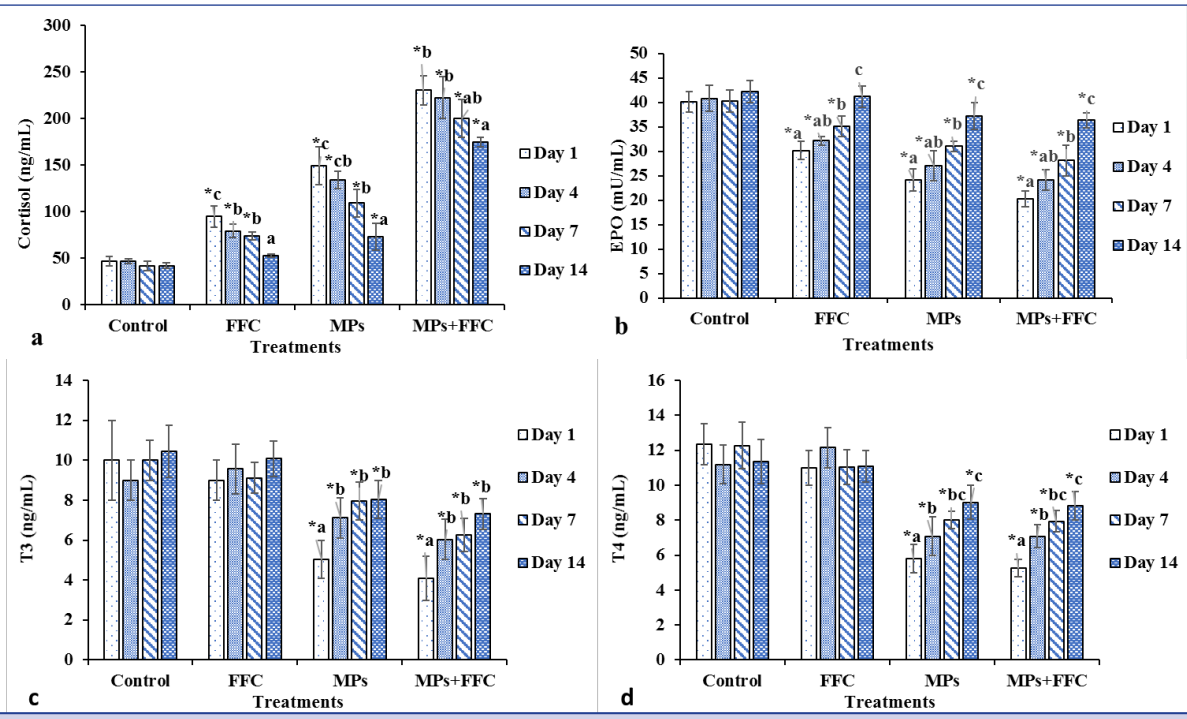

Figure 4. The impact of feeding yellowfin seabream with FFC and MPs, either separately or together, on cortisol (a), erythropoietin (EPO; b), triiodothyronine (T3; c), and thyroxine (T4; d) (Mean ± SD; n=9). The fish that were tested on various days within the same groups showed notable differences, which were marked by different letters. An asterisk (*) indicated a meaningful difference between the treated group and the control group ($p < 0.05$).

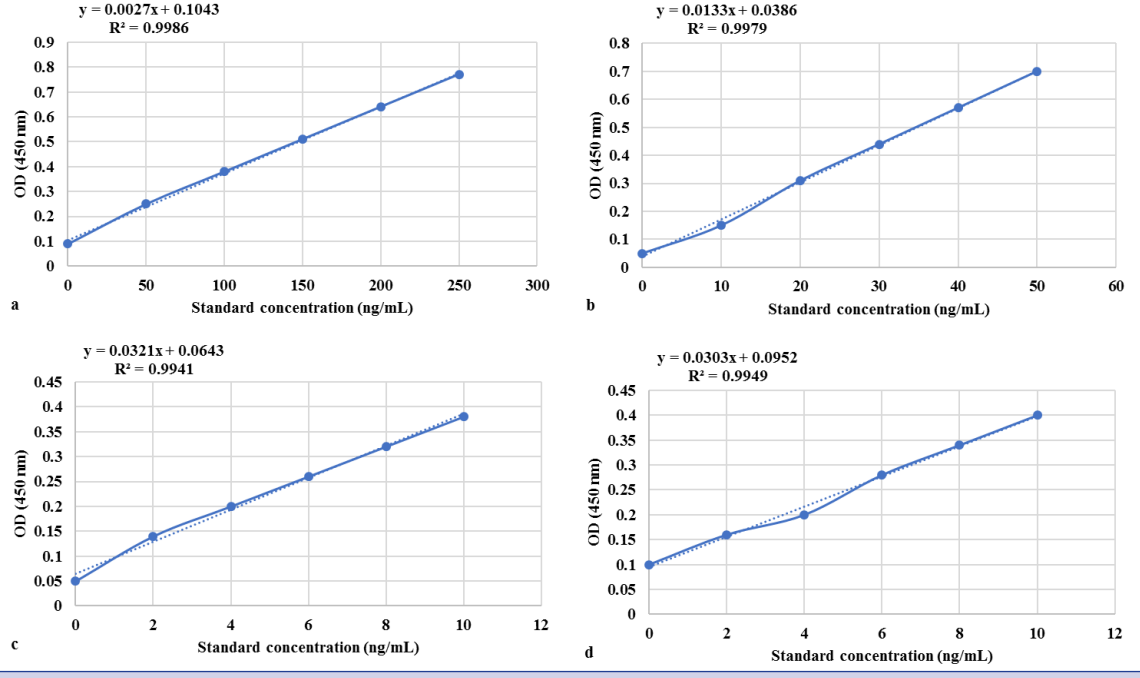

Figure 5. Standard curve of ELISA method for hormones including cortisol (a), erythropoietin (EPO; b), triiodothyronine (T3; c), and thyroxine (T4; d)

Following a two-week period, all hematological and hormonal parameters in the FFC-only group reverted to their baseline levels, likely attributed to the metabolism of FFC (Bardhan et al., 2023). Conversely, the groups exposed to MPs and FFC+MPs exhibited only partial recovery, with Ht and MCH levels returning to normal in the MPs group and MCH levels stabilizing in the mixed group. This indicates that MPs may induce lasting damage, possibly due to their accumulation in various tissues and their function as carriers for antibiotics (Hossain et al., 2023). Our findings align with earlier studies on juvenile common carps exposed to MPs (Ammar et al., 2023). The group that experienced both MPs and FFC exhibited the most significant changes in blood and hormone levels, highlighting the synergistic effects of these factors.

The lack of notable improvement in most hematological and hormonal indicators among the MPs and mixed groups highlights the persistent ecological effects of MP pollution in aquaculture systems. These findings emphasize the need to improve waste disposal methods to minimize MP contamination in the environment. While this study provides important insights into the synergistic effects of MPs and antibiotics, there are limitations that future research should explore. Specifically, it is important to examine the toxicity of MPs and FFC in relation to genomic abnormalities in blood cells of commercially significant species

CONCLUSION

The current study focuses on the impact of FFC and MPs on A. latus over a 14-day period, both individually and in conjunction. Significant alterations in hematological markers, EPO, cortisol, T3, and T4 hormones, along with FFC accumulation were observed in the plasma of the fish. The changes were more noticeable following exposure to a combination of MPs and FFC. The groups that received MPs, either alone or with FFC, did not show normalization in most parameters after 14 days, unlike the group exposed only to FFC, indicating persistent toxicity from MPs. Although the toxic effects of FFC were observed in the present study, these changes were temporary, indicating that this antibiotic is safe for use for this species. However, the continued presence of MPs and their role as carriers of antibiotics highlights the need for improved contamination management strategies. The ELISA data confirmed our hypothesis and underscored its importance in hormone research.

Conflict of interests: The authors report there are no competing interests to declare

Ethics committee approval: The experiments were conducted in accordance with the guidelines of the National Ethical Committee for Animal Research in Iran

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Review

The Role of Microalgae in Enhancing Anaerobic Digestion: A Bibliometric Review

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ABSTRACT

Anaerobic digestion (AD) is a well-established and extensively explored technology for waste management. However, the literature review related to the effect of microalgae on biogas production in the anaerobic co-digestion with different substrates is limited. Using scientometric tools can offer valuable insights into research gaps and emerging trends, facilitate the updating of scientific datasets, and expand knowledge in this field. Therefore, this bibliometric review will focus on the investigation of the advancements, trends, and recent updates in the co-digestion of microalgae with different substrates. The Web of Science database was used for document selection, and the bibliometric analysis was conducted using the VOSviewer version 1.6.19 software. The findings of this study reveal that, up until 2024, the major focus areas in the field are environmental studies related to biogas production, emphasizing microbiological and engineering aspects. Key opportunities and trends identified include the integration of feedstock pretreatment before AD to enhance biogas yield and quality. Adding microalgae as a co-substrate in anaerobic reactors has emerged as a promising strategy to boost AD process efficiency. Microalgae contribute additional organic matter and nutrients for AD and provide environmental benefits such as carbon sequestration and wastewater treatment, aligning with Sustainable Development Goals (SDGs). Gaining a deeper understanding of the role of microalgae in the systems is essential to establishing AD as a profitable and sustainable waste management solution, offering substantial economic and environmental advantages.

Keywords: Anaerobic, Microalgae, Co-digestion, Bibliometric, Sustainable Development Goals

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INTRODUCTION

The increasing energy demand and interest in sustainable solutions emphasize the importance of renewable energy sources (Muhammad Nasir et al., 2012). The production of biogas through anaerobic digestion (AD) should be particularly encouraged in both low- and middle-income countries because of its ability to reduce dependence on fossil fuels and positive contribution to waste management (Rahman et al., 2015). The biogas systems, which produce renewable energy, decrease greenhouse gases, improve waste management, provide nutrient-rich fertilizer, enhance sustainable agriculture, support local economies, create jobs, reduce pollution pressure of the wastes on land and surface waters. The systems also serve most of the United Nations (UN) Sustainable Development Goals (SDGs). Increasing regulatory pressures (Collivignarelli et al., 2015), and the cost of conventional wastewater treatment systems necessitate investigation of the alternative processes such as microalgae. The produced microalgae can be used as a co-substrate in anaerobic processes.

The inclusion of microalgae in AD is frequently emphasized in the literature for its potential to increase methane production. Microalgae,

which can adapt to different environments (freshwater, saltwater, and wastewater) with autotrophic, heterotrophic, and mixotrophic feeding modes, stand out with their biomass production capacities and environmental adaptation characteristics (Bingül et al., 2021; Xue et al., 2020). Microalgal biomass increases the biodegradability of waste in addition to being an ideal substrate for bacteria in anaerobic reactors, thereby enhancing methane yield (Costa et al., 2022). It has been shown that protein-rich species, in particular, can accelerate the hydrolysis and methanogenesis stages (Magdalena et al., 2018).

Microalgae like *Chlorella vulgaris* and *Scenedesmus* have been reported to be used for optimizing methane production by increasing the biological degradability of organic waste. For example, the use of *Chlorella vulgaris* together with wastewater treatment sludge, increases methane production while reducing ammonia toxicity, thereby improving process efficiency (Solé-Bundó et al., 2019a). Additionally, the mixture of *Chlorella vulgaris* biomass with cattle manure provides high methane yield (Mahdy et al., 2017). Under mesophilic conditions, *Scenedesmus* biomass has shown promising results with 70% chemical oxygen demand removal and high biogas production (Greses et al., 2017). These findings indicate that both species can be effectively used in anaerobic reactors.

The use of microalgae for bioenergy and alternative material production has been a subject of intense research for decades. However, the practical applications of microalgae for bioenergy purposes have mostly remained at the laboratory scale (Zhu et al., 2020). Despite limited full-scale applications, microalgae have the potential to increase biogas in anaerobic reactors, and they can be used as a valuable substrate. Nevertheless, to increase methane yield, microalgae may need to be pretreated or co-digested with different substrates (Vargas-Estrada et al., 2022). Studies in the literature also include the use of microalgae with sewage sludge, fertilizers, food waste, and industrial waste (Solé-Bundó et al., 2019b).

Microalgae stand out as a valuable resource not only in biogas systems but also in the production of various biofuels (biohydrogen, syngas, biobutanol, and bioethanol) and bio-products (Nagarajan et al., 2021; Dolganyuk et al., 2020). The presence of high amounts of biologically active compounds in their structures, such as proteins, polysaccharides, lipids, vitamins, pigments, and enzymes, makes them an ideal candidate for many sustainable applications (Dolganyuk et al., 2020). Furthermore, it has been shown that microalgae have higher CO_2 capture capacities compared to terrestrial plants (Cea-Barcia et al., 2018). The contribution of microalgae cultivation to processes such as wastewater treatment, nutrient recovery, and CO_2 capture, offers a synergistic approach for environmental sustainability.

This study aims to systematically evaluate research focused on the role of microalgae in AD processes through bibliometric analysis. The bibliometric technique can provide a comprehensive mapping of the existing literature and identify unrecognized gaps in the research field. Despite the significant interest in the potential of microalgae to enhance methane production for biogas production, there has been a lack of comprehensive research on this topic in the literature. This study aims to contribute to the literature by carefully evaluating the effect of microalgae on AD, analyzing microalgae potential in the system and identifying research gaps.

MATERIALS AND METHODS

This study used the Web of Science (WoS) database to obtain the scientific output data. The WoS database search was accessed from 2009 up to 1 January 2024. The search keywords used were 'microalgae' and 'biogas' and 'co-digestion,' excluding 'Document Types: Review Article,' 'Publication Years: 2024,' and 'Document Types: Book Chapters.' Our keyword-based search yielded a total of 228 publications in the form of articles. Using VOSviewer version 1.6.19 software, the data was examined for the most productive countries, the most cited documents, co-occurrence networks, keyword clusters of authors, publication years, and major countries involved in research. These components provide a comprehensive perspective on the state of research, highlighting the significance of bibliometric studies. The VOS viewer software used in the study is an open-source and user-friendly program widely used for network creation and visualization of bibliometric data (Van & Waltman, 2010).

In the WoS database, the search results can be refined by the UN SDGs. In our study, the search results are exported to an Excel program, and the percentage of each SDG to the total number of papers was calculated.

All data (n=288) were searched for the specific microalgae species used. This search was carried out through paper abstracts. The data obtained were recorded separately for each microalgae species. A separate classification was made according to the habitats of these microalgae. In addition to this classification for microalgae species, pretreatment methods preferred in the literature were examined over all data (n=228) through the abstracts. The collected data were categorized separately for each pretreatment.

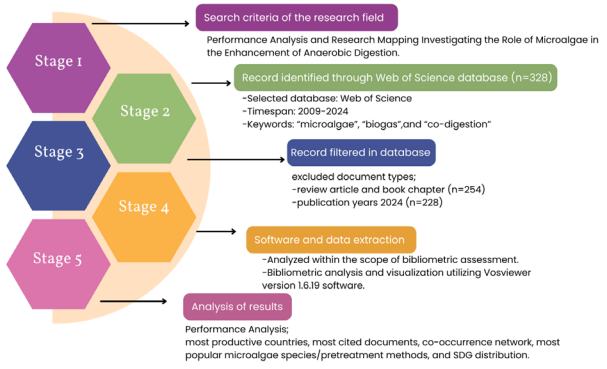

Figure 1. Research methodology.

RESULT AND DISCUSSION

In this study, the bibliometric analysis related to the use of microalgae in AD are presented, focusing on prominent countries, publication numbers by year, most cited articles, and journals. Additionally, the analyses were carried out for determining the

relationship between keywords, the most commonly used microalgae species, frequently applied pretreatment methods, and the SDG distributions of the publications. Each section of the results was discussed to provide detailed data on the research progress and trends in the AD technology of microalgae.

Leading countries for studies on the use of microalgae in AD

The distribution by country of scientific studies examining the contribution of microalgae to AD is an important tool to understand the research intensity and international trends in the field. In Figure 2, the number of papers published by specific countries is shown on a colour-coded world map. This analysis is derived from bibliometric data and reveals regional differences in scientific productivity in the field.

The map shows that Spain is in the leading position with 64 papers. Spain is followed by China with 28 papers. The USA, which ranks third, makes a significant contribution with 25 articles thanks to diversified academic studies and funding sources in this field. The USA was followed by India (21 papers), Brazil's (18 papers), Poland (12 papers). The countries have more interest in anaerobic co-digestion systems with microalgae. Australia (7 papers), and Egypt (7 papers) contribute to the field with a more limited number of publications. The low number of publications in these countries can be attributed to the interest for other research priorities.

This analysis provides a basis for understanding the geographical differences in scientific research on the contribution of microalgae to AD. The map reveals a significant geographical disparity in scientific publications on microalgae and AD, particularly in Africa and the Middle East. It has been determined that scientific publications are high in some countries and quite low in others. The reason for this is that, despite extensive laboratory-scale studies in this identified field of research, full-scale studies are lacking. Therefore, the studies conducted in this area cannot be applied to real systems, which limits interest for research. This geographical analysis provides a broader perspective on studies conducted on the contribution of microalgae to AD. In future studies, including the missing regions in the research network will contribute to a more balanced increase in knowledge in the field.

Figure 2. The world map displays the major countries involved in research on the use of microalgae in anaerobic studies.

The most cited publications on the use of microalgae in AD

Table 1 presents a summary of the top 10 most efficient sources in publishing literature focused on AD with microalgae. This summary includes the total citation count, sources, type of microalgae and co-digestion, and finally, the highest methane yield.

Publications on AD with microalgae have concentrated in the high-impact scientific journal "Bioresource Technology." This journal has 9 publications and a total of 551 citations. The closely following journals are "Science of the Total Environment," "Waste Management," "Biomass Conversion and Biorefinery," and "Water Science and Technology," which have 4, 2, 2, and 2 publications, respectively, and 190, 96, 40, and 27 total citations respectively. These journals have played an important role in publishing academic studies on AD by microalgae. Overall, these top references have contributed to the dissemination of knowledge obtained by researchers and to the advancement of studies on the use of microalgae in anaerobic systems, and represent important platforms for them to promote innovation in this field.

Preferred microalgae species in bibliometric analysis

Table 1, which presents the top 10 most cited articles, also highlights the microalgae species preferred in the studies. In order to examine the effective role of microalgae in AD in more detail, the most preferred microalgae species among all bibliometric data (n=228) are given in Figure 3. Publications on AD by microalgae generally focused on *Chlorella* microalgae. 48 articles preferred this microalgae species. *Scenedesmus, Nannochloropsis, Tetraselmis, Spirulina platensis* and *Chlamydomonas* microalgae, which closely follow this microalgae species, were included in 23, 13, 4, 3 and 3 publications, respectively. *Isochrysis galbana, Dunaliella salina, Selenastrum capricornutum, Pinnularia sp., Synechococcus sp.* and *Desertifilum tharense* microalgae are reported in less than 3 publications.

In the light of the data obtained for WoS search, the co-digestion studies focused mainly on *Chlorella* and *Scenedesmus* species, with a smaller number of studies testing *Nannochloropsis* and *Tetraselmis* microalgae. However, considering that microalgae are very diverse with different characteristics, it is important to test the

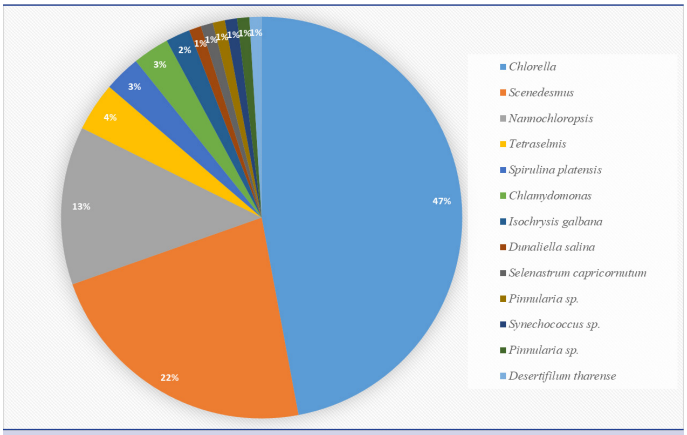

Figure 3. Distribution of microalgae used in studies on the role of microalgae in AD.

Table 1. The top 10 most cited articles on the role of microalgae in AD.

Ranking	Citation	Source	Type of microalgae	Waste type use in the co-digestion	Highest Methane yield	References
1st	130	Bioresource technology	*Chlorella*	Waste sludge	468 mL/g VS	Wang et al., 2013
2nd	92	Science of the Total Environment	*Chlorella sp.*	Primary sludge	-	Solé-Bundó et al., 2017
3rd	91	Bioresource technology	Microalgae mixture	Sewage sludge	408 ± 16 Ncm^3 $g\ VS^{-1}$	Olsson et al., 2014
4th	85	Energy Conversion and Management	*Scenedesmus*	Opuntia maxima	308 ± 22 LCH_4 $kgVS^{-1}$	Ramos-Suárez et al., 2014
5th	82	Renewable Energy	*Isochrysis galbana & Selenastrum capricornutum*	Sewage sludge	566 ± 5 mLBiogas/gSV	Caporgno et al., 2015
6th	73	Bioresource Technology	-	Barley straw, beet silage and brown seaweed	404 $L_N\ kg^{-1}$ VS	Herrmann et al., 2016
7th	62	Bioresource technology	*Nannochloropsis salina*	Corn silage	0.33 m^3 kg VS^{-1}	Schwede et al., 2013
8th	57	Renewable and Sustainable Energy Reviews	-	-	-	Solé-Bundó et al., 2019b
9th	56	Bioresource Technology	*Dunaliella salina*	Olive mill solid waste	48.1 mL CH_4/ (g VS day)	Fernández-Rodríguez et al., 2014
10th	54	Waste Management	*Chlorella sp.*	Chicken manure	31.62 $mL.g^{-1}$ VS	Li et al., 2017

potential of other microalgae species in anaerobic digestion. The effect of different microalgae species on methane yield in anaerobic co-digestion systems has not yet been fully studied. In addition, this study was conducted only for the WoS database. Future studies can also be conducted to include other databases.

Table 2 shows the natural habitats of microalgae preferred in the studies obtained as a result of bibliometric analysis. Generally, microalgae species living in freshwater were preferred in the studies. Since they are widely used, the selection of certain microalgae species can affect the efficiency of anaerobic systems. Therefore, it seems that the selection of both microalgae species and waste type in anaerobic co-digestion is important for the efficiency of the systems.

Preferred pretreatment methods in bibliometric analysis

Anaerobic digestion processes are more efficient if pretreatment methods are properly determined. Bibliometric analysis shows a range of methods to optimise the treatment of biomass (Figure 4). Thermal pretreatment has been reported as one of the most preferred of the pretreatment methods in the literature (Schwede et al., 2013; Mahdy et al., 2015; Chen et al., 2017; Passos et al., 2017; Wang et al., 2017; Cheng et al., 2018; Solé-Bundó et al., 2018; Passos et al., 2018; Solé-Bundó et al., 2020; Llamas et al., 2021; Vassalle et al., 2021). In comparison to other methods, thermal pretreatment is preferred since it is capable of destroying the structure of the biomass and increasing the amount of biogas produced (Solé-Bundó et al., 2018). However, in the studies, the thermal pretreatment process has not been evaluated in terms of energy usage or sustainability in general. A few studies that have evaluated it have shown that some part of the energy required by the pretreatment process can be maintained from the produced biogas. Carrillo-Reyers et al. (2021) assessed the thermal energy required for thermophilic digestion (50 kWh d-1) and stated that the energy can be provided by the wastewater treatment plant. Vassalle et al. (2022) performed an energy assessment in terms of the energy input and output values for the anaerobic reactor. Another method, alkaline pretreatment, is the second most common approach in five studies (Cheng et al., 2018; Panyaping et al., 2018; Wannapokin et al., 2018; Du et al., 2020; Fardinpoor et al., 2022). Ultrasonication with three papers is the third most common approach (Caporgno et al., 2016; Saleem et al., 2020; Debowski et al., 2022). These methods are useful due to their ability to dissolve organic matter and increase microbial accessibility. Enzymatic pretreatment offers a biotechnological alternative in three studies (Prajapati et al., 2015; Avila et al., 2021; Llamas et al., 2021). Another two studies combine the advantages of thermal and alkaline approaches (Solé-Bundó et al., 2017; Fu et al., 2023). The various pretreatment techniques, each reported in a separate study, illustrate the experimental diversity in the field. Acid hydrolysis (Cheng et al., 2018), autohydrolysis (Arias et al., 2018), urea treatment (Yu et al., 2021), hydrothermal treatments (Bohutskyi et al., 2019), microwave-assisted pretreatments (Feng et al., 2019), and thermo-anaerobic treatment (Damtie et al.,

Table 2. Habitats of microalgae included in the studies used in bibliometric analysis.

Types of microalgae	Number of documents	Fresh water	Saline water
Chlorella	48	+	
Scenedesmus	23	+	
Nannochloropsis	13		+
Tetraselmis	4		+
Spirulina platensis	3	+	
Chlamydomonas	3	+	
Isochrysis galbana	2		+
Dunaliella salina	1		+
Selenastrum capricornutum	1	+	
Pinnularia sp.	1	+	
Synechococcus sp.	1	+	+
Desertifilum tharense	1	+	

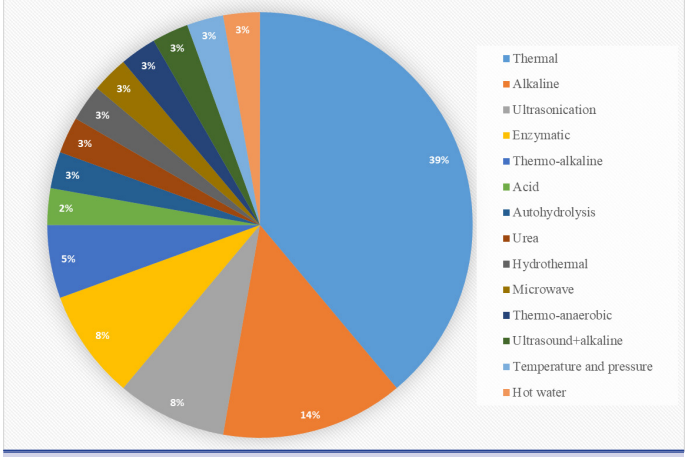

Figure 4. Distribution of pretreatment methods used in studies on the role of microalgae in AD.

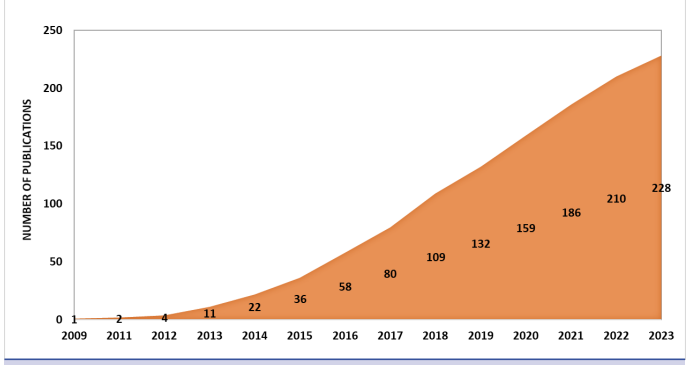

Figure 5. Evolution of the number of publications over the years (2009–2023 and 2009–2023).

2021) are some of them. Hybrid strategies utilizing ultrasound and alkaline treatment (Caporgno et al., 2016), as well as variations in temperature and pressure (Arelli et al., 2020), demonstrate novel methods to enhance substrate degradability. Ultimately, hot water pretreatment (Saleem et al., 2020) is evaluated as a cost-effective and eco-friendly solution. This distribution highlights the dynamic and exploratory aspects of pretreatment research on anaerobic digestion.

Yearly Trends and Keyword Co-Occurrences in Bibliometric analysis

Figure 5 shows data from the WoS related to the distribution trend of all articles on the use of microalgae in AD until the beginning of 2024. The results show a consistent upward trend in research on the use of microalgae in AD from 2009 to 2021. A noticeable decrease in publication output occurred from 2021 to the beginning of 2024. It has been stated that there is a restricted applicability of microalgae in full-scale anaerobic systems (Díez-Montero et al., 2020) and this decrease in the publications may be linked to this situation. In addition, an analysis of studies on microalgae over the last five years shows a strong research focus on bioengineering, ecology and pharmaceutical sciences. A search of the WoS database using the only keyword as "microalgae" revealed nearly 15,000 publications between 2021 and 2025, excluding review articles and book chapters (WoS link for microalgae search). When the research areas were analyzed for the microalgae, the results highlighted not only the continued importance of microalgae in the energy sector but also their growing importance in ecological applications, food production, and pharmaceutical research. Therefore, the observed decline in research publications can also be attributed to a shift in research priorities for microalgae.

This study uses a keyword co-occurrence map to examine the scientific literature on the role of microalgae in AD and biogas production (Figure 6). The VOSviewer software produces a map showing microalgae, biogas, co-digestion, and AD as the main themes explored in the literature. The colour pattern of the map indicates the frequency of terms over a given time period. Blue shades indicate publications from 2014 and earlier. Within this period, the terms 'waste-activated sludge' and 'anaerobic digestion' are prevalent. The green shades highlight topics such as 'biomass', 'methane production' and 'C/N ratio', all of which were analysed from 2016 to 2018. The yellow tones indicate topics that appeared in the literature from 2020 onwards, including the keywords "biomethane potential", "mixture design" and

"microalgal biomass". Researchers have investigated methods to optimize processes (C/N ratio, methane potential) and the digestion of microalgae using different biomasses (sewage sludge, chicken manure). The analysis concludes that microalgae are considered an innovative substrate for biogas production and represent a crucial area of research for sustainable energy production.

Figure 7 shows the importance levels of keywords in the bibliometric analysis according to the criteria of "occurrences" (frequency) and "total link strength". This study, when evaluating the role of microalgae in AD and biogas production through keyword analysis, shows that the keyword 'microalgae' is the most frequently found in the literature (n = 25) and has the strongest association strength (114). On the other hand, the term 'biogas' appears 22 times and has a connection strength of 106, suggesting that microalgae play an important role in biogas production. The terms 'co-digestion' and 'anaerobic co-digestion' stand out with n=19 and n=18 occurrences respectively and have high connection values (83-84). This finding indicates that extensive research has been conducted on microalgae in digestion processes with different biomass. On the contrary, the term 'anaerobic digestion' is observed with lower frequency (n=11) and connection strength (55). This indicates that microalgae research is concerned with AD processes, but the main focus is on co-digestion and biogas production. Finally, the study shows that microalgae are an important area of research for biofuel production and sustainable energy systems. They are also very important for biogas production and especially for co-digestion studies.

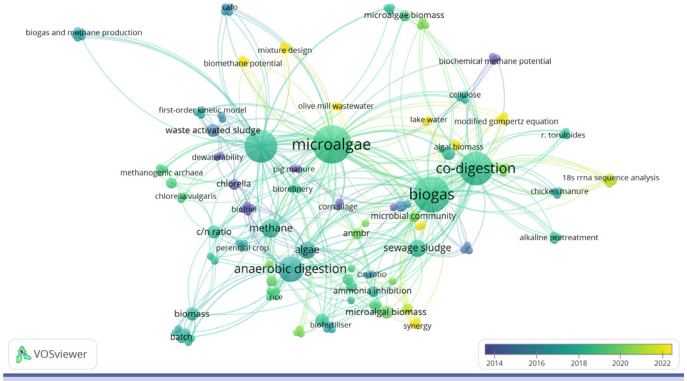

Figure 6. Role of microalgae in AD: Bibliometric Keyword Co-Occurrence Network (2009-2023).

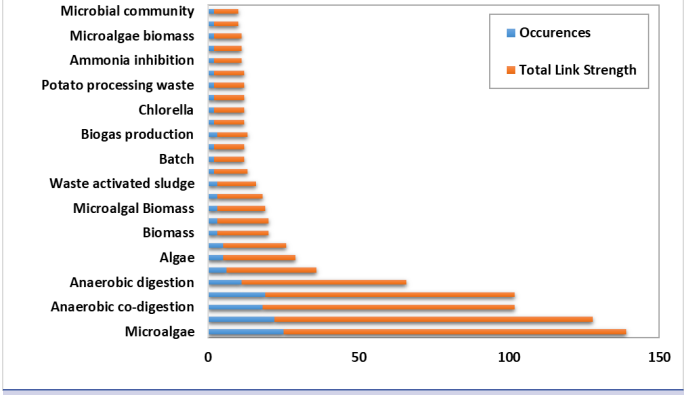

Figure 7. Distribution of Keywords by Occurrence and Total Link Strength.

Alignment of Microalgae and Anaerobic Digestion Studies with SDGs

Figure 8 also illustrates the strong relevance of studies on the role of microalgae in AD to the SDGs. According to the data, a very high proportion (98.25 percent) of studies using microalgae in AD processes were associated with "clean water and sanitation" (SDG 6). For "affordable and clean energy" (SDG 7), AD offers a significant strategy to provide clean energy through biogas production. Additionally, in line with the goals of "responsible production and consumption" (SDG 12), AD supports the recovery of biomass and waste, contributing to responsible consumption targets. Although "climate action" (SDG 13) has a low representation rate of 0.44%, the goal of "increasing climate resilience" stated in SDG 13.2.1 can be supported by the adaptability of microalgae-based anaerobic systems to waste sources and energy security. AD processes provide contributions to this target by providing carbon emission reduction and renewable energy generation. The lack of studies directly related to climate change mitigation provides an opportunity for future research in this field. The underrepresentation of SDG 13 in the context of microalgae and anaerobic digestion research may highlight the gap in carbon footprint studies for microalgae-based systems. Addressing these gaps will require a concerted effort to prioritize research that assesses the environmental impacts of integrating microalgae into anaerobic systems, as well as fostering interdisciplinary collaboration and funding for studies that emphasize climate action. In this way, the potential of microalgae to contribute to sustainable development and climate change mitigation can be more effectively realized.This study is an important resource to assess the contribution of AD and microalgae to sustainable development goals. Future research should focus on further unlocking the potential of AD in the context of climate change and energy efficiency.

To summarise the study in general, this bibliometric analysis covers the studies on this subject in the literature. In addition to other bibliometric analysis data, the study was detailed in terms of microal-

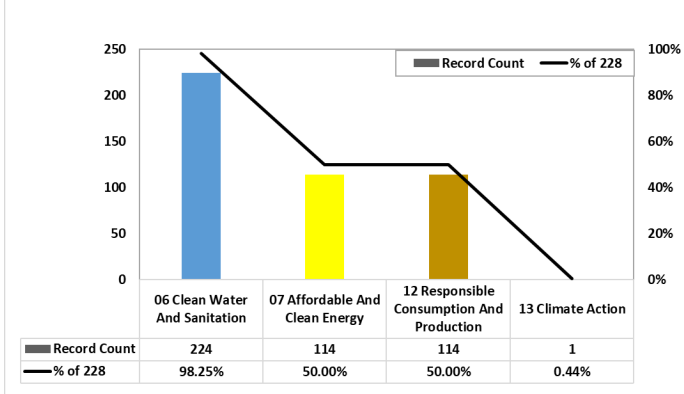

Figure 8. SDG Distribution of Microalgae and AD Studies.

gae species used in the studies, pre-treatment methods, and link to the SDGs. Some research gaps were identified in the literature. The primary limitations are that most studies are confined to laboratory-scale experiments, with a focus on certain preferred microalgae species. Additionally, there is a lack of optimization studies, integration of new technologies into existing systems, and sufficient evidence to demonstrate sustainability and economic feasibility. This bibliometric analysis is expected to play an important role in the development of future studies in this field.

CONCLUSION

This research is a bibliometric analysis of the research on the use of microalgae as co-digestion in anaerobic systems and the resulting biogas production. From the bibliometric analysis, it can be concluded that 70% of the studies on the use of microalgae in anaerobic systems were published until 2021. Most of the research was linked to the SDGs on "clean water and sanitation" (SDG 6), "affordable and clean energy" (SDG 7) and "responsible production and consumption" (SDG 12). The most commonly used microalgae species are *Chlorella, Scenedesmus, Nannochloropsis, Tetraselmis, Spirulina platensis*, and *Chlamydomonas* microalgae. Microalgae living in fresh waters were generally preferred. In some of the anaerobic systems where microalgae were used, pretreatment was preferred. The most commonly used pretreatments were thermal, alkaline, ultrasonication, enzymatic, and thermo-alkaline, respectively.

Given the current findings, future studies should not be limited to lab-scale, and full-scale studies should be attempted. In addition, in anaerobic systems where certain microalgae species are favoured, more comprehensive studies are needed to understand the process mechanism of other microalgae species. Little information is available in the literature on the microalgae-methanogen relationship, and further studies are needed to understand this. Future studies with the integration of advanced technological applications (smart sensor and automation technologies, etc.) may provide more insights into biogas volume and efficiency in these systems.

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