WASTEWATER TREATMENT TECHNOLOGIES BY ALGAE-BACTERIA CONSORTIA

by

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WASTEWATER TREATMENT TECHNOLOGIES BY ALGAE-BACTERIA CONSORTIA

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ABSTRACT

WASTEWATER TREATMENT TECHNOLOGIES BY ALGAE-BACTERIA CONSORTIA

Micropollutants (MPs) are a wide variety of chemicals that should be appropriately treated in wastewater before discharge into the environment. In this dissertation, an algae-bacteria consortium system that could effectively treat benzalkonium chloride (BAC) and copper (Cu) together in the same operation was proposed. For this, various microalgae species were screened for their tolerance to various heavy metals (HMs) and BAC. *Tetraselmis suecica* was the most resilient against BAC, cadmium (Cd), Cu, and zinc (Zn). Later, HM tolerance of a BAC-degrading bacterium, *Pseudomonas sessilinigenes* BIOMIG1, was determined in terms of the metal effect on growth and BAC degradation. Copper ions inhibited the BAC degradation but not the growth on rich organics. A consortium of these species was established and BAC and Cu were treated by the consortium. Efficient removal of BAC by bacteria and partial removal of copper via adsorption and assimilation were observed at 20 ppm BAC and 1 ppm Cu in synthetic medium. The presence of algae contributed to the survival of bacteria under increasing copper concentrations, and the presence of bacteria supported the algal growth at inhibitory BAC concentrations. The effects of copper at 1 mg Cu/L on *T. suecica* were also investigated through transcriptomic analysis. Carbohydrate catabolism, nitrogen assimilation, and fatty acid biosynthesis & elongation were upregulated and the expression of metal transporters was altered. The outcomes of this study can be utilized in industrial wastewater treatment using microalgae-bacteria consortia as a sustainable biological remediation approach.

ÖZET

ALG-BAKTERİ KONSORSİYUMU VASITASIYLA ATIKSU ARITIM TEKNOLOJİLERİ

Mikrokirleticiler, çevreye deşarj edilmeden önce atıksuda uygun şekilde arıtılması gereken çeşitli kimyasal maddelerdir. Bu tezde, benzalkonyum klorür (BAK) ve bakır gibi kirleticileri etkili bir şekilde aynı işlemde arıtabilecek bir yosun-bakteri konsorsiyum sistemi önerilmiştir. Bu amaçla çeşitli mikroalglerin farklı ağır metallere ve BAK'a karşı toleransları taranmış ve denenen mikroyosunlar arasında deniz mikroyosunu *Tetraselmis suecica*, BAK, kadmiyum, bakır ve çinkoya karşı en dayanıklı tür olarak tespit edilmiştir. Daha sonra, BAK parçalayabilen bir bakteri olan *Pseudomonas sessilinigenes* BIOMIG1'in ağır metal toleransı, büyüme ve BAK parçalanması üzerindeki metal etkisi bakımından belirlenmiştir. Bakır iyonlarının, sadece BAK parçalanmasını etkilediği, ancak zengin organik içeren ortamda büyümeye etki etmediği tespit edilmiştir. Bu türlerin bir konsorsiyumu BAK ve bakır arıtımı için kullanılmış ve 20 ppm BAK ile 1 ppm bakır içeren sentetik ortamda BAK'ın bakteriler tarafından etkili bir şekilde giderildiği, bakırın ise adsorpsiyon ve asimilasyon ile kısmi olarak uzaklaştırıldığı gözlemlenmiştir. Alglerin varlığının, bakterilerin artan bakır konsantrasyonlarında hayatta kalmasına katkıda bulunduğu ve bakterilerin varlığının BAK maruziyetinde alglerin büyümesini desteklediği tespit edilmiştir. Bakırın *T. suecica* üzerindeki etkisi *de novo* transkriptomik analiz ile incelenmiştir. Analiz sonucunda karbonhidrat katabolizması, azot asimilasyonu ve yağ asidi biyosentezi ve uzaması yolaklarının ekspresyonlarının arttığı ve metal kanallarının ekspresyonunun değiştiği tespit edilmiştir. Bu çalışmanın sonuçları, endüstriyel atıksu arıtımında mikroalg-bakteri konsorsiyumu kullanılarak sürdürülebilir bir biyolojik iyileştirme yaklaşımının uygulanabileceğini göstermektedir.

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LIST OF SYMBOLS/ABBREVIATIONS

Symbol	Explanation	Unit
k'	Cell specific substrate utilization rate	mg/h
Ks	Half saturation constant	mg
μ	Growth rate	1/d

Abbreviation	Explanation
ACN	Acetonitrile
ANOVA	Analysis of variance
APX	Ascorbate peroxidase
ATP	Adenosine triphosphate
ATPF-	F-type ATPase subunit
ATPV-	V-type ATPase subunit
BAC	Benzalkonium chloride
BDMA	Benzyldimethylamine
BP	Biological process
CC	Cellular component
CCP	Cytochrome c peroxidase
CFU	Colony forming unit
CPM	Counts per million
Ctr	Copper transporter
DDT	Dichlorodiphenyltrichloroethane
DEG	Differentially expressed gene
DHAP	Glycerone phosphate
DNA	Deoxyribonucleic acid
EC	Emerging contaminants
Eq.	Equation
EtAc	Ethyl acetate
ETC	Electron transport chain
FDR	False discovery rate
G3P	Glyceraldehyde-3-phosphate
GO	Gene ontology
GPX	Glutathione peroxidase

GRX	Glutaredoxin
GSH	Glutathione
HFPW	Hydraulic fracturing produced water
HM	Heavy metals
HMA	Heavy metal ATPase
HPLC	High performance liquid chromatography
HRAP	High rate algal ponds
HRT	Hydraulic retention time
IAA	Indole-3-acetic acid
IC_{50}	Half-maximal inhibitory concentration
ICP/MS	Inductively coupled plasma mass spectrometry
IDL	Instrumental detection limit
IQL	Instrumental quantification limit
katG	Catalase-peroxidase
KEGG	Kyoto Encyclopedia of Genes and Genomes
LB	Luria-Bertani medium
logCPM	Logarithm (base 2) of counts per million
logFC	Logarithm (base 2) of fold change
M9	Minimal salt medium
MATE	Multidrug and toxic compound extrusion
MB3N	Modified Bold 3N Medium
METE	B12-independent methionine synthase
METH	B12-dependent methionine synthase
MF	Molecular function
MH-II	Mueller Hinton II broth
MP	Micropollutants
MTP	Metal transport protein
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NDUF-	NADH dehydrogenase (ubiquinone) flavoprotein subunit
NES	Normalized enrichment score
OAA	Oxaloacetate
OD	Optical density

OD_{680}	Optical density at 680 nm
PAHs	Polycyclic aromatic hydrocarbons
PBR	Photobioreactor
PC	Phytochelatin
PCR	Polymerase chain reaction
PCS	Phytochelatin synthase
Pet-	Photosynthetic electron transport protein
PPCPs	Pharmaceuticals and personal care products
Psa-	Photosystem I subunit
Psb-	Photosystem II subunit
PUFAs	Polyunsaturated fatty acids
QAC	Quaternary ammonium compounds
RNA	Ribonucleic acid
ROS	Reactive oxygen species
ROS	Reactive oxygen species
RuBisCO	Ribulose-bisphosphate carboxylase
SBR	Sequencing batch reactor
SOD	Superoxide dismutase
Trp	Tryptophan
TRX	Thioredoxin
ZIP	Zrt- and Irt-like protein

1. INTRODUCTION

The majority of the Earth's surface is covered with water. Yet 96.5% of the water is located in the oceans as saline water. When the water in the glaciers and saline groundwater are subtracted, less than 1% of the water on Earth is left as freshwater for use (Shikimanov I, 1993). In 2022, 2.2 billion people lacked access to safe drinking water, of which over 700 million cannot reach even at least basic drinking water services (UNICEF/WHO, 2023). Considering seasonal fluctuations, about 4 billion people around the world experience at least one month of severe water scarcity (Mekonnen & Hoekstra, 2016). Due to the increase in shortwave radiation as a result of climate change, water scarcity is expected to rise globally in the near future (Denissen et al., 2022). The number of people living in regions with absolute water scarcity (<500 m^3/year per capita) is expected to be 1.8 billion in 2025 (FAO). A forecast indicates that the count of major urban centers facing water scarcity is expected to rise to a range of 193–284, encompassing 10–20 megacities by the year 2050 (C. He et al., 2021). On the other hand, the demand for freshwater is still high. The volume of water withdrawn per capita for domestic purposes is estimated around 480 liters per day in the US by 2020, and this value rises above 3600 liters when withdrawal of industry, agriculture, public supply, and thermoelectrical power generators are also considered (Knoema, 2022). Most of the water utilized for various purposes eventually turns into wastewater and is discharged into the environment treated or untreated. For instance, in 2022, 197 liters of wastewater per capita was discharged daily in Türkiye (TURKSTAT, 2023). On the verge of water scarcity affecting humankind in the near future, wastewater treatment plays a significant role in sustaining water sources.

The variety of contaminants within wastewater diversifies through the years resulting in hard-to-treat compositions. As the contaminants persist longer in receiving environments due to their gross amount or high obstinacy, they pose not only environmental hazards but also threaten humans and other living organisms. The European Union recognizes 68 key pollutants that need to be routinely monitored due to their significant risks to human health and the natural biodiversity of exposed environments (Backhaus, 2023). The network of reference laboratories, research centres and related organisations for monitoring of emerging environmental substances extends the list of emerging contaminants (ECs) to tens of thousands of chemicals, mainly comprised of pharmaceuticals, pesticides, per- and polyfluoroalkyl substances, natural toxins, and priority contaminants of emerging concern (NORMAN Network et al., 2022). The list spans contaminants of global concern, including micropollutants (MPs) which are present in treatment facilities and aquatic systems at low

concentrations yet carry high toxicities (Schwarzenbach et al., 2006). Removal of these contaminants from wastewater systems carries great importance to protect both the environment and human health.

Among MPs, organic pollutants and heavy metals (HMs) are of essential concern and are widely found in industrial wastewater. Industrial wastewater streams are more complicated compared to municipal wastewater and require several physicochemical and biological treatment processes (Rathod et al., 2016). Chemical precipitation, coagulation with aluminum sulfate or iron salt, and adsorption onto activated carbon are among the most preferred physicochemical processes that are conventionally applied to treat industrial wastewater (Otto & Bajpai, 2007). Recent technological advances and increased availability have facilitated the application of various advanced physicochemical processes. These include the use of titanium dioxide and other nanoparticles (Pattnaik, Sahu, Poonia, & Ghosh, 2023; Sabri, Habibi-Yangjeh, Rahim Pouran, & Wang, 2023), various special catalysts such as Fenton derivatives (Nidheesh et al., 2023), electrocoagulation coupled with adsorption (Graça & Rodrigues, 2022), adsorption to micro-particles from plant material (Chiban, Soudani, Sinan, & Persin, 2012), the application of ozone technologies and photolysis (Mecha & Chollom, 2020), sonolysis (Ryu et al., 2021; Ziylan-Yavas & Ince, 2018) and advanced oxidation processes involving hydroxyl radical formation, comprising the applications of multiple of those processes (Agopcan Cinar, Ziylan-Yavaş, Catak, Ince, & Aviyente, 2017). Despite their many advantages, physicochemical treatment methods require high amounts of energy, and they may either alter sludge characteristics (Nawaz & Ahsan, 2014) or yield unwanted residuals. Due to the oxidation of matrix components in water, metabolites may arise from a contaminant originally less harmful than the products of its chemical reactions during the processes (Sullivan, 2008).

A biological treatment method is to utilize living organisms for the removal of contaminants from the wastewater. Biological applications were introduced into sewage systems successfully with what is now known as activated sludge operation, an established and satisfactory system for the efficient removal of biodegradable organic contaminants (Nelson, 2016). Today, most of the wastewater treatment facilities involve an activated sludge operation in their secondary treatment units. Despite its commonality and sufficiency for decreasing the total organic load, activated sludge systems are not specialized for certain MPs. The importance of MPs derives from their persistence in wastewater treatment plants, the downstream aquatic environment, their toxicity to aquatic life, and their potential impact on drinking water sources (Schwarzenbach et al., 2006).

Biological treatment for the removal of MPs requires specific organisms of various taxa. Specifically termed as bioremediation, natural and/or engineered organisms are utilized for breaking

down pollutants into either less toxic or non-toxic forms. In recent years, microorganisms have successfully been demonstrated for the bioremediation of MPs (Gavrilescu, Demnerová, Aamand, Agathos, & Fava, 2015; Mali et al., 2023; Narayanan et al., 2022). Intuitively, bioremediation operations require careful control of the conditions for the optimal removal of the contaminants. As part of a remediation process, bacteria may be used for biotransformation, biodegradation, biosorption, or bioaccumulation of the contaminant. Microbial biotransformation occurs via the biochemical transformation of the contaminant into another compound by enzymatic activity (Mahesh et al., 2022). If the contaminant is completely metabolized to inorganic base components such as water and carbon dioxide, the process is called biodegradation. In cases where the contaminant is neither biodegradable nor bio-transformable, bacteria might still be utilized for the removal of the contaminant by sorption of the contaminant onto the cell surface (Mahesh et al., 2022). If successful, the contaminant is removed from wastewater when the bacteria are collected. The removal of HMs by bacteria can be through the accumulation of HM inside cells and changing the complexation status of the metal which leads to precipitation (Reith, Lengke, Falconer, Craw, & Southam, 2007). Bioremediation by bacteria is constrained to physical and chemical parameters such as pH, temperature, degradation rate, oxygen concentration, moisture content, nutrient ratio, aqueous solubility and bioavailability of the contaminants, biochemical redox potential, and the toxicity of the contaminants (Das, 2014; Dua, Singh, Sethunathan, & Johri, 2002).

The use of macro- and microalgae for the remediation of contaminated environments is referred to as phycoremediation, a promising alternative for the treatment of wastewater (Kaplan, 2013). Microalgae in particular are suitable for wastewater treatment applications due to several advantages as listed below:

- Microalgal cells grow much faster (2-3 weeks) relative to higher plants (2-6 months) which are also used for phytoremediation (Ali, Khan, & Sajad, 2013; McIntyre, 2003).
- There is no need for arable land to grow microalgal biomass.
- Microalgae can be cultivated in brackish or marine water.
- Phycoremediation offers sustainability metrics as microalgae are autotrophic and capture carbon dioxide (CO_2).
- Multivalorization to biofuels, biofertilizers, etc. can be coupled with phycoremediation processes (Rawat, Kumar, Mutanda, & Bux, 2011).

Microalgae play a crucial role in wastewater treatment, specifically in the removal of nutrients such as nitrogen (N) and phosphorus (P) (Ajala & Alexander, 2020). High rate algal ponds (HRAPs)

are commonly employed for biomass production, utilizing wastewater as a nutrient source and solar energy for algal growth (Robles et al., 2020). In the realm of EC removal, microalgae demonstrate the ability to transform common xenobiotics and efficiently remove ECs from wastewater (Z. Wang et al., 2022; J.-L. Zhou, Yang, Huang, Chen, & Gao, 2022). Synthetic hormones, while more challenging to eliminate, may benefit from coexistence with natural hormones (P. Wang, Wong, & Tam, 2016). Algal bioreactor systems are proposed for advanced treatment of wastewater, successfully removing pharmaceuticals through various mechanisms (de Wilt et al., 2016). Microalgae also demonstrate potential for heavy metal removal through adsorption, where factors such as pH, algal strain, and biomass concentration influence the sorption capacity (Kaplan, 2013). Algal biotransformation detoxifies heavy metal ions, involving complexation with amine or hydroxide groups (Essa & Mostafa, 2013).

There are several examples of the usage of algae and bacteria consortia together for wastewater treatment. Early examples of the algae-bacteria relationship in wastewater treatment are algal turfs, operated for the removal of nutrients along with the removal of organic load (Craggs et al., 1996). Recent studies have examined the removal of organics and nutrients by a single-species alga in consortium with bacteria, aiming to enhance algae growth for potential exploitation (Cho, Ramanan, Heo, Lee, et al., 2015; Gonçalves, Pires, & Simões, 2016; Jasper, Jones, Sharp, & Sedlak, 2014; Lee, Oh, Oh, Kim, & Ahn, 2016; X. Yang et al., 2015). Jasper et al. investigated the construction of wetlands using *Staurosira construens* and bacteria for the removal of pharmaceutical compounds, where the consortium increased the sum of biotransformation and photolysis 10 to 100-fold (Jasper et al., 2014). The authors also reported 90% removal for atenolol, metoprolol, propranolol, trimethoprim, and sulfamethoxazole. There are other studies indicating the removal of hazardous contaminants, i.e., heavy metals (Gu, Li, Zhang, Zheng, & Luo, 2020; Nagarajan et al., 2022). Algae are favored by CO_2 and cofactors such as vitamin B_{12}, which is produced by bacteria; and bacteria, in turn, utilize organic matter generated by algae (Croft, Lawrence, Raux-Deery, Warren, & Smith, 2005; Fuentes et al., 2016). de-Bashan et al. stated that synthetic mutualism can be achieved between algae and bacteria that do not share an evolutionary relationship (de-Bashan et al., 2016). Hence, there is a need for further research to understand how the relationship between algae and bacteria actually occurs, and to what extent this symbiosis goes in a mutualistic way rather than competition. Dittami et al. pointed out that eukaryote-microbe interactions in the case of algae-bacteria consortia are not analyzed in detail, such as in physiological studies, as other eukaryote-microbe interactions such as gut microbiome or plant roots (Dittami, Eveillard, & Tonon, 2014).

In this dissertation, the main objective is to develop a consortium of microalgae and bacteria, which can efficiently treat different kinds of micropollutants like heavy metals and surfactants from wastewater simultaneously. Various microalgae species were challenged and screened for their tolerance against heavy metals and benzalkonium chloride (BAC), a common surfactant found in detergents and disinfection agents (for the discussion about BAC, see Sections 2.1.2 and 6.1). The algae species with the highest tolerance against BAC, cadmium (Cd), copper (Cu), and zinc (Zn) was determined as *Tetraselmis suecica*. This species was cultured in a consortium with *Pseudomonas sessilinigenes* BIOMIG1, a bacterial strain that can degrade BAC, in a synthetic media containing BAC and Cu to evaluate the removal capacity. Both species assisted each other against contaminant stress, specifically the bacteria supported algae against BAC stress and the algae supported bacteria against Cu stress. The metabolic changes in *T. suecica* at the transcriptomic level were evaluated following Cu exposure to gain insights into its systems-level mode of action against a heavy metal.

2. LITERATURE BACKGROUND

In this chapter, the answer to the question "Why choose algae-based systems for the treatment of micropollutants in wastewater streams?" is discussed. The impacts of MPs on the environment and human health are briefly explained. Microalgae-based wastewater treatment and the symbiosis mechanisms between microalgae and bacteria in co-culture treatment systems are reviewed. Three key points needed clarification before further discussion. First, I employed both "EC" and "MP" concepts. EC, in this context, refers to various organic emerging contaminants, while MP collectively refers to organic pollutants and HMs. Second, I utilized the terms "algae" and "microalgae". "Algae" here refers to either both micro- and macroalgae in general, or, more commonly, "algae" and "microalgae" are used interchangeably to refer to microalgae. The term "microalgae" refers to a collective of eukaryotic microalgae from different phyla and cyanobacteria. Occasionally, both "microalgae" and "cyanobacteria" are used to emphasize the eukaryotic aspect. The third point is that I used the concept of "microalgae-based wastewater treatment" to refer to both axenic and co-culture studies.

2.1. Impacts of Micropollutants on the Environment

The impacts of MPs on the environment are multifaceted and can manifest in various detrimental ways. MPs, often present in trace concentrations, encompass a diverse range of substances such as pharmaceuticals, personal care products, pesticides, flame retardants, nanoparticles, and so on (Tolboom et al., 2019). In the year 2019, Europe alone utilized 294.6 million tons of chemical substances, with approximately 74% of the overall quantity constituting hazardous chemicals (Ozcelik, Sakarya, Tezel, & Haznedaroglu, 2022). Dispersion of the MPs in receiving environments is problematically global. The toxicological info of a small portion of the MPs is studied and regulated, but most of the tens of thousands of MPs remain outside of legislative control (Rogowska, Cieszynska-Semenowicz, Ratajczyk, & Wolska, 2020). The impacts of the main groups of MPs are going to be reviewed in the next subsections.

2.1.1. Pharmaceuticals & Personal Care Products

Pharmaceuticals cover a wide variety of substances including antibiotics, analgesics, and endocrine disruptors. The global use of pharmaceuticals and personal care products (PPCPs) has been increasing with 5.8 billion prescriptions in the US in 2018 (Adeleye et al., 2022). These products end

up in wastewater excreted by users and discharged into the environment with wastewater effluent. Pharmaceuticals have been found in the natural environment across over 70 countries, spanning all continents (aus der Beek et al., 2016). Residues of pharma products, specifically, may be found in surface water, groundwater, influent, effluent, and sludge of the wastewater treatment plants, biosphere, geosphere, and even polar regions (Patel et al., 2019). Pharmaceuticals are formulated to attach to particular receptors in humans and animals. Consequently, these substances can also engage with analogous receptors found in other organisms present in the environment (Fent, Weston, & Caminada, 2006). Effects reported in the literature include growth malformations in rats, fish, and frogs, growth inhibition of *Daphnia*, microalgae, and cyanobacteria, and inhibition of protein, nucleic acid, or cell envelope synthesis in prokaryotes (Patel et al., 2019). Exposure to these compounds, outside of therapeutic contexts, is believed to have a more detrimental impact on environmental microorganisms compared to aquatic vertebrates like fish (Le Page, Gunnarsson, Snape, & Tyler, 2017). In aquatic ecosystems, hormonally active MPs, a group of natural hormones and pharmaceuticals, can disrupt the endocrine system of aquatic vertebrates, and their effect on invertebrates is unclear (Sumpter, 2008). Certain antibiotics such as diclofenac occur in wastewater at chronic toxicity dose for fish (Schwaiger, Ferling, Mallow, Wintermayr, & Negele, 2004).

2.1.2. Surfactants

Surfactants, or surface-active agents, are compounds that lower the surface tension between two phases, typically between a liquid and a solid or between two liquids. These compounds commonly have hydrophobic and hydrophilic groups, allowing them to interact with both oil and water. They are named after their hydrophilic groups, categorized as anionic, nonionic, or cationic. Global surfactant production is predominantly led by anionic surfactants, constituting 60%, while nonionic surfactants comprise 30%, with cationic and amphoteric surfactants representing 10% (Palmer & Hatley, 2018). Due to their persistence in the environment and higher toxicity to aquatic organisms, cationic surfactants, especially quaternary ammonium compounds (QACs), used in disinfectant formulations, pose greater environmental concerns (Tezel & Pavlostathis, 2015). The use of disinfection products containing QACs has increased during the recent SARS-CoV-2 pandemic (Hora, Pati, McNamara, & Arnold, 2020). Studies reported that 75% of QACs enter wastewater treatment systems, while the remaining 25% is directly discharged into the environment (Mohapatra et al., 2023). QAC toxicity occurs through the interaction between the cationic quaternary nitrogen and negatively charged structures on the bacterial cell membrane. QACs induce aquatic toxicity via oxidative stress, disruption of the cell membrane, or molecular defects causing apoptosis (Qian et al., 2022). A major concern about QACs is the potential dissemination of antimicrobial resistance in

bacteria. Upon exposure to sublethal concentrations of BAC, the most widely used QAC, bacteria may develop cross-resistance against certain antibiotics by altering membrane permeability, increasing efflux pumps, undergoing horizontal gene transfer, or acquiring adaptive mutations (Mohapatra et al., 2023).

2.1.3. Pesticides

Pesticides are widely used agents in agriculture for pest and disease management to enhance crop yields. The global use of pesticides has substantially increased from 2.3 million metric tons in 1990 to 4.1 million metric tons in 2018 (Degrendele et al., 2022). Due to their significant environmental and human health hazards, certain pesticides are banned in the previous century. Well-known examples include dichlorodiphenyltrichloroethane (DDT), chlordane, and dieldrin, collectively named legacy pesticides, which are reported to still persist in aquatic environments (Y. Yang et al., 2022). The main groups of pesticides currently in use include organophosphates, triazines, pyrethroids, and triazoles. These pesticides may be present in aquatic systems, the atmosphere, or soil, where they can impact agricultural soil quality and biodiversity (Tang & Maggi, 2021). Mevinphos, esfenvalerate, cyfluthrin, diuron, terbuthylazine, glyphosate, and atrazine are among the most commonly applied pesticides. According to a recent study, either these pesticides or their transformation products were detected in soil frequently around the globe (Sabzevari & Hofman, 2022). Many pesticides act on the nervous system of insects, posing a risk of neurotoxicity in exposed humans (Chetty-Mhlanga et al., 2021; Foong et al., 2020). Other health hazards include hepatorenal, endocrine, metabolic disorders, and various types of cancers (Sabzevari & Hofman, 2022).

2.1.4. Heavy Metals

HMs are characterized by their high density and atomic weight. Certain HMs, including cobalt (Co), Cu, nickel (Ni), iron (Fe), manganese (Mn), molybdenum (Mo), selenium (Se), and Zn, are essential for various organisms but can become harmful in excessive amounts. On the other hand, HMs like arsenic (As), Cd, chromium (Cr), and lead (Pb) are non-essential and can cause severe health complications even at low concentrations (Munir et al., 2022). These metals originate from both anthropogenic and natural sources, with rapid urbanization, alterations in land use, and industrialization in developing countries with large populations being significant contributors to HM pollution (Rai, Lee, Zhang, Tsang, & Kim, 2019).

The bioaccumulation of HMs across trophic levels poses a significant risk to organisms in higher trophic levels. Unlike organic pollutants, HMs do not degrade but undergo transformations into different oxidation states. This ability to transform into a toxic or more mobile state under changing environmental conditions makes HMs a persistent risk factor. Cd, mercury (Hg), and Pb may enter into the atmosphere, degrading air quality. Along with arsenic, these HMs are poisonous to humans upon intake. Cadmium, in particular, lacks attributes that favor plant growth or metabolism unlike other metals (Hayat, Nauman, Nazir, Ali, & Bangash, 2019). Arsenic and cadmium have been shown to have neurodegenerative effects (Mitra et al., 2022). Methylmercury, a product of mercury contact with aquatic sediments, is highly toxic to both humans and the environment (Gworek, Dmuchowski, & Baczewska-Dąbrowska, 2020). Oxidized chromium (Cr VI) is a more toxic form of chromium, primarily contributed by the ferrochrome industry (Coetzee, Bansal, & Chirwa, 2020). Although copper and zinc are essential micronutrients, elevated levels can disrupt cellular processes and lead to adverse health effects in organisms. For a more in-depth exploration of the adverse effects of HMs, comprehensive reviews are available elsewhere (Mitra et al., 2022; Munir et al., 2022).

The environmental impacts of HMs in wastewater are profound and extend beyond the immediate aquatic environment. Discharged into natural water bodies, HMs can persist and accumulate in sediments, impacting benthic organisms and the broader aquatic ecosystem. Furthermore, heavy metals may undergo leaching and migration, affecting groundwater quality. The persistence of heavy metals in the environment raises concerns about their long-term impact on ecosystems, biodiversity, and human health, necessitating comprehensive wastewater management strategies to mitigate these risks (Mishra et al., 2019).

2.2. Wastewater Treatment by Microalgae

Algae has been utilized in the treatment of domestic and industrial wastewater and agricultural runoff for the removal of nutrients like nitrogen (Garcia, Mujeriego, & Hernandez-Marine, 2000) and phosphorus (González, Cañizares, & Baena, 1997). HRAPs are the common method for algae production for biofuels where the feed is wastewater and algae supply its energy for growth from the sun (Park, Craggs, & Shilton, 2011). The removal of nutrients in a batch reactor depends on the initial concentration of the nutrient and is effective when the nutrient concentration is below a certain level (Aslan & Kapdan, 2006). Higher concentrations cannot be efficiently removed because the growing algae limit the light coming into the reactor. Dissolved oxygen and pH are important factors for nitrogen and phosphorus removal and should be monitored and optimized (Cai, Park, & Li, 2013). The affinity of the nutrient-to-be-treated to algae is also an important parameter for removal. For

instance, ammonium is more preferred by some types of algae as a nitrogen source than nitrate, and the presence of ammonium represses nitrate removal (I. Morris & Syrett, 1963).

Algal wastewater treatment as EC removal is widely studied in the literature. *C. vulgaris* is tolerant to the common xenobiotic, pesticide diazinon, and can transform it into a less toxic product, 2-isopropyl-6-methyl-4-pyrimidinol (Kurade, Kim, Govindwar, & Jeon, 2016). Natural hormones can be easily removed from wastewater systems within several days with the help of algal biotransformation (Peng et al., 2014). The removal of synthetic hormones is harder, however, might be facilitated when in a mixture with natural hormones (P. Wang et al., 2016). The biotransformation itself may not be enough to remove toxicity, i.e., the by-products in the water stream may bear toxicity, as well (Peng et al., 2014). de Wilt et al. propose an algal bioreactor system for advanced treatment of separated wastewater systems to remove pharmaceuticals as well as to recover common nutrients nitrogen and phosphorus (de Wilt et al., 2016). The authors discovered that most of the tested pharmaceuticals were removed via photodegradation except for metoprolol and paracetamol, whose removal was through biodegradation by algae along with photolysis. Photodegradation by-products can also be toxic, and they are shown to be matrix composition dependent (Fatta-Kassinos, Vasquez, & Kümmerer, 2011). Studies on the algal treatment of ECs have primarily focused on synthetic media, but it is essential to investigate real wastewater applications, considering the fluctuations in parameters such as pH or temperature in HRAPs (Z. N. Norvill, Shilton, & Guieysse, 2016).

Another major pollutant type which microalgae can be used for removal is HMs. Microalgae can adsorb HMs due to the characteristics of their cell wall. The sorption potential is dynamic, and HM concentrations affect biosorption capacity through alteration in the expression of extracellular polymeric substances (EPS) (Mota et al., 2015). Metal sorption occurs owing to the interaction between negatively charged side chains of EPS and positively charged heavy metal ions (Monteiro, Castro, & Malcata, 2012). The sorption to algae typically occurs rapidly, and its extent is influenced by several parameters such as pH, algal strain, the type of heavy metal, and the biomass concentration (Kaplan, 2013). When employing microalgae cultures for the phycoremediation of HMs, the algal capacity to sequester metals and their tolerance to specific metals become decisive factors in the selection of suitable algae (Hashemi, Leppard, & Kushnert, 1994). The utilization of microalgae for HM treatment is a preferable choice, especially considering their remarkable removal efficiency, even at low metal concentrations (Monteiro et al., 2012). Sorption using dead algal cells is also applicable, where problems in sustaining the culture in the contaminated media are not of concern during application (Suresh Kumar, Dahms, Won, Lee, & Shin, 2015).

2.2.1. Microalgae Selection

The selection of microalgae for wastewater treatment is a critical aspect of developing efficient and environmentally sustainable treatment strategies. Species choice depends on the type of contaminants to be removed from the system. Microalgae has been a prime choice for the removal of nutrients from wastewater effluents for decades (Vo et al., 2019). *Chlorella sp.* and *Scenedesmus sp.* are the most extensively studied microalgae genera for wastewater treatment, as they grow well on nutrients in the wastewater (de Morais, Murillo, Lens, Ferrer, & Uggetti, 2022; Z. He et al., 2023). Particularly in the context of MPs, microalgae tolerance to the contaminant of interest should be screened for resistant algae. The screening process involves the identification and cultivation of microalgal species with inherent resistance or tolerance to specific MPs. Half-maximal inhibitory concentration (IC_{50}) derived from a dose-response model can be used as an indicator for the sensitivity of microalgae to the toxic chemical (Ritz, Baty, Streibig, & Gerhard, 2016). Factors such as the ability to uptake, sequester, and metabolize hazardous contaminants are pivotal considerations in the selection criteria. Additionally, the choice of microalgae must align with the targeted pollutants, ensuring an effective removal process (Sutherland & Ralph, 2019).

A significant concern in microalgal photobioreactors (PBRs) is the occurrence of grazers such as protists, which can deplete up to 90% of the algae biomass within just a few days (Maryjoseph & Ketheesan, 2020). While strictly controlled closed PBRs may be employed to prevent grazer contamination, they are expensive for wastewater treatment operations. Microalgae that exhibit tolerance to extreme conditions, such as extreme pH or temperature, could offer a viable solution. For example, *Galdieria* can thrive at pH levels as low as 0.0 and survive thermophilic conditions that protists cannot endure (Kharel, Shrestha, Tan, & Selvaratnam, 2023). *Arthrospira* culture pH increases above 11.0 during growth with externally supplied nutrients, establishing a natural barrier by the microalgae itself against predators (W. Liu, Wang, & Liu, 2019). Marine microalgae inherently thrive in high salinity, which provides a barrier against grazers. However, when utilizing extreme conditions to prevent predation, careful monitoring of the impact of these conditions on the overall efficiency of bioremediation is essential (Henkanatte-Gedera et al., 2015).

The selection process necessitates a comprehensive understanding of the physiological and biochemical characteristics of various microalgal strains, ensuring the development of robust and tailored wastewater treatment solutions capable of addressing the challenges posed by micropollutants, including those of a hazardous nature.

2.2.2. Pollutant Removal Mechanisms by Microalgae

Microalgae utilize nutrients such as nitrogen, phosphorus, potassium (K), or sulfur (S) species for growth. Removal of those nutrients by algae from wastewater occurs through assimilation into organic matter. Algae utilizes a variety of direct or indirect mechanisms for the removal of ECs. Direct mechanisms include bioadsorption, bioaccumulation, biodegradation, and hydrolysis by algae (J.-L. Zhou et al., 2022). Indirect mechanisms involve photodegradation, volatilization, and biodegradation by bacteria in consortium with algae (Gu et al., 2020; J.-L. Zhou et al., 2022). Algae can biodegrade various types of PCPPs (Nguyen, Yoon, Ngo, & Jang, 2021). Since those contaminants occur at very low levels in wastewater, biodegradation may occur through co-metabolism, where another substrate is present for maintaining the microorganisms' growth (Maryjoseph & Ketheesan, 2020). Hydrolysis is a type of transformation of chemicals. Studies in the literature reported that hydrolysis facilitated the subsequent redox reactions and biodegradation of certain compounds (R. Jiang et al., 2019; C. Zhang, Zhang, Dong, & Zhou, 2021). The predominant removal mechanism is adsorption for hydrophobic compounds, and volatilization for the ECs that have high Henry's law constant (Matamoros, Gutiérrez, Ferrer, García, & Bayona, 2015). Photolysis is the direct breakdown of the compounds by light energy. It is the main approach for the removal of fluoroquinolones, cephalosporins, and sulfonamides in microalgae-based wastewater treatment systems (R. Liu, Li, Tu, & Hao, 2021). Certain chemicals, such as the antibiotic norfloxacin, are recalcitrant to photolysis. Their photodegradation is facilitated by reactive oxygen species (ROS) produced by algae (Leng et al., 2020). Certain types of contaminants, such as pesticides, HMs, or antibiotics like triclosan also accumulate in microalgae cells. They enter the cells via passive diffusion, facilitated diffusion, or active transport across the cell membrane (Sutherland & Ralph, 2019). The potential for bioaccumulation and the subsequent fate of ECs within the microalgae biomass must be thoroughly evaluated to prevent unintended ecological consequences (Maryjoseph & Ketheesan, 2020).

Algae can be employed for metal removal through biosorption. This can be achieved either by using dead cells, where only adsorption onto the lifeless biomass occurs, or by using live algae cells, where adsorption is followed by the assimilation of metals. The presence of functional groups, such as hydroxyl (-OH), carboxyl (-COOH), amino ($-NH_2$), and sulfhydryl (-SH) on the surface of microalgae cells facilitates the adsorption of positively charged metal ions onto dead biomass (Chai et al., 2021). Biosorption is contingent on abiotic and biotic factors such as pH, temperature, contact time of biomass to metals in the matrix, biomass concentration, and initial metal ion concentration (Zeraatkar, Ahmadzadeh, Talebi, Moheimani, & McHenry, 2016). Different algae species exhibit

varying biosorbent capacities for different metals (Çetinkaya Dönmez, Aksu, Öztürk, & Kutsal, 1999). The biosorption capacity of algae may be altered in the presence of multiple metals in the matrix (Saavedra, Munoz, Taboada, Vega, & Bolado, 2018).

Heavy metal ions can be detoxified with the help of algal biotransformation (Gadd, 2004). Transformation of heavy metals in microalgal cells may occur as complexation with nitrogen or hydroxyl groups (Essa & Mostafa, 2013). Sulfur species of heavy metals, i.e., silver sulfide (Ag_2S), may also occur and accumulate at low metal concentrations (Leonardo et al., 2016). Algae has its own complexation proteins, phytochelatins (PCs) for the chelation of heavy metals to increase mobility. PCs are a group of cysteine-rich proteins belonging to class III metallothioneins (Suresh Kumar et al., 2015). The enzyme phytochelatin synthase is responsible for phytochelatin biosynthesis from glutathione (GSH) and its homologs (Gupton-Campolongo, Damasceno, Hay, & Ahner, 2013). The general formula of PC is $(\gamma\text{-Glu-Cys})_n\text{-Gly}$, where n varies between 2 to 11. Abboud and Wilkinson showed increased PC synthesis in *Chlamydomonas reinhardtii* after short-term exposure to Cd or Cu (Abboud & Wilkinson, 2013). In another study pretreatment with Zn is shown to induce tolerance to Cd, Cu, Hg, Pb, and As in *Dunaliella tertiolecta* (Tsuji et al., 2002). Authors suggest that PC synthesis was induced by Zn treatment which might have played a role not only in the detoxification of heavy metals but also in the mitigation of oxidative stress as algae exhibit increased tolerance to hydrogen peroxide (H_2O_2) and paraquat (Tsuji et al., 2002).

2.2.3. Environmental and Economic Benefits of Using Microalgae in Wastewater Treatment

Microalgae efficiently remove pollutants such as nitrogen and phosphorus, playing a pivotal role in nutrient removal from wastewater. This not only mitigates environmental pollution but also helps prevent issues like eutrophication in water bodies (Leong, Huang, & Chang, 2021). Additionally, the biomass produced during the treatment process can be harnessed for bioenergy production, contributing to the development of renewable energy resources (Mohsenpour, Hennige, Willoughby, Adeloye, & Gutierrez, 2021). A study reported that in an HRAP for wastewater treatment coupled with biofuel production, 800–1400 GJ/ha/year of energy is produced in the form of harvested algal biomass (Mehrabadi, Farid, & Craggs, 2017). The cultivation of microalgae in wastewater systems also acts as a carbon capture mechanism, reducing greenhouse gas emissions. A recent study revealed that CO_2 emissions from HRAP are significantly lower (0.15 kg CO_2e/m^3) than those of a conventional system (0.46 kg CO_2e/m^3), as well as operational costs (Kohlheb et al., 2020). Economically, the integration of microalgae in wastewater treatment systems provides a dual benefit by simultaneously treating wastewater and generating valuable biomass, potentially offsetting

operational costs further. Biomass can be utilized in downstream processes to produce value-added compounds such as pigments or lipids, or directly utilized as a fertilizer (Flores-Salgado, Thalasso, Buitrón, Vital-Jácome, & Quijano, 2021). Specifically, antioxidant compounds such as astaxanthin and β-carotene, pigments phycocyanin and phycoerythrin, omega-3, omega-6, and other polyunsaturated fatty acids (PUFAs), and collagen or anti-aging agents can be synthesized from microalgae biomass (Gu et al., 2020). Overall, the use of microalgae in wastewater treatment aligns with sustainable practices, offering a multifaceted approach that addresses environmental concerns while showcasing economic viability.

2.3. Symbiosis Mechanisms between Microalgae and Bacteria

Microalgae and bacteria have complex symbiotic connections that are essential to the health of many ecosystems. These relationships affect the cycling of nutrients, environmental remediation, and overall ecosystem health (Xiao et al., 2023). The symbiosis between algae and bacteria is characterized by reciprocal exchanges, where microalgae benefit from bacterial nutrients and protection, while bacteria receive organic carbon compounds. Understanding these intricate relationships is crucial for harnessing the synergistic potential of microalgae and bacteria in applications ranging from wastewater treatment to bioenergy production. In this section, diverse mechanisms underpinning the symbiotic relationship between microalgae and bacteria are reviewed to shed light on their ecological significance and potential applications in sustainable biotechnological processes.

2.3.1. Carbon-Oxygen Exchange

Bacteria utilize oxygen (O_2) to convert fixed carbon into energy for maintaining themselves while biologically treating organic load in wastewater. This phenomenon makes the aeration of the wastewater systems a prime bottleneck for cost and CO_2 emission. It has been stated that the aeration cost of a typical wastewater treatment plant is 50% of the overall operational cost (Muñoz & Guieysse, 2006). Effective utilization of photosynthetic microalgae in such systems may overcome external aeration necessity as well as decrease the carbon footprint as algae utilize CO_2 produced in the system. Indeed, treatment systems utilizing photosynthetic oxygenation have been applied for almost 60 years (Flores-Salgado et al., 2021). It has been shown that efficient treatment can be accomplished without an external O_2 supply in such photosynthetically aerated systems (Gu et al., 2020).

In algae-bacteria wastewater treatment systems, the integration of carbon mitigation strategies is crucial for enhancing overall efficiency and sustainability. Algae-bacteria interaction in such systems proceeds with algae utilizing CO_2 to photosynthetically produce O_2 and fixed carbon, which are utilized by bacteria and converted into CO_2 and inorganic nutrients. The latter is used by algae to further grow and continue the cycle. The main apparent outcomes of this cycle, apart from photosynthetic oxygenation, are the net yield of biomass and net zero CO_2 emission. The CO_2 fixation efficiency of algae is manifold higher than that of plants (Xu, Gu, Wang, Shatner, & Wang, 2019). A consortium of *Chlorella vulgaris* and *Pseudomonas sp.* was shown to treat a mixture of phenol and pyridine, with almost complete CO_2 mitigation (Essam, ElRakaiby, & Hashem, 2013). Theoretically, linking this system with carbon capture from, i.e., flue gas might decrease the operational net emission below zero. The integrated algae-bacteria wastewater treatment system highlights the potential for carbon-neutral or even carbon-negative wastewater treatment processes, aligning with the broader goals of environmental sustainability.

2.3.2. Exchange of Growth Promoting Factors

Reciprocal benefit exchange between algae and bacteria in a co-culture is not limited to the recycling of carbon and oxygen. There are diverse types of factors such as vitamins of B complex, indole-3-acetic acid (IAA), metabolites in amino acid biosynthesis pathways, lycopene, squalene, iron chelators, or organosulfur compounds interchanged between algae and bacteria (Rambo, Dombrowski, Constant, Erdner, & Baker, 2020; Wienhausen, Noriega-Ortega, Niggemann, Dittmar, & Simon, 2017). One of the well-studied growth-promoting factors for algae supplied by bacteria is vitamin B_{12} (Cooper et al., 2019). Over half of the microalgae species are B_{12} auxotrophs, whereas the portions of thiamine and biotin auxotrophs are substantially lower, around 20% and 5%, respectively (Croft Martin, Warren Martin, & Smith Alison, 2006). Vitamin B_{12} is solely synthesized by prokaryotes, and algae acquire it through a symbiotic relationship with bacteria (Helliwell, Wheeler, Leptos, Goldstein, & Smith, 2011). In return for B_{12} from *Ruegeria pomeroyi*, the marine diatom *Thalassiosira pseudonana* selectively feeds a sulfur-rich molecule called 2,3-dihydroxypropan-1-sulfonate, which serves as an organic carbon as well as a sulfur source for bacteria (Durham et al., 2015). Bacteria may also provide micronutrients indirectly through, i.e., synthesizing chelators for trace metals that are essential for algae. Iron uptake of dinoflagellate *Scrippsiella trochoidea* increased by 20-fold when co-cultured with *Marinobacter sp.*, which produced an iron chelator, vibrioferrin (Shady A. Amin et al., 2009).

Constructing co-cultures with such nutrient exchange in wastewater treatment systems may be beneficial as it can decrease resource material amounts necessary for maintaining the biotic factor, as well as it may elevate the produced biomass which can be a resource for downstream applications.

2.3.3. Toxicity Reduction

In the process of MP treatment from wastewater, these contaminants bear toxicity to the microorganisms utilized in the treatment. Another support mechanism for consortium members to each other may be detoxification of the contaminant that would be harmful to the other members of the consortium and would reduce the overall treatment efficiency. For instance, antibiotics are harmful to most bacterial strains. Microalgae are not as sensitive to antibiotics as bacteria, considering that antibiotics are a part of the defense mechanism of fungi against bacteria (Ozcelik et al., 2022; van der Grinten, Pikkemaat, van den Brandhof, Stroomberg, & Kraak, 2010). The removal of antibiotics by algae might favor the survival capacity of bacterial partner(s). The vice versa may also occur: Antibiotic-resistant bacteria may increase the tolerance of algae to antibiotics, such as in the case of sulfonamide tolerance of *S. obliquus*, which tolerated up to 160 mg/L sulfonamide antibiotics in the presence of bacterial consortium (H. Wang et al., 2022). Bacteria grow very rapidly, faster than algae, and can acclimate to hazardous chemicals through the acquisition of resistance genes (Q. Xiong et al., 2021). This feature of bacteria can be utilized in the consortium for the protection of algae from hazardous contaminants in the treatment process. For instance, the introduction of *Achromobacter xylosoxidans* eliminated the harmful effects of BAC on microalgae in an oxidation pond (Liang, Liu, Ge, Liu, & Wong, 2015). Bacteria may also increase the tolerance of algae in consortium against heavy metals like copper (Jacqueline L. Levy, Stauber, Wakelin, & Jolley, 2009). Bacteria not only provide protection against contaminants in the growth environment but also against other invasive bacteria. For instance, the antibiotic tropodithietic acid synthesized by *Phaeobacter inhibens* protects the associated microalgae, *Emiliania huxleyi,* from bacterial invasions (Celik et al., 2017).

2.3.4. Metabolic Complementation

Metabolic complementation refers to a phenomenon in which two or more microorganisms collaborate to complete metabolic processes that none of them can carry out independently (Mori, Ponce-de-León, Peretó, & Montero, 2016). This process involves interdependent species where the product of one strain becomes the substrate of another. A prime example is the formation of methane from organic matter: various taxa of microorganisms play distinct roles in different stages of methane production, none of which could accomplish the entire pathway alone. For microalgae-bacteria

interactions, metabolic complementation can be considered as a set of mutually beneficial interactions between microalgae and bacteria, where each organism complements the metabolic needs of the other. In this context, metabolic complementation may refer to something as basic as the interchange of one or a few metabolites, or more generalized interactions where microalgae supply oxygen and organic carbon to the bacteria, and bacteria reciprocate by providing carbon dioxide and nutrients through the biodegradation of organic carbon.

Before continuing on specific examples of metabolic complementation between algae and bacteria, the concept of the parasite-mutualist continuum should be introduced. Parasitic or mutualistic interactions are defined based on the impact of the interaction on a partner's evolutionary fitness as positive or negative (Drew, Stevens, & King, 2021). The continuum states that through certain conditions such as environmental or biotic factors (Ramanan, Kim, Cho, Oh, & Kim, 2016), the interrelation may shift from one end to the other. On one end of the continuum resides parasitism, on the other end, mutualism; and commensalism sits at the center. One might consider why or how mutualism through metabolic complementation benefits an organism. It might be due to the high cost of one process, an organism should avoid it because the benefit is too low compared to the cost, a phenomenon known in the literature as the Black Queen Hypothesis (E. Kazamia, Helliwell, Purton, & Smith, 2016). For instance, neither of the strains of the *Prochlorococcus* genus carries the *katG* gene, encoding for the heme-dependent catalase-peroxidase enzyme, which catalyzes H_2O_2 (J. J. Morris, Kirkegaard, Szul Martin, Johnson Zackary, & Zinser Erik, 2008). This enzyme has a high iron cost, and leaving the neutralization of H_2O_2 to heterotrophic bacteria in the niche favors this alga genus (J. J. Morris, Johnson, Szul, Keller, & Zinser, 2011). In the context of metabolic complementation, to lose a gene might mean to gain a genome (Hom, Aiyar, Schaeme, Mittag, & Sasso, 2015).

An example of metabolic complementation potentially very common in the coastal environments is IAA - tryptophan (Trp) signaling between the diatom *Pseudo-nitzschia multiseries* and *Sulfitobacter*. IAA synthesized from Trp and excreted by *Sulfitobacter* promotes cell division of diatom, which synthesizes Trp from IAA, and excretes it from the cell along with organosulfur compounds, which are uptaken by bacteria (S. A. Amin et al., 2015). B_{12} vitamin dependence of certain algae is another example. Cobalamin is an essential cofactor of methionine synthase (METH). Certain algae utilize a B_{12}-independent methionine synthase (METE) while others synthesize methionine via METH. It has been shown that B_{12} dependence in microalgae evolved recently, and growth in the presence of cobalamin might lead to repression of METE expression (Helliwell et al., 2011). Another study showed that B_{12} auxotroph microalgae could be co-cultured with B12-

synthesizing bacteria that lack complete pathways to synthesize other B vitamins, which can be produced by algae (Cooper et al., 2019). In that study, the authors showed that a stable long-term co-culture could be constructed without the supply of any external B vitamins. Another example is the nitrogen metabolism of *Chlamydomonas*. Calatrava et al. co-cultured *Chlamydomonas* with *Methylobacterium spp.* on a proline-derived nitrogen source, which could not be utilized by *Chlamydomonas* (Calatrava, Hom, Llamas, Fernández, & Galván, 2018). The microalgae grew well on the ammonium biotransformed from proline by bacteria.

Up to here, metabolic complementation as an interspecific concept was covered. In fact, different tropic phases of microalgae may also complement each other. Algae utilize photoautotrophy in the presence of light, and heterotrophy in the dark. A synergy between phototrophic energy metabolism and heterotrophic carbon metabolism can be constructed with certain algae via culturing in mixotrophic conditions, where both light and external organic carbon source are supplied (Z. Zhang et al., 2017). Compared to photoautotrophy, mixotrophy may give a higher growth yield, through higher photosynthesis performance, decreased light damage, and substantial upregulation in the glucose metabolism (Y. Liu et al., 2022). Many species of microalgae, including *Auxenochlorella protothecoides*, *Chlamydomonas reinhardtii*, *Chlorella pyrenoidosa*, *Chlorella sorokiniana*, *Chromochloris zofingiensis*, *Galdieria sulphuraria*, *Haematococcus pluvialis*, have been demonstrated to be cultured under mixotrophic conditions for various purposes. Along with the growth boost, mixotrophy may favor nutrient recovery from certain kinds of substrates such as biogas slurry (Han et al., 2022), increase toxicity tolerance against pharmaceutical pollutants (H. Wang et al., 2022), and elevate EPS production in cyanobacteria (Ghorbani, Nowruzi, Nezhadali, & Hekmat, 2022).

2.3.5. Factors Affecting Symbiosis Dynamics

Algae-bacteria co-culture systems could be applied for the efficient treatment of various types of wastewater. To maintain this, various biotic and abiotic factors should be considered in the system design. In a stagnant pond, for instance, temperature and light intensity, alkalinity, turbidity, initial ratio of biomass, algal cell size, and pond depth were all found to significantly affect the removal of substrates and microalgal biomass productivity (Cervantes-Gaxiola et al., 2020). Temperature and illumination of the system should be carefully controlled, as the alteration of those might cause a population shift. Low light conditions might lead to oxygen depletion, where denitrifying bacteria might prevail and compete for limited nutrients with microalgae (Ozcelik et al., 2022). High temperature and low light may favor nitrifying bacteria, competing with microalgae for ammonium,

which leads to nitrate accumulation (Gonzalez-Camejo et al., 2018). Gonzalez-Camejo et al. showed almost complete removal of nitrogen under high light intensity at 22 °C by a microalgae-bacteria consortium (Gonzalez-Camejo et al., 2018). On the other hand, other studies reported better removals at lower light intensity (Arcila & Buitrón, 2017; Pastore, Santaeufemia, Bertucco, & Sforza, 2018). Another important factor is pH. Increasing pH from acidic to neutral increased ammonia removal efficiency from 78% to 86% (Liang et al., 2015). It should be noted that the effect of the pH should be examined with care, as it is difficult to differentiate between the direct effect of pH on the consortium members, and the effect on abiotic conditions such as carbonate or ammonia equilibria, available phosphorus or trace metals (Muñoz & Guieysse, 2006).

An important factor in continuous algae-bacteria consortium systems is hydraulic retention time (HRT). Algae grow relatively slower than bacteria. The HRT of the HRAPs typically varies between 2-6 days (Muñoz & Guieysse, 2006). Decreased HRT may lead to poor treatment efficiency for nutrients and chemical oxygen demand (Arcila & Buitrón, 2016), or washout of microalgae (Pastore et al., 2018). Wastewater or contaminant feeding regime to operating HRAP may also affect the removal efficiency of contaminants or nutrients. In their study for surfactant treatment, Serejo et al. found better biomass yield for microalgae and better removal efficiencies when feed was shifted from continuous (24h/d) to semi-continuous (12h/d or 1 h/d) at similar HRT of 7.0 days (Serejo, Farias, Ruas, Paulo, & Boncz, 2020). Cho et al. indicated that wastewater treatment efficiency and algae biomass production in an HRAP that contains an algae-bacteria consortium were significantly affected by cyanobacteria in the influent (Cho, Ramanan, Heo, Kang, et al., 2015).

As stated here, the dynamics of algae-bacteria consortia are intricately influenced by a multitude of biotic and abiotic factors. Understanding the intricate interdependencies and responses to these biotic and abiotic factors is fundamental for optimizing the performance of algae-bacteria consortia in various applications, ranging from wastewater treatment to sustainable bioenergy production.

3. MATERIALS AND METHODS

3.1. Solutions and Media Preparation

All algae media was prepared from stock chemicals according to the recipes given in Table 3.1. The recipes were taken from The Culture Collection of Algae at the University of Texas at Austin (https://utex.org/pages/algal-culture-media). Freshwater media was prepared from ultrapure water (>18.2 MΩ). For seawater media, seawater from the Black Sea was collected (41°14'44.7"N, 29°00'50.2"E) with 20 L carboys and stored at +4 °C until use. Seawater was filtered, and the pH was adjusted to 6.8 before autoclaving for 15 minutes at 121°C (All the autoclave processes throughout this dissertation were conducted under these conditions unless stated otherwise). A commercial sea salt mixture (Red Sea Salt) was added to achieve 32‰ salinity. All ingredients of F/2 medium were added aseptically after autoclave. ¼ F/2 (8‰) was prepared by diluting seawater. The pH of ¼ F/2 was further adjusted to 7.0 after autoclave with sterile 1.5 N HCl. For BG-11 medium preparation, all ingredients were added, and the pH was adjusted to 7.5 before autoclave. 1 mM sodium thiosulfate pentahydrate ($Na_2S_2O_3.5H_2O$) was added to the BG-11 agar medium. Modified Bold 3N medium (MB3N) was prepared by adding all the ingredients except vitamins before autoclave. Soilwater was prepared as follows: 9 g greenhouse soil and 5 mg $CaCO_3$ were added to 1 L of deionized water. It was pasteurized in a 98 °C water bath for 3 hours for 2 consecutive days. The medium was then filtered and autoclaved.

Minimal salt medium (M9) ingredients were as follows: 7.4 g/L K_2HPO_4, 3.0 g/L KH_2PO_4, 0.5 g/L NaCl, 1.00 g/L NH_4Cl, 0.1 M $MgSO_4.7H_2O$, 0.01 M $CaCl_2.2H_2O$ and 0.1% (v/v) trace metal solution (0.5 g/L $ZnCl_2$, 0.30 g/L $MnCl_2.4H_2O$, 3.0 g/L H_3BO_3, 2.0 g/L $CoCl_2.6H_2O$, 0.10 g/L $CuCl_2.2H_2O$, 0.20 g/L $NiSO_4.6H_2O$ and 0.30 g/L $Na_2MoO_4.2H_2O$). All nutrients except for $MgSO_4.7H_2O$ and $CaCl_2.2H_2O$ were added before autoclave, these two nutrients were added aseptically. For Luria-Bertani (LB) medium, 10 g tryptone, 5 g yeast extract, and 5 g NaCl were added into 1 L ultrapure water and autoclaved. LB-200 BAC medium was prepared by adding 20 mL 10000 ppm BAC into sterile LB medium under aseptic conditions. M9-200 BAC agar medium was prepared as follows: All the ingredients of M9 except for $MgSO_4.7H_2O$ and $CaCl_2.2H_2O$ were added into 100 mL ultrapure water in 10-fold concentration. 15 g agar was added into 900 mL ultrapure water. These two solutions were autoclaved separately and mixed after autoclave. $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, and BAC were added before pouring onto 90 mm petri plates. Mueller Hinton II (MH-

II) broth medium was prepared by the addition of 22 g of MH-II powder (beef extract 3g/L, acid hydrolysate of casein 17.5g/L, starch 1.5g/L, BD Difco) into 1 L ultrapure water and autoclave.

Table 3.1. Ingredients of microalgae culture media

	Concentration (mg/L)		
	BG-11	MB3N	F/2
$NaNO_3$	1500	750	75
K_2HPO_4	40	75	-
KH_2PO_4	-	175	-
$NaH_2PO_4.H_2O$	-	-	5
$MgSO_4.7H_2O$	75	75	-
$CaCl_2.2H_2O$	36	25	-
NaCl	-	25	-
Citric acid.H_2O	6	-	-
Ferric ammonium citrate	6	-	-
$Na_2SiO_3.9H_2O$	-	-	30
$Na_2EDTA.2H_2O$	1	4.5	4.4
Na_2CO_3	20	-	-
H_3BO_3	2.86	-	-
$FeCl_3.6H_2O$	-	0.582	-
$Fe(NH_4)_2.(SO_4)_2.6H_2O$	-	-	4.6
$MnCl_2.4H_2O$	1.81	0.246	-
$MnSO_4.H_2O$	-	-	0.152
$ZnSO_4.7H_2O$	0.22	-	0.023
$ZnCl_2$	-	0.03	-
$Na_2MoO_4.2H_2O$	0.39	0.024	0.0073
$CuSO_4.5H_2O$	0.079	-	-
$CuCl_2.2H_2O$	-	-	0.0068
$Co(NO_3)_2.6H_2O$	0.0494	-	-
$CoCl_2.6H_2O$	-	0.012	-
$CoSO_4.7H_2O$	-	-	0.014
Soilwater GR+ medium	-	40 mL/L	-
Vitamin B_{12}	-	0.135	0.135
Biotin	-	0.025	0.025
Thiamin	-	0.335	0.335
$Na_2S_2O_3.5H_2O$ (agar only)	249	-	-

A 50% benzalkonium chloride (BAC) mix was used for BAC stock solution preparation. The mixture contains 76.2% C_{12}BDMA-Cl (benzyldimethyldodecylammonium chloride) and 23.8% C14BDMA-Cl (benzyldimethyltetradecylammonium chloride), which was verified with high performance liquid chromatography (HPLC) analysis. For the preparation of 10 g/L BAC stock solution 5 g 50% BAC mix was dissolved in 250 mL ultrapure water, and the solution was filter-sterilized.

Stock solutions of metal salts were prepared in the concentrations of 5.28 g/L $As2O_3$, 3.26 g/L $CdCl_2$, 5.37 g/L $CuCl_2.2H_2O$, 2.68 g/L $PbCl_2$, 3.20 g/L $Pb(NO_3)_2$, and 4.17 g/L $ZnCl_2$ to achieve 4 g/L arsenic and 2 g/L other metals in metal basis. The metal concentrations in the stock solutions were verified with inductively coupled plasma mass spectrometry (ICP/MS) analysis. Heavy metal stocks were filter-sterilized with 0.45 µm syringe filters and kept at room temperature. EDTA wash buffer for adsorbed heavy metal analysis was prepared as follows: 3.72 g Na_2EDTA was dissolved in around 800 mL ultrapure water. 11.74 g KH_2PO_4 and 2.39 g K_2HPO_4 were added, pH was checked, and the final volume was completed to 1 L. The final solution contains 0.01 M EDTA and 0.1 M phosphate buffer at pH=6.0.

Aseptic conditions were maintained by either a Bunsen burner or a biosafety cabinet (class II, type A). Chemicals used in media preparation were at least purum grade. Heavy metal salts used in the study were analysis grade. Ethyl acetate (EtAc) and acetonitrile (ACN) used for BAC extraction and analysis were puriss p.a. and gradient grade for liquid chromatography, respectively. Nitric acid used in metal analysis sample preparation was analytical grade.

3.2. General Analytical Methods

Bacterial colony forming units (CFUs) were determined as follows: 900 µL sterile saline solution (NaCl, 0.85%) was added to sterile microcentrifuge tubes. 100 µL bacterial sample was mixed into the first tube and mixed by pipetting. 100 µL of this tube was taken, added to the next tube, and mixed. The desired dilution was made in the powers of 10 with this serial dilution method. 50 or 100 µL of diluted sample was pipetted onto an agar plate and spread with a glass spreader. Algae density was determined via microscopy or absorbance. For cell count determination, a 250 µL sample was loaded onto a Neubauer improved hemocytometer, and the cells were counted under a standard light microscope (Boeco, Hamburg, Germany). Spectrophotometric absorbance (optical density, OD) of algae and bacteria samples were determined at wavelengths 680 nm and 600 nm, respectively, on the HACH DR3900 laboratory spectrophotometer (Hach Lange GmbH, Düsseldorf, Germany). The pH and salinity were measured with a portable multimeter (HandyLab 680, SI Analytics, Mainz, Germany) using ScienceLine Plus A 7780 IDS pH probe and LF 413T IDS conductivity probe.

3.3. Optical Density and Fluorescence Measurements on Multiwell Plates

OD and fluorescence of the algae were also measured on multiwell plates using a plate reader (SpectraMax i3, Molecular Devices, Silicon Valley, CA, United States) with the following

parameters: For OD; wavelength, 680 nm; pathcheck, on; shake, 10 seconds. For fluorescence; excitation, 488 nm (9 nm bandwidth); emission, 680 nm (25 nm bandwidth); 3 flashes per read; read from the top; read height, 1.54 mm; shake, 10 seconds; in black opaque well-plate. Fluorescence intensity bandwidth was optimized with the optical density of dilutions of an algae sample at 680 nm (OD_{680}). 9 and 15 nm bandwidth for excitation and 15 and 25 nm bandwidth for emission were tested for fluorescence response. The best linearity with OD_{680} was when the excitation bandwidth was 9 nm, and the emission bandwidth was 25 nm (Figure 3.1).

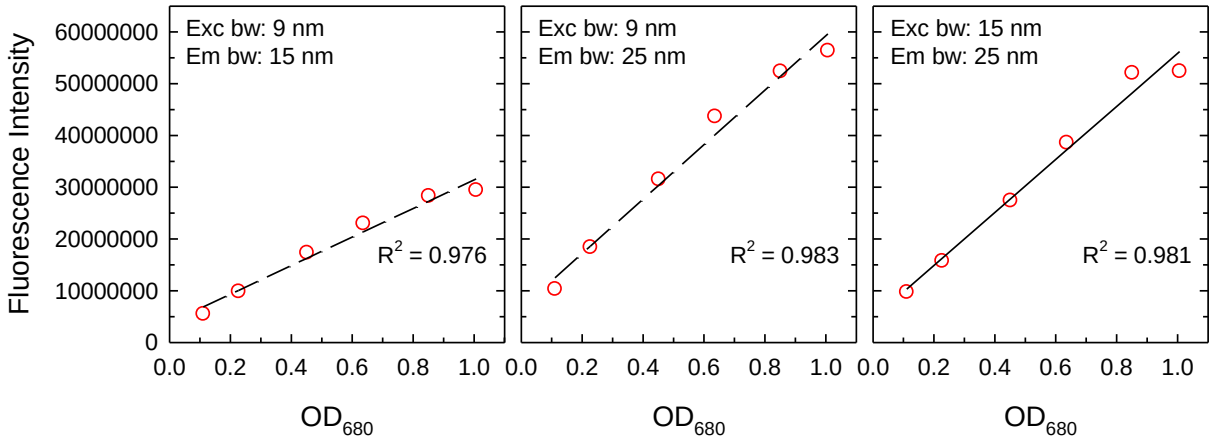


Figure 3.1. Fluorescence intensity optimization for bandwidth. Exc bw: excitation bandwidth. Em bw: emission bandwidth.

3.4. BAC Measurement

For BAC analysis, 0.6 mL sample was mixed with 0.6 mL HPLC eluent used in the analysis (60% ACN, 40% phosphate buffer, pH: 2.5; final pH: 3.5). Samples mixed with the eluent were centrifuged at 10000 g for 10 mins at 4 °C. 0.6 mL of supernatant was taken into a GC vial for analysis. BAC was measured via HPLC. Unless otherwise stated, all analyses of BAC throughout this dissertation used this sampling and analysis methodology.

Extraction of BAC from the sample was done according to a protocol explained elsewhere (Tezel, 2009) as follows: 2 mL of sample was mixed with 0.8 mL 100 mM $AgNO_3$ and 1.2 mL ACN in 10 mL chromatography vials. The mixture was vortexed, and 2 mL EtAc was added. The cap of the final mixture was carefully closed to prevent a leak. The mixtures were mixed overnight on a rotator shaker at 45 rpm. The next day, samples from the EtAc (upper) phase were taken for HPLC analysis.

Parameters for BAC analysis on HPLC were as follows: A Phenomenex LUNA SCX (strong cation exchange) column was serially connected with the Polaris C18 column for separation of BACs. Eluent was 60% ACN + 40% 50 mM phosphate buffer. The column temperature was 35 °C. BACs were detected at 210 nm with a UV-Vis diode array detector. The detector temperature was 35 °C. The flow rate of the eluent was 1 mL/min. The sample injection volume was 50 uL.

3.5. Metal Analysis

2 or 5 mL sample was taken for metal analysis throughout the different parts of this dissertation. Heavy metal analysis was done following a protocol explained elsewhere (J. L. Levy et al., 2008). The sample was centrifuged at 3000 g for 5 mins. The supernatant was collected. The pellet was resuspended in equivolume isotonic seawater, and centrifuged again. Both supernatants were identified as dissolved metal fractions. The pellet was then resuspended in equivolume EDTA wash solution and incubated for 20 mins. The sample treated with EDTA wash solution was centrifuged, supernatant taken, and resuspended in isotonic seawater. It was centrifuged again. Both supernatants were identified as adsorbed fractions. The final pellet was digested with 2 mL concentrated HCl overnight until the solution became clear. It was identified as assimilated metal fraction, and diluted to 20 mL. 0.2 mL conc. HNO_3 was added into 1 mL from dissolved and adsorbed fractions, and the samples were diluted to a final volume of 10 mL. The sample preparation workflow is shown in Figure 3.2. HM standards were prepared with an addition of 1 mL seawater and 0.2 mL conc. HNO_3 to avoid the matrix effect. For metals with low concentrations, calibration curves included 1 µg/L, 2 µg/L, 5 µg/L, 10 µg/L, 20 µg/L, 50 µg/L, and 100 µg/L points. For metals with high concentrations, calibration curve also included 200 µg/L, 500 µg/L, and 1000 µg/L points. To determine instrument's detection and quantification limits, 10 samples were prepared via dissolving 0.1 µg/L metals in UPH_2O. Those 10 samples were analyzed, mean and standard deviation values of the intensities were determined. Instrumental detection limit (IDL) and instrumental quantification limit (IQL) were calculated from Eq. 3.1 and Eq 3.2, respectively, where m is the slope of the calibration curve. The IDL and IQL values are listed in Table 3.2.

$$\mathrm{IDL} = 3 * \mathrm{SD}/\mathrm{m} \tag{3.1}$$

$$\mathrm{IDL} = 10 * \mathrm{SD}/\mathrm{m} \tag{3.2}$$

The diluted samples were analyzed on ICP/MS (iCAP RQ, ThermoFisher Scientific, Waltham, MA, USA) with the following parameters: Plasma cool gas, 14 L/min, auxiliary gas, 0.8 L/min,

plasma power, 1550 W, nebulizer gas, 1 L/min, cooling water flow, 0.68 L/min, interface temperature, 30 °C, vacuum pirani pressure, 1.9 mbar, penning pressure 10^{-6} mbar, vacuum turbo pump speed, 1000 Hz, peristaltic pump speed, 40 rpm, and spray chamber temperature, 2.5 °C.

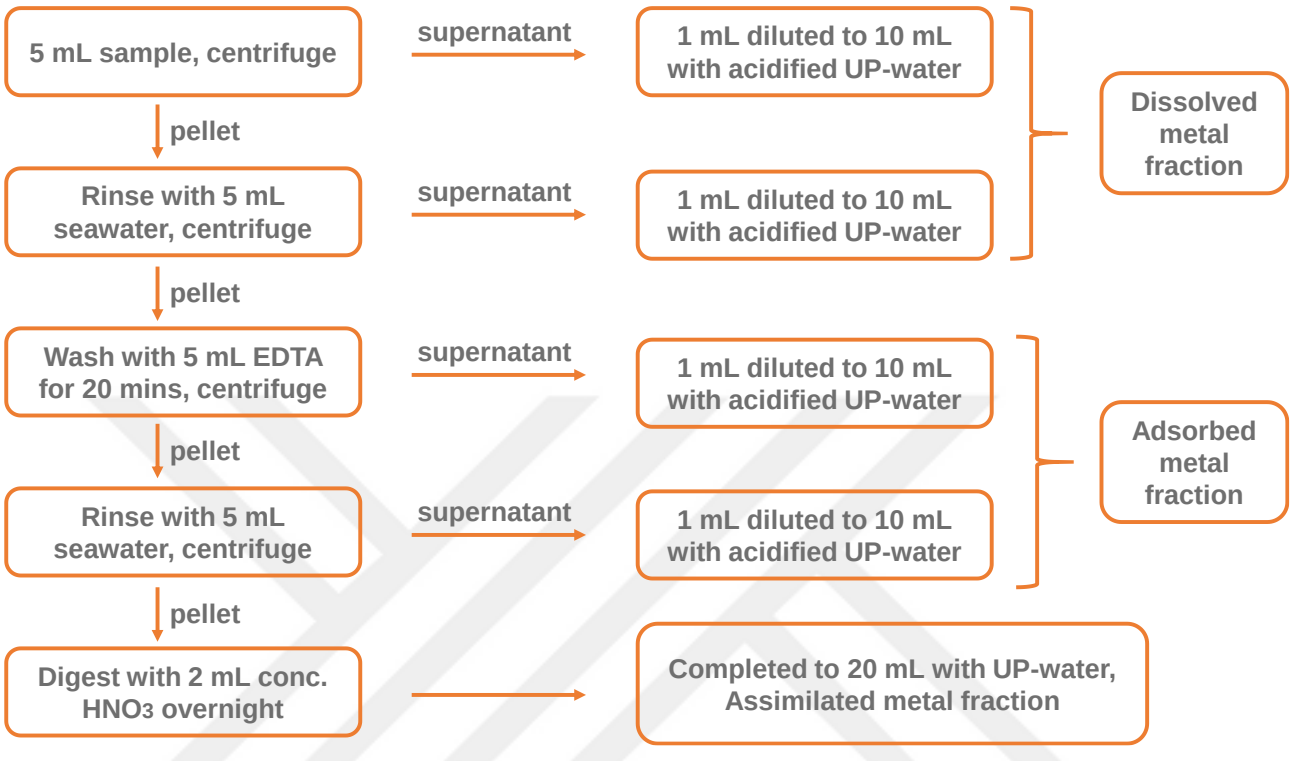


Figure 3.2. Sample preparation workflow for heavy metal analysis on ICP/MS.

Table 3.2. The IDL and IQL values of the analyzed metals

Elements	Isotope for analysis	IDL (µg/L)	IQL (µg/L)
Arsenic	75As	0.032	0.108
Cadmium	111Cd	0.011	0.036
Copper	63Cu	0.016	0.054
Lead	208Pb	0.015	0.050
Zinc	66Zn	0.488	1.625

3.6. Culture Maintenance

For culture maintenance, 100 µL dense stock of *P. sessilinigenes* BIOMIG1 was added into 50 or 100 mL M9 medium with 200 ppm BAC and grown for 3-5 days. Complete BAC degradation was verified with HPLC analysis. This culture was utilized as the stock for the growth experiments in MH II or M9-BAC media. 4 mL BIOMIG1 culture grown in M9-200BAC was added into 8‰ salinity F/2 containing 200 ppm BAC, and grown until all the BAC was degraded. This culture was utilized as the stock for seawater experiments. 50 mL culture grown in M9-200BAC was centrifuged at 10000

g for 10 minutes, and the pellet was resuspended in 1 mL M9-200BAC solution and kept at +4 °C until the next culturing.

Algae strains utilized in this dissertation were all maintained in their respective culture medium. Media for the culture stocks were replenished every other week. To determine the growth phases of the strains, cultures were grown in 100 mL - 2 L batch cultures several times for periodical cell count and OD_{680} data. To utilize these data interchangeably, we plotted the OD-cell count curves for each strain (Figure E.1 in Appendix E). Before the experiments, each algae strain was grown for 5-10 days starting from an OD_{680} value around 0.05 - 0.2, accordingly with the growth rate until it reached the mid- or late-exponential phase. All cultures were grown under 67 μmol photons/m^2·s white cool light at 25 °C, on an orbital shaker at 90 rpm, unless otherwise stated. A reference toxicant was not employed throughout the research to assess whether there was a change in the culture's response. It was assumed that the cultures remained consistent throughout the research timespan.

3.7. Protocols Related to RNA-Seq

For the RNA extraction protocol described in Chapter 7, DNA contamination checks and RNA quantifications were applied. RNA was quantified and A_{260}, A_{280}, and A_{230} values were determined on a NanoDrop microvolume spectrophotometer (Implen GmbH, Munich, Germany). RNA quantification before library preparation and DNA quantification before and after pooling were conducted on a QFX fluorometer (DeNovix, Wilmington, DE, US) using the manufacturer's quantification assay kits according to the manufacturer's protocol. Extracted RNA was also checked for DNA contamination through conducting polymerase chain reaction (PCR) for eukaryotic algae 18S rDNA and bacteria 16S rDNA. Primers used for algae PCR are as follows (Maistro, Broady, Andreoli, & Negrisolo, 2009): X11SF (forward), 5'- TGACTCAGGGAGGTAGTGACA-3', X12SR (reverse), 5'- TCAGCCTTGCGACCATAC-3'. 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) universal primers were used to check for bacterial DNA (dos Santos, Argolo, Argôlo-Filho, & Loguercio, 2019). The PCR products were run on an agarose gel with a positive and a negative control (An example is shown in Figure F.1 in Appendix F).

4. HEAVY METAL AND BAC TOLERANCE OF ALGAE AND BACTERIA

4.1. Introduction

When present in aquatic environments, MPs pose serious threats to the environment and human health. Microalgae have been investigated for their potential in bioremediation, specifically for the removal of HMs and ECs from contaminated water. Utilization of microalgae for the treatment of such contaminants promises good sustainability metrics, as they are photosynthetic microorganisms (Mojiri, Zhou, Ratnaweera, Rezania, & Nazari V, 2022). Optimizing their utilization in environmental restoration depends on knowing how these pollutants affect microalgae.

Trace amounts of metals are required for microalgae cells to sustain core metabolic functions. For instance, copper can be utilized in electron transport systems of photosynthesis (Andersen, 2005), Cu-Zn superoxide dismutases (Okamoto, Asano, Aidar, & Colepicolo, 1996), and copper reduction on cell surface might be associated with nitrate reductase activity (Kong, 2022). Core enzymes such as carbonic anhydrase of CO_2 fixation, and RNA polymerase, which is responsible for transcription of DNA, contain zinc in their structures (Moroney, Bartlett, & Samuelsson, 2001; Sunda, 2012). Although essential at a low level, higher levels of those and non-essential HMs may be toxic to microalgae. High concentrations of HMs may decrease the growth rate and final cell density of the microalgae by altering chlorophyll density (Buayam, Davey, Smith, & Pumas, 2019). HMs at high levels may cause the formation of ROS such as hydrogen peroxide, hydroxyl radicals, or superoxide radicals, which bear oxidative stress on key metabolic and structural molecules of microalgal cells (Kramarova, Fargasova, Molnarova, & Bujdos, 2012). Other harmful effects may include cell membrane lysis, altered/dysfunctional morphology, or alteration in flagella form and length (Miazek, Iwanek, Remacle, Richel, & Goffin, 2015).

The impacts of MPs passing through wastewater, on algae growth and metabolism extend beyond their effects on algae cultivation for the treatment of MPs, encompassing various environmental aspects. Microalgae species are crucial players in their ecosystems; hence, the disruption in their health may indicate harm to the entire ecosystem and biodiversity. Since microalgae are involved in the removal of nutrients, alteration in their metabolic function may affect water quality. They might also accumulate MPs, which can be transferred to higher tropic levels and eventually to humans via the food chain causing potential risks for human health. ECs such as PPCPs, pesticides, or antibiotics, if observed in wastewater streams, should be monitored in terms of

inhibitory effect on microalgae to evaluate environmental impact. IC_{50} is a parameter frequently used for the effect of ECs and HMs on microalgae growth. IC_{50} values of PPCPs and antibiotics can be found in articles by (Miazek & Brozek-Pluska, 2019) and (J. Q. Xiong et al., 2019) that listed IC_{50} of several such compounds to various microalgae strains.

In this chapter, the objective was to determine HM and BAC (a representative EC) tolerance of selected microalgae. A microalgae strain with high metal removal capacity was sought; therefore, the microalgae strains were selected based on their potential for HM sequestration. The main metal sequestration mechanism of algae is through the synthesis of PCs (Gekeler, Grill, Winnacker, & Zenk, 1988). The microalgae having at least a hypothetical phytochelatin synthase (PCS) gene in their genome were searched using online databases. A bacterial strain was also sought, which could degrade BAC. *P. sessilinigenes* BIOMIG1, which can completely mineralize BAC (Ertekin, Hatt, Konstantinidis, & Tezel, 2016) was selected and evaluated for its metal tolerance.

4.2. Materials and Methods

4.2.1. Selection of Microalgae Species

Algae species that contain PCS or putative (presumably) PCS genes in their genome were searched in the UniProt database (https://www.uniprot.org) to choose potential metal accumulators, and the taxonomy data was gathered to find PCS-bearing microalgae and cyanobacteria genera. The algae with possible PCS enzymes were listed in Appendix A (Table A.1). The strains used in the experiments were selected from those genera (at least in the same genus).

4.2.2. Challenge Test Design

For the effect of HMs on algae growth to be determined, the metal challenge tests were conducted. Algae species were tested against arsenic, cadmium, copper, lead, or zinc in the salt forms As_2O_3, $CdCl_2$, $CuCl_2$, $PbCl_2$, or $ZnCl_2$, respectively. The metal concentrations were 1, 10, and 100 mg/L (2, 20, 200 mg/L for arsenic) as metals basis (i.e., 100 mg/L $ZnCl_2$-Zn). Algae were incubated in 12-well plates at a volume of 2 mL with metals. OD_{680} of the multiwell plates were determined using a plate reader with a cross-section well scan option, 5 points, 2.55 µm distance. Blanks for varying HM concentrations were measured and deducted from the absorbance results. Media blank absorbance values are given in Table 4.1. To observe the effect of different lead species on toxicity, the lead challenge of *T. suecica* was repeated with $PbCl_2$ and $PbNO_3$ with the same experimental

design. To evaluate the effect of algae density on metal tolerance, the zinc challenge was repeated with a lower cell density of *T. suecica*.

Table 4.1. Media blanks with metals

	As_2O_3	$CdCl_2$	$CuCl_2$	$PbCl_2$	$ZnCl_2$
BG-11 media blanks (media absorbance: 0.075)					
Low	0.077	0.078	0.077	0.076	0.077
Med	0.076	0.077	0.08	0.077	0.076
High	0.078	0.079	0.086	0.103	0.079
F/2 media blanks (media absorbance: 0.075)					
Low	0.079	0.079	0.075	0.077	0.078
Med	0.077	0.076	0.08	0.079	0.078
High	0.078	0.077	0.086	0.087	0.076
MB3N media blanks (media absorbance: 0.078)					
Low	0.078	0.079	0.079	0.078	0.079
Med	0.078	0.078	0.081	0.078	0.079
High	0.08	0.083	0.088	0.102	0.08

For the BAC tolerance of algae to be determined, BAC challenge tests were conducted. Serial dilutions of a factor of 2 for BAC concentrations were applied starting from 32 ppm to 0.25 ppm. Algae were incubated in 12-well plates at a volume of 2 mL with specified concentrations of BAC. The tests were conducted as triplicates. Example photos of cyanobacterial species *A. flos-aquae* at the start and the end of challenge tests were depicted in Figure 4.1.

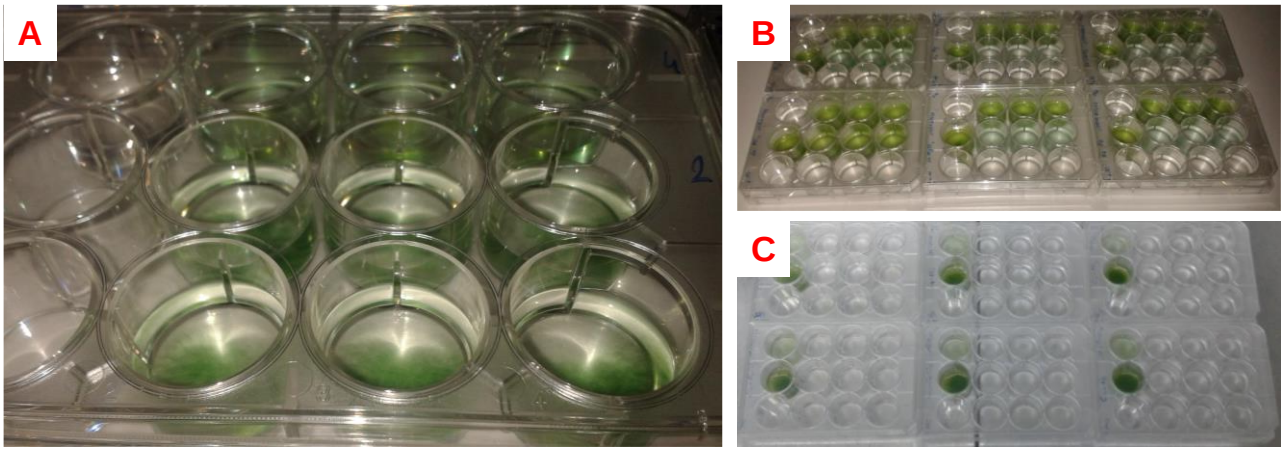


Figure 4.1. Challenge tests in 12-well plates. The starting culture of *A. flos-aquae* challenged with BAC (A), and the *A. flos-aquae* cultures in the end of zinc challenge (B) and copper challenge (C).

For the metal challenge of bacteria, a culture of *P. sessilinigenes* BIOMIG1 was grown in modified M9 medium with 200 ppm BAC. After BAC was completely degraded by bacteria (verified by HPLC), 1 mL of culture was added into LB-200BAC medium, and incubated overnight. Biomass was centrifuged, washed with 0.85% isotonic sodium chloride solution, and resuspended in either

MH II medium or modified M9 in an optical density of 0.1 at 600 nm wavelength for growth or BAC degrading activity tests, respectively. For the growth challenge, 1/100 dilutions of culture in MH II medium were added to MH II media containing varying concentrations of individual metals. The next day the optical densities at 600 nm wavelength were measured against MH II blanks with the same metal concentrations to determine the metal effect on bacterial growth. For the metal challenge on BAC degradation, culture resuspended in M9 was added into an equivolume of M9 containing 20 ppm BAC and a varying amount of individual metal concentrations (concentrations halved in final medium). BAC was measured the next day on HPLC to evaluate the 24-hour effect of metals on bacterial BAC degradation activity.

4.2.3. IC_{50} Determination

From the growth data taken from the previous tasks, growth rates (μ, day-1) were determined for control groups and algae stressed with heavy metals and BAC, from the growth rate formula given in Eq. 4.1.

$$\mu = \frac{\ln(X_n) - \ln(X_0)}{t_n - t_0} \tag{4.1}$$

To determine the effect of different contaminants on algae, IC_{50} values were calculated. For this, first, growth inhibition (Ig, %) was determined from the growth rates of control and exposed groups (Eq. 4.2). The average growths on the first 7 days for green algae and the first 6 days for cyanobacteria were measured. Then a 4-parameter logistic equation was utilized to calculate IC_{50} values (Neubig, Spedding, Kenakin, & Christopoulos, 2003) from %-inhibitions at certain contaminant doses (Eq. 4.3).

$$I_g(\%) = \frac{\mu_{control} - \mu_{test}}{\mu_{control}} \cdot 100 \tag{4.2}$$

$$I_g(\%) = \frac{I_{gmax}(\%)}{1 + \left(\frac{[A]}{IC_{50}}\right)^{-b}} \tag{4.3}$$

In Eq. 4.2, $I_g(\%)$ is the growth inhibition percent at contaminant concentration [A], $\mu_{control}$ is the growth rate of algae that was not exposed to contaminants, μ_{test} is the growth rate of algae that was exposed to contaminant at concentration [A]. $I_{gmax}(\%)$ represents the growth inhibition as the

concentration approaches infinity, IC_{50} is the half-maximal inhibitory concentration of the contaminant, and b is the hillslope of the expected S-curve in Eq. 4.3. Percent inhibitions at different concentrations were utilized for predicting IC_{50} values on SigmaPlot software (Systat Software, San Jose, California, United States) (Millán de Kuhn et al., 2006).

4.2.4. Incubation of *T. suecica* Culture with a Mixture of Five Metal Salts

T. suecica was cultivated in F/2 (16‰ salinity) medium spiked with As_2O_3, $CdCl_2$, $CuCl_2$, $PbCl_2$, and $ZnCl_2$ to determine the metal removal capacity of algae from the mixture of five metal salts. Concentrations of each metal in the experiments are given in Table 4.2. The concentrations were chosen to resemble the amounts in hydraulic fracturing produced water (HFPW) (L. Hu et al., 2022; W. Jiang et al., 2022). There were 3 test reactors containing algae, 2 reactors for control without algae, and 2 algae reactors without metals for growth control. The initial cell density of the algae was set around 10^6 cells/mL. Cell density was measured daily via microscopy. Metal concentrations as dissolved in matrix, adsorbed on the algae, and assimilated in the algae were daily monitored following the protocol described in Section 3.5.

Table 4.2. Metal concentrations in the reactors

Metal	Salt	Added amount (metals basis)
Arsenic	As_2O_3	2 mg/L As
Cadmium	$CdCl_2$	0.5 mg/L Cd
Copper	$CuCl_2$	0.5 mg/L Cu
Lead	$PbCl_2$	0.25 mg/L Pb
Zinc	$ZnCl_2$	0.5 mg/L Zn

4.3. Results & Discussion

4.3.1. Algae Growth in Different Metals

The effect of individual metals in dissolved salt forms on the growth of algae was evaluated. Algae tested were *A. flos-aquae* (UTEX B1444), *C. vulgaris* (CCAP 211/11b), *C. subellipsoidea* (UTEX 278), *E. oleoabundans* (UTEX 1185), *Cyanobacterium sp.* AV1403, and *T. suecica* (Istanbul Medeniyet University, IMU). The stock cultures were grown with aeration in their preferred culture medium until reaching the mid-exponential phase. Each biomass was then transferred to a culture medium with either individual HMs or BAC during the mid-exponential phase and incubated for several days at 25 °C until the stationary phase was achieved, under continuous illumination with

white cool fluorescent light (67 μmol photons/m^2·s). The alteration in growth rate was assessed by measuring absorbance at 680 nm. Initial cell densities and culture media are listed in Table 4.3.

Cd and Cu were completely inhibitory to tested cyanobacteria species, *A. flos-aquae* and *Cyanobacterium sp.* (Figure 4.2.B, 4.2.C, Figure 4.3.B, 4.3.C). The inhibitory effect of Pb was only observable at the highest concentration tested (100 mg/L) for these strains (Figure 4.2.D, Figure 4.3.D). Zn was completely inhibitory above 10 mg/L to both cyanobacteria strains (Figure 4.2.E, Figure 4.3.E). Arsenic was completely inhibitory to *A. flos-aquae* at the highest level (200 mg/L), but slightly affected growth at lower levels (Figure 4.2.A). Almost no effect of arsenic was observed on *Cyanobacterium sp.* (Figure 4.3.A). Literature shows that many cyanobacteria species are resistant to arsenic exposure (Y. Wang et al., 2015). For instance, *Synechocystis sp.* PCC 6803 contains arsenite carrier and arsenate reductase genes which may help the cells of this strain bear resistance to higher levels of arsenic (López-Maury, Florencio Francisco, & Reyes José, 2003).

The growth profiles of *C. vulgaris* (Figure 4.4) and *E. oleoabundans* (Figure 4.6) show arsenic and zinc were completely inhibitory to these strains at the highest level. No arsenic or zinc levels were completely inhibitory to *C. subellipsoidea* (Figure 4.5). Cadmium and copper were completely inhibitory at the medium level (10 mg/L) to those three green algae species. Lead was only effective at the highest level on *C. vulgaris* and had almost no effect on *E. oleoabundans* and *C. subellipsoidea*. For *T. suecica* growth, arsenic, copper, and zinc at the highest level, and cadmium at the medium level (10 mg/L) were completely inhibitory (Figure 4.7).

Analysis of the growth curves indicates that cadmium and copper are the determinant metals for the optimal performance of algae. This is evident from the fact that salts containing these metals had the most significant impact on the growth profiles of the selected algae. In the subsequent step, IC_{50} values were calculated for each metal concerning the growth of each algae strain, and these findings will be discussed in Section 4.3.3.

Table 4.3. Initial cell densities and growth media of algae for metal and BAC challenges

Species	Strain ID	Culture media	Initial cell density (x10^6 cells/mL)
Anabaena flos-aquae	UTEX B1444	BG-11	4.5
Chlorella vulgaris	CCAP 211/11b	MB3N	6.7
Coccomyxa subellipsoidea	UTEX 278	MB3N	4.5
Ettlia oleoabundans	UTEX 1185	MB3N	8.6
Cyanobacterium sp.	AV1403	BG-11	18.2
Tetraselmis suecica	IMU	F/2	0.8

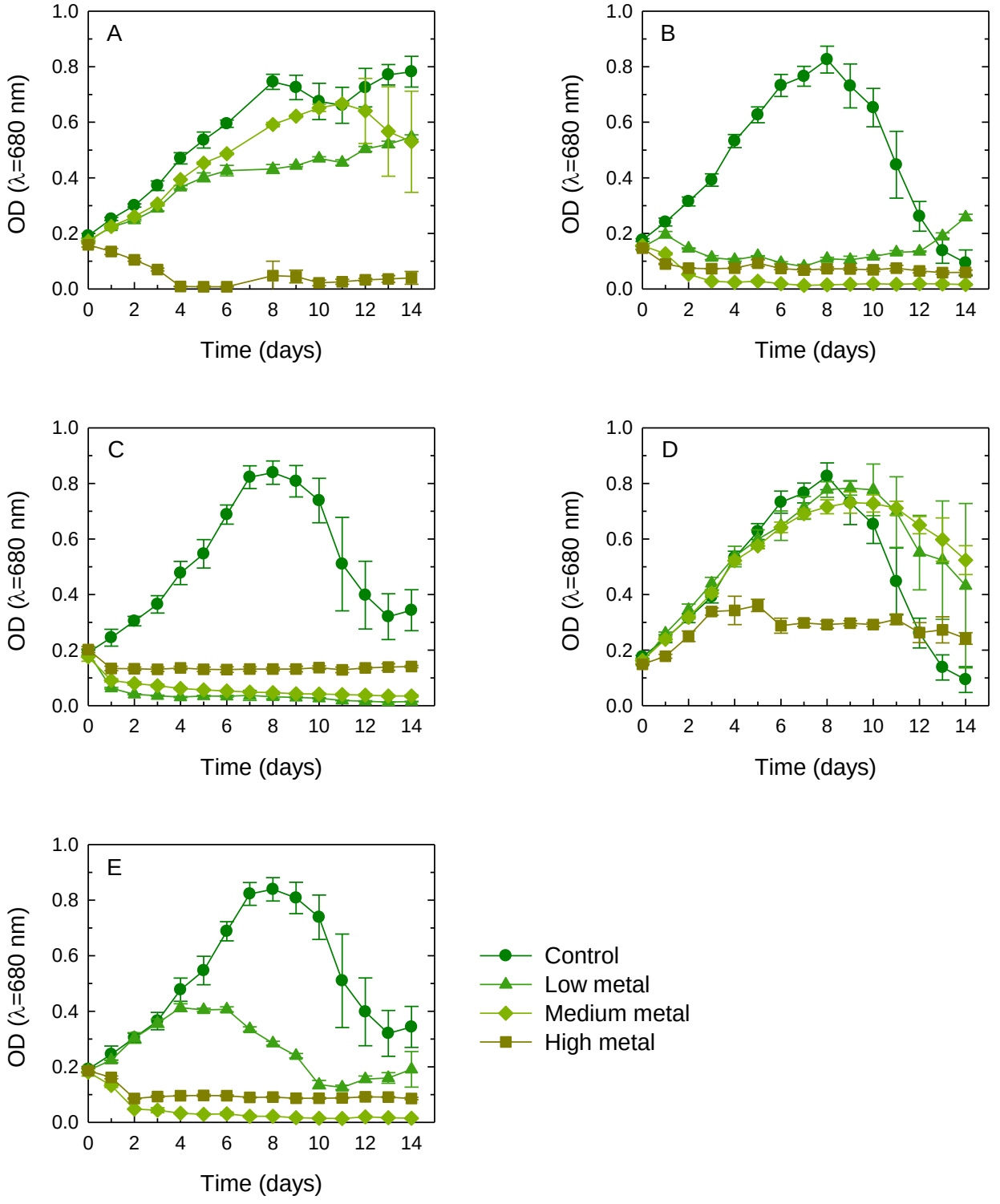


Figure 4.2. The effect of individual heavy metals on the growth of *A. flos-aquae*. Metal salts tested were A) As_2O_3, B) $CdCl_2$, C) $CuCl_2$, D) $PbCl_2$, and E) $ZnCl_2$.

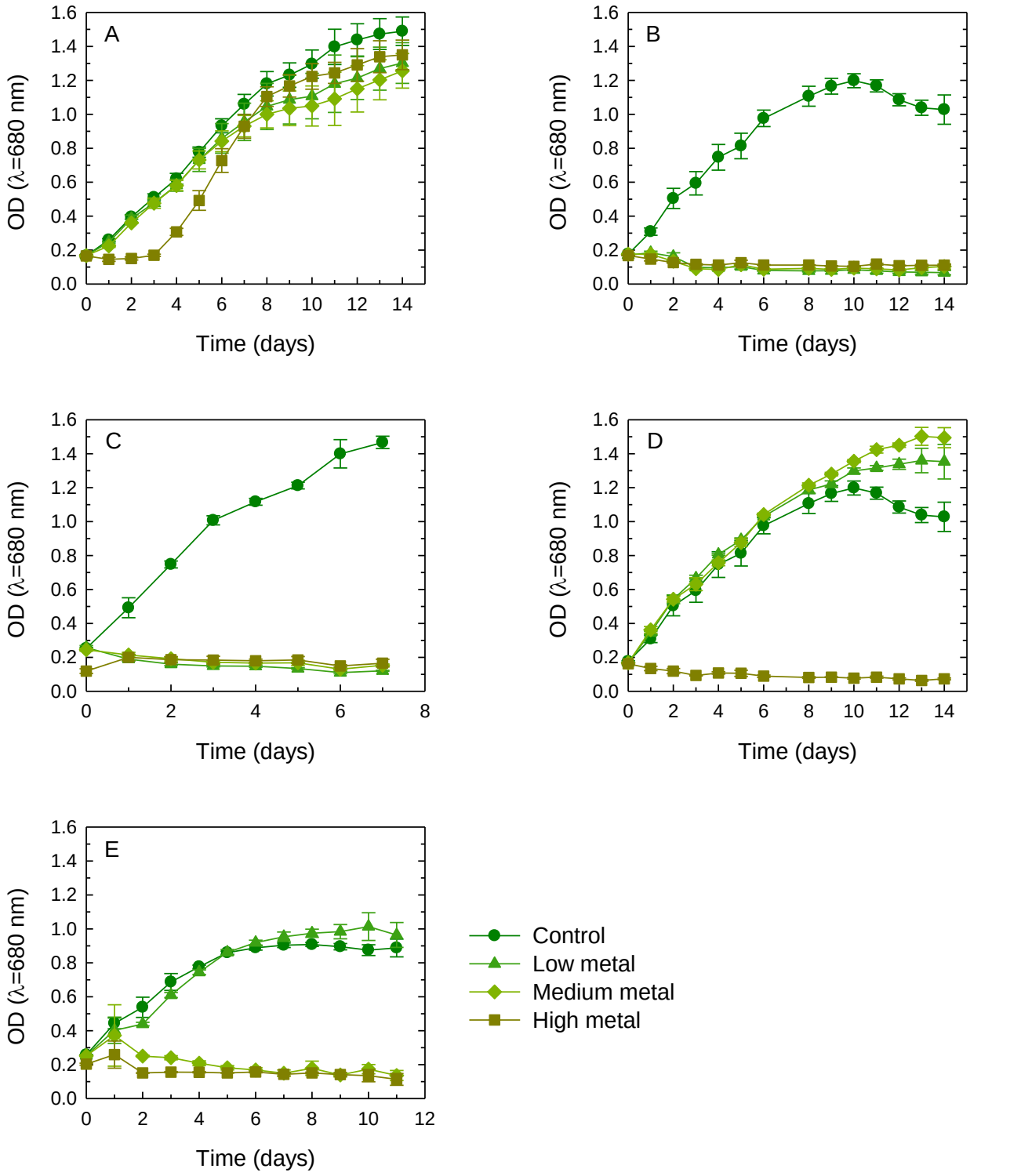


Figure 4.3. The effect of individual heavy metals on the growth of *Cyanobacterium sp*. Metal salts tested were A) As_2O_3, B) $CdCl_2$, C) $CuCl_2$, D) $PbCl_2$, and E) $ZnCl_2$.

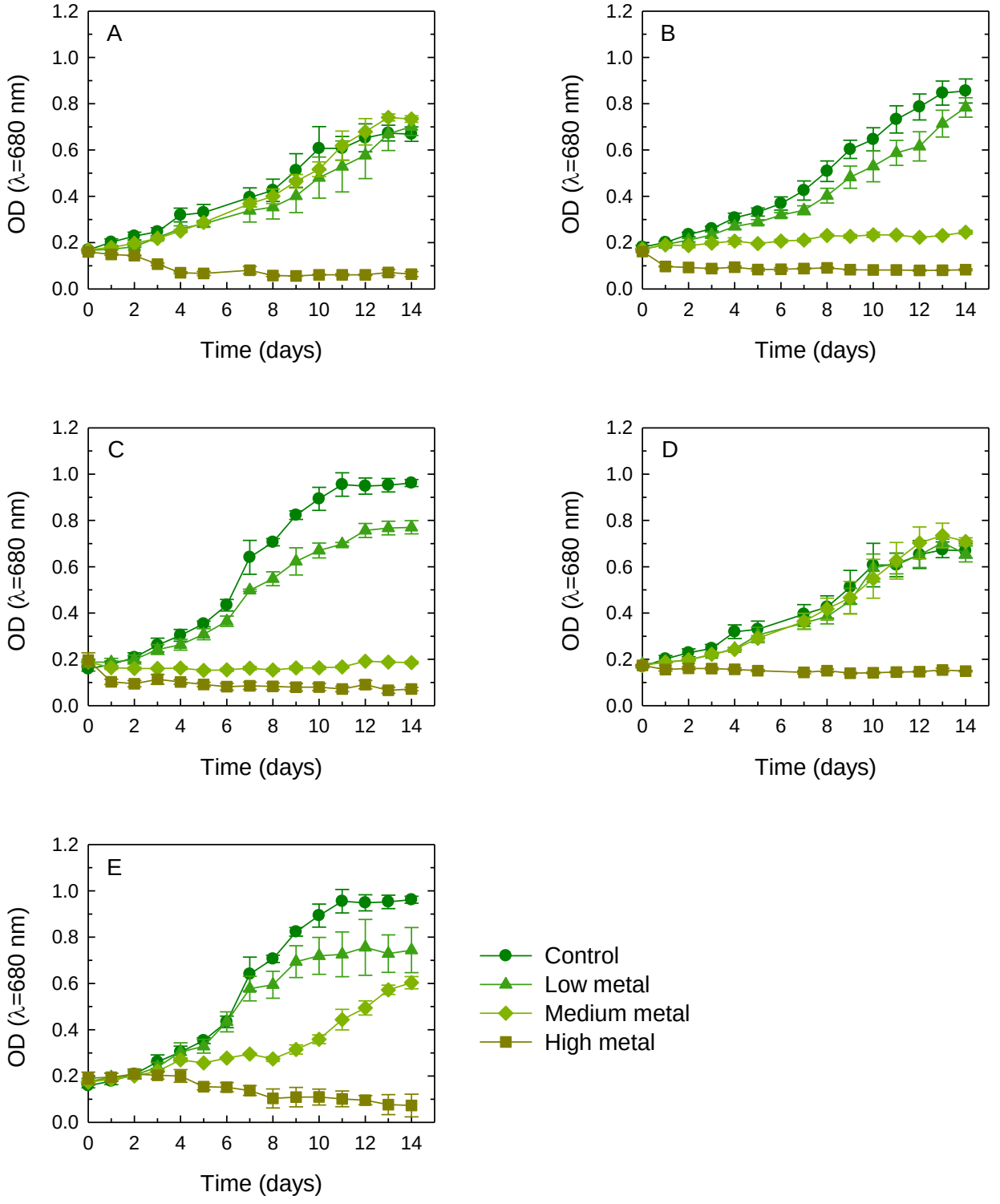


Figure 4.4. The effect of individual heavy metals on the growth of *C. vulgaris*. Metal salts tested were A) As_2O_3, B) $CdCl_2$, C) $CuCl_2$, D) $PbCl_2$, and E) $ZnCl_2$.

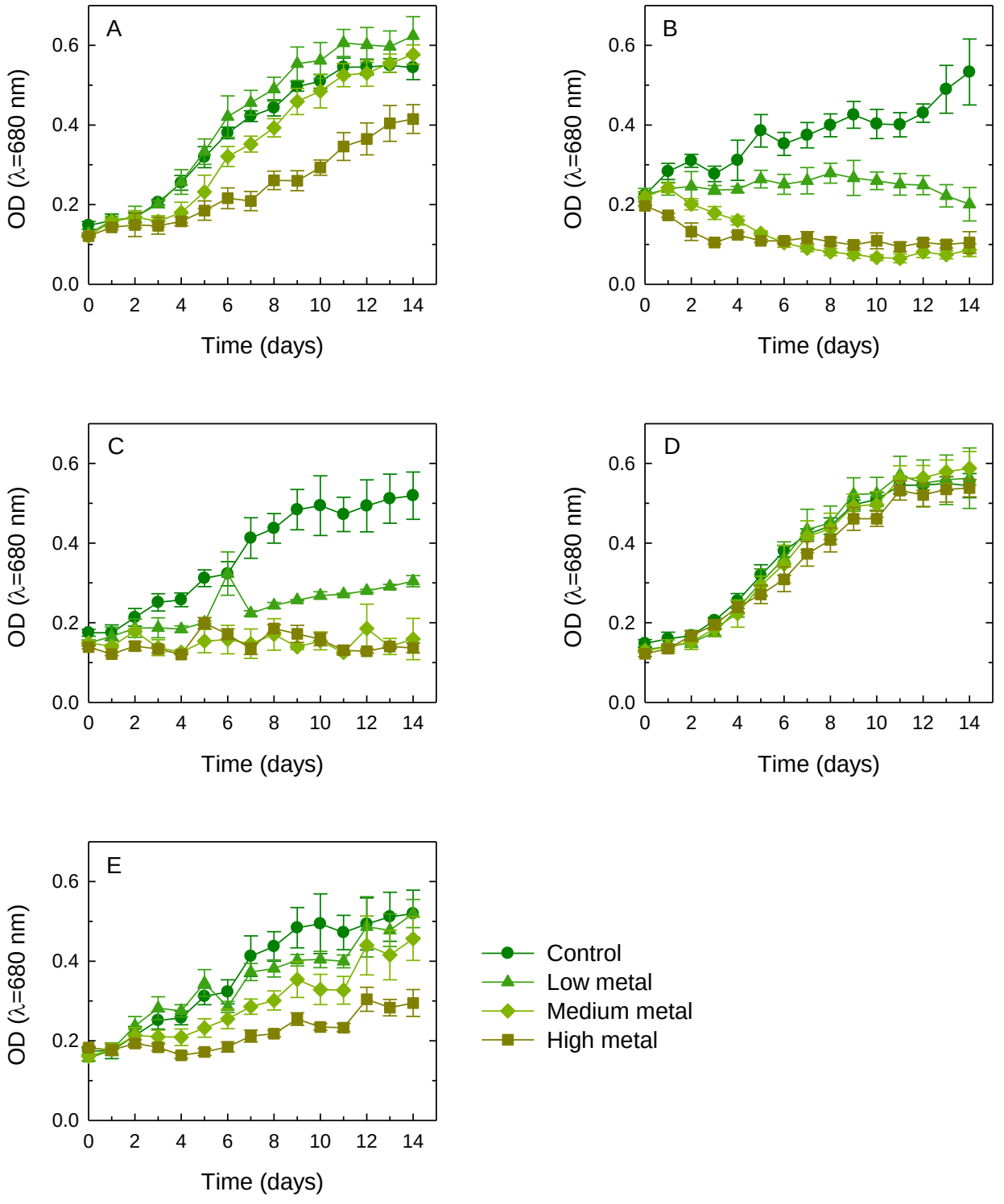


Figure 4.5. The effect of individual heavy metals on the growth of *C. subellipsoidea*. Metal salts tested were A) As_2O_3, B) $CdCl_2$, C) $CuCl_2$, D) $PbCl_2$, and E) $ZnCl_2$.

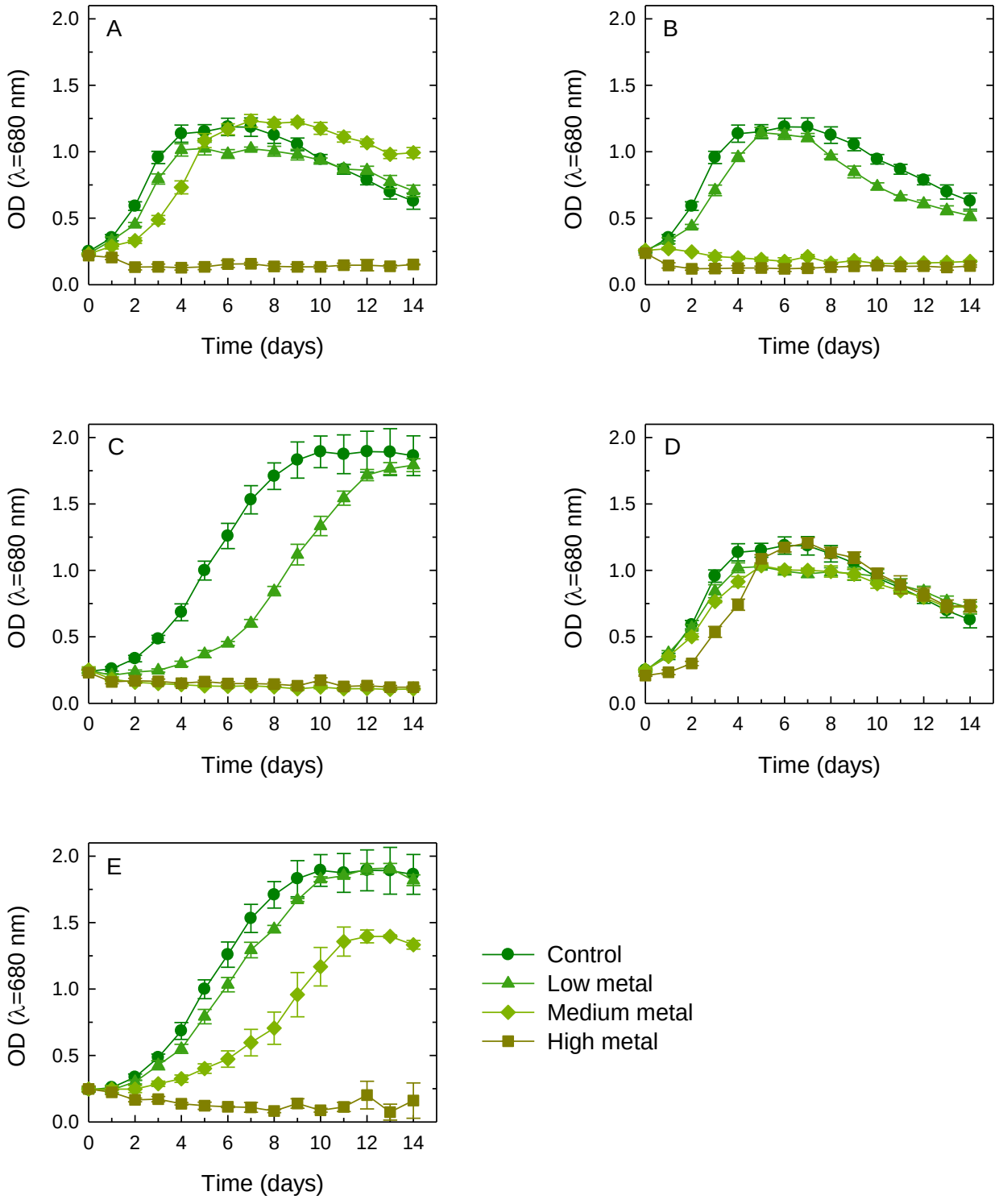


Figure 4.6. The effect of individual heavy metals on the growth of *E. oleoabundans*. Metal salts tested were A) As_2O_3, B) $CdCl_2$, C) $CuCl_2$, D) $PbCl_2$, and E) $ZnCl_2$.

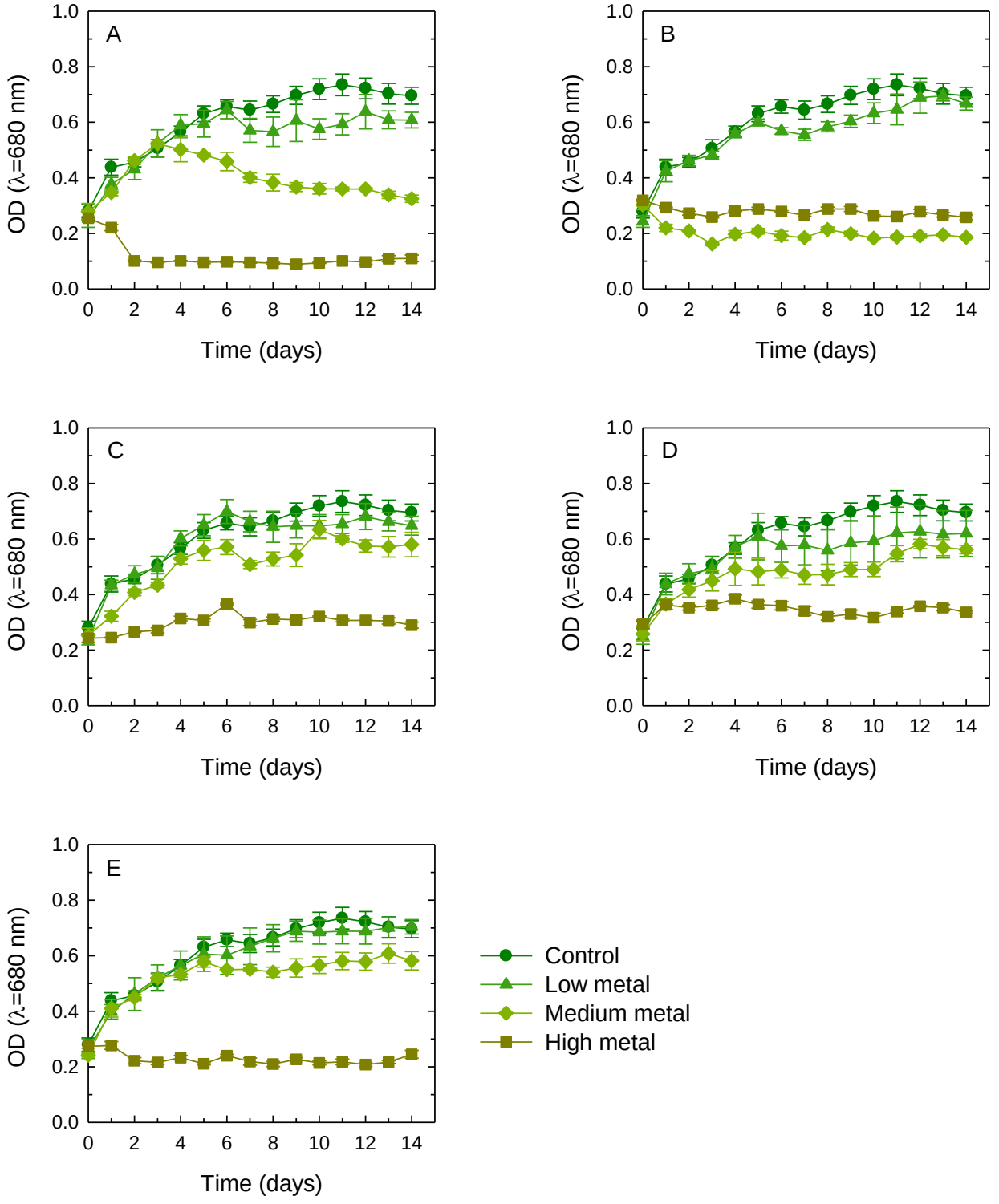


Figure 4.7. The effect of individual heavy metals on the growth of *T. suecica*. Metal salts tested were A) As_2O_3, B) $CdCl_2$, C) $CuCl_2$, D) $PbCl_2$, and E) $ZnCl_2$.

4.3.2. Bacterial Growth in Different Metals

The inhibitory effect of arsenic and cadmium on both BAC degradation and growth of *P. sessilinigenes* BIOMIG1 was high at high concentrations (Figures 4.8 & 4.9). Pb and Zn were non-inhibitory to BAC biodegradation up to 64 mg/L and bacteria grew in MH II medium up to 128 mg/L, respectively (Figures 4.11 & 4.12). A study on bacteria isolated from a sequencing batch reactor (SBR) revealed that the IC_{50} value of 30 min Pb exposure on *Pseudomonas* was >1500 times higher than that of 30 min Cd exposure (Zare et al., 2015). In another study, Zn was shown to have an insignificant effect on biomass growth up to 1 mM (65 mg/L) (Hassen, Saidi, Cherif, & Boudabous, 1998).

A different pattern was observed when BIOMIG1 was challenged with $CuCl_2$ (Figure 4.10). An inhibitory effect significant effect was observed when BIOMIG1 was exposed to Cu in carbon-rich media. However, Cu inhibited the biotransformation of BACs. Heavy metal inhibition in bacteria might occur through alteration of varying metabolisms, i.e., via the replacement of essential metal in enzymes (Olaniran, Balgobind, & Pillay, 2013). Such impact also decreases the bacteria growth. For BACs as cationic surfactants, Cu^{2+} ions may have reduced the sorption capacity to bacteria, which had a negatively charged surface, decreasing bioavailability to bacteria (Pateiro-Moure, Arias-Estévez, & Simal-Gándara, 2013). This might explain why BAC degradation was affected without a decrease in growth.

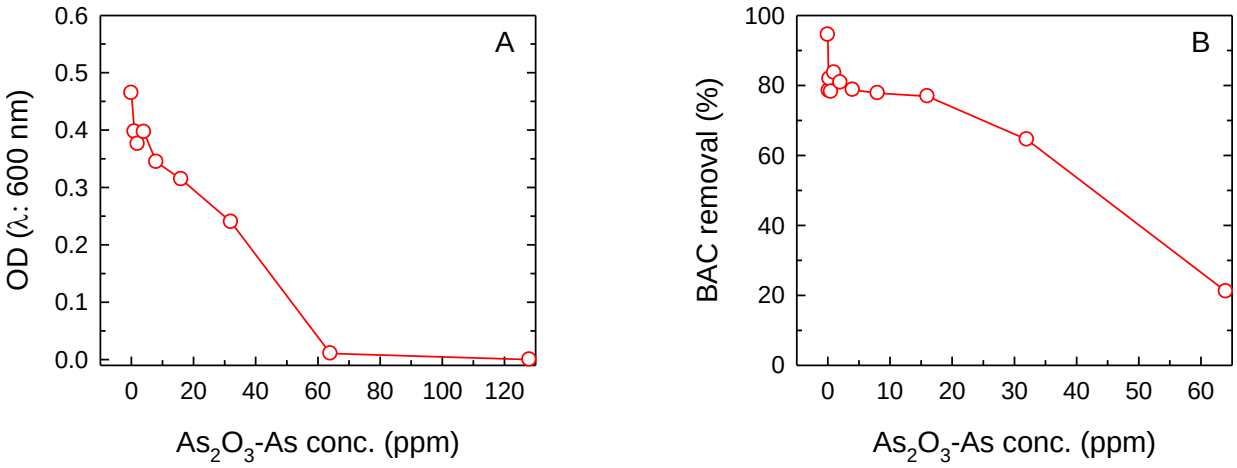


Figure 4.8. The effect of arsenic on bacterial growth (A) and BAC degradation activity (B).

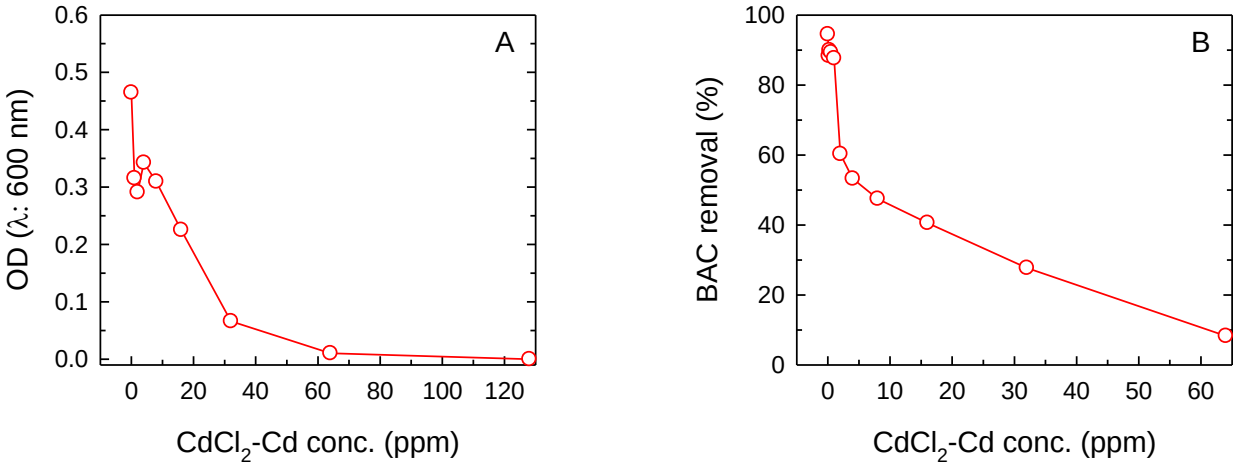


Figure 4.9. The effect of cadmium on bacterial growth (A) and BAC degradation activity (B).

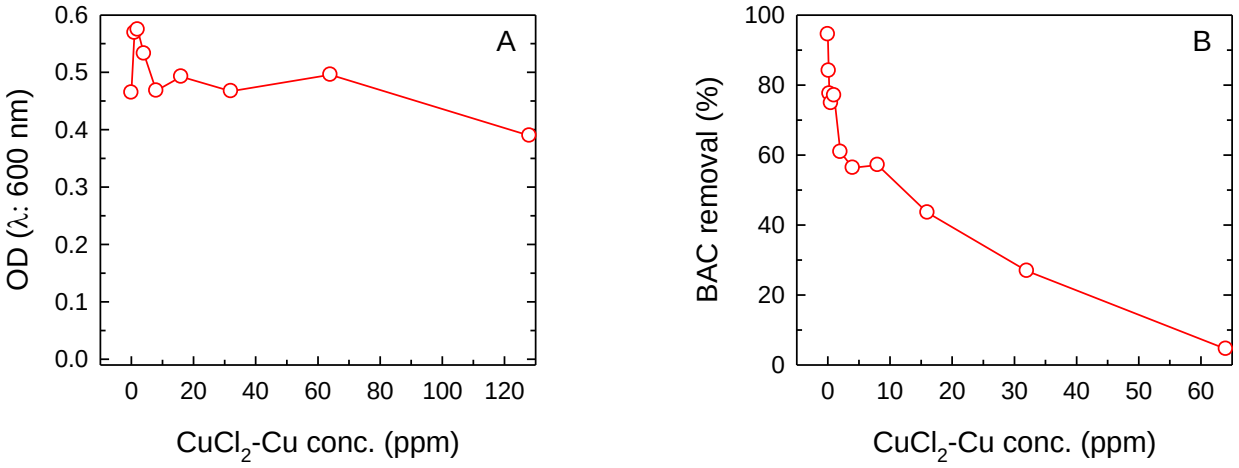


Figure 4.10. The effect of copper on bacterial growth (A) and BAC degradation activity (B).

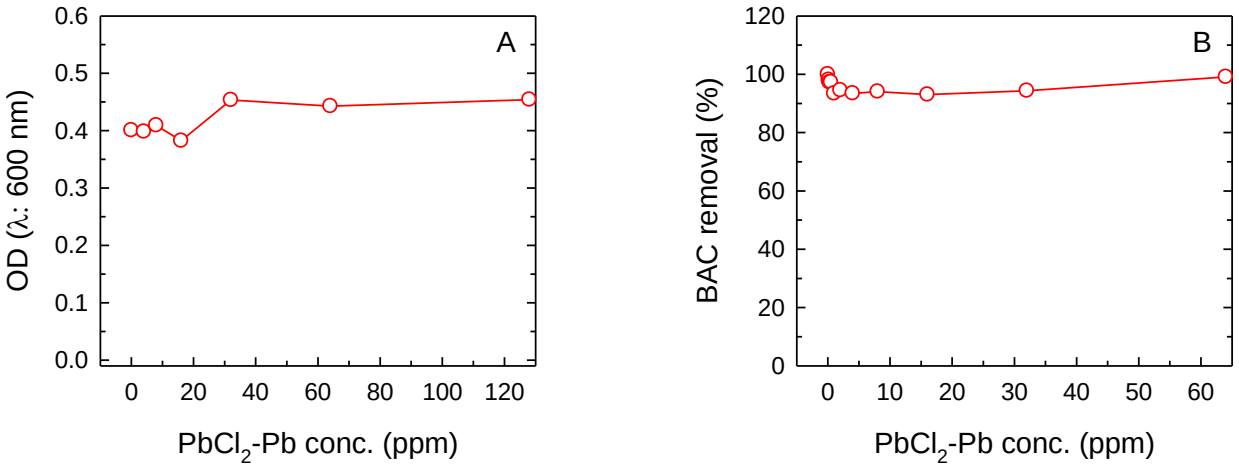


Figure 4.11. The effect of lead on bacterial growth (A) and BAC degradation activity (B).

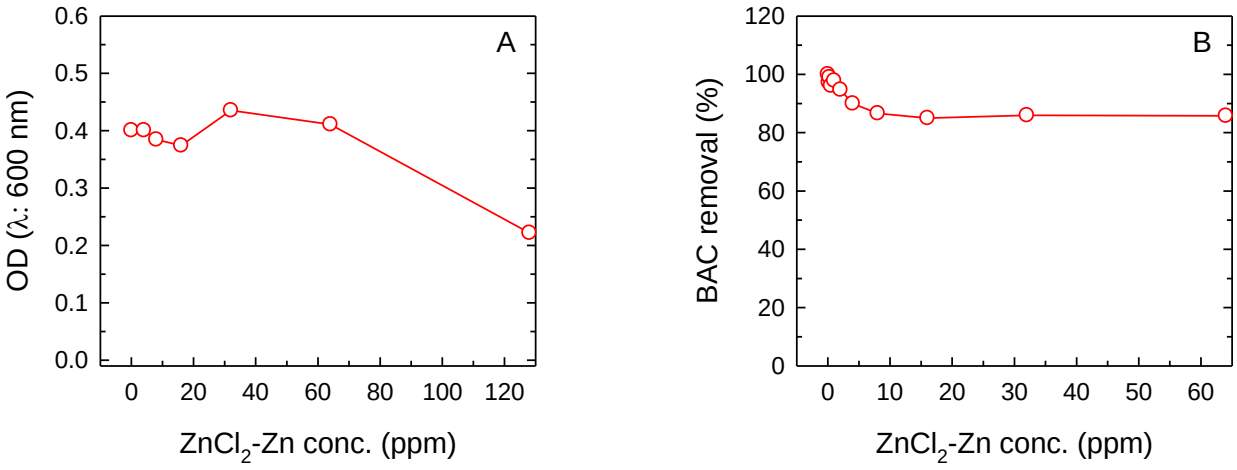


Figure 4.12. The effect of zinc on bacterial growth (A) and BAC degradation activity (B).

4.3.3. Tolerance of Algae against Heavy Metals and BAC by Means of IC_{50}

To determine the tolerance of algae against HMs and BAC, IC_{50} values were determined. *Cyanobacterium sp.* was the most tolerant against arsenic as the highest concentration was not inhibitory at all. This strain was not in the IC_{50} graph of arsenic because its IC_{50} could not be calculated. *T. suecica* was most tolerant to salts of Cd, Cu, and Zn (Figure 4.13). *C. subellipsoidea* and *E. oleoabundans* were resistant to Pb at all tested levels and their IC_{50} values were not calculated. Two cyanobacterial species, *A. flos-aquae* and *Cyanobacterium sp.*, were excluded from the calculation of Cd and Cu IC_{50} values because salts of these metals were completely inhibitory at the minimum tested level (1 mg/L).

Tolerance to HMs depends on many biotic factors such as cell size, which affects surface-to-volume ratio, type of cell wall, antioxidant activity, motility, bearing stress-related genes and/or metabolisms (Hamed, Selim, Klöck, & AbdElgawad, 2017; Jacqueline L. Levy, Stauber, & Jolley, 2007). Debelius et al. (2009) found that larger cells were more tolerant to Cu and Pb (Debelius, Forja, DelValls, & Lubián, 2009). An increase in surface volume ratio may lead to higher uptake, and thus higher toxicity (Quigg, Reinfelder, & Fisher, 2006). In the present study, the biggest cell size belonged to *T. suecica*, followed by other three green algae, and the smallest ones being the cyanobacteria strains. According to present IC_{50} values, the tolerance against Cd, Cu, and Zn was correlated with cell size.

T. suecica was most tolerant amongst tested algae strains against BAC with an IC_{50} value of 8.0±0.02 mg/L (Figure 4.14).

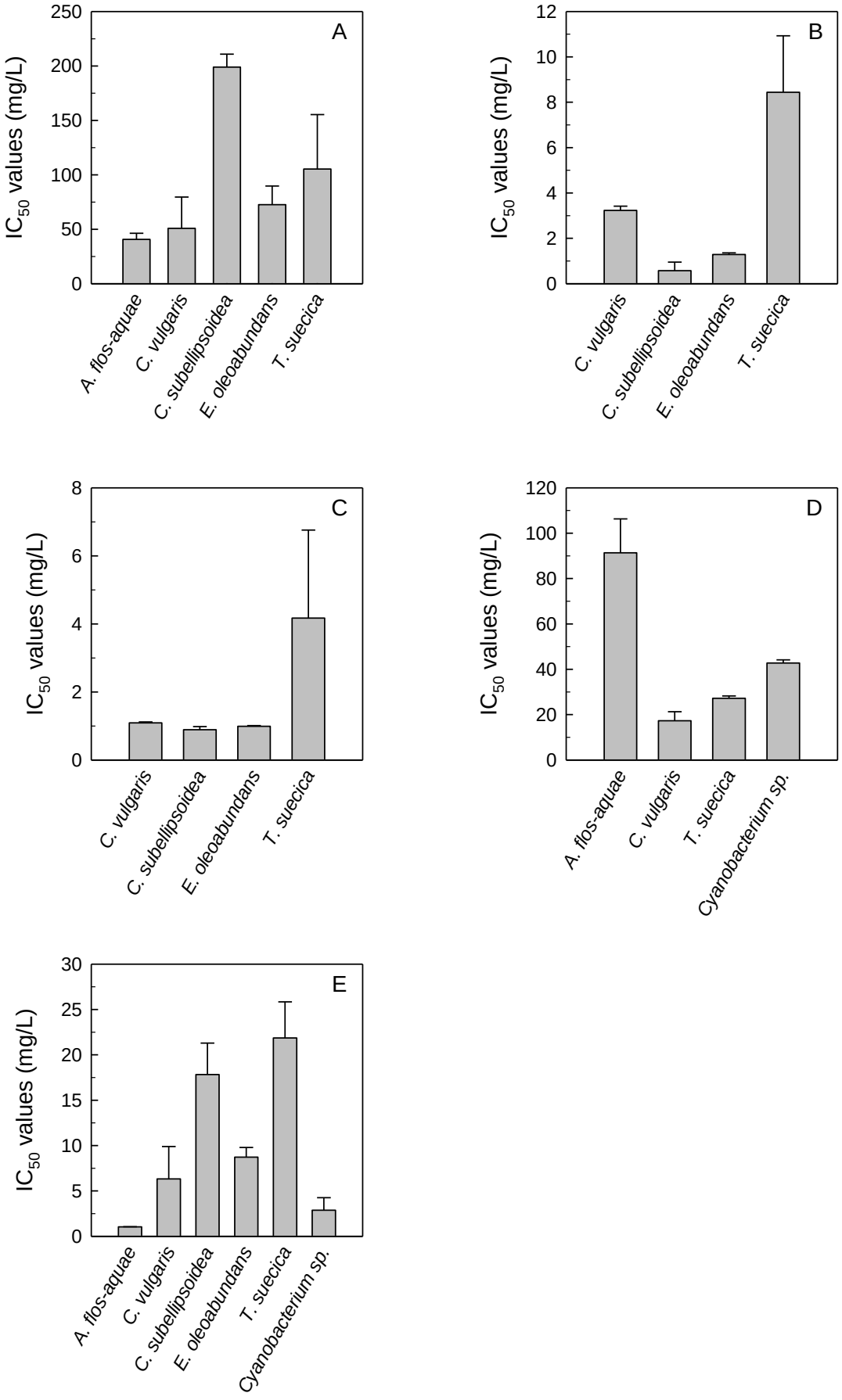


Figure 4.13. IC_{50} values of arsenic (A), cadmium (B), copper (C), lead (D), and zinc (E) against tested microalgae species.

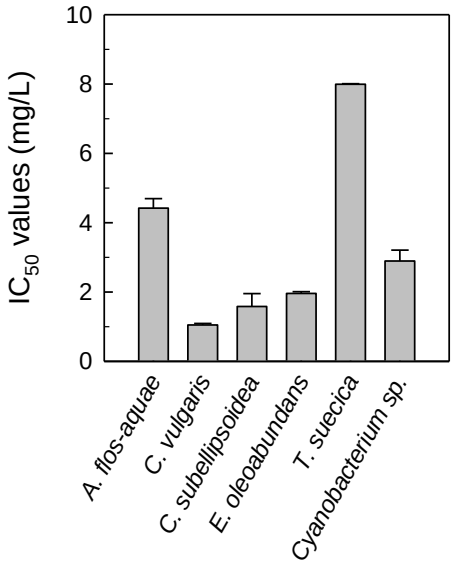


Figure 4.14. IC_{50} values of BAC against tested microalgae species.

Those tolerance tests have some limitations such as:

1) The added concentrations of metals might be complexed with ingredients of the media utilized in growth challenge tests,
2) The algae concentration might be so high that the effectivity of the metals was lowered because of complexation with the present biomass (some cells might have survived and divided since some of the aqueous metal species were bound to solid biomass)
3) $PbCl_2$ might have very low solubility so it could not affect the growth of algae cultures. As specified in Section 3.1, metal concentrations in stock solutions were verified with ICP/MS analyses.

For (2) and (3), experiments were conducted with lower algae density to observe the effect of algae density on tolerance, and a different Pb species was tested to evaluate the effect of Pb species on inhibitory effect. The results of these experiments are discussed in the next section. For (1), Visual Minteq software (https://vminteq.lwr.kth.se/) was utilized to evaluate the speciation of metal salts spiked into algae and bacteria media. In algae media 60-70% of cadmium was in Cd^{2+} form. This value was determined to range between 40-80% for Zn^{2+}, 12.5-20% for Pb^{2+}, and 1.2-4.6% for Cu^{2+}. From these values, it can be concluded that, for instance, copper would have been more toxic to algae if the ionic form was higher in abundance. The IC_{50} values were accepted as the effect of total added metal salt (as metals basis).

Selected studies for heavy metal effect on microalgae were listed in Table 4.4 for comparison with the experimental findings of this study.

Table 4.4. Effects of heavy metals on microalgae

Algae	Environment	Metal	Initial cell density	Exposure time	Effect	Reference
Chlorella vulgaris	Freshwater	Cu, Co, Zn	N/A (Chla Chlb measurement)	7 days	Total cell death at 10^{-5} M Cu, 10^{-4} M Co or Zn	(Afkar, Ababna, & Fathi, 2010)
Chlorella vulgaris	Freshwater	As (V)	10^7 cells/mL	7 days	No inhibitory effect at 100 mg/L	(Maeda et al., 1983)
Chlorella vulgaris	Freshwater	Cd, Cu, Hg, Pb, Zn	2-3.5x10^5 cells/mL	33 days	IC_{50} Cd 0.06±0.001 mg/L, IC_{50} Cu 0.18±0.003 mg/L, IC_{50} Hg 1.03±0.03 mg/L, IC_{50} Pb >100 mg/L, IC_{50} Zn 5.1±0.09 mg/L	(Rosko & Rachlin, 1977)
Chlorella vulgaris	Freshwater	Various elements	Low (transparent initial culture)	Several weeks	Lowest inhibitory concentration of Cu 0.04 mg/L, Zn 3.5 mg/L, Cd 0.14 mg/L, Pb 3 mg/L, As 0.06 mg/L	(den Doore de Jong & Roman, 1965)
Chlorella salina	Marine	As (III) as $NaAsO_2$ As (V) as KH_2AsO_4	10^5 cells/mL	72 h	IC_{50} As (III) 0.18±0.04 mg/L, IC_{50} As (V) 0.20±0.01 mg/L	(Karadjova, Slaveykova, & Tsalev, 2008)
Chlorella sp. CE-35	Freshwater	As (III) as $NaAsO_2$ As (V) as $Na_2HAsO_4.7H_2O$	3x10^4 cells/mL	72 h	IC_{50} As (III) 27±10 mg/L, IC_{50} As (V) 19±3 mg/L	(Rahman et al., 2014)
Chlorella pyrenoidosa	Freshwater	Cd, Pb, Co, Ni, Zn	1.5-1.8x10^6 cells/mL	24 h	Continuous reactor IC_{50} Cd 0.028 mg/L, IC_{50} Pb 0.14 mg/L, IC_{50} Co 0.098 mg/L, IC_{50} Ni 0.09 mg/L, IC_{50} Zn 0.057 mg/L	(Lin, Lee, & Chen, 2007)
Chlorella sp.	Freshwater	Cu, Zn	2-4x10^4 cells/mL	72 h	IC_{50} Cu 0.0073 mg/L, IC_{50} Zn 0.11 mg/L	(Johnson, Stauber, Adams, & Jolley, 2007)
Chlorella sp.	Freshwater	As (III), As (V)	2-4x10^4 cells/mL	72 h	EC_{50} As (III) 25.2 mg/L, EC_{50} As (V) 25.4 mg/L	(Jacqueline L. Levy et al., 2005)
Tetraselmis sp. CS-87	Marine	Cu	3x10^3 cells/mL	72 h	IC_{50} Cu 0.047 mg/L	(J. L. Levy et al., 2008)

Table 4.4. Cont.

Algae	Environment	Metal	Initial cell density	Exposure time	Effect	Reference
Tetraselmis suecica	Marine	Cd, Cu	10^4 cells/mL	72 h	EC_{50} Cu 1.19 mg/L, EC_{50} Cd >100 mg/L	(Macken et al., 2009)
Tetraselmis tetrathele	Marine	Cd, Cu, Pb in 2% HNO_3, As (III) as H_3AsO_4	0.1-0.25 µg Chla/mL	72 h	IC_{50} As 3.2 mg/L, IC_{50} Cd 9.8 mg/L, IC_{50} Cu 7.4 mg/L, IC_{50} Pb 8.6mg/L	(Satoh, Vudikaria, Kurano, & Miyachi, 2005)
Tetraselmis suecica	Marine	Cd	$25x10^4$ cells/mL	6 days	EC_{50} Cd 7.9±1 mg/L	(Pérez-Rama, Abalde Alonso, Herrero López, & Torres Vaamonde, 2002)
Tetraselmis suecica	Marine	Cu	N/A	N/A	EC_{50} Cu for motility 40 mg/L	(Millán de Kuhn et al., 2006)
Tetraselmis suecica	Marine	Cu, Cd	10^4 cells/mL	96 h	IC_{50} Cd 9.4 mg/L, IC_{50} Cu 0.17 mg/L	(Nassiri, Ginsburger-Vogel, Mansot, & Wéry, 1996)
Tetraselmis suecica	Marine	Cu, Ni, Pb	10^6 cells/mL	72 h	IC_{50} Cu 43.03±0.154 mg/L, IC_{50} Ni 16.11±0.187 mg/L, IC_{50} Pb 9.62±0.135 mg/L	(Ebenezer & Ki, 2013)
Tetraselmis chuii	Marine	Cu, Pb	10^4 cells/mL	72 h	IC_{50} Cu 0.33±0.1 mg/L, IC_{50} Pb 2.64±0.2 mg/L	(Debelius et al., 2009)
Neochloris sp.	Freshwater	Zn	$7.75x10^4$ cells/mL	2 h	IC_{10} Zn >1300 mg/L	(Novis, Halle, Wilson, & Tremblay, 2009)

4.3.4. Effect of Culture Density and Lead Species on IC_{50} Value

The effect of algae density on tolerance to heavy metals was tested. Experiments were done using $ZnCl_2$ and *T. suecica* whose initial density was 10^5 cells/mL. IC_{50} of Zn for low-density algae was 20.3±1.7 mg/L, which was comparable to what was found in the previous challenge tests with 10^6 cells/mL culture density, 21.9±4.0 mg/L. Additionally, the activities of $PbCl_2$ and $Pb(NO_3)_2$ on *T. suecica* culture growth were compared. Their IC_{50} values did not change significantly (41±18 mg/L for $Pb(NO_3)_2$-Pb, 55±43 mg/L for $PbCl_2$-Pb).

From the challenge tests with HMs and BAC, marine microalgae *T. suecica* was selected for the next experiments as it had the utmost growth performance against Cd, Cu, and Zn salts, and BAC stress.

4.3.5. *T. suecica* Metal Removal Capacity When Present in a Media with a Mixture of Five Metal Salts

T. suecica was cultured with a mixture of five metals to determine removal capacity in a synthetic media. The concentrations of added metals were determined to resemble HFPW. Hydraulic fracturing is the process of unconventional shale gas or shale oil extraction by cracking the deep rocks (shale) with the help of a water-based liquid. This cracking fluid consists of 99% water and sand, and the remaining 1% is comprised of the additives that regulate pH and viscosity, and reduce friction, precipitation, scaling, and biocides for biological control (Akyon, Stachler, Wei, & Bibby, 2015; Kahrilas, Blotevogel, Stewart, & Borch, 2014). After shale oil or shale gas is extracted, some of the fluid is drawn back which is called produced water, with high salinity, heavy metals, radionuclides (T. Zhang, Gregory, Hammack, & Vidic, 2014), additives or transformation products of the additives (Hoelzer et al., 2016) in the original fracking water. Considering that shale gas fulfills approximately 50% of natural gas production in the US (G.-F. Feng, Wang, Chu, Wen, & Chang, 2021), the handling, discharge, and disposal of the produced water become important issues, posing risks such as ecological impacts, public health risks, socio-economical risks, and cumulative risks involving multiple aspects (Vengosh, Jackson, Warner, Darrah, & Kondash, 2014).

The culture growth with or without the presence of metals is shown in Figure 4.15. The mix of metals did not dramatically alter the growth of algae. Biomass yield of test reactors as cells/mL decreased by 27% from algae control on Day 15. The culture was in the stationary phase after Day

10. Day 1 to Day 10 growth rates were 0.16±0.02 and 0.15±0.03, for control without metals and culture in the test reactors, respectively.

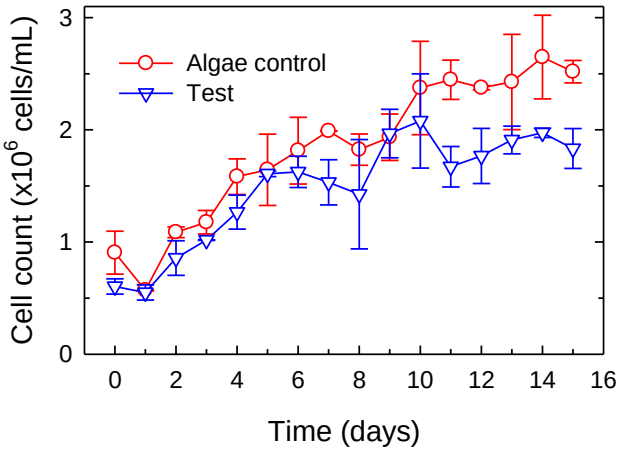


Figure 4.15. Growth of the culture incubated with a mixture of five metals compared to the control culture.

The fate of five metals was tested in media without algae, as well as algae-containing reactors, and the results are presented in Figures 4.16 - 4.20, including removal percent and removal per cell graphs. Assimilations of arsenic and zinc were poor, not exceeding 2% for arsenic and remaining around the initial assimilated value for zinc. On Day 15, 18% of lead was assimilated into algae. The primary removal mechanism for arsenic appeared to be adsorption, reaching up to 10% on Day 15. Per cell adsorption of arsenic remained stable after Day 1, suggesting increased removal due to increased cell density. High removals of Pb and Zn through adsorption may be attributed to precipitation, as the method employed could not differentiate between precipitation and adsorption. Adsorbed Pb was higher in the first seven days, but decreased afterward, indicating bioleaching of Pb towards the end.

Assimilated Cd and Cu increased consistently throughout the experiments. Per cell Cu assimilation remained stable after the initial days, indicating that the increase in assimilation was due to cell proliferation. Per cell Cd assimilation continuously increased until the end of the experiments. The daily assimilation rates were 0.013, 0.071, 0.191, 0.013, and 0.002 µM/day for arsenic, cadmium, copper, lead, and zinc, respectively. In total, 25% of metals were removed from the aqueous phase on a molar basis. These results indicate that metal removal by *T. suecica* from a matrix containing these five metals is limited for arsenic, lead, and zinc through assimilation. Partial removal of cadmium and copper could be accomplished through adsorption and assimilation. The assimilated

and total removals for cadmium were 25.9% and 27.3%, and for copper were 46.9% and 52.6%, respectively, on Day 15.

In their study exploring the metal effect on algae, Shivaji and Dronamaraju observed over 60% and around 60% total removal through adsorption and assimilation of cadmium and zinc, respectively (Shivaji & Dronamaraju, 2019). They investigated removals after exposure to individual metals. The lower removal levels observed in my experiments may be attributed to mixed exposure. The results of another study suggest around 600 fg/cell adsorption and 60 fg/cell assimilation after 72 h of exposure of *D. tertiolecta* to copper at a concentration of 500 µg/L (J. L. Levy et al., 2008). The results of the present study indicate a stable assimilation of copper per cell at around 96 fg/cell between days 2 and 15.

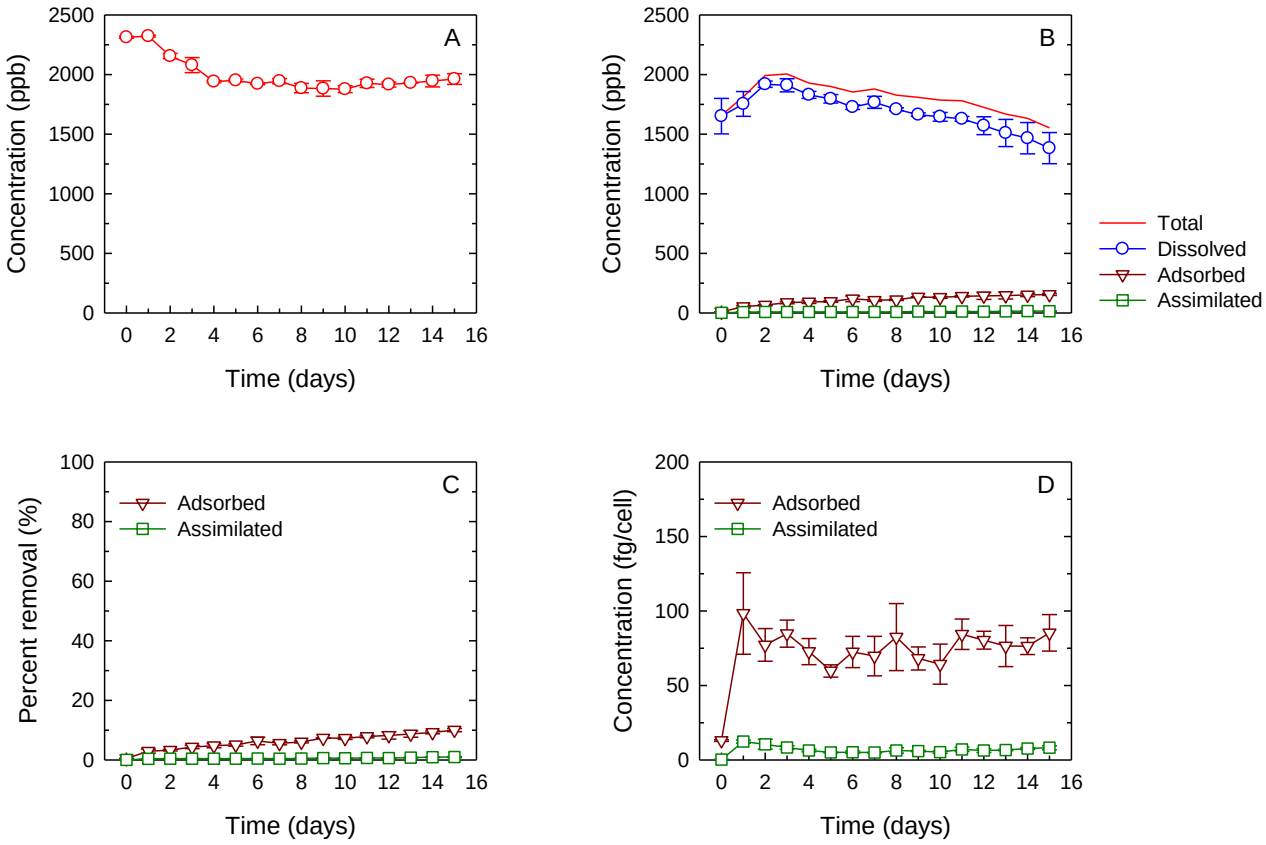


Figure 4.16. The fate of arsenic in the reactors. The concentration of arsenic in abiotic control (A), in different phases when algae is present (B), percent removal by algae with respect to removal mechanism (C), and per cell removal by algae (D).

In this part of the study, metal removal capacity of *T. suecica* was demonstrated when cultured with a mix of five metals commonly found in HFPW, in a saltwater media. HFPW actually contains much more diverse ingredients of contaminants, such as oil and grease, BTEX compounds (benzene,

toluene, ethylbenzene, and xylenes), polycyclic aromatic hydrocarbons (PAHs), organic acids, phenols, naturally occurring compounds originated from shale rock such as calcium, magnesium, and barium, radionuclides that can bear radioactivity hazard, and additives of fracturing fluid (W. Jiang et al., 2022; Jiménez, Micó, Arnaldos, Medina, & Contreras, 2018). A simplified synthetic wastewater was modeled here to evaluate metal removal along with algae growth.

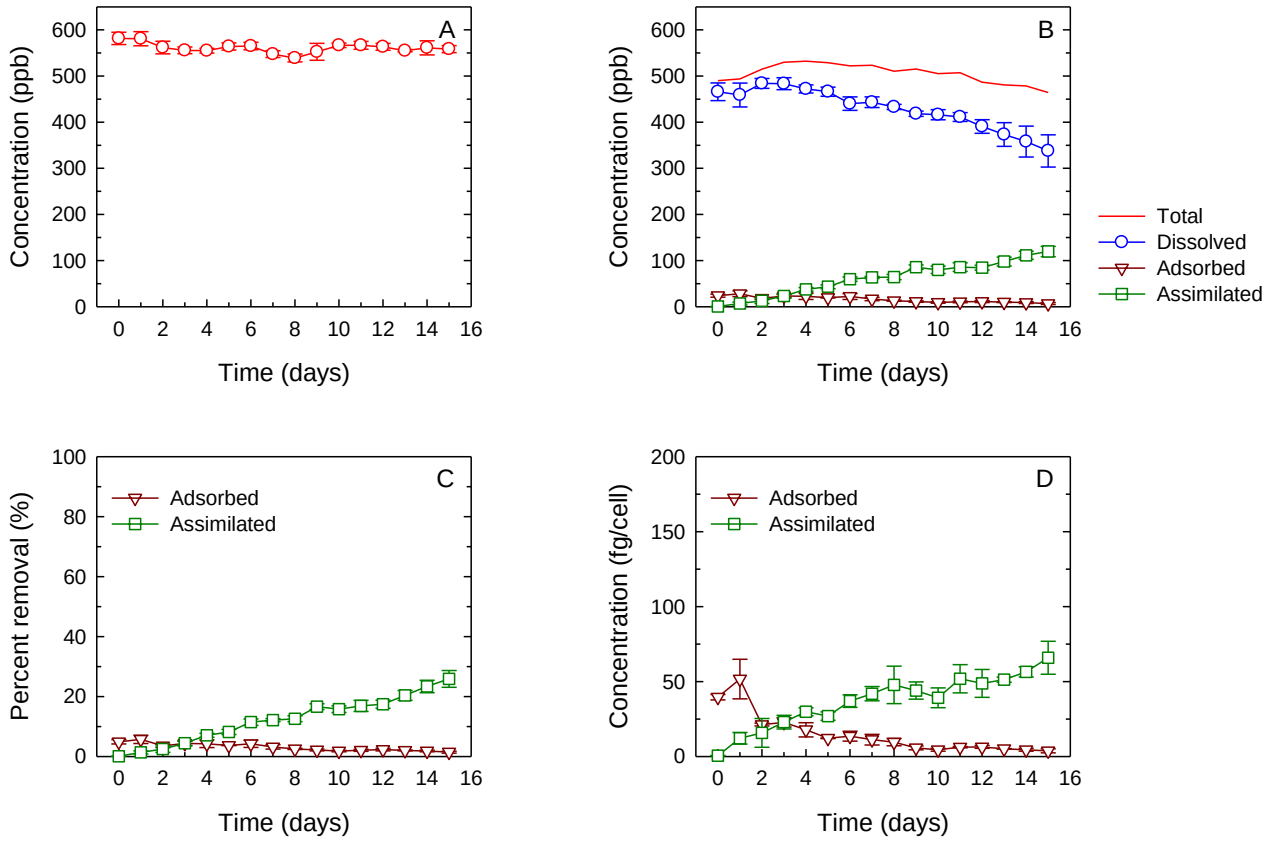


Figure 4.17. The fate of cadmium in the reactors. The concentration of Cd in abiotic control (A), in different phases when algae are present (B), percent removal by algae with respect to removal mechanism (C), and per cell removal by algae (D).

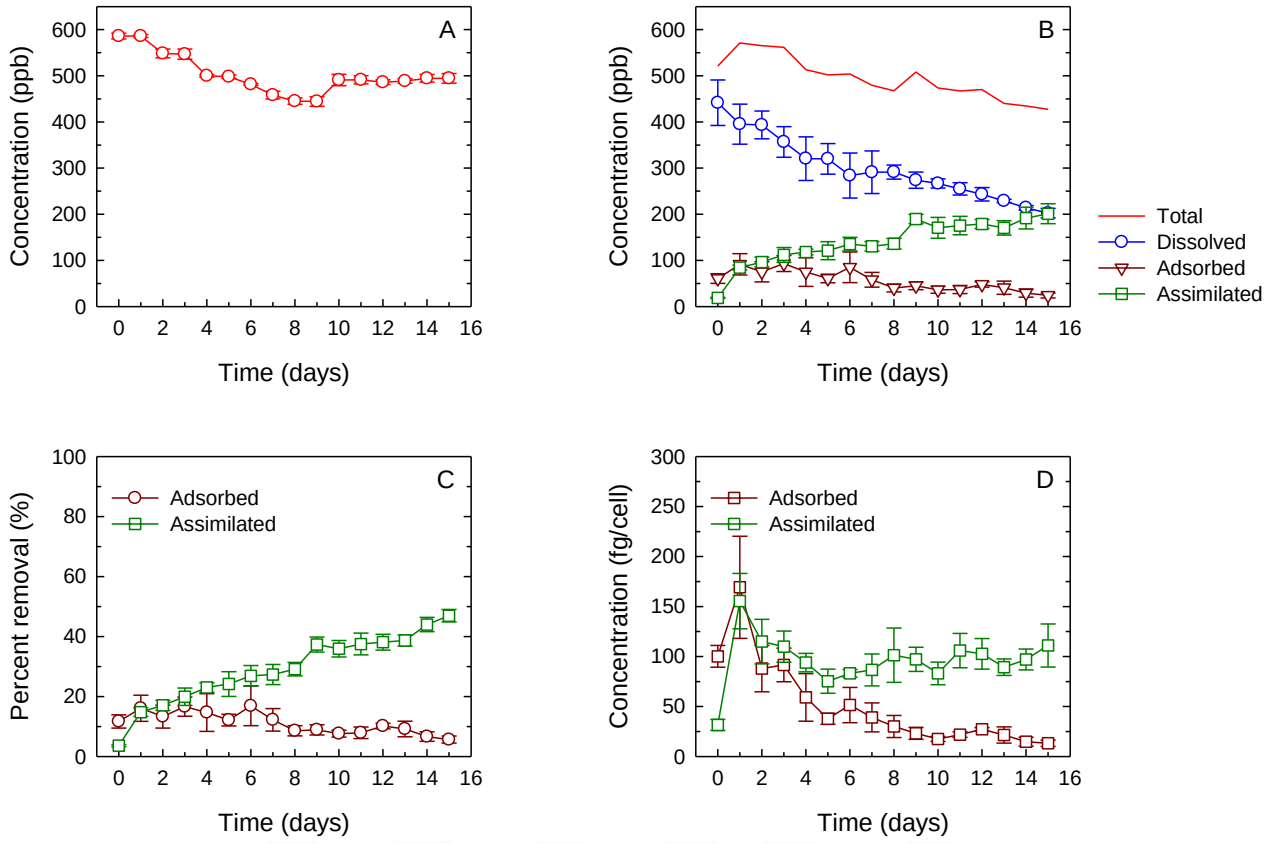


Figure 4.18. The fate of copper in the reactors. The concentration of Cu in abiotic control (A), in different phases when algae are present (B), percent removal by algae with respect to removal mechanism (C), and per cell removal by algae (D).

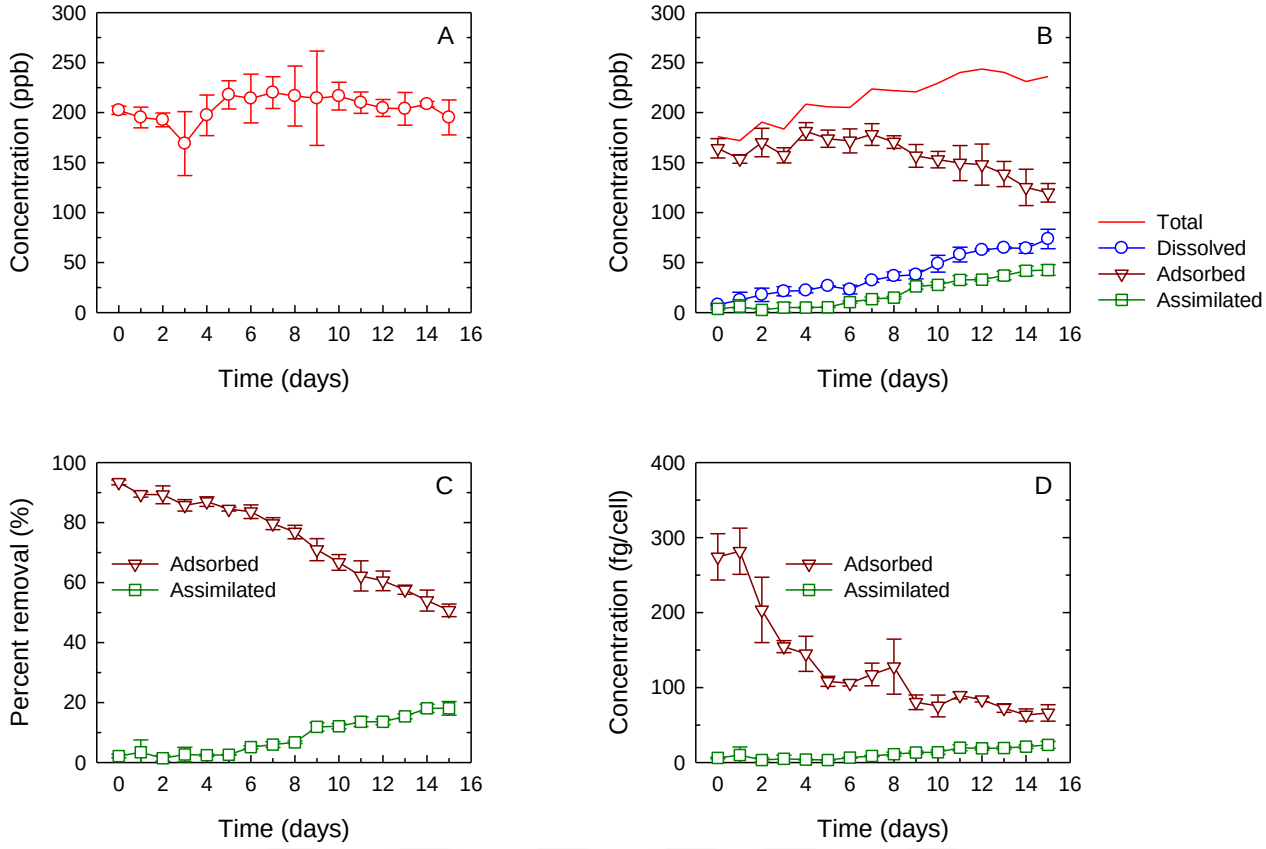


Figure 4.19. The fate of lead in the reactors. The concentration of Pb in abiotic control (A), in different phases when algae are present (B), percent removal by algae with respect to removal mechanism (C), and per cell removal by algae (D).

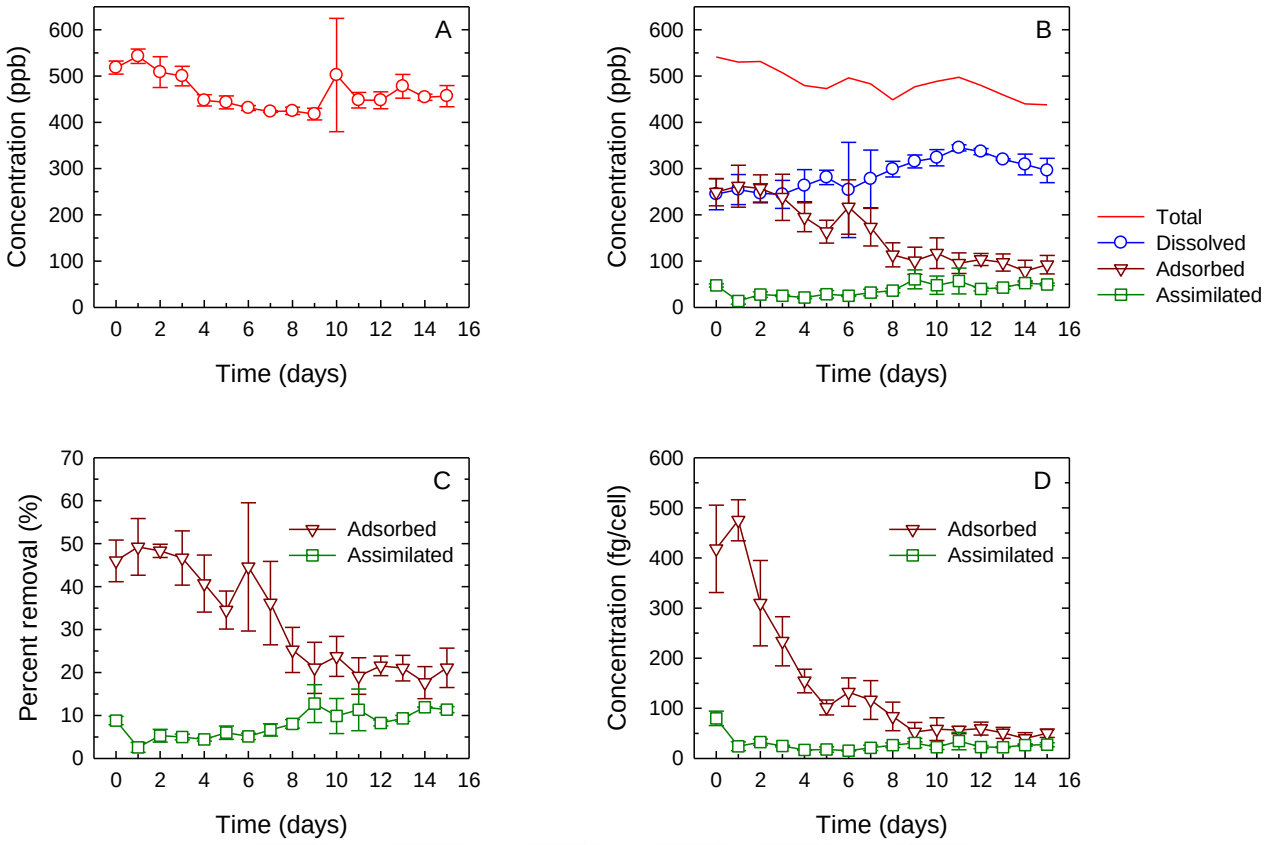


Figure 4.20. The fate of zinc in the reactors. The concentration of Zn in abiotic control (A), in different phases when algae are present (B), percent removal by algae with respect to removal mechanism (C), and per cell removal by algae (D).

4.4. Summary

In this chapter, several microalgae and cyanobacteria species were challenged against HMs and BAC to determine a strain with the highest tolerance to metals and BAC. *Tetraselmis suecica* was most tolerant against cadmium, copper, zinc, and BAC. A BAC-degrading bacterium, *P. sessilinigenes* BIOMIG1, was also challenged against individual HMs for the effect on growth and BAC degrading activity. Arsenic and cadmium affected both growth and BAC degradation, lead and zinc affected neither process, and copper did not affect growth but affected BAC degradation. *T. suecica* was also experimented for metal removal capacity from a synthetic wastewater containing a mix of five metal salts. This strain was not very effective for the removal of arsenic and zinc, but it might remove up to 27.3% of cadmium and 52.6% of copper.

5. ESTABLISHMENT OF A CONSORTIUM OF *T. suecica* AND BIOMIG1

5.1. Introduction

Algae and bacteria have a long history of evolutionary companionship (Ramanan et al., 2016). Their reciprocal significance in each other's evolutionary paths has made this concept a focal point in literature for understanding the biogeochemical history of the Earth. It has also paved the way for the development of new methods to harness this symbiotic relationship for purposes such as algal biomass production and the generation of value-added compounds (Fuentes et al., 2016), wastewater treatment (Cho, Ramanan, Heo, Kang, et al., 2015), and the removal of specific contaminants (Mahdavi, Prasad, Liu, & Ulrich, 2015; Manser et al., 2016). If the symbiotic relationships between algae and bacteria are well understood, treatment processes utilizing both algae and bacteria as a consortium could be operated more effectively. In this context, one of the key objectives is to establish algae-bacteria consortia where at least one of the species benefits from the association, i.e., provision of oxygen by algae for bacterial biodegradation of the compound of interest, and utilize this relationship for the biological treatment of industrial wastewater streams.

In the environment, the interrelationships among organisms are based on trophic interactions such as predation, parasitism, mutualism, or commensalism (Drew et al., 2021). In a predatory association, one species feeds on another. In parasitism, one partner benefits from the relationship while the other suffers. When both species mutually benefit from the interaction, it is called mutualism; and when only one partner benefits and the other is unaffected, it is commensalism. These types of associations result from a long evolutionary history. Constructed associations without this evolutionary history have loose bounds which can be in a competitive, commensal, or cooperative manner (Lindemann et al., 2016). In a competitive manner, partners compete for nutrients or space when those resources are limited. Cooperative associations occur like mutualism but they could change when the alterations in the environment occur. For instance, it has been shown that between two species, each of which requires the metabolites synthesized by the other, interaction shifts to cooperation when metabolites are absent, and become competition when metabolites are provided exogenously (Momeni, Brileya, Fields, & Shou, 2013).

Algae co-culture with bacteria offers several benefits compared to the axenic culturing of algae. Many algae species are auxotrophic for vitamin B_{12}, and bacteria can provide this essential vitamin (Croft et al., 2005). Indeed, a co-culture of B_{12}-synthesizing bacterium and B_{12}-dependent microalgae

was developed, as reported in the literature, providing neither vitamin B_{12} nor any organic carbon source (Elena Kazamia et al., 2012). In such cultures where nutrients are supplied by the individual species of co-culture, the growth dynamics are strictly regulated by the other's growth in an interdependent manner (Cooper et al., 2019). The presence of selected bacteria in co-culture with algae may also prevent contamination by other invasive bacteria since that niche is already occupied (C. A. Santos & Reis, 2014). Another favorable aspect of the presence of bacteria might be the removal of oxygenic tension on the algae. Bacteria, while growing, may remove excess O_2 produced by algae through photosynthesis, thereby ameliorating stress on algae growth caused by high O_2 concentrations (Mouget, Dakhama, Lavoie, & de la Noüe, 1995).

In the context of wastewater treatment, bacteria utilize O_2 and remove organic contaminants through biodegradation, producing CO_2. When algae come into play, they convert CO_2 into O_2 and organic matter through photosynthesis. The combination of these two processes yields a net zero balance of O_2/CO_2 production or consumption. This phenomenon renders the algae-bacteria co-culture system an attractive option for wastewater treatment due to its ability to reduce costs associated with O_2 supply and decrease CO_2 emissions. These aspects make the co-culture system a sustainable eco-friendly approach.

Benzyldimethylamine (BDMA) is a tertiary amine compound with the chemical formula of $C_9H_{13}N$ and molecular weight of 135.2 g/mole. It is used as an initiator in the formation of epoxy polymers, as a less volatile and environmentally friendly catalyst (Firouzmanesh & Azar, 2003; Hulme, Muha, & Harakal, 1994; Messersmith & Giannelis, 1994; Sun et al., 2007). Those polymers can be utilized as a resin in sample preparation for electron microscopy or Raman microscopy (Freeman & Spurlock, 1962; Hardcastle & Harris, 2015; Hether, Misono, & Lessard, 2013). BDMA itself is not a compound of environmental concern as it is easily biodegradable in wastewater treatment systems. However, it can turn into nitrosodimethylamine upon contact with chloramines, which are formed in the chlorination of ammonia bearing water in drinking water reservoirs (Bei et al., 2016). Nitrosodimethylamine belongs to the nitrosamines, a compound group recognized for its genotoxic carcinogenic properties (Tricker & Preussmann, 1991). BDMA is the first metabolite of BAC biodegradation by *P. sessilinigenes* BIOMIG1 and other bacteria (Ahn et al., 2016; Ertekin, Konstantinidis, & Tezel, 2017).

In this chapter, the objective was to establish a consortium involving the microalgae *T. suecica* and the bacterium *P. sessilinigenes* BIOMIG1. The BAC degrading activity of the bacteria and the growth of the algae were assessed in an optimized medium. Subsequently, these two species were co-

cultured with various organic carbon sources. Bacterial activity was monitored using BDMA as an organic carbon source, and the kinetic of bacterial BDMA removal was determined in both the presence and absence of algae in the co-culture.

5.2. Materials & Methods

5.2.1. Media Optimization for Bacterial BAC Degradation

P. sessilinigenes BIOMIG1 culture that was grown in modified M9 containing 200 ppm BAC was inoculated into either F/2 medium, NH_4Cl enriched F/2 medium, or F/2 medium prepared with diluted seawater. ½ or ¼ dilutions of seawater were used to prepare 16‰ and 8‰ F/2, respectively. Other ingredients were the same as the original tariff. 10 ppm BAC was added to all tested media. BAC measurements were done after 24 or 48 hours via HPLC. M9 with 10 ppm BAC was also tested as a positive control for BAC degradation activity. After algae culture media was optimized for bacterial BAC degradation, algae growth in this media was monitored via absorbance at 680 nm and via cell count.

5.2.2. Consortium Culturing with Different Organic Carbon Sources

BIOMIG1 culture was incubated with *T. suecica* in 8‰ F/2 media with either no organic carbon source, or the addition of one of the following: 50 ppm BDMA, 100 ppm BDMA, 200 ppm BDMA, 200 ppm glucose, or 200 ppm sodium acetate. The starting algae density was $0.6x10^6$ cells/mL. Two bacteria densities were tested, $1.2x10^6$ CFU/mL and $2.9x10^7$ CFU/mL. Algae density was determined through microscopic cell count. The density of the bacteria was determined through serial dilution and spreading onto LB-200BAC agar, and M9-200BAC agar plates. BDMA was measured through HPLC. When BDMA was depleted, it was spiked to maintain an organic source. On Day 5 and Day 9, 200 ppm glucose and 200 ppm sodium acetate were spiked in corresponding culture flasks.

Incubation of *P. sessilinigenes* BIOMIG1 and *T. suecica* together in 8‰ F/2 with 100 ppm BDMA was repeated with varying algae and bacteria initial densities. Culture densities were as follows:

Only algae (10^6 cells/mL)
Algae 10^6 cells/mL, bacteria 10^7 cells/mL (Algae-bacteria mix)
Algae 10^7 cells/mL, bacteria 10^7 cells/mL (Mix with 10-fold algae)

Algae 10^6 cells/mL, bacteria 10^8 cells/mL (Mix with 10-fold bacteria)

Only bacteria (10^7 cells/mL)

BDMA was spiked daily in the first 8 days and every other day after Day 8. Algae and bacteria densities were monitored every other day. On Day 15, 20 ppm BAC was spiked into each vessel. BAC was analyzed on HPLC 24 hours and 48 hours after BAC addition. Tests were conducted in triplicates.

5.2.3. Kinetic Analysis of BDMA Removal

A kinetic experiment was set to determine the BDMA biotransformation rate. 3 parallels of one set consisting of the algae-bacteria mix with 10^6 cells/mL algae and $3x10^{10}$ CFU/mL bacteria, and another set consisting of only bacteria ($3x10^{10}$ CFU/mL) were conducted (Figure 5.1). Initial BDMA was 40 mg/L. Data was taken every 30 minutes, and BDMA concentrations were measured in HPLC. The specific substrate utilization rate was calculated using a modified Monod kinetic equation. There are two ordinary differential equations in Monod kinetics (Eq. 5.1 and Eq. 5.2) representing substrate and biomass change through time. In these equations, S is substrate concentration, t is time, k is maximum specific substrate utilization rate, X is biomass density, K_s is half saturation constant, Y is biomass yield from unit substrate, and k_d is the decay rate of bacteria. Assuming biomass is constant, the first equation can be simplified by replacing k*X with k' (Eq. 5.3), where k' is the cell-specific substrate utilization rate at specific bacteria density. In my model, I used only the bacteria concentration for X.

$$\frac{d(S)}{dt} = -k * X * \frac{S}{K_s + S} \tag{5.1}$$

$$\frac{d(X)}{dt} = -Y * \frac{d(S)}{dt} - k_d * X \tag{5.2}$$

$$\frac{d(S)}{dt} = -k' * \frac{S}{K_s + S} \tag{5.3}$$

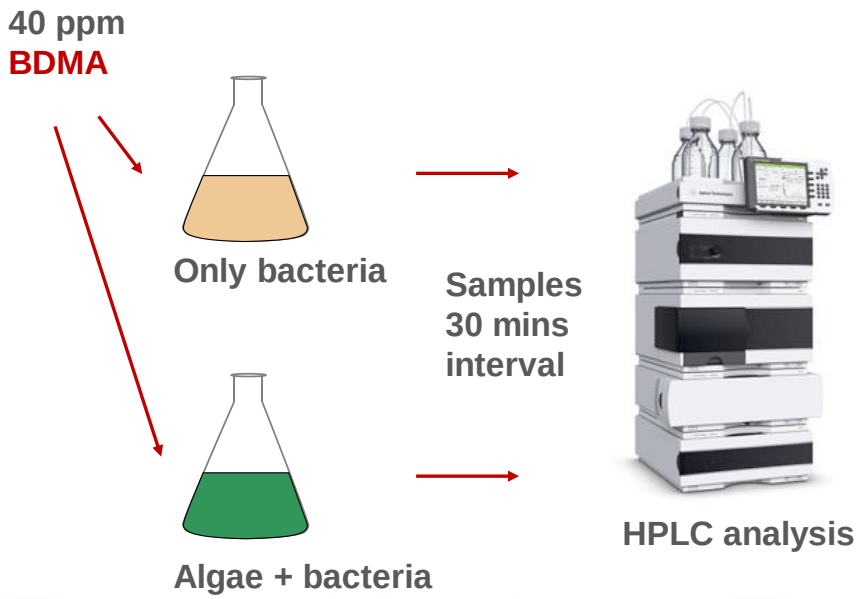


Figure 5.1. Schematic representation of BDMA removal kinetic experimental design.

5.3. Results & Discussion

5.3.1. BAC Degradation in Algae Media

The culture medium of *T. suecica*, F/2 medium, was chosen as the matrix for consortium development. It is a marine algae medium, prepared with 32‰ seawater. The bacterial strain used for consortium development, *P. sessilinigenes* BIOMIG1, was isolated from sewage; its activity in seawater was not known. On the first hand, the BAC degrading activity of this strain was evaluated in F/2 media. BAC degradation was poor in this media. A bacterial nitrogen source, NH_4Cl, was added to F/2; hence, the biotransformation activity was still poor (Figure 5.2). Poor removals of BAC might be related to polyvalent ionic strength in saline F/2 media. Indeed, flocs of bacterial biomass were observed after a few days of inoculation. The ionic strength of the media was reduced by diluting the seawater used for the preparation of F/2. Media prepared with ½ or ¼ diluted seawater, corresponding to 16‰ and 8‰ salinity, were tested for bacterial BAC degradation activity (Figure 5.3). Complete removal of BAC was observed after 48 hours and after 24 hours in 16‰ and 8‰ salinity media, respectively. As the removal rate was closer to that in M9 media, 8‰ salinity F/2 (will be referred to as 8‰ F/2 from now on) was chosen as the media for consortium development.

T. suecica growth in 8‰ F/2 was evaluated in terms of OD_{680} and microscopic cell count (Figure 5.4). 8-days growth rates (μ, 1/d) were 0.27±0.009 and 0.23±0.006 in terms of absorbance change, and 0.24±0.04 and 0.23±0.04 in terms of increase in cell counts, for algae grown in 32‰ and 8‰

F/2, respectively. Growth inhibition of algae in 8‰ F/2 media compared to original F/2 media was 14% in terms of absorbance, and 7% in terms of cell count.

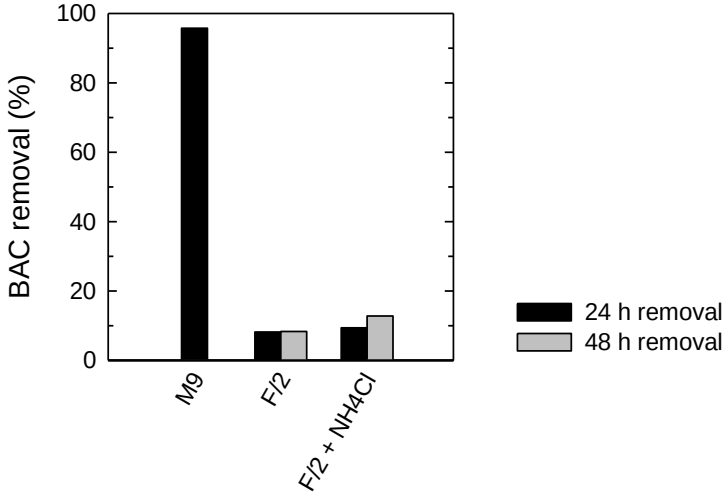


Figure 5.2. BAC removal by bacteria in original F/2 media. F/2 media was also tested with the addition of NH_4Cl for bacterial nitrogen source. *P. sessilinigenes* BIOMIG1 was also grown in M9 media + BAC as a positive control for BAC removal activity.

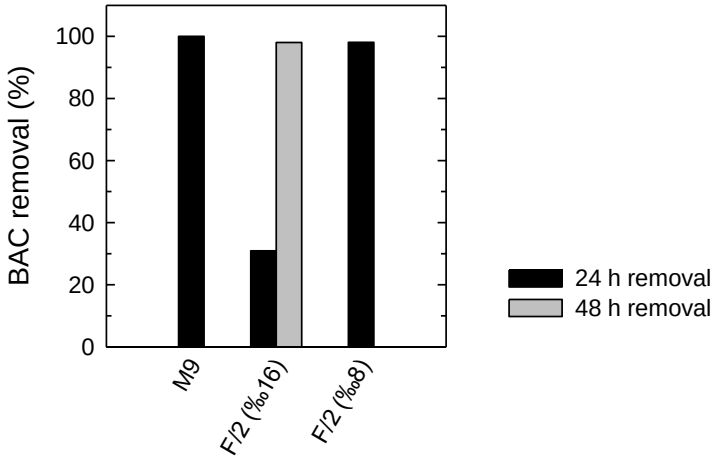


Figure 5.3. BAC removal by bacteria in F/2 media prepared with diluted seawater. *P. sessilinigenes* BIOMIG1 was also grown in M9 media + BAC as a positive control for BAC removal activity.

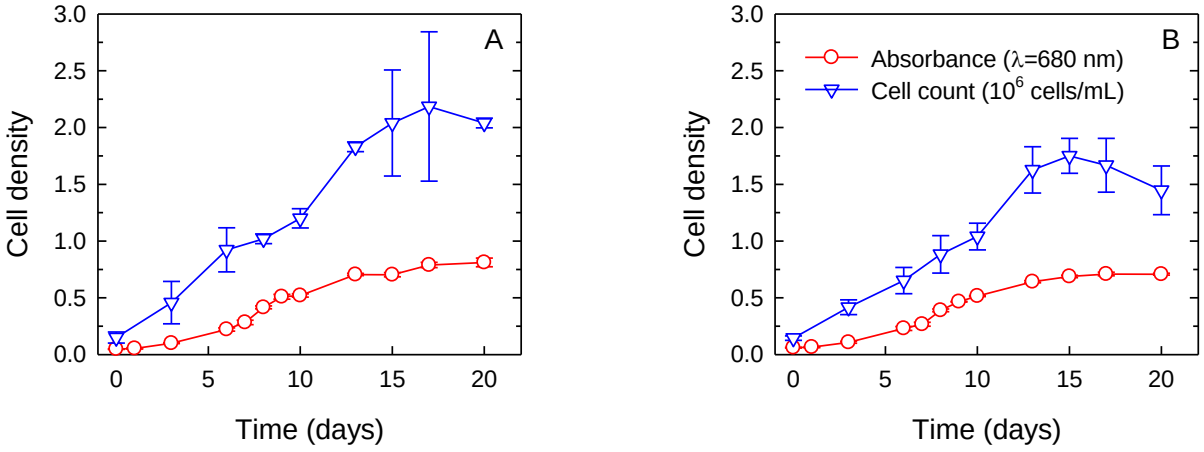


Figure 5.4. Algae growth in the media 32‰ F/2 (A) and 8‰ F/2 (B) in terms of OD_{680} (red, circle) and microscopic cell count (blue, triangle down).

5.3.2. Growth of Algae and Bacteria in Consortium with Organic Carbon Source

T. suecica and *P. sessilinigenes* BIOMIG1 were incubated in 8‰ F/2 medium without adding an organic carbon source. Algae growth was similar to that without bacteria addition, but the bacteria stopped growing. After a few days, there were no colonies observed on the LB-200 BAC agar plates. Bacteria require an organic carbon source, thus an experiment was designed with various types of organic carbon sources. These carbon sources were BDMA, glucose or sodium acetate. BDMA is the first metabolite of BAC when BAC is degraded by BIOMIG1. IC_{50} value of BDMA to *T. suecica* was determined before the start of the experiments as 12±0.4 mg/L. 50, 100, or 200 ppm BDMA, 200 ppm glucose, or 200 ppm sodium acetate were added into the media as the organic carbon source of the bacteria. M9-200 BAC agar plates were utilized, which contained BAC as the sole organic carbon source to determine the presence of BAC-degrading bacteria in the consortium. When colonies were formed on the plate, it would imply that there were bacteria with BAC biotransformation activity in the sample. Two initial bacterial densities for each set were tested, $1.2x10^6$ CFU/mL and $2.9x10^7$ CFU/mL.

In the vessels fed with glucose or sodium acetate, there were no colonies observed on M9-200 BAC agar plates on Day 7, despite the initial bacterial density. Indeed the gene responsible for the oxidation of BAC to BDMA, the first step of BAC degradation by *P. sessilinigenes* BIOMIG1, was shown to be on a composite transposon on the genome (Ertekin et al., 2017). When not exposed to BAC for a while, the culture of the bacteria loses its BAC degradation ability. In the sets fed with BDMA, the presence of BAC-degrading activity of the bacteria was verified with the colonies observed on M9-BAC agar plates.

BDMA removal and survival of the algae in the sets fed with 50 ppm, 100 ppm, and 200 ppm BDMA were determined as a preliminary step for consortium development. The initial pH values were between 8.0-8.5 for all the sets. On Day 5, the pH values of the low initial bacterial density reactors were very close to each other, 9.53±0.06. The pH value for high initial bacterial density sets were 8.99±0.13. On Day 13, pH decreased for all the sets, to a value of 8.89±0.39 and 8.61±0.43 for low and high initial bacterial density sets, respectively. Change in BDMA concentrations and algae density throughout the experiments were shown in Figure 5.5 and Figure 5.6, for $1.2x10^6$ CFU/mL and $2.9x10^7$ CFU/mL initial bacterial density, respectively. Very similar BDMA removal patterns were observed in the sets with low and higher initial bacterial density. Algae cell density differed between low and higher initial bacterial density sets, especially after Day 5. From Day 5 to Day 13 in the sets that started with $1.2x10^6$ CFU/mL bacterial density, algae density increased when the consortium was fed with 50 ppm BDMA, stayed similar when fed with 100 ppm BDMA, and decreased when fed with 200 ppm BDMA. In the sets that started with $2.9x10^7$ CFU/mL bacterial density, within the same timespan algae density increased slightly at all tested BDMA concentrations.

The experiments were repeated with 100 ppm BDMA to observe the effects of initial bacteria and algae density on consortium development. This time vessels were set up with 10^6 cells/mL algae and 10^7 CFU/mL bacteria. Additional sets with 10-fold algae concentration, 10-fold bacteria concentration in the consortium, only algae, and only bacteria were also tested for BDMA removal and algae and/or bacteria density monitoring. The pH values of the individual reactors of the sets were between 7.59 and 8.96 throughout the course of the experiments. The mean pH values of different sets were listed in Table 5.1.

Table 5.1. The pH values of the sets fed with 100 ppm BDMA.

Time (days)	Only algae	SD	Consortium	SD	Consortium 10-fold algae	SD	Consortium 10-fold bacteria	SD
0	8.55	0.02	8.45	0.05	8.28	0.03	7.72	0.20
2	7.78	0.08	8.03	0.08	8.50	0.40	8.22	0.04
4	8.17	0.01	8.26	0.16	8.49	0.38	8.35	0.10
6	7.94	0.10	8.34	0.14	8.39	0.28	8.41	0.07
8	7.99	0.07	8.31	0.29	8.85	0.13	8.39	0.31
10	7.91	0.04	8.58	0.05	8.86	0.09	8.63	0.00
12	8.05	0.08	8.63	0.15	8.71	0.05	8.73	0.04
15	7.96	0.08	8.62	0.15	8.63	0.08	8.69	0.07

For algae, the added level of BDMA was completely inhibitory to growth and led to the death of most of the cells in the culture in the absence of BIOMIG1 (Figure 5.7.A). In the presence of bacteria when the initial algae density was 10^6 cells/mL, algae survived for a longer time but there was no observable growth (Figures 5.8.A, 5.10.A). In the set with 10 times the initial algae density (~10^7

cells/mL), algae did not grow but sustained its density (Figure 5.9.A). At the end of these experiments, algae cells were the healthiest in the set containing 10-fold initial algae density.

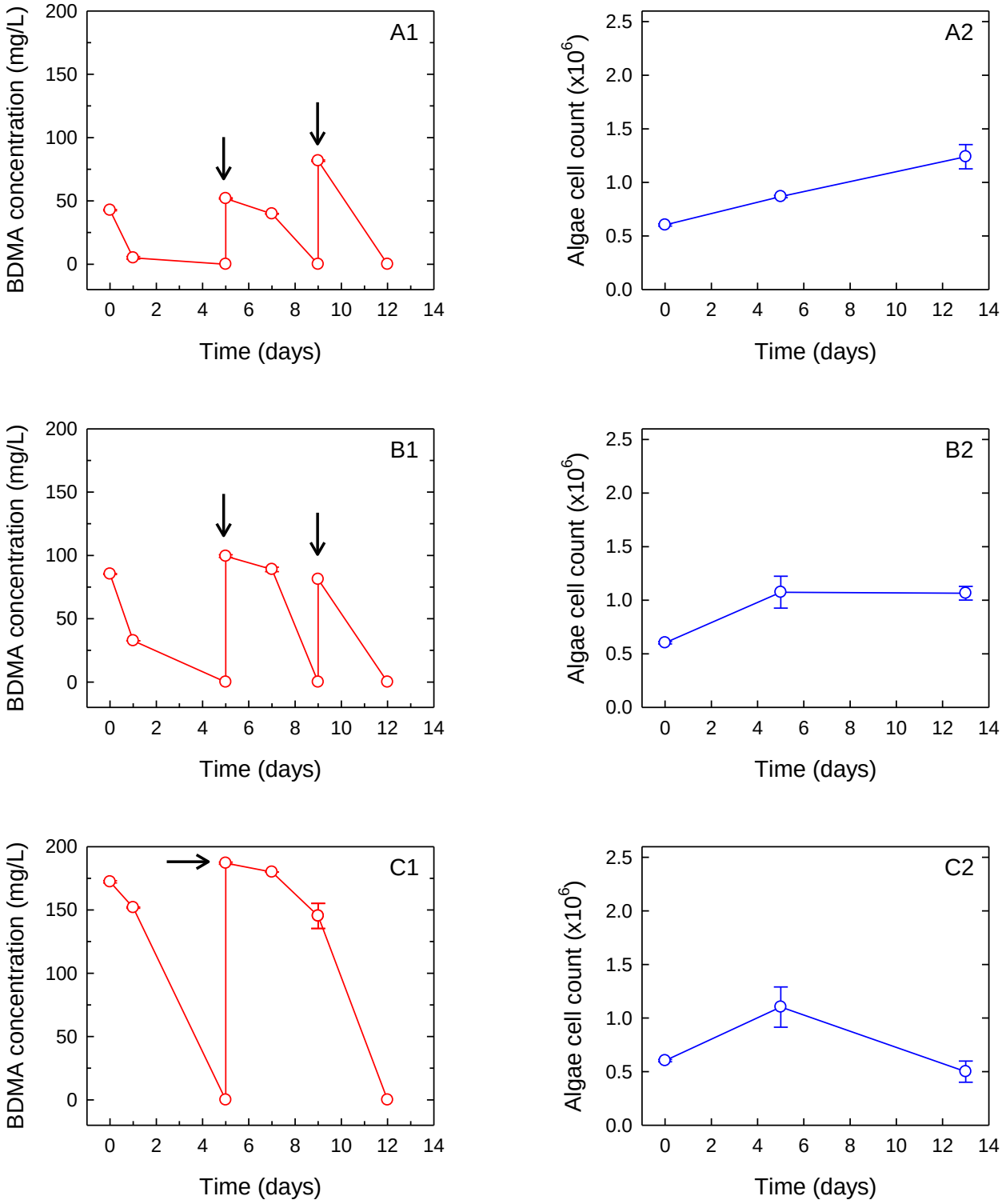


Figure 5.5. BDMA concentrations (1) and algae cell counts (2) in the reactor sets fed with 50 ppm (A), 100 ppm (B), and 200 ppm (C) initial BDMA at an initial bacterial density of 1.2×10^6 CFU/mL. BDMA was spiked as depleted (represented by arrows).

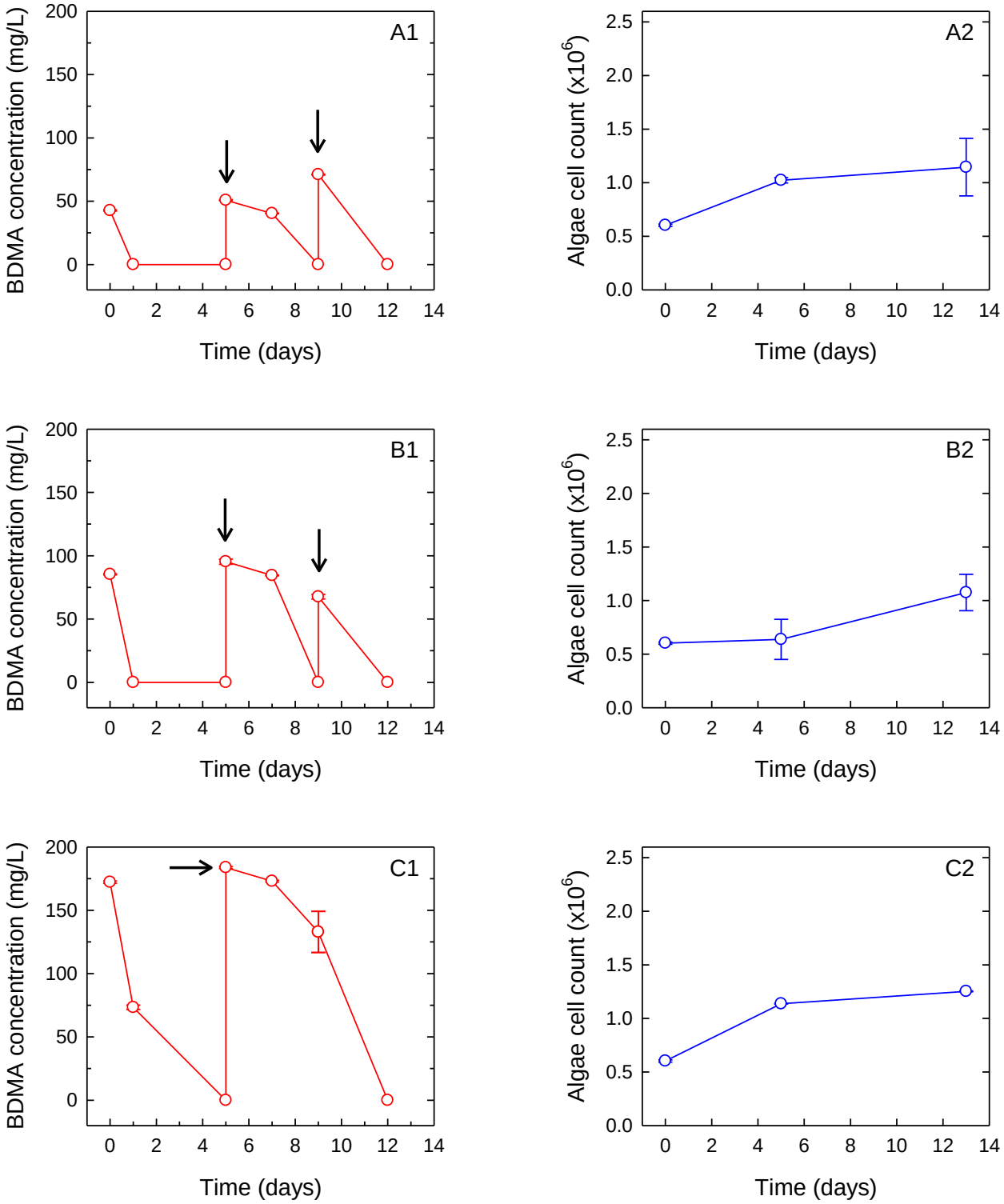


Figure 5.6. BDMA concentrations (1) and algae cell counts (2) in the reactor sets fed with 50 ppm (A), 100 ppm (B), and 200 ppm (C) initial BDMA at an initial bacterial density of 2.9×10^7 CFU/mL. BDMA was spiked as depleted (represented by arrows).

The bacterial cell density was bounded by the total substrate influx, as inferred from the data. When the substrate, BDMA, was daily spiked at 100 ppm (first 8 days), bacterial density was increased. When the spike frequency was per 48 hours, bacterial densities in the mixed sets were

gradually decreased down to 10^7 CFU/mL (Figures 5.8.B, 5.9.B, 5.10.B). The bacterial density in the set with no algae somewhat differed. The increase in the first days was more rapid, and the concentration equilibrated in the magnitudes of 10^{11} CFU/mL by Day 12 (Figure 5.11.A). In the set with only algae and no initial bacteria added, no colonies were observed on the LB-200BAC agar plates.

In the set containing only algae, there was no BDMA spike and the BDMA concentration was almost stable throughout the experiments (Day 16 BDMA conc. was 82% of the initial BDMA conc., Figure 5.7.B). In the sets containing 10^6 cells/mL algae and 10^7 or 10^8 CFU/mL bacteria BDMA was completely consumed daily until Day 7 and Day 6, respectively. Afterwards, in either one or two parallels of those sets BDMA was not totally consumed overnight (Figures 5.8.C**,** 5.10.C). On the contrary, in the set containing 10-fold algae density (10^7 cells/mL), incomplete consumption was observed in one or two replicates during the first 6 days. From day 7 on, BDMA was consumed overnight in the vessels of this set until the end of the experiments (Figure 5.9.C). In the set with only bacteria between Days 2 and 6, complete or almost complete removal of BDMA was observed. However, on the subsequent days, the bacteria were unable to consume BDMA up overnight (Figure 5.11.B).

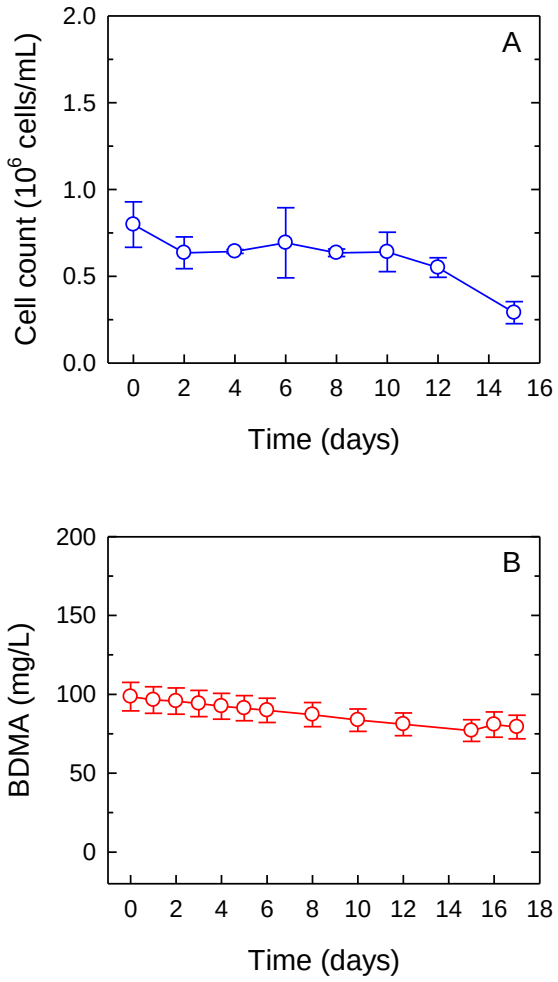


Figure 5.7. Algae density (A) and BDMA concentration (B) in the reactors seeded only with algae as 10^6 cells/mL.

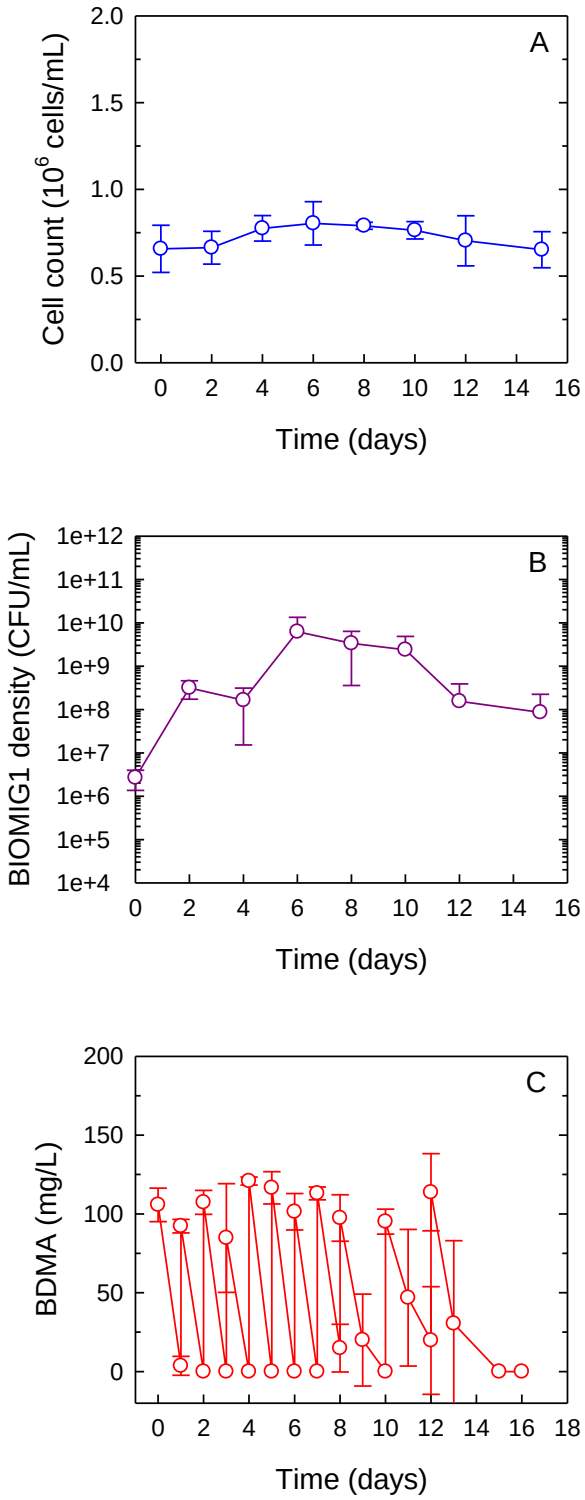


Figure 5.8. Algae density (A), bacteria density (B), and BDMA concentration (C) in the reactors seeded with 10^6 cells/mL algae and 10^7 CFU/mL bacteria. BDMA was spiked as depleted.

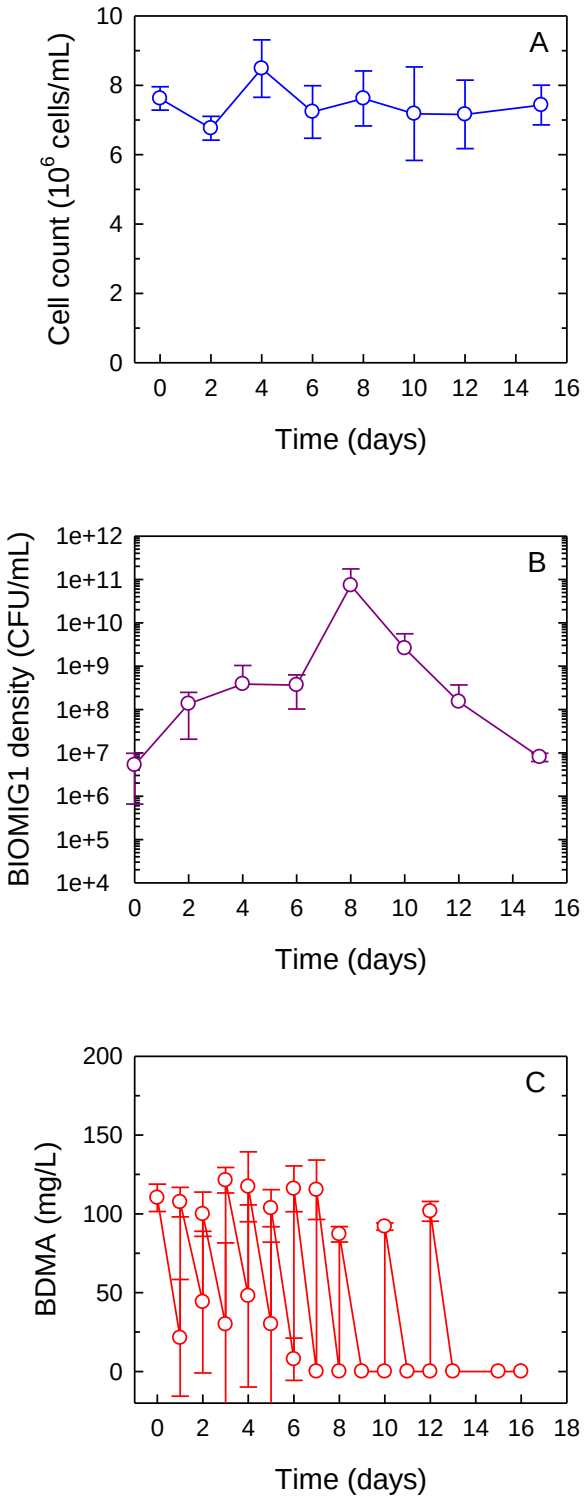


Figure 5.9. Algae density (A), bacteria density (B), and BDMA concentration (C) in the reactors seeded with 10^7 cells/mL algae and 10^7 CFU/mL bacteria. BDMA was spiked as depleted.

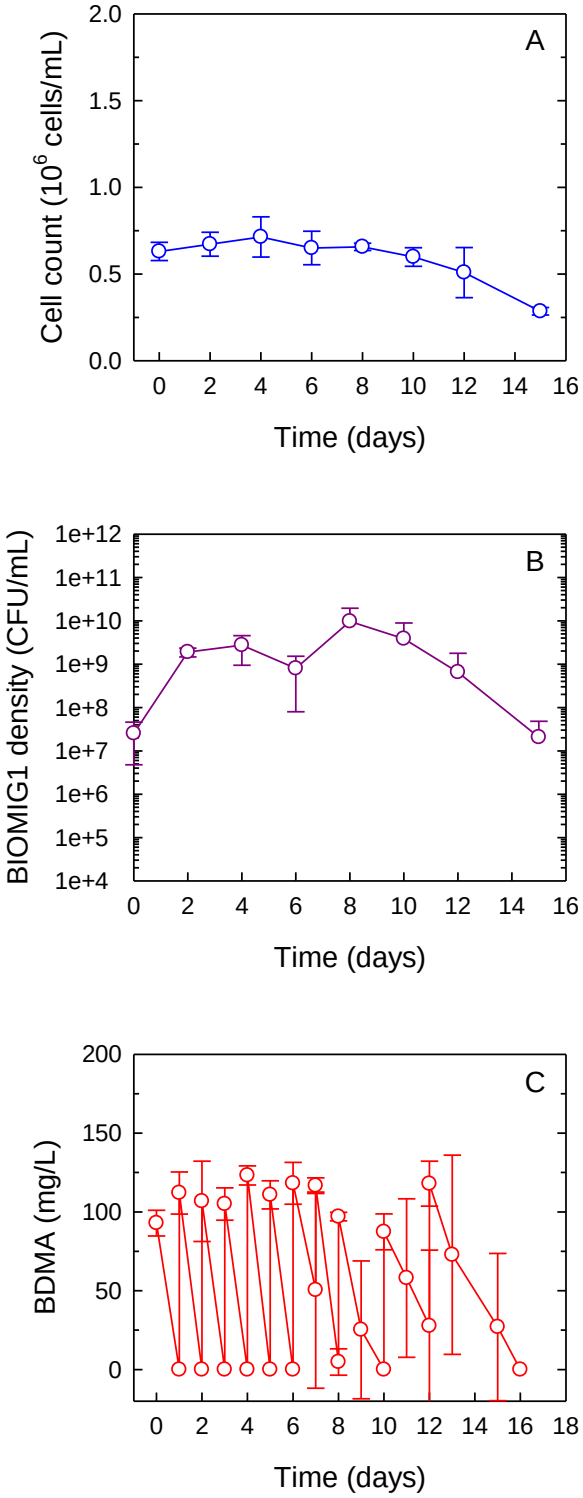


Figure 5.10. Algae density (A), bacteria density (B), and BDMA concentration (C) in the reactors seeded with 10^6 cells/mL algae and 10^8 CFU/mL bacteria. BDMA was spiked as depleted.

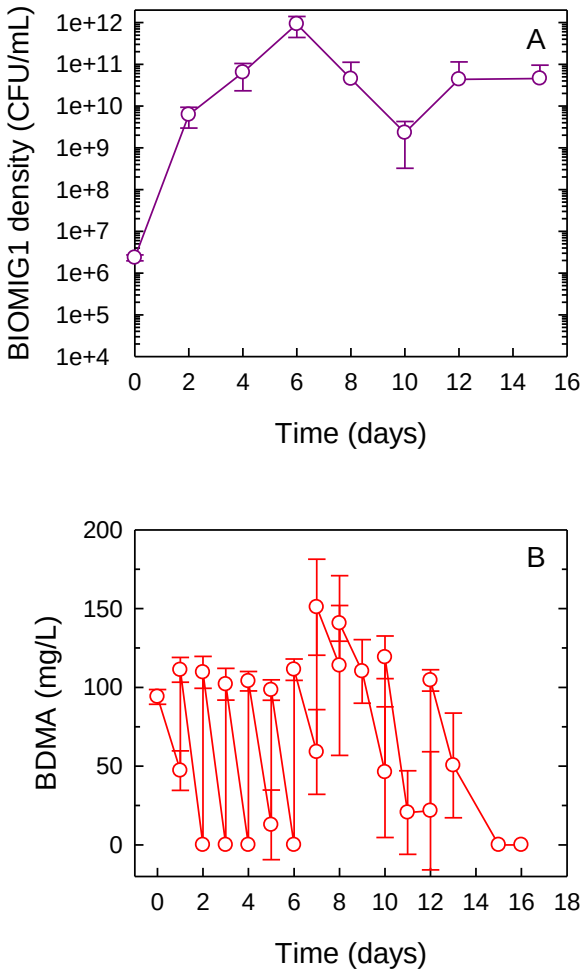


Figure 5.11. Bacteria density (A) and BDMA concentration (B) in the reactors seeded only with bacteria as 10^7 cells/mL. BDMA was spiked as depleted.

On Day 15, 20 ppm BAC was added to the vessels of each set. BAC removals were observed in the two subsequent days as 24-hour removal or 48-hour removal (Figure 5.12). Efficient removal of BAC was observed in 24 hours in the sets containing 10^6 cells/mL algae in the consortium, and the set containing only bacteria. When algae density was 10 times higher in the consortium, partial BAC removal happened in 24 hours, but most of the BAC was eliminated in 48 hours. BAC removal was less than 10% in the set containing only algae.

For the mixed culture to work efficiently for BAC degradation, a stable bacterial culture is necessary. 10^7 CFU/mL bacterial density was observed to be sufficient for BAC and BDMA removal. Daily spikes of 100 ppm were high as it was toxic to algae and it led to overexpansion of bacterial culture followed by a decrease in bacteria concentration because of the insufficiency of the substrate to high density of bacteria. In the set without algae inoculum, the bacterial density in the later phase of the experiments was 1000-fold higher than that of mixed sets, although the BAC removal

performance was slightly worse implying the positive effect of the presence of algae in the reactor.

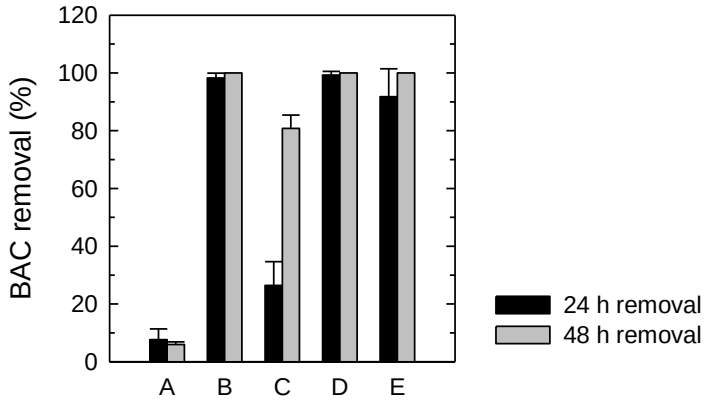


Figure 5.12. BAC removal efficiencies of the sets after spiked with 20 ppm BAC on Day 15. A: Only algae, B: Algae-bacteria mix, C: Mix with 10-fold initial algae concentration, D: Mix with 10-fold initial bacteria concentration, E: Only bacteria.

5.3.3. BDMA Removal Kinetic in The Presence of Algae

BDMA removal kinetic by *P. sessilinigenes* BIOMIG1 was determined in the presence or absence of *T. suecica*. The fit curves of the model for sole bacteria and algae-bacteria consortium were shown in Figure 5.13. K_s was calculated as 2.36 mg from the model applied to the set with only bacteria. It was utilized as constant in the model of mixed culture, was not calculated again from the model fit. Cell-specific utilization rates (k' values) for BDMA were determined as 31.0 and 32.1 mg/h, corresponding to 230 and 237 µM/h, for sole bacteria and consortium sets, respectively. BDMA degradation rate of BIOMIG1 did not change between the presence and absence of algae in co-culture.

In previous studies, the cell-specific BAC degradation rate by *P. sessilinigenes* BIOMIG1 was determined to be 4 - 7 µM/h (Tezel, Tandukar, Martinez, Sobecky, & Pavlostathis, 2012; Yılmaz, Ertekin, & Tezel, 2019). It was also shown that the BDMA biotransformation rate was faster than BAC conversion to BDMA (Ertekin et al., 2017), which is in line with the high BDMA utilization rates found in the present study. These findings shed light on the dynamic interactions between algae and bacteria in the consortium, demonstrating the potential for efficient organic carbon degradation and nutrient utilization. These results contribute to the understanding of the practical applications of this symbiotic relationship in the context of wastewater treatment and resource management.

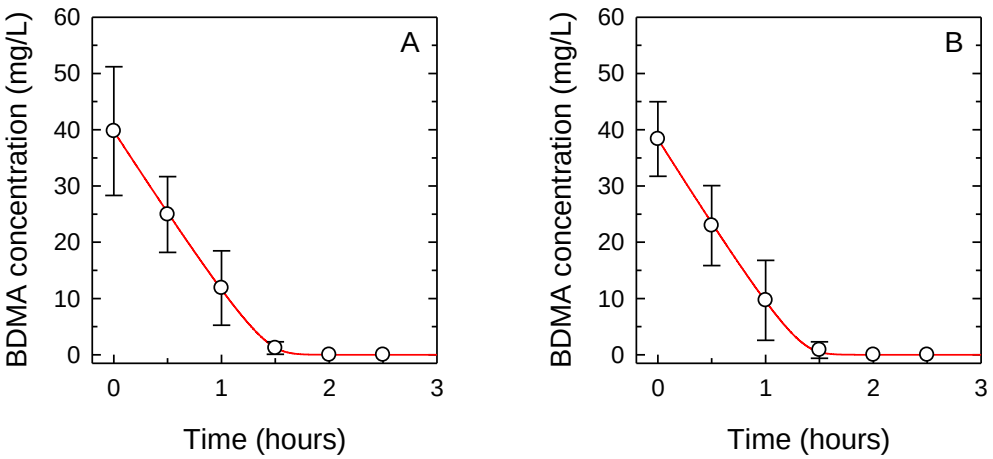


Figure 5.13. BDMA biotransformation kinetics of only bacteria (A) and algae-bacteria mix (B). The red line indicates the biodegradation model; circular symbols with error bars represent the actual observed data.

5.4. Summary

In this chapter, a consortium of *Tetraselmis suecica* and *P. sessilinigenes* BIOMIG1 was developed using the growth medium of *T. suecica*, F/2. BIOMIG1 culture was unable to degrade BAC in this media. Media was optimized by decreasing the ionic strength. Algae growth in the optimized media, 8‰ F/2, was comparable to growth in the original F/2. A consortium of algae and bacteria was established using BDMA as the organic carbon source. Without an organic carbon source or with different types of organic carbon sources such as glucose or sodium acetate, the bacteria diminished or lost BAC degrading ability. At 100 ppm BDMA, algae growth was almost completely inhibited after 6-7 days. The BDMA removal rate of the consortium was similar to the sole bacteria BDMA removal rate.

6. TREATMENT OF COPPER AND BAC BY *T. suecica* AND BIOMIG1

6.1. Introduction

The presence of ECs in wastewater has become a significant concern in recent years due to their potential environmental and public health impacts. ECs encompass a broad range of chemicals, including PPCPs, industrial chemicals, and pesticides (Tolboom et al., 2019). These substances often enter wastewater through various sources, including municipal, industrial, and agricultural discharges (García-Galán et al., 2020; Z. Norvill, 2016; C. E. Santos, de Coimbra, Bermejo, Pérez, & Cabero, 2017). While wastewater treatment plants are designed to remove conventional pollutants, they may not effectively eliminate ECs, allowing these compounds to persist in treated effluents and potentially enter natural water bodies.

Mistreatment or inadequate removal of ECs in wastewater can pose several risks. Firstly, these substances can have adverse ecological effects, leading to disruptions in aquatic ecosystems (Mojiri et al., 2022). Additionally, some emerging contaminants are known to be persistent and bioaccumulative, which means they can accumulate in organisms and eventually find their way into the food chain, potentially impacting human health. Furthermore, the presence of certain pharmaceuticals in treated wastewater may contribute to the development of antibiotic resistance in microbial populations, posing a global public health threat. BAC, which is widely used as a surfactant and disinfectant, is one of them (Tezel & Pavlostathis, 2011; K. Yang, Chen, & Zhu, 2023). BAC concentration in the wastewater influent is reported as up to 6 mg/L, which decreases to <4.1 μg/L in wastewater effluent and subsequent receiving water bodies (Mahony, McNamara, & Arnold, 2023; Merchel Piovesan Pereira & Tagkopoulos, 2019). Since its presence in the environment at sublethal concentrations bears a risk of the emergence of multi-compound resistant “super-bugs”, its complete removal in wastewater treatment systems is necessary.

Biotransformation, particularly through the usage of microorganisms, offers a promising approach to remediate wastewater contaminated with BAC. Biological removal of BAC has been studied extensively in the literature (Belter, McCarlie, Boucher-van Jaarsveld, & Bragg, 2022). It starts with N-dealkylation, which is accomplished by a Rieske-type oxygenase (Ertekin et al., 2017). Even though biodegradation of BAC is shown to be possible in municipal wastewater by certain types of degrader bacteria (Sakarya, Haznedaroglu, & Tezel, 2021), removal from complex matrices such as industrial wastewater which contains other types of hazardous contaminants could be problematic.

For instance, a study found complete removal of BAC by the green microalgae *T. suecica* in seawater, but only partial removal in HFPW (Jaén-Gil et al., 2020).

Heavy metals consist of elements that have a density higher than 3.5 g/cm^3 (Aijaz et al., 2022). Because of their non-biodegradable nature, they pose a risk to the environment in terms of bioaccumulation towards higher tropic levels of biota. They may enter the receiving environments through water disposal, industrial discharge, or mining activities. Their presence in wastewaters is also problematic since they might bear the risk of mistreatment of other pollutants in wastewater treatment systems (Sinharoy, Kumar, Chaudhuri, Saikia, & Pakshirajan, 2022). Many physicochemical techniques have been utilized to date for heavy metal removal from wastewater, including adsorption, ion exchange/electrochemical precipitation, membrane filtration/ultrafiltration, combined oxidation/coagulation, and photocatalysis if the metal is photoactive (Ab Hamid et al., 2022; Aijaz et al., 2022). Disadvantages of these processes might be high operating costs, excessive hazardous sludge production, or high costs.

Copper is among the most common metals found in industrial wastewater, such as acid-mine drainage (Masindi et al., 2022), petrochemical wastewater (Mirbagheri & Hosseini, 2005), and hydraulic fracturing wastewater (Jiménez et al., 2018). Humans have utilized copper since ancient times, and its main source is the copper deposits on Earth's crust. The utilization of copper has been increasing in the past decades. The main sectors include electronic product manufacturing, the manufacturing of vehicles or industrial machinery, the paint and pigment industry, construction, electricity generation and transmission of buildings, and the fertilizer industry (Ab Hamid et al., 2022; Mirbagheri & Hosseini, 2005). Copper levels in wastewater varies depending on the source of wastewater, local industrial activities, and the use of copper-containing consumer products in households. Industrial wastewater such as HFPW contains 0.24-1.46 mg/L copper, whereas the concentration may rise to around 40 mg/L in acid mine tailings (Hu, Yang, Wu, Fang, & Tan, 2022; W. Jiang et al., 2022). Most of the copper entering wastewater partition in the sludge, where it is the second most abundant HM after zinc (J. Feng, Burke, Chen, & Stewart, 2023). Copper in fertilizers and plant protection agents is the main source of copper deposition into the soil (Husak, 2015). The copper level in soil ranges between 2-100 ppm, 70% of which reside in the organic fraction (Guemiza et al., 2017; Mulligan, Yong, & Gibbs, 2001). Copper is toxic to aquatic organisms at even low levels; it is a known biocide and algicide (Allen et al., 2021). Copper in freshwater is highly variable, ranging between 0.02 to 133 mg/L in aquatic environments in the UK and between 0.5 to 4 μg/L median concentrations in the rivers around Europe (Comber et al., 2023; Donnachie, Johnson, Moeckel, Pereira, & Sumpter, 2014). For the prevention of environmental harm and human health hazards,

copper removal from wastewater is necessary.

Microalgae can be utilized for heavy metal removal from wastewater. Their potential for faster growth rate than macroalgae and higher plants, and the variety of biochemical compounds produced by microalgae make them an important alternative for biocatalytic applications. Integrated systems including multiple operations like wastewater treatment by microalgae and valorization of biomass have been getting increased attention (Hussain et al., 2021). Along with generating biomass as a raw material for downstream processes, microalgae can be utilized for metal recovery. A major application of algae for heavy metal removal is adsorption, as the living or non-living biomass is a source of cheap biosorbent (Al-Saydeh, El-Naas, & Zaidi, 2017).

In this chapter, the aim was an efficient BAC and copper removal by a consortium of *T. suecica* and *P. sessilinigenes* BIOMIG1. The fate of BAC was determined in the presence of *T. suecica*. The alleviating effects of *T. suecica* on copper inhibition of BIOMIG1 and of BIOMIG1 on BAC inhibition of *T. suecica* were evaluated.

6.2. Materials & Methods

6.2.1. Bacteria Copper Challenge in Optimized Media

P. sessilinigenes BIOMIG1 was grown in M9-200 BAC medium until all the BAC was finished (verified via HPLC). Afterwards, 5 mL of the culture was added into 200 mL 8‰ F/2 containing 200 ppm BAC. After BAC was fully degraded, biomass was centrifuged, washed with 0.85% isotonic sodium chloride solution (saline), and resuspended in 8‰ F/2 medium at an OD_{600} of 0.1. The seed was 1/100 diluted with 8‰ F/2 medium containing 1/5 diluted MH II for growth challenge. Serial dilutions of 2X concentrations of $CuCl_2$ in 8‰ F/2 medium were prepared as Cu stocks ranging from 32 ppm to 0.25 ppm $CuCl_2$-Cu in the medium. 1/100 diluted seed was mixed with Cu stocks to have around 10^6 CFU/mL bacteria in 8‰ F/2 : 1/10 MH II medium containing 0.125 ppm to 16 ppm Cu. The next day OD_{600} values were measured against 8‰ F/2 : 1/10 MH II medium blanks with the same metal concentrations to determine the metal effect on bacterial growth. For the metal challenge on BAC degradation, seed culture resuspended in 8‰ F/2 was added into an equivolume of 8‰ F/2 containing 20 ppm BAC and 2X $CuCl_2$ ranging from 0.25 ppm to 32 ppm Cu. BAC was measured the next day on HPLC to evaluate the 24-hour effect of Cu on bacterial BAC degradation activity.

6.2.2. BAC Analysis in the Presence of Algae and Copper

The possible impacts of microalgae *T. suecica*, Cu, or both on BAC analysis were determined in 8‰ F/2 medium. Four sets of experiments were conducted: 1) BAC varying Cu set, 2) Cu varying BAC set, 3) Algae varying BAC set, and 4) Algae+Cu varying BAC set. Vessels were prepared with 100 mL media containing 20 ppm BAC and varying $CuCl_2$ concentrations (0.5, 1, 2, 5 ppm Cu) for BAC varying Cu set. For varying BAC sets, 10, 20, 50 or 100 ppm BAC was incubated with 1 ppm $CuCl_2$-Cu (Cu varying BAC), 3 million cells/mL algae (Algae varying BAC), or both (Algae+Cu varying BAC set). BAC was measured via HPLC. For extraction of BAC, $AgNO_3$-ACN-EtAc extraction (described in Section 3.4) was employed. Briefly, 2 mL of homogenous sample was taken and mixed with equivolume of 40:60 100 mM AgNO3:ACN and equivolume of EtAc; the mixture was agitated overnight on a rotator. The next day 0.6 mL from the EtAc phase was collected for HPLC analysis.

6.2.3. Analysis of BAC Biotransformation by Algae

T. suecica was incubated with 10 ppm or 20 ppm BAC in 100 mL 8‰ F/2 medium, at algae cell densities as $3x10^6$ cells/mL or $6x10^6$ cells/mL, respectively. The set containing 10 ppm BAC was spiked on Day 4 and Day 8 with 10 ppm BAC. The set initially containing 20 ppm BAC was spiked with 20 ppm BAC on Day 6. BAC analysis was conducted through $AgNO_3$-ACN-EtAc extraction as described in the previous section. BAC was measured via HPLC.

6.2.4. Analysis of BAC and Copper in the Presence of Bacteria or Consortium of Algae-Bacteria

Two experiments were conducted for BAC and copper treatment by the consortium of *T. suecica* and *P. sessilinigenes* BIOMIG1:

1) A preliminary experiment used vessels containing 5 and 10 ppm $CuCl_2$-Cu along with 20 ppm BAC. Algae densities were set at either 10^6 cells/mL or 10^7 cells/mL. Bacteria density was 10^7 CFU/mL.

2) A second experiment involved concentrations up to 1 ppm $CuCl_2$-Cu and 20 ppm BAC. The biotic factor was either only bacteria or the algae-bacteria consortium. Algae density at the start was around $3x10^6$ cells/mL. Bacteria density was $9x10^8$ CFU/mL. There were control

groups that did not contain any $CuCl_2$ for sets containing only bacteria and consortium. BAC was replenished as depleted. Reactors containing consortium had initial pH values between 7.7 and 7.9.

Algae density was measured via microscopic cell counts for both experiments. Bacteria density was determined via serial dilution and spreading onto LB-200 BAC agar, and reported as CFU/mL. BAC was determined via HPLC. For the determination of adsorbed BAC, the extraction protocol described in previous sections was employed. Total BAC removal (%), removal through adsorption (%), and biotransformation/biodegradation (%) were calculated according to Eq. 6.1, Eq. 6.2, and Eq. 6.3. Metal concentrations as dissolved in matrix, adsorbed onto algae, and assimilated into algae were determined via ICP/MS following the protocol described in Section 3.5.

$$\text{Total BAC removal (\%)} = \frac{\text{Total BAC input} - \text{BAC level on Day 9}}{\text{Total BAC input}} \text{x100} \quad (6.1)$$

$$\text{Adsorption (\%)} = \frac{\text{Extracted BAC on Day 9} - \text{BAC level on Day 9}}{\text{Total BAC input}} \text{x100} \quad (6.2)$$

$$\text{Biotransformation (\%)} = \frac{\text{Total BAC input} - \text{Extracted BAC on Day 9}}{\text{Total BAC input}} \text{x100} \quad (6.3)$$

6.2.5. Analysis of Bacterial BAC Degradation under Increasing Copper

P. sessilinigenes BIOMIG1 was fed with 20 ppm BAC in four flasks containing 100 mL of 8‰ F/2 medium each. It completely consumed all the BAC daily and was spiked again the following day with 20 ppm BAC. This was done for 3 consecutive days. On the 3rd day of the experiment, 2 ppm $CuCl_2$-Cu was added with 20 ppm BAC. After BAC was consumed in the next few days, another 2 ppm $CuCl_2$-Cu was spiked with 20 ppm BAC on Day 7. On Day 9, around $3x10^6$ cells/mL algae was added to two of the flasks to buffer Cu inhibition on bacteria. On Day 14, 4 ppm $CuCl_2$-Cu was spiked with 20 ppm BAC. Initial bacterial density was around 10^8 CFU/mL. A low bacterial density set was also conducted to observe the Cu effect on BAC degradation by bacteria at lower bacterial density. Culture flasks were set up by diluting the cultures from the other set by 1/100 on Day 1, after confirming that all the cultures in the other set removed BAC. The starting bacterial density of this set was 10^5 CFU/mL. The initial BAC concentration was 20 ppm. After 2 complete cycles of BAC degradation, on Day 3, this set was also added 2 ppm $CuCl_2$-Cu along with 20 ppm BAC. On Day 6, $2x10^6$ cells/mL algae were added to ameliorate Cu inhibition on bacteria. On Day 8 and Day 12,

another 2 ppm and 4 ppm $CuCl_2$-Cu, respectively, were spiked with 20 ppm BAC. Afterwards, BAC was replenished as it was depleted in all the sets. BAC was measured via HPLC. Cu was measured via ICP/MS. Samples for Cu analysis were prepared as described in Section 3.5. EDTA chelation for adsorbed metal determination in the procedure was omitted. Cu levels were reported as dissolved, or removed by biomass, in percentages. Experimental design is illustrated in Figure 6.1.

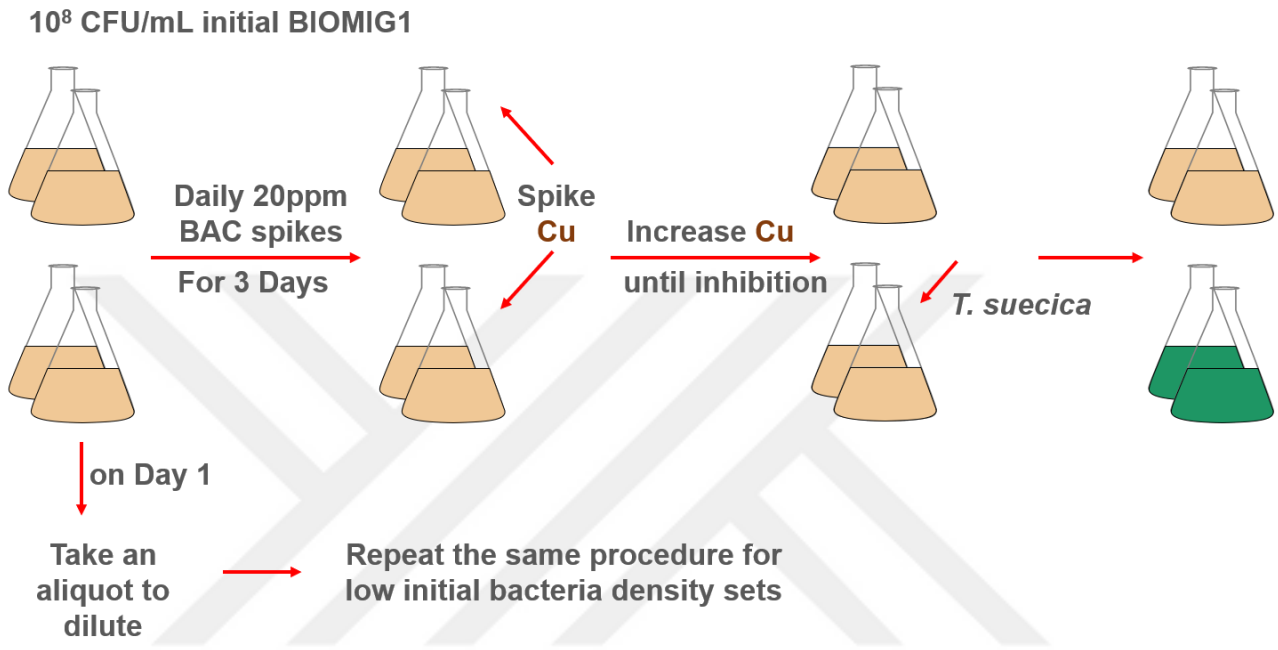


Figure 6.1. Schematic representation of the experimental design for bacterial BAC-degrading activity under increasing copper.

6.2.6. Analysis of Bacteria's Effect on BAC Inhibition to Algae Growth

The effect of the presence of bacteria on algae inhibition by BAC was tested. *T. suecica* was spiked 10 or 20 ppm BAC with or without co-culture with *P. sessilinigenes* BIOMIG1. To visualize the adverse effects of BAC on algae, and how the situation changes when bacteria is in play, the fluorescence of algae was measured along with microscopic cell count and optical density. OD_{680} and fluorescence were measured on a plate reader as described in Section 3.3. Bacteria density was measured via serial dilution and spreading onto LB-200 BAC agar.

6.2.7. Statistical Analysis

T-tests were conducted for comparison of two independent sets of samples (i.e., algae density with or without bacteria present). ANOVA tests with Bonferroni post-hoc analysis were conducted for comparison of the 48-h change in the fluorescence in the presence of various amounts of BAC

between only algae and consortium sets. The statistical tests were conducted using SPSS software V.21 (SPSS Inc., Chicago, USA). Statistical significance was reported with 95%, 99%, or 99.9% confidence intervals with corresponding probability values $p<0.05$, $p<0.01$, or $p<0.001$, respectively.

6.3. Results & Discussion

6.3.1. Copper Challenge of Bacteria in Optimized Algae Media

The development of a consortium of *T. suecica* and *P. sessilinigenes* BIOMIG1 in 8‰ F/2 medium was discussed in the previous chapter. Before testing the treatment efficiency of the consortium by means of BAC and Cu removal from this media, BIOMIG1 was challenged against Cu in the optimized media in terms of growth and BAC degrading activity. Growth was tested in carbon-rich medium, MH II, which was 1/10 diluted in 8‰ F/2. BAC degrading activity was tested in 8‰ F/2 containing 20 ppm BAC as the sole carbon source.

The results of the Cu challenge tests of BIOMIG1 are shown in Figure 6.2. Growth was not affected by Cu up to 16 ppm. 24-hour BAC degradation was completely inhibited when the Cu level was 2 ppm and above. This finding is different from the effect of Cu on BAC degradation in M9, where inhibition started at 8 ppm, and almost complete inhibition was observed at 64 ppm (Discussed in Section 4.3.2). This phenomenon has probably resulted from the difference in media recipes. M9 contains a high level of phosphate, which might have complexed with the copper ions, which in turn lowered the inhibitory activity of Cu.

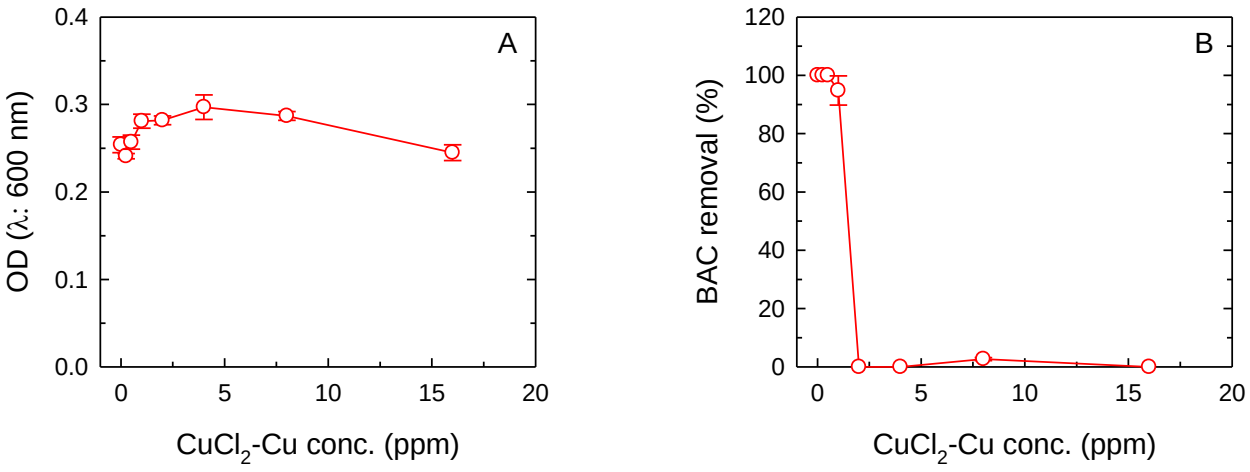


Figure 6.2. The effect of $CuCl_2$ on bacterial growth (A) and BAC degradation activity (B) in the optimized media.

6.3.2. The Fate of BAC in the Presence of Algae and Copper

Most microalgae cells have a negatively charged cell surface (N. Li et al., 2022). Cations might adsorb strongly or weakly to algae biomass. For this reason, the fate of BAC was determined in 8‰ F/2 medium in the presence of algae, Cu, or both. Two sets of experiments were conducted; an abiotic set for changes in BAC concentration at varying Cu levels, and another set where algae biomass, 1 ppm Cu, or both were present at varying BAC levels. In the abiotic environment, BAC concentration in the media stayed the same as the initial value at all tested Cu concentrations when 20 ppm BAC was added initially (Figure 6.3). At 1 ppm Cu without the presence of algae, BAC levels in the media were also steady at all tested BAC concentrations (Figure 6.4).

In the presence of algae, BAC levels decreased to a portion of the initial BAC concentration in the sets fed with 20 ppm, 50 ppm, and 100 ppm BAC (Figure 6.4). In the set fed with 10 ppm BAC, a continuous decrease of BAC was observed. At all tested BAC levels BAC was measured lower in the sets containing algae and Cu than in the ones with only algae. These results indicated a possible BAC adsorption to algae biomass. To verify BAC adsorption to algae, an extraction protocol was conducted with $AgNO_3$, ACN, and EtAc. The sets were almost one month old, to observe the degradation of BAC if occurred.

Extraction efficiency was calculated by dividing the BAC concentration found from EtAc extraction by the results of regular measurement for the Cu+BAC set for the sets with varying initial BAC concentrations. Recovery of BAC measurement in the presence of algae (Algae+BAC, Algae+Cu+BAC sets) was determined by dividing measured BAC concentrations to extraction results in algae-containing sets. Extraction recovery was around 100% for Cu+BAC set at any BAC concentration (Table 6.1). Little decreases in the efficiencies on increasing BAC concentrations were attributed to instrumental error, as HPLC measurements would be more precise in the increased concentrations due to less background noise interference. BAC measurement recovery was around 60% in algae-contining sets whereas 30-35% in the sets containing Cu in addition to algae (Table 6.2). These results indicated that the addition of copper chloride affected BAC adsorption onto algae. The results of extracted and non-extracted BAC measurements are illustrated in Figure 6.5. Almost complete BAC removal by algae was observed in reactors containing 10 ppm BAC. In 20-ppm BAC reactors, there was a substantial amount of BAC removal. This phenomenon was attributed to BAC biotransformation by algae. There were newly formed peaks with a considerably large area in the HPLC chromatogram; those peaks were absent in the chromatograms of Day 0 measurements (data not shown).

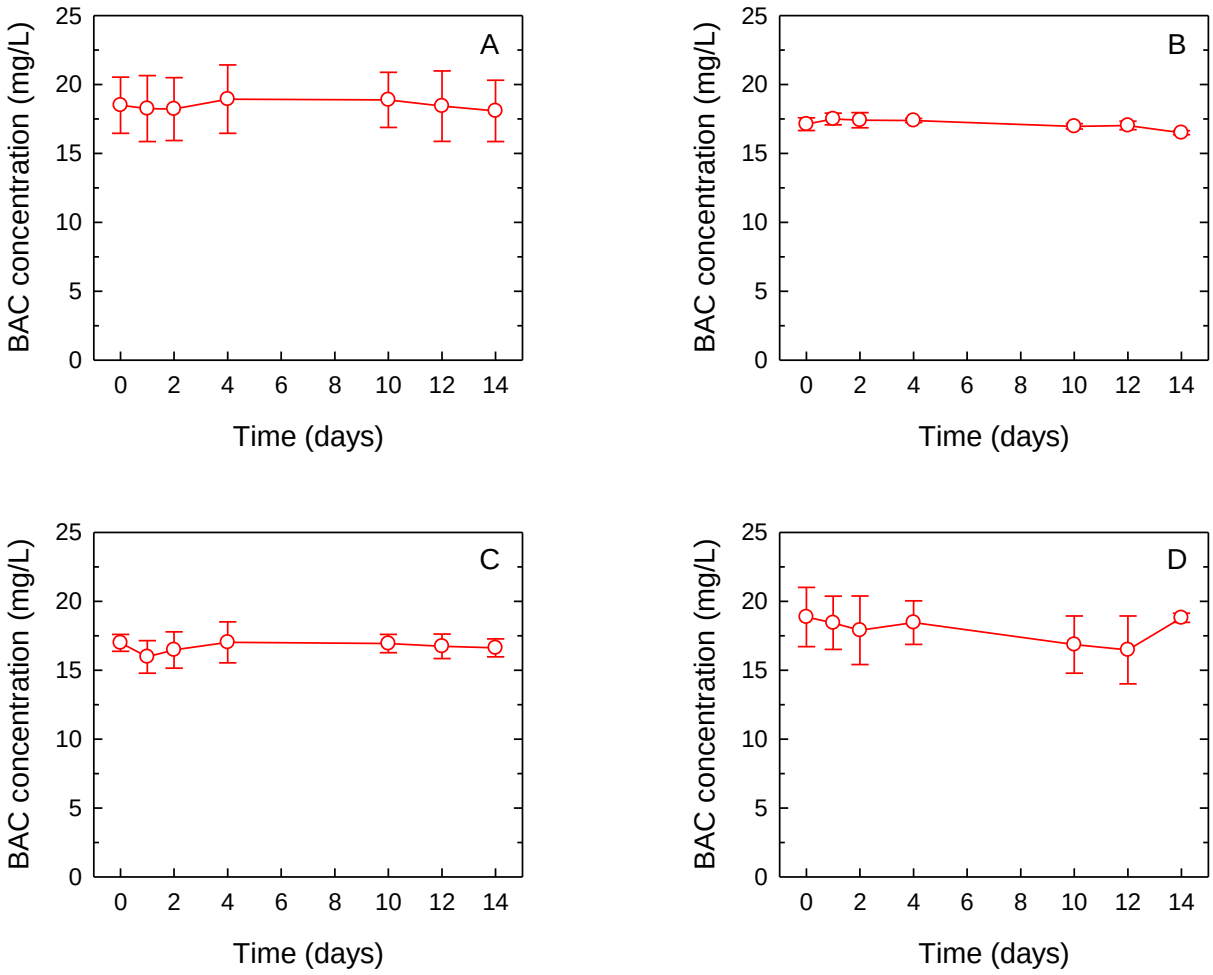


Figure 6.3. The fate of BAC in the abiotic environment in the presence of Cu at the concentrations of 0.5 ppm (A), 1 ppm (B), 2 ppm (C), and 5 ppm (D). The initial BAC concentration was 20 ppm.

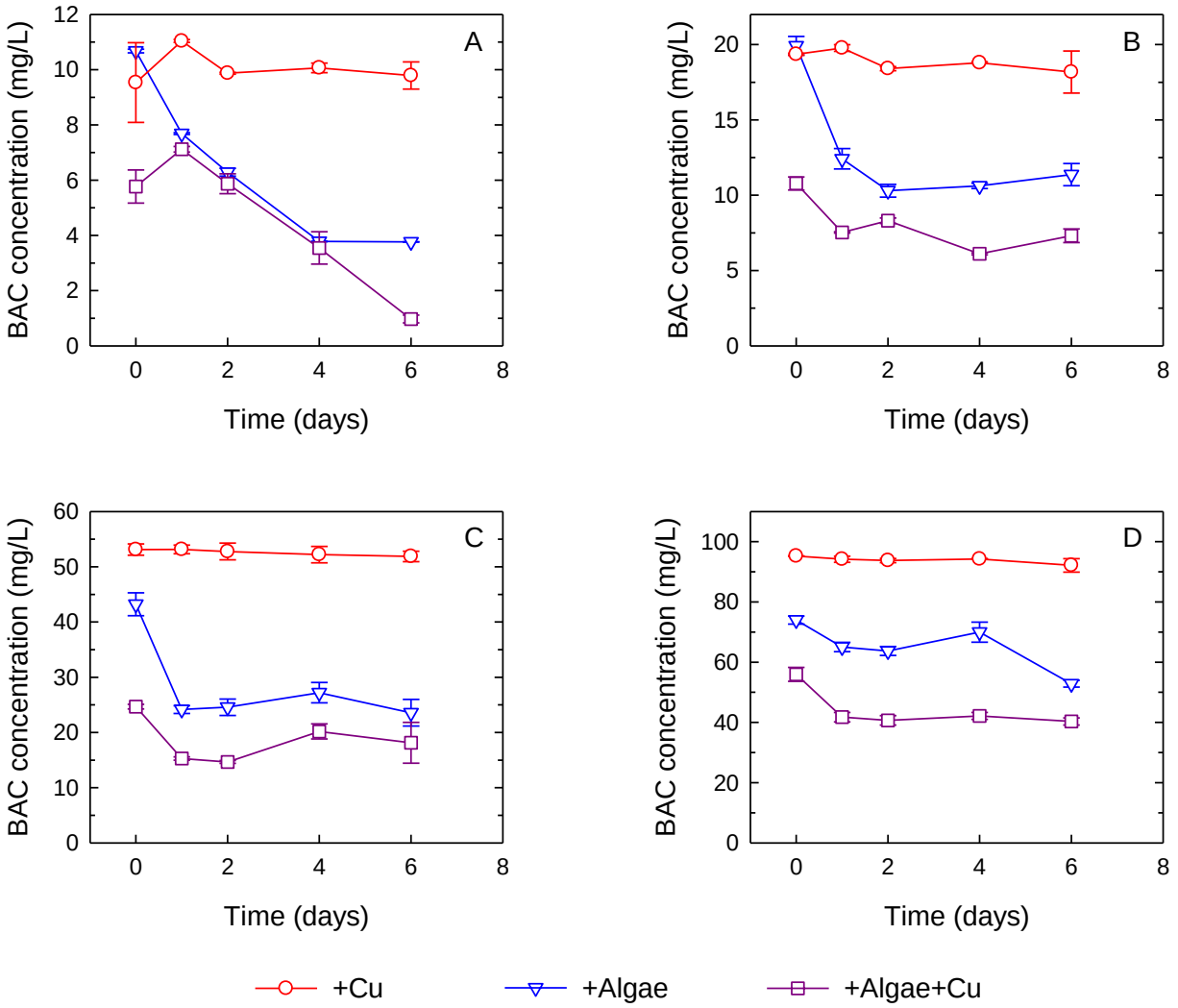


Figure 6.4. The fate of BAC in the presence of algae and Cu in varying BAC concentrations of 10 ppm (A), 20 ppm (B), 50 ppm (C), and 100 ppm (D). The initial Cu concentration was 1 ppm.

Table 6.1. BAC extraction efficiency by EtAc-ACN-$AgNO_3$ method

BAC conc. (ppm)	Mean efficiency	Std dev.
10	107%	6%
20	105%	4%
50	102%	2%
100	101%	2%
Concentration independent efficiency	103%	4%

Table 6.2. BAC measurement recoveries of Algae and Algae+Cu containing sets (10 and 20 ppm BAC levels were not added since there were little or no BAC measured by both regular BAC measurement method and extraction)

BAC conc. (ppm)	Alg	Alg-Cu	Alg SD	Alg-Cu SD
50 ppm	59.5%	31.7%	2.8%	0.3%
100 ppm	58.8%	36.4%	1.8%	0.3%
General recovery	59.2%	34.0%	2.0%	2.7%

SD: standard deviation

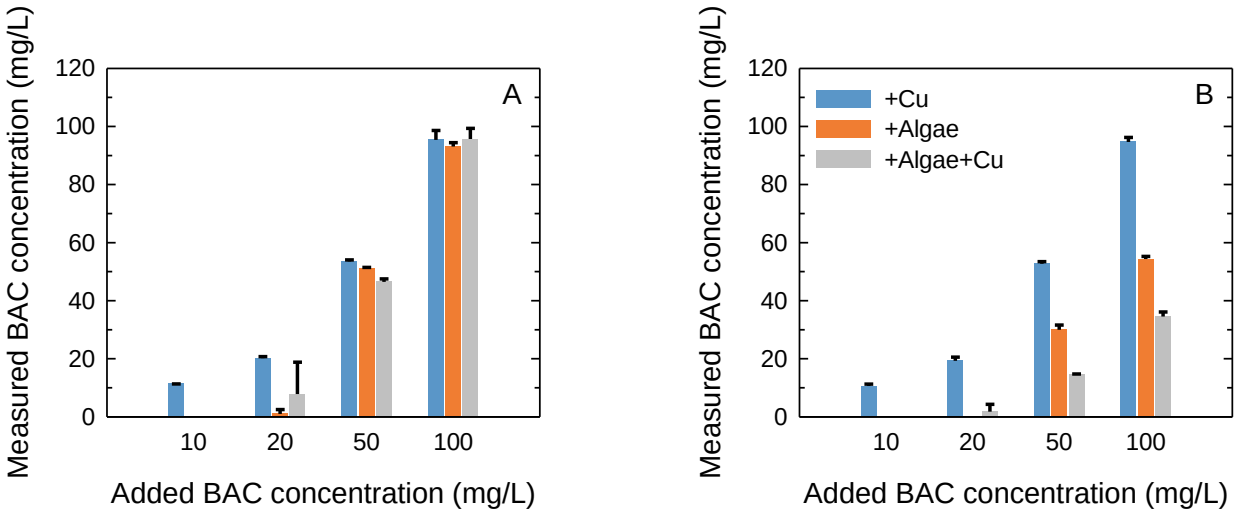


Figure 6.5. BAC concentrations in the sets with (A) or without (B) $AgNO_3$-ACN-EtAc extraction.

6.3.3. BAC Biotransformation by Algae

In the previous section biotransformation of BAC by the marine microalgae, *T. suecica* was detected. The sets fed with 10 ppm and 20 ppm BAC were repeated with algae densities of $3x10^6$ cells/mL, and $6x10^6$ cells/mL, respectively, without the addition of the copper salt. $AgNO_3$-ACN-EtAc extraction was employed to observe BAC removal due only to biotransformation. Biotransformation was observed in both concentrations of BAC (Figure 6.6). BAC was spiked twice in the 10-ppm BAC set, and once in the set containing 20 ppm BAC.

BAC biotransformation starts with hydroxylation and dehydrogenation of the compound in the presence of oxygen (Tezel & Pavlostathis, 2015). There are many studies involving BAC biotransformation by bacteria, but eukaryotic biotransformation was not studied well. A recent study shows a green algae, *T. suecica*, can remove BAC from industrial wastewater via consecutive hydroxylations (Jaén-Gil et al., 2020). This is the only eukaryotic example of BAC biotransformation in the literature, to date. Several studies report *Pseudomonas* type bacteria responsible for BAC biotransformation (Ertekin et al., 2016), though other genera such as *Aeromonas* and *Xanthomonas*

have been reported for aerobic BAC biodegradation (Patrauchan & Oriel, 2003; Tezel & Pavlostathis, 2015). A Rieske-type oxygenase, QxyA (previously reported as OxyBAC) was characterized in the last decade and was responsible for the first step in BAC biotransformation where BAC was turned into BDMA by *P. sessilinigenes* BIOMIG1 (Ertekin et al., 2017). The gene responsible for the expression of this enzyme is transferred via transposable elements. To determine whether *T. suecica* genome contains the *QxyA* gene, PICOTA (Altinbag & Tezel, 2022), a pipeline for de-novo identification of composite transposons, was utilized on publicly available sequence read archive (SRA) of *T. suecica* complete genome (SRR14100057, https://www.ncbi.nlm.nih.gov/sra/SRX10471995). *QxyA* gene was not found in the specified reads. *T. suecica* BAC biotransformation is slower than that of *P. sessilinigenes* BIOMIG1 as the data implies, and should be in a different mechanism.

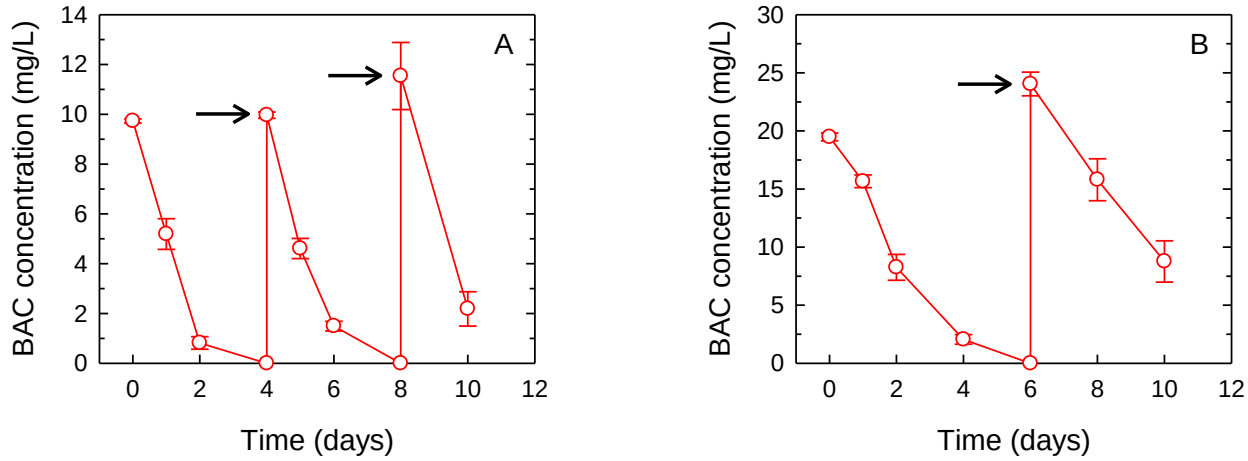


Figure 6.6. BAC biotransformation by algae as revealed by $AgNO_3$-ACN-EtAc extraction. A) 10 ppm initial BAC treated by 3 million cells/mL *T. suecica*, B) 20 ppm initial BAC treated by this algae strain at a cell density of 6 million cells/mL. Arrows represent BAC spikes.

6.3.4. Consortium Treatment Performance at High Copper Level

Preliminary experiments were conducted for BAC and Cu removal from synthetic media by the consortium with initial algae cell densities of 1 million and 10 million cells/mL, and an initial BIOMIG1 density of 10^7 cells/mL. The initial BAC concentration was 20 ppm; two copper concentrations, 5 and 10 ppm were tested. Tests were conducted without replica, just to observe if consortium works in the media containing BAC and Cu.

BAC degradation was not observed in either of the sets. From colony counts, *P. sessilinigenes* BIOMIG1 was also not detected in the reactors. Stagnant growth curves for T. suecica were observed

in the reactors (Figure 6.7). 43-68% and 47-68% of Cu were removed from the media through adsorption and assimilation in the sets initially containing 5 ppm or 10 ppm Cu, respectively (Figure 6.8). The assimilated copper amounts were interestingly not varying between 5 and 10 ppm initial Cu levels when the initial algae concentrations were the same. On Day 8, assimilation of around 0.7 ppm and 2.3 ppm Cu was observed into algae biomass with 10^6 cells/mL and 10^7 cells/mL density, respectively, independent from the initial Cu concentration.

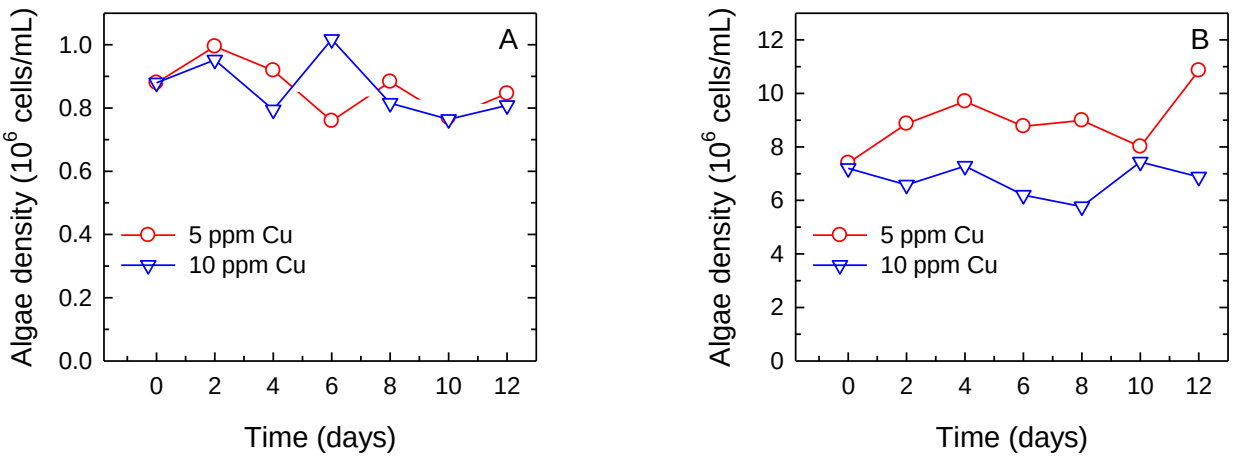


Figure 6.7. Algae growth in the presence of 20 ppm initial BAC and varying Cu. The initial algae densities were 1 million cells/mL (A), and 10 million cells/mL (B).

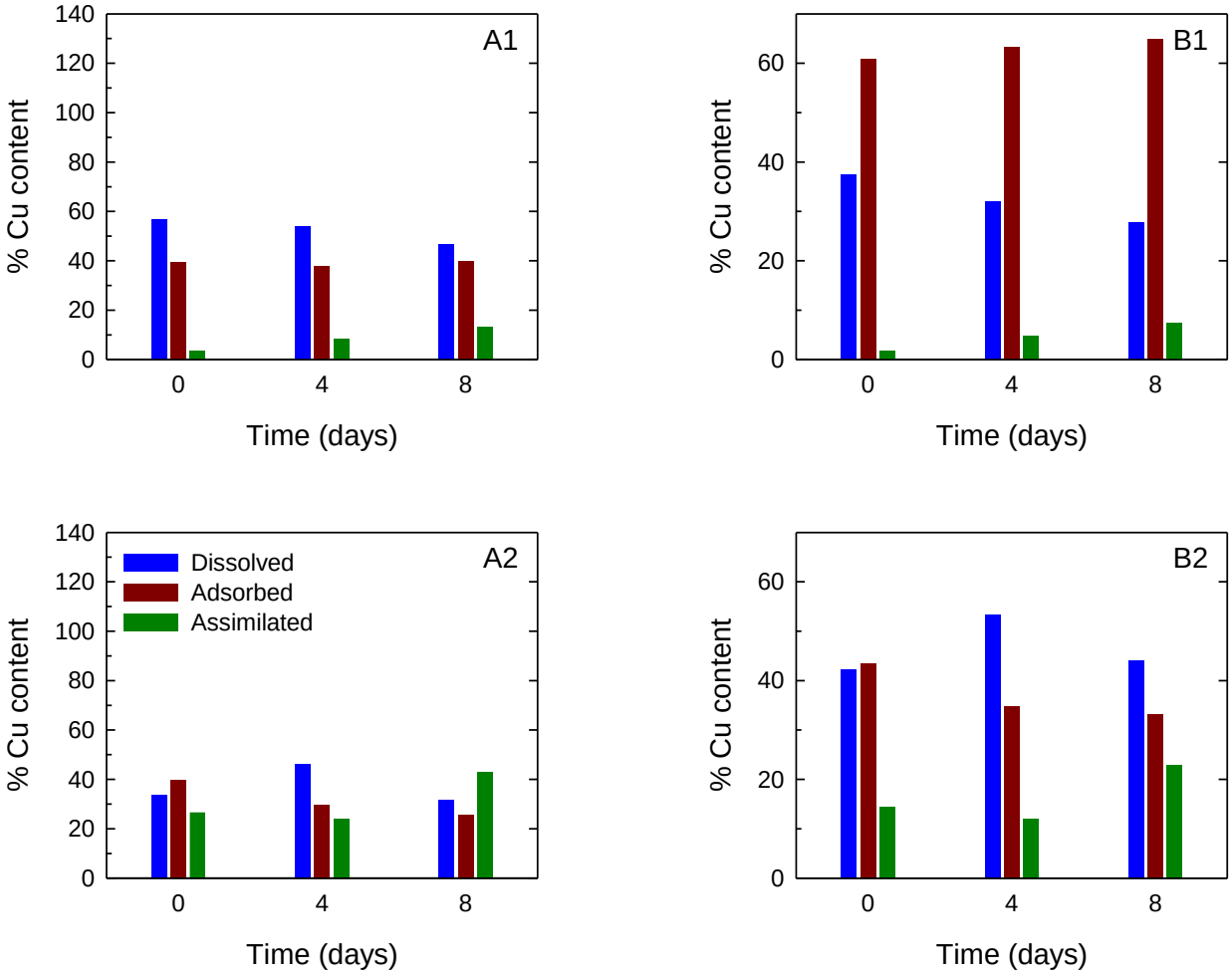


Figure 6.8. Cu removal from synthetic media by the consortium in the presence of 20 ppm initial BAC at 5 ppm initial Cu (A), and 10 ppm initial Cu (B), with 1 million cells/mL (1) or 10 million cells/mL (2) algae density.

6.3.5. BAC Removal Activity at Up to 1 ppm Copper

BAC removal by bacteria was not observed at 5 and 10 ppm Cu. The experiments were repeated with 0.1 ppm, 0.5 ppm, and 1 ppm Cu concentrations. Algae density was $3x10^6$ cells/mL. An intermediate concentration between million and 10 million cells/mL was chosen, because, when algae density was low, BAC toxicity affected algae faster, and when it was higher, adsorption highly interfered with biotransformation. Experiments with sets containing only bacteria and a consortium of algae-bacteria were conducted. BAC and Cu removal from these experiments are going to be discussed in this and the next sections.

BAC treatment performance of the sets containing only bacteria and algae-bacteria consortium were illustrated in Figure 6.9 and Figure 6.10. BAC was replenished every other day. Stable BAC

removal from the media was observed at all tested Cu levels. BAC removal was mostly within 24 hours and a few times within 48 hours of BAC addition. The total added BAC, total removal of BAC, and removal mechanisms are listed in Table 6.3. On Day 9, $AgNO_3$-ACN-EtAc extraction was employed to differentiate between adsorbed and biodegraded BAC. In the sets containing only bacteria, all of the input BAC was eliminated via biodegradation. 80-96% of BAC removal was attributed to biotransformation or biodegradation in the sets containing algae-bacteria consortium. 3-14% of removal in consortium sets was through adsorption onto algae biomass. Total BAC removal by consortium through adsorption and biotransformation varied between 92% and 99%. Increasing Cu concentration seemed to be irrelevant to BAC removal by consortium up to 1 ppm Cu.

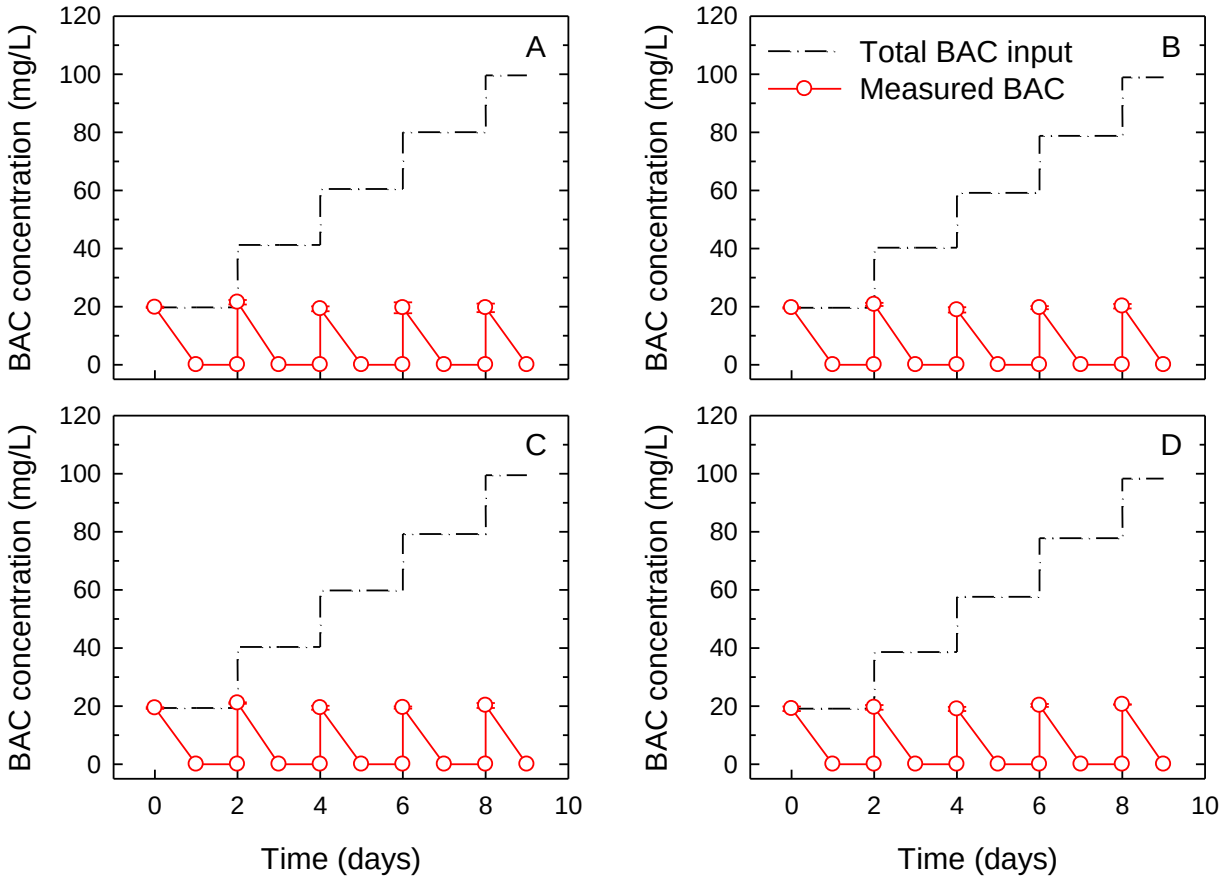


Figure 6.9. BAC removal from media by sole bacteria under 0 ppm (A), 0.1 ppm (B), 0.5 ppm (C), and 1 ppm Cu (D). Experiments were started with 20 ppm BAC and reactors were spiked with 20 ppm BAC every other day. The dotted line represents the cumulative BAC concentrations if there was no removal activity.

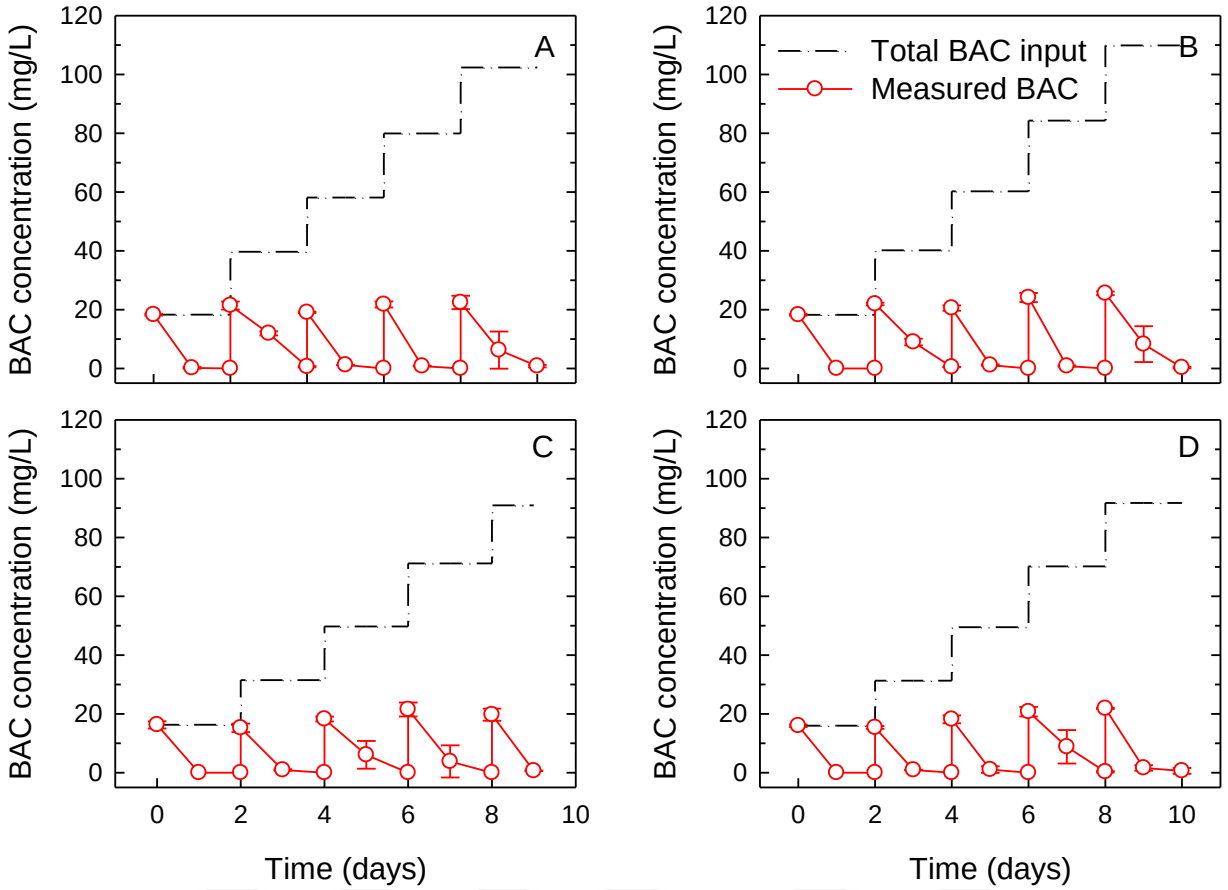


Figure 6.10. BAC removal from media by algae-bacteria consortium at 0 ppm (A), 0.1 ppm (B), 0.5 ppm (C), and 1 ppm Cu (D). Experiments were started with 20 ppm BAC and reactors were spiked with 20 ppm BAC every other day as soon as BACs were consumed. The dotted line represents the cumulative BAC concentrations if there was no removal activity.

Table 6.3. Removal of BAC by sole bacteria or algae-bacteria consortium from start to Day 9.

	No Cu	0.1 ppm Cu	0.5 ppm Cu	1 ppm Cu
Only bacteria				
Total BAC input (ppm)	99.6±3.6	98.9±0.8	99.4±1.3	98.3±3.0
Total BAC removal (%)	100%	100%	100%	100%
Removal through biotransformation/biodegradation (%)	100%	100%	100%	100%
Removal through adsorption (%)	0%	0%	0%	0%
Consortium				
Total BAC input (ppm)	102.3±2.1	109.8±2.1	90.9±3.3	91.7±3.3
Total BAC removal (%)	93.8±6.3	92.5±5.5	99.3±0.1	98.3±1.0
Removal through biotransformation/biodegradation (%)	79.6±4.3	86.8±8.9	96.1±0.5	94.3±0.3
Removal through adsorption (%)	14.3±10.6	5.7±3.4	3.2±0.4	4.0±0.8

6.3.6. Copper Removal by Algae Under BAC Stress

Removal of copper from the sets containing 1 ppm Cu was analyzed. Sets contained only algae, the consortium of algae-bacteria, or only bacteria in 8‰ F/2 containing 20 ppm BAC and 1 ppm initial $CuCl_2$-Cu. In only bacteria and consortium sets, BAC was replenished every other day as discussed in the previous section. There were no additional BAC spikes in the set containing only algae. Changes in algae density and Cu levels in different phases as dissolved in the media, adsorbed onto biomass, and assimilated into algae cells were depicted in Figure 6.11, in the absence or presence of bacteria. Algae density slightly increased in the presence of bacteria (Figure 6.11.B1). Algae density was significantly higher in the presence of bacteria on Day 6 ($p<0.05$) and Day 9 ($p<0.01$).

Total Cu removal in the presence of only algae was highest on Day 1 and on Day 4, being 43.7±1.5%, and 43.8±0.4%, respectively. Total removals on these two days were significantly higher than the removals on other days ($p<0.001$). Assimilation rates were also significantly higher on Day 1 and Day 4, compared to other days ($p<0.001$), where Day 1 was highest (Day 1 > Day 4, $p<0.001$) in the absence of bacteria. This decrease was attributed to the inhibition of algae growth and Cu assimilation capacity through time due to the BAC effect. Total Cu removal was highest on Day 4 in the presence of bacteria with algae (57.4±3.5%) and it was significantly higher than that on Day 1 ($p<0.001$) and Day 9 ($p<0.001$). The consortium outperformed the sole-algae set in terms of total removal on each measurement day ($p<0.01$), except for Day 1, where no significant difference was observed. Assimilation of Cu when the bacteria were also present was higher in all the measurements ($p<0.01$).

Total Cu removal by sole bacteria in the presence of 20 ppm BAC was also determined (Figure 6.12). 1 ppm Cu and 20 ppm BAC were added to this set at the start, and BAC was replenished every other day. Total removal of Cu by sole bacteria was 11.6±0.3% on Day 4, and 9.1±0.5% on Day 9. When the difference in total removals between the set containing only algae and the set containing consortium were compared, it was 13.6±3.5% on Day 4, and 9.0±3.1% on Day 9, with the consortium set showing a higher percentage. However, these values were not significantly greater than the removal achieved by sole bacteria on the same days. It cannot be conclusively stated that the efficiency of the algae-bacteria consortium in copper removal was higher than the combined removals achieved by algae and bacteria individually. Nevertheless, in the presence of BAC, algae in the consortium removed copper to a level comparable to sole algae and sole bacteria, and the consortium also achieved the removal of over 90 ppm of BAC.

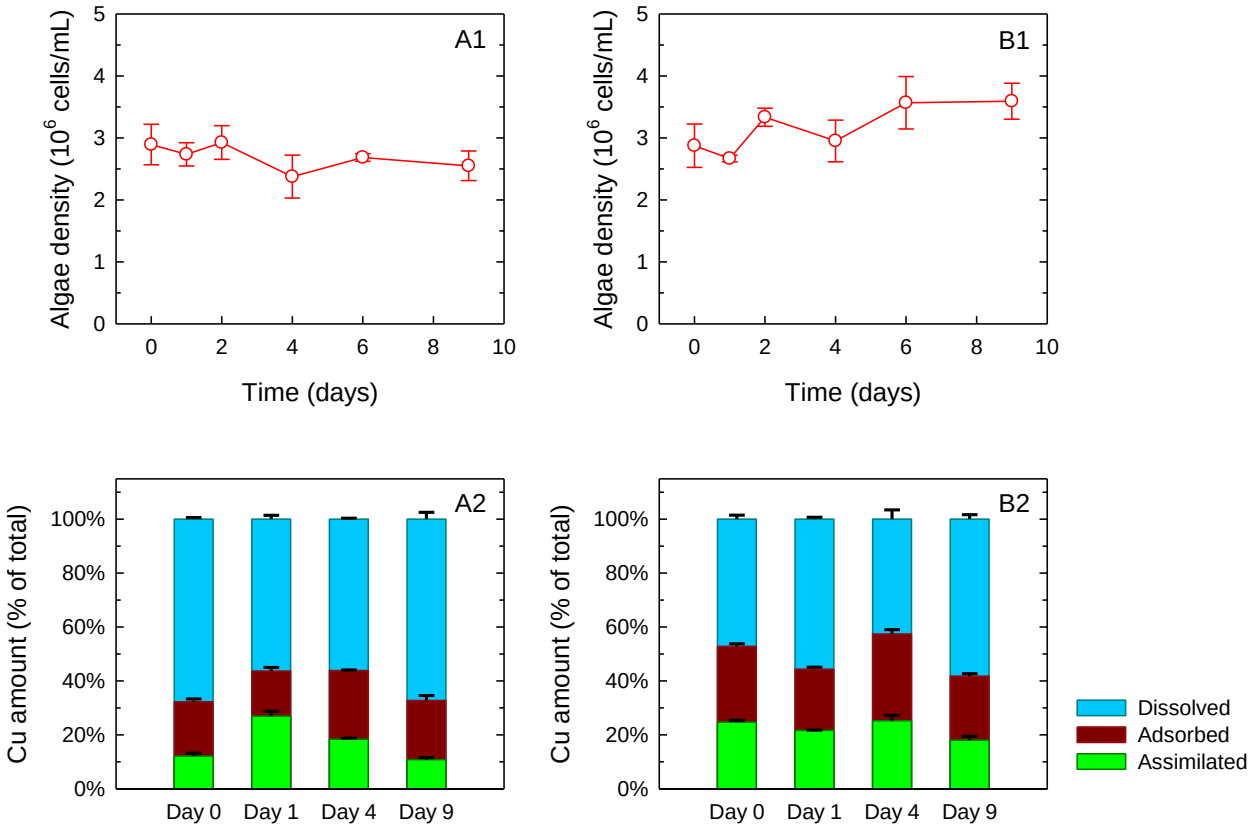


Figure 6.11. Cu removal from media when only algae (A) or algae-bacteria consortium (B) were present in the media fed with 1 ppm Cu and 20 ppm BAC. Algae cell count (1) and partitioning of Cu within the media, whether it was dissolved, adsorbed to algae, or assimilated by algae (2). The set inoculated with only algae was fed once with BAC on Day 0, whereas the set inoculated with consortium was spiked with 20 ppm BAC every other day.

In these experiments, the removal of copper and BAC by algae and bacteria in a consortium was evaluated using a mechanistic approach to determine removal mechanisms. The scenario would change when experimenting with actual wastewater, as wastewater contains various other organic and inorganic contaminants that can affect the consortium's performance. In the literature, EC removal was mostly studied in a mechanistic manner (R. Liu et al., 2021). While wastewater treatment by algae or consortia has been extensively studied for the removal of nutrients and COD, the treatment of industrial wastewater is still emerging in the literature with relatively few comprehensive studies (Ozcelik et al., 2022; Sakarya, Ertekin, & Haznedaroglu, 2023). Safonova et al. constructed a consortium immobilized onto various solid carriers for the removal of phenols (85%), anionic surfactants (73%), oil spills (96%), along with metals such as copper, iron, manganese, nickel, zinc, as well as the efficient removal of BOD (97%) from industrial zone wastewater (Safonova et al., 2004). Maza-Márquez et al. operated a full-scale PBR for the treatment of oil washing wastewater, where up to around 4000 L of wastewater could be treated daily, achieving effective removals of

BOD, COD, phenols, color, and turbidity at a 3-days HRT (Maza-Márquez, González-Martínez, Rodelas, & González-López, 2017).

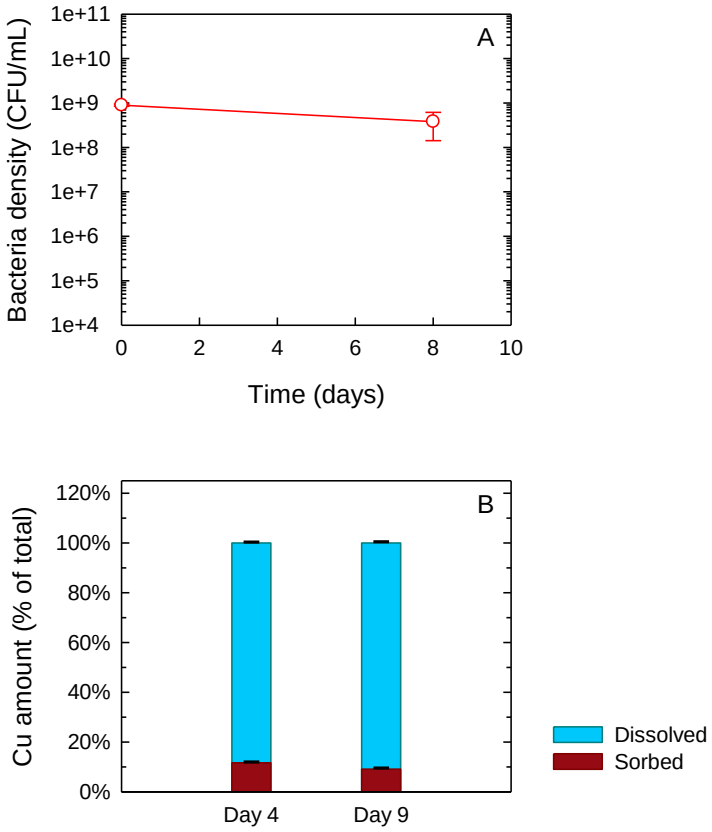


Figure 6.12. Cu removal from media by the sole bacteria. Bacteria density as CFU/mL (A), and Cu partitioning as dissolved in the media, or on bacterial biomass (B). Initial Cu and BAC amounts were 1 ppm and 20 ppm, respectively, with BAC spiked at 20 ppm every other day.

6.3.7. Effect of the Algae on the Inhibitory Effect of Copper on Bacterial BAC Degradation

In the previous section, the treatment of a synthetic media containing BAC and Cu by the algae-bacteria consortium was tested and up to 1 ppm Cu, bacterial BAC degradation activity was not affected by copper. This copper concentration was utilized in the experiments, as it is the level found in HFWP (W. Jiang et al., 2022). There was no positive effect of the presence of algae on BAC degradation at up to 1 ppm Cu levels. To evaluate whether algae protect bacteria from Cu stress, another set of experiments was designed. *P. sessilinigenes* BIOMIG1 were incubated at a density of 10^8 CFU/mL in two separate sets of duplicate vessels with 20 ppm BAC, replenished as depleted. On the third day, 2 ppm Cu was added, and the copper concentration was increased until BAC degradation was inhibited. When the Cu level was 4 ppm and BAC degradation activity was interrupted by Cu toxicity, 3 million cells/mL *T. suecica* was added to one of the sets. BAC removals

through adsorption and biotransformation were monitored to see the amelioration effect of the algae. One of the resilience parameters of microorganisms to a toxic compound is the density of microorganisms. To evaluate the effect of the culture density of BIOMIG1 on copper inhibition on the BAC degradation activity, another low-density culture was prepared by taking 1 mL seed from the original sets on Day 1 into 100 mL culture media containing 20 ppm BAC. The same protocol was followed for the new sets: for the initial 3 days they were fed with 20 ppm BAC daily, and then an increasing amount of Cu was added to each set. At the point where BAC degradation activity was interrupted by Cu toxicity at 2 ppm Cu, algae were added to one of the sets.

Input Cu concentration, bacteria density, BAC concentration in the media, algae density in the sets where algae were introduced, and adsorbed BAC concentrations were shown in Figure 6.13 and Figure 6.14, for 10^8 CFU/mL and 10^5 CFU/mL initial bacteria density, respectively. Total Cu levels were raised to 8 ppm Cu in all sets. For both high and low initial bacterial density, there was a similar pattern; in the sets where algae were not added, the bacteria could not survive upon high Cu concentrations, and BAC biotransformation stopped. In the sets where algae were added, bacteria growth was restored and stable BAC degradation was observed. Adsorbed BAC concentrations measured after algae addition were stable, around 6 ppm BAC for the sets started with high bacterial density, and below 5 ppm BAC for those started with low bacterial density. The major removal mechanism for BAC was through biotransformation.

Copper concentrations were determined both on biomass and in the dissolved phase throughout the study (Figure 6.15). In the high-initial density bacteria cultures, the concentration of dissolved Cu ranged from 76% to 87% during the period between Day 9 and Day 18, where no algae were introduced. In the set where algae were added within the same time interval, dissolved Cu spans from 38% to 66% of total Cu input. A decreasing trend was observed in dissolved Cu fraction in the set where algae was introduced by Day 9. The dissolved Cu in the low-initial density bacteria cultures ranged from 84% to 98% during the period between Day 8 and Day 17 when no algae were introduced, it ranged from 42% to 63% when algae were introduced. The presence of algae contributed to the partial removal of Cu, and this in turn increased the survivability of the bacteria and remediated the BAC biodegradation activity.

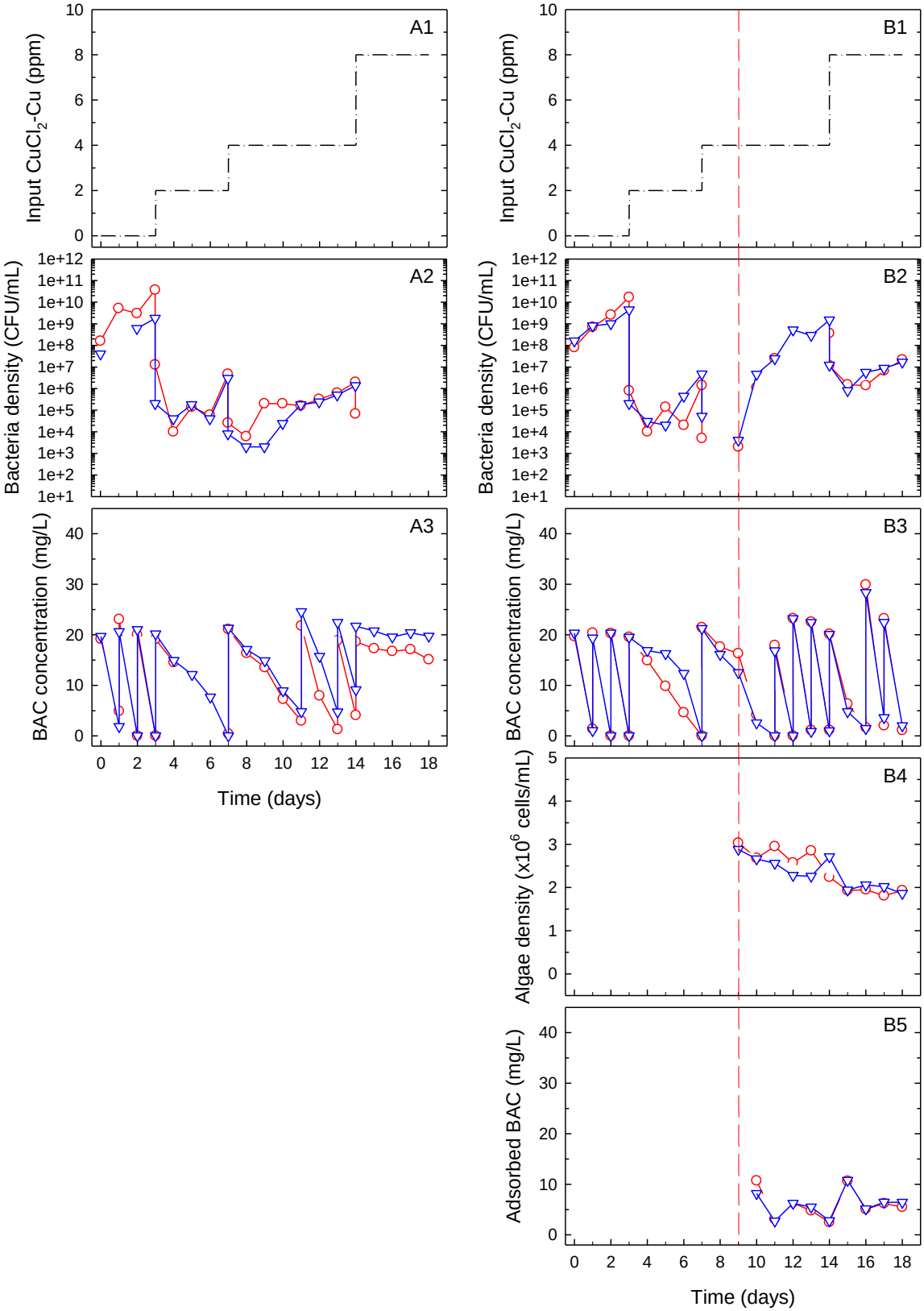


Figure 6.13. Bacterial response to increasing Cu without or with algae present at 10^8 CFU/mL initial bacterial density. Input copper level (1), bacterial density (2), and BAC concentration (3) in the set in the absence (A) or presence (B) of algae. Algae density (B4), and BAC adsorbed to algae biomass (B5). Individual curves in Graphs A2, A3 and B2 to B5 represent the replicates. The dashed red line indicates the day of algae addition.

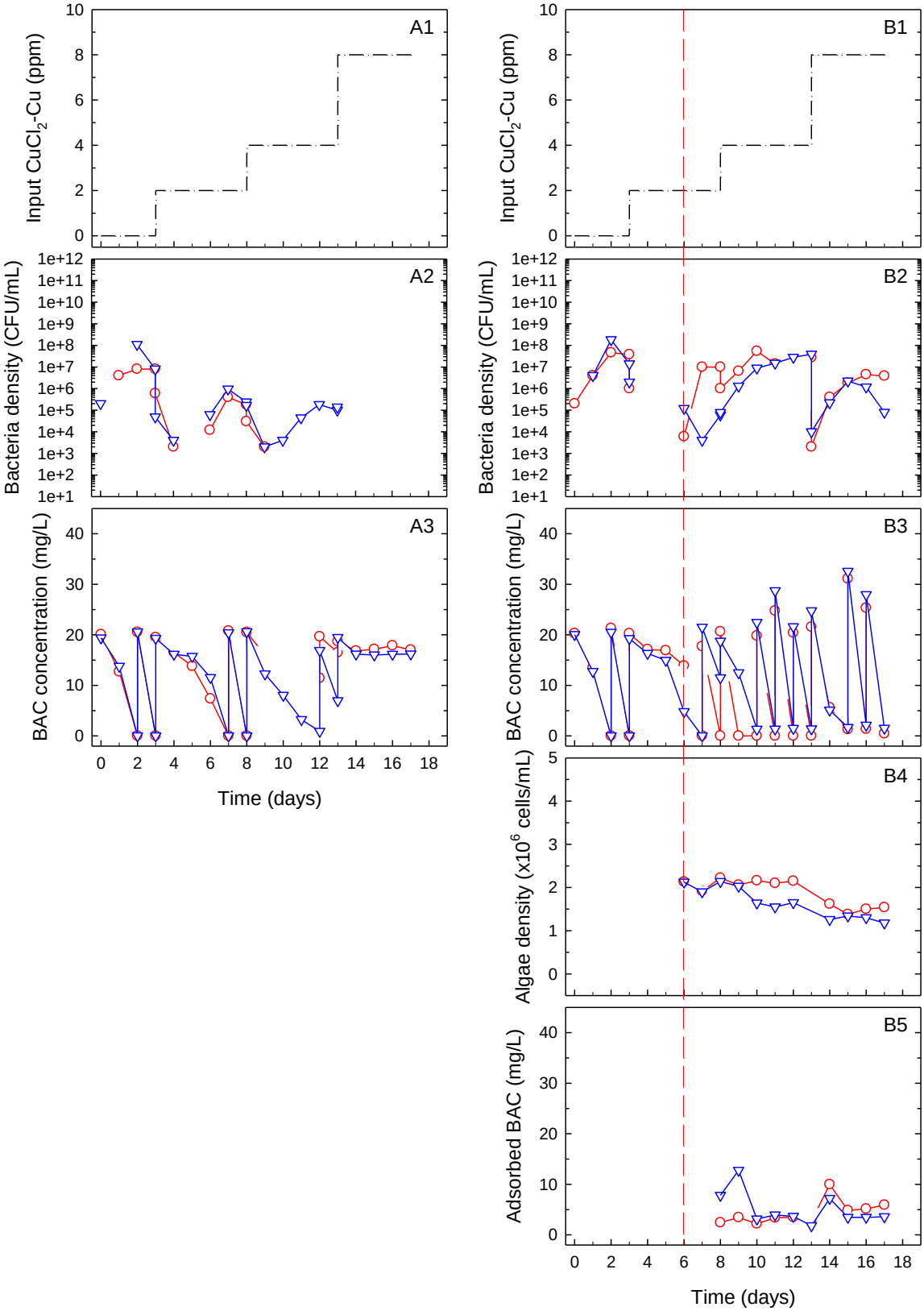


Figure 6.14. Bacterial response to increasing Cu without or with algae present at 10^5 CFU/mL initial bacterial density. Input copper level (1), bacterial density (2), and BAC concentration (3) in the set in the absence (A) or presence (B) of algae. Algae density (B4), and BAC adsorbed to algae biomass (B5). Individual curves in Graphs A2, A3 and B2 to B5 represent the replicates. The dashed red line indicates the day of algae addition.

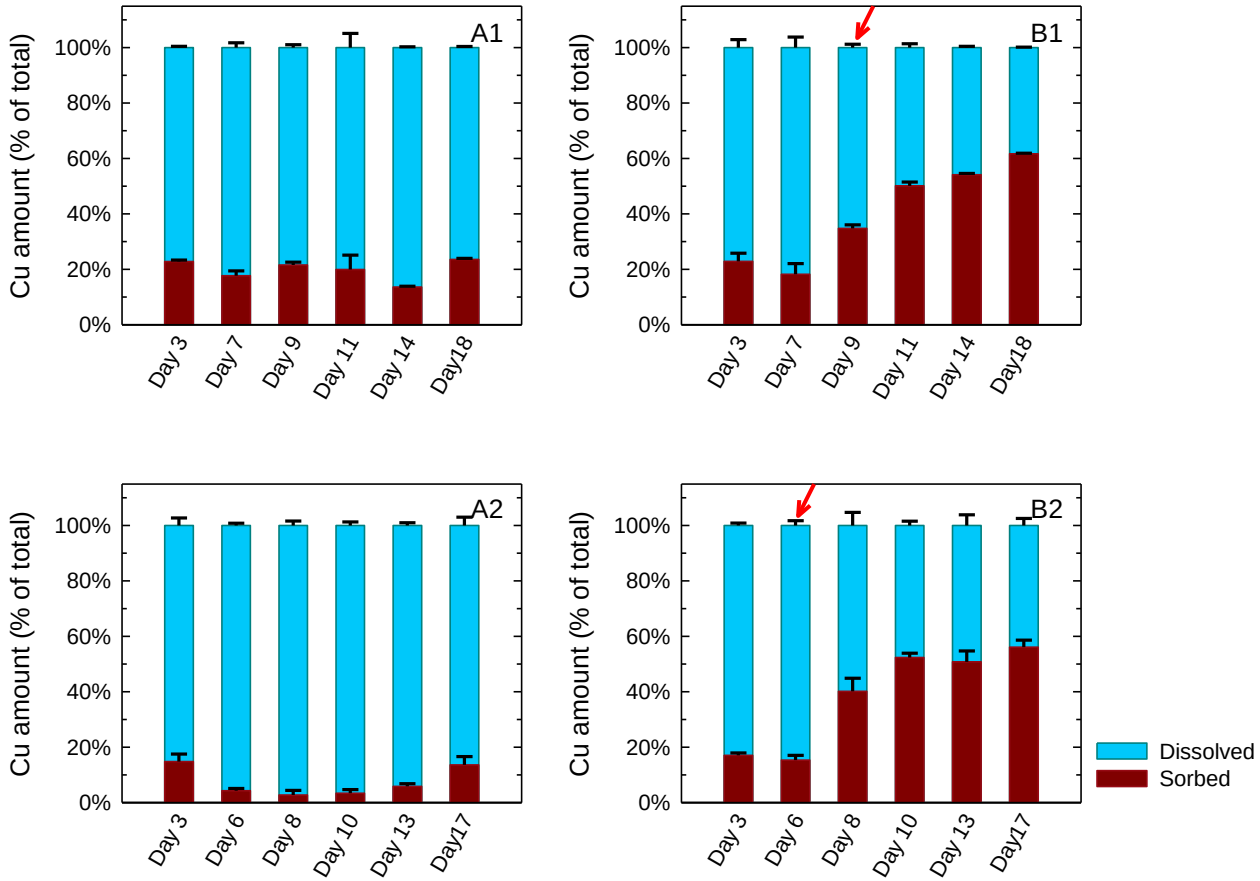


Figure 6.15. Copper fate in the reactors in the presence of only bacteria (A) or bacteria with algae introduced at some point (B). The sets were conducted with a bacterial density of 10^8 CFU/mL (1) or 10^5 CFU/mL. Red arrows indicate the time of algae introduction.

6.3.8. Effect of Bacteria on Inhibitory Impact of BAC on Algae Growth

In Section 6.3.6, the presence of *P. sessilinigenes* BIOMIG1 was observed to increase algae growth resilience against the stress of BAC (See Figure 6.11.A.1 and 6.11.B.1, and related text). In this section, the effect of the presence of bacteria on algae growth and photosynthetic parameters are going to be discussed.

An experimental setup was designed to evaluate the effect of the presence of bacteria on algae growth and fluorescence in a media containing varying concentrations of BAC. Sets containing no BAC, 10 ppm, or 20 ppm BAC were incubated with only algae or with the algae-bacteria consortium. Algae density was determined via cell counts or OD_{680}. Algae fluorescence was also measured throughout the study to evaluate the health of algae biomass. The experiments were conducted for 48 hours because BAC in the media would be consumed afterward. The effect of the same amount of initial BAC present in the media on the algae was observed, whether the bacteria were present or not.

Algae density increased in terms of both cell counts and OD_{680} value when no BAC was introduced, independent of the presence of bacteria (Figure G.1.A1 and G.1.B1, see Appendix G). Algae fluorescence also increased (Figure G.1.A2 and G.1.B2). Because there was no organic carbon source introduced, bacterial density decreased (Figure G.1.B3). At 10 ppm BAC, algae cell count was stationary, but OD_{680} decreased through time, more sharply in the set where no bacteria were present (Figure G.2.A2 and G.2.B2). The fluorescence of the algae, similar to OD_{680}, decreased in both bacteria-present and absent sets (Figure G.2.A3 and G.2.B3). At 20 ppm BAC in the absence of bacteria, a dramatic decrease of approximately 15-fold was observed in algae fluorescence intensity (Figure G.3.A3). The change in algae fluorescence from start to 48 hours under various BAC concentrations were compared statistically between the sets containing only algae and the consortium of algae-bacteria. The presence of BAC caused a sharp decrease in algae fluorescence compared to the change in fluorescence in no BAC sets ($p<10^{-4}$). When the biotic factor was compared, the 48-hour change in the fluorescence did not vary between the only algae and consortium sets in the absence of BAC or in the presence of 10 ppm BAC. The decrease in fluorescence intensities after 48 hours were significantly higher in only algae set than consortium set at 20 ppm BAC ($p<0.01$). The 48-hour change in algae fluorescence intensities upon BAC exposure were illustrated in Figure 6.16.

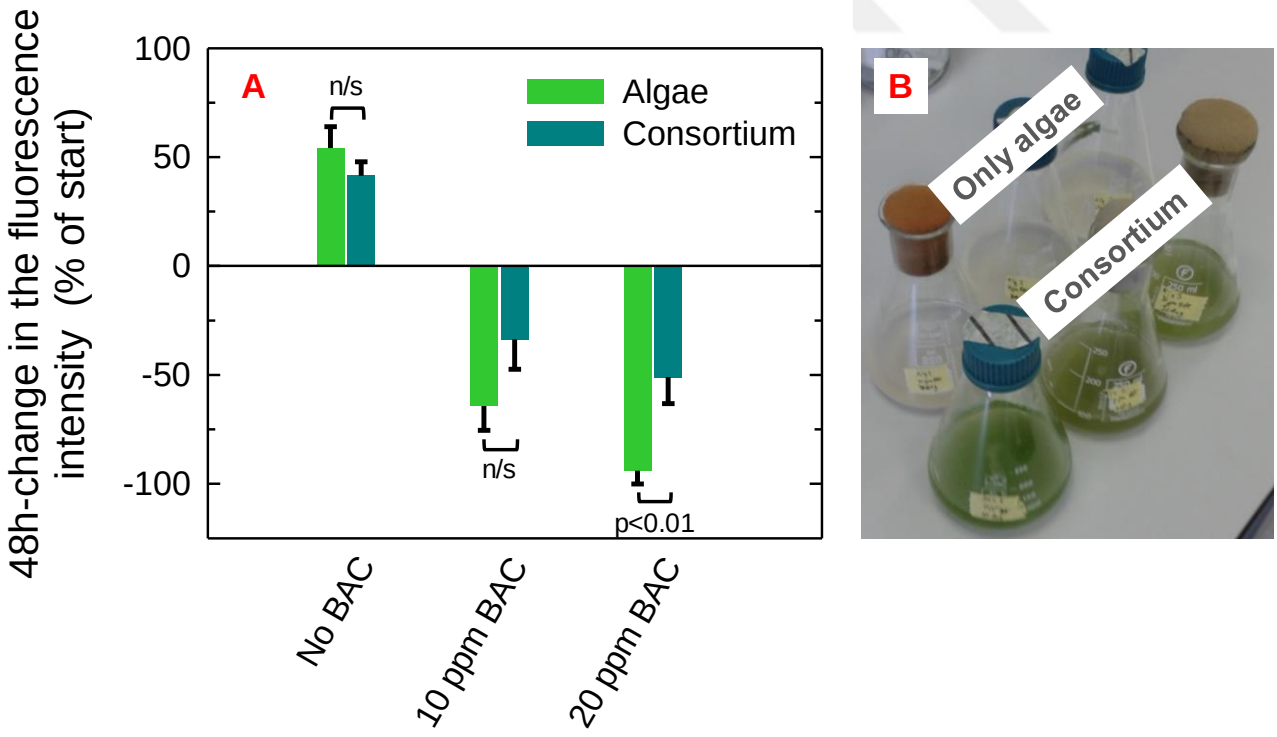


Figure 6.16. The change in fluorescence intensity of *T. suecica* after 48-hour BAC exposure, either with algae alone or in consortium with BIOMIG1 (A) and the photos of the vessels containing 20 ppm BAC in the end of 48 hours (B).

Consensus in algae cell counts with differences in the fluorescence indicates a change in pigment concentration in algae. With the results documented in this section, it was determined that the presence of BIOMIG1 in consortium with *T. suecica* under BAC stress did not yield a recovery in algal cell count, however, resulted in the preservation of algae fluorescence. Fluorescence intensity was measured with excitation at 488 nm and emission at 680 nm. Fluorescence at this excitation and emission wavelengths are directly proportional to chlorophyll a level (Gregor, Jančula, & Maršálek, 2008). Hence, the fluorescence intensity at these specific wavelengths is positively correlated with the photosynthetic activity of the algae. This phenomenon is of great significance since the photosynthetic activity of algae directly influences the amount of oxygen they provide to a mixed-culture wastewater treatment system. The supply of O_2 represents a major cost factor and a source of CO_2 emissions in wastewater treatment plants. Reducing the O_2 demand of a treatment plant is a favorable option for improving sustainability metrics.

6.4. Summary

In this chapter, the consortium performance for BAC and Cu removal was evaluated in optimized media (8‰ F/2), and the favor of the consortium participants, the algae *T. suecica* and the bacterium *P. sessilinigenes* BIOMIG1, to each other against BAC and Cu toxicity was assessed. Firstly, the effect of Cu on bacteria growth and BAC degradation in 8‰ F/2 was determined. Over 2 ppm Cu was completely inhibitory to 24-hour BAC degradation. The assessment of BAC fate in the presence of the algae revealed that adsorption to algae biomass served as a removal mechanism for BAC. Additionally, under suitable conditions, *T. suecica* demonstrated the ability to remove BAC through biotransformation.

The consortium was not effective for BAC and Cu removal at high Cu levels (5 and 10 ppm). At these Cu levels, the amount of copper assimilated by algae was independent of the Cu level and correlated to the amount of algae biomass. Stable BAC removal by sole bacteria and by consortium was observed at up to 1 ppm Cu. At 1 ppm Cu concentration, the consortium removed more Cu than the sole algae; however, consortium performance was not significantly higher than the sum of copper removals by the sole algae and the sole bacteria. Finally, it was demonstrated that in the consortium, the algae *T. suecica* aided the bacterium *P. sessilinigenes* BIOMIG1 in mitigating Cu stress, while the bacterial partner assisted the microalgae in alleviating BAC stress.

7. EFFECTS OF COPPER ON THE METABOLISM OF *T. suecica*

7.1. Introduction

Copper is an essential micronutrient for green algae and plants. Copper ions play a crucial role in aerobic respiration by aiding the transfer of electrons to molecular oxygen. They also function as cofactors in superoxide dismutases and are an essential component of plastocyanin, an electron carrier utilized in oxygenic photosynthesis (Bertoni, 2011). Excess copper can be toxic to algae. An inhibitory effect of copper was found on *Tetraselmis chuii* growth, although this effect was less pronounced at copper levels below 1 mg/L (Cordero, Guevara, Morales, & Lodeiros, 2005). Copper may also inhibit the activity in photosystem II redox reactions of algae (Juneau, El Berdey, & Popovic, 2002). The effect of copper on photosynthesis might also be through the replacement of Mg^{2+} in chlorophyll or via a decrease in photosynthetic pigment content (Xiao et al., 2023). Copper leads to oxidative stress in algae through the production of ROS, which might result in an increase in oxidative stress-related enzymes upon exposure to copper at sublethal doses (Hamed et al., 2017). The formation of hydroxyl radical, the most dangerous ROS, can be directly catalyzed by Cu (I) ions (Nowicka, 2022). Microalgae utilize antioxidants and chelators like GSH, ascorbate, or PCs to tolerate copper-induced oxidative stress (Danouche, El Ghatchouli, & Arroussi, 2022). Other copper stress-related mechanisms include the production of EPS in cyanobacteria (Gu et al., 2020), modification of cell ultrastructure (G.-J. Zhou, Peng, Zhang, & Ying, 2012), or alterations to metal transporters (Tripathi & Poluri, 2021).

To the best of my knowledge, there are very few transcriptomic studies of *Tetraselmis* in the literature. Two of such studies are related to nitrogen starvation (Lauritano et al., 2019; Lim et al., 2017), and one of them is related to the effect of temperature variations on metabolism (Shin et al., 2016). Two studies are about starch hyperaccumulation (Shi et al., 2023), and biohydrogen generation (Cao et al., 2014). There is only one study about copper's effect on the metabolism of *T. suecica* at the transcriptome level with pyrosequencing (Sathasivam, Guo, Wang, Lim, & Ki, 2018). Although with limited data, the authors showed increased expression of heat shock proteins, namely, HSP70 and HSP90. The copper effect on green microalgae has been studied by other authors in the last decade. Olsson et al. found that excess copper upregulated photosynthesis, carbohydrate metabolism, and stress response-related transcripts in *Chlamydomonas acidophila* (Olsson, Puente-Sánchez, Gómez, & Aguilera, 2015). Beauvais-Flück et al. showed that the response of *C. reinhardtii* to copper stress is different from the aquatic plant *Elodea nuttallii* (Beauvais-Flück, Slaveykova, & Cosio,

2019). In another study, copper stress increased the expression of transcripts related to photosynthesis, carbon assimilation and carbohydrate metabolism (Ling et al., 2021). The copper effect on the transcriptome of cyanobacteria is a different story. Copper was found to attenuate the expression of genes related to photosynthesis in *Microcystis aeruginosa* (T. Wang, Hu, Zhu, & Yin, 2020).

In this chapter, the changes in *T. suecica* in transcriptomic level when exposed to copper stress were analyzed, while it was also removing copper from the media through adsorption and assimilation. *T. suecica* is a microalga belonging to the class *Chlorodendrophyceae*. It has a good removal capacity for heavy metals (Pérez-Rama et al., 2002), and it can be considered a microalgae with heavy metal tolerance (see Chapter 4). With a specific focus on the transcriptomic responses of *T. suecica* under copper stress, this chapter aimed to shed light on the molecular intricacies of this interaction, exploring how the microalga copes with elevated copper concentrations while concurrently acting as a bio-remediator by removing copper ions from the surrounding environment. Through this investigation, I sought to contribute to a comprehensive systems-level understanding of copper's impact on microalgae, particularly in the context of wastewater treatment strategies.

At the end of this chapter, a mechanism for the interactions within the algae-bacterium consortium is suggested, combining the findings of Chapter 6 and Chapter 7.

7.2. Materials & Methods

7.2.1. Culture Growth Conditions & Copper Removal

Around 3 million/mL *T. suecica* cells were cultured in 8‰ F/2 medium under 67 μmol photons/m^2·s illumination with white cool light on a 12h:12h light:dark regime at 25 °C in 50 mL batch cultures in 250 mL Erlenmeyer flasks in five biological replicates for control and Cu-exposed sets. $CuCl_2$ was added to the media of the Cu-exposed set as 1 mg Cu/L. Samples from three of the batches were collected every other day for microscopic cell count to determine culture density. Determination of copper in dissolved, adsorbed, and assimilated phases was done according to a protocol modified from elsewhere (see Section 3.5) (J. L. Levy et al., 2008). Briefly, 2 mL sample was centrifuged; the pellet was rinsed with isotonic salt water, and centrifuged again. The supernatants of these processes were identified as dissolved fractions. Then the pellet was resuspended in EDTA wash buffer (0.01 M EDTA, 0.1 M KH_2PO_4/K_2HPO_4 buffer, pH: 6.0) and incubated for 20 min. The sample was then centrifuged, the pellet rinsed with isotonic salt water, and

centrifuged again. The supernatants of these processes were identified as adsorbed fractions. The dissolved and adsorbed fractions were acidified with 0.2 mL conc. HNO_3, and diluted to 10 mL. The final pellet was digested overnight in 2 mL conc. HNO_3 and the clear digestate was diluted to 20 mL the next day. This digestate was identified as the assimilated fraction. All fractions were analyzed for copper concentration on ICP/MS (iCAP RQ, ThermoFisher Scientific, Waltham, MA, USA). Samples for copper analysis were collected on the 0^{th}, 1^{st}, 4^{th}, and 9^{th} days, as biological triplicates.

7.2.2. RNA Extraction, Library Preparation & Sequencing

On the first day of the experiments (around 20 h), samples for RNA extraction were collected to a total biomass of 10^7 cells each. The samples were centrifuged and frozen immediately in liquid nitrogen, and stored at -80 °C until RNA extraction, which was done using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA quality (RINe value, Table B.1 in Appendix B) was determined for each sample with a 4200 TapeStation electrophoresis system (Agilent Technologies, Santa Clara, CA, USA). The libraries of mRNA were constructed using TrueSeq Stranded mRNA Sample Prep Kit (Illumina, San Diego, CA, USA) according to the protocol supplied by the manufacturer. Briefly, RNA concentrations were normalized to 400 ng total; poly-A containing mRNA was first purified using poly-T oligo attached magnetic beads, and fragmented. SuperScript II Reverse Transcriptase was used for first strand synthesis. The second strand was synthesized using DNA polymerase I. cDNA was purified using AMPure XP beads (Beckman Coulter), 3' ends were adenylated, and cDNA libraries were indexed using TruSeq RNA Single Indexes Set A & Set B. Indexed cDNA libraries were purified again using AMPure XP beads, and enriched with a 15-cycles PCR. The DNA concentrations in the cDNA libraries were measured with a fluorometer. The average base distribution was evaluated with TapeStation for each sample. Twenty-four cDNA library samples were pooled with normalized molarity, and 1% PhiX control was added. 101-bp paired-end sequencing of the cDNA libraries was then performed on an Illumina NextSeq 2000 sequencing platform.

7.2.3. Functional Annotation

Reads obtained from sequencing underwent adapter removal and quality trim via Cutadapt and Trimmomatic software, respectively (Bolger, Lohse, & Usadel, 2014; Martin, 2011). The quality of the reads was checked before and after trimming via FastQC software (Andrews, 2010). A sample figure for FastQC quality reports of raw and trimmed reads of a library was shown in Appendix C (Figure C.1). High quality reads were assembled *de novo* on Trinity v2.15 (Grabherr et al., 2011).

CD-HIT-EST software was utilized for creating a set of non-redundant contigs (W. Li & Godzik, 2006). The basic local alignment search tool (Blastx for nucleotide to protein) was utilized for the annotation of non-redundant sequences in the final assembly, searching against the libraries in the order listed in Table 7.1 (see Section 7.3.2), with an E-value cutoff of 10^{-3} (Altschul et al., 1997; Goodstein et al., 2012). Sequences without a hit were selected at each step and blasted against the next database. Omicsbox software was utilized for further annotation of the whole assembly utilizing InterProScan and eggNOG-Mapper (Blum et al., 2021; Götz et al., 2008; Huerta-Cepas et al., 2017; Kanehisa & Goto, 2000).

7.2.4. Quantitative Gene Expression Analysis

Reads were mapped to the assembly via HISAT2 software and count files were generated via HTseq (Anders, Pyl, & Huber, 2015; Kim, Langmead, & Salzberg, 2015). Then edgeR package of R was utilized for differential expression analysis assuming a negative binomial distribution (Robinson, McCarthy, & Smyth, 2010). TMM normalization was applied on logarithm (base 2) of count per million (logCPM) data, and sequences without meaningful expression in at least four samples were discarded from the expression analysis. Filtered and TMM normalized logCPM data is shown in Figure C.2 (see Appendix C). Read counts before and after filtering are shown in Figure C.3. The false discovery rate (FDR) values were utilized in omicsbox as a rank metric for gene set enrichment analysis. Combined pathway analyses were conducted in omicsbox, and from these analyses, lists of transcripts belonging to KEGG pathways were pulled. Transcripts not annotated on omicsbox but annotated through Blast were searched manually in Excel. CPM values of each enzyme or protein were summed up to determine that protein's expression value. Pathways were illustrated with draw.io (https://app.diagrams.net/). For oxidative phosphorylation, photosynthesis, and light harvesting proteins the figures of relevant pathways in the KEGG database were modified (https://www.genome.jp/kegg/pathway.html).

7.2.5. Statistical Analysis

For copper removal data, one-way ANOVA was utilized with Bonferroni or Tamhane's post-hoc according to the homogeneity of variance on SPSS software v21. In differential expression statistics, both FDR and p values were utilized. FDR<0.05 was utilized as a significance threshold for the expression of differentially expressed transcripts (DETs, i.e.: transport channels). T-tests were conducted for the expression of enzymes or proteins with more than one transcript. There were single and multiple transcript enzymes in the same pathway in the data. For analysis to be uniform, p values

were utilized instead of FDR for those enzymes with single transcripts, since those values are closer to the t-test p value. The data of enzyme expressions were reported with two confidence intervals; $p<0.05$ and $p<0.01$. To be convenient, $p<0.05$ and $p<0.01$ corresponds to $FDR<0.21$ and $FDR<0.072$, respectively, in the dataset.

7.3. Results & Discussion

7.3.1. Algae Growth and Copper Removal

The effect of copper on the growth of *T. suecica*, and the extent of copper removal by these algae through adsorption and assimilation were evaluated under 1 mg Cu/L initial copper concentration. The growth on Cu-spiked media was comparable to growth in the control group, the cell density even got higher after 6 days of growth (Figure 7.1.A). The copper removal rate was highest on the first day as observed by the decrease in the fraction dissolved in the media (Figure 7.1.B). The decrease was significant between Days 0-1, Days 0-4, and Days 1-4 ($p<0.001$). Adsorption was at its peak on the first day, slowly decreasing in the subsequent days. Adsorbed fractions on Days 1 and 4 were significantly higher than those on Day 0 and Day 9 ($p<0.001$). A continuous increase in assimilated copper was observed, reaching 18.4±1.8%, 35.7±1.3%, and 48.6±1.6% of total copper in the system on Days 1, 4, and 9, respectively. These values correspond to 0.17±0.02 ppm (2.7±0.28 µM), 0.33±0.02 ppm (5.2±0.24 µM), and 0.47±0.003 ppm Cu (7.4±0.04 µM). Each day's assimilation data were significantly different from each other ($p<0.05$). A cadmium removal of 59.6% by *T. suecica* at 6 mg/L Cd was observed in the literature, where 38.6% was assimilated into the cells (Pérez-Rama et al., 2002). Over 80% of copper removal was observed in another study with *Chlorella minutissima* at 0.2 or 0.4 mM Cu, yet the assimilated ratio was around 10% (J. Yang, Cao, Xing, & Yuan, 2015).

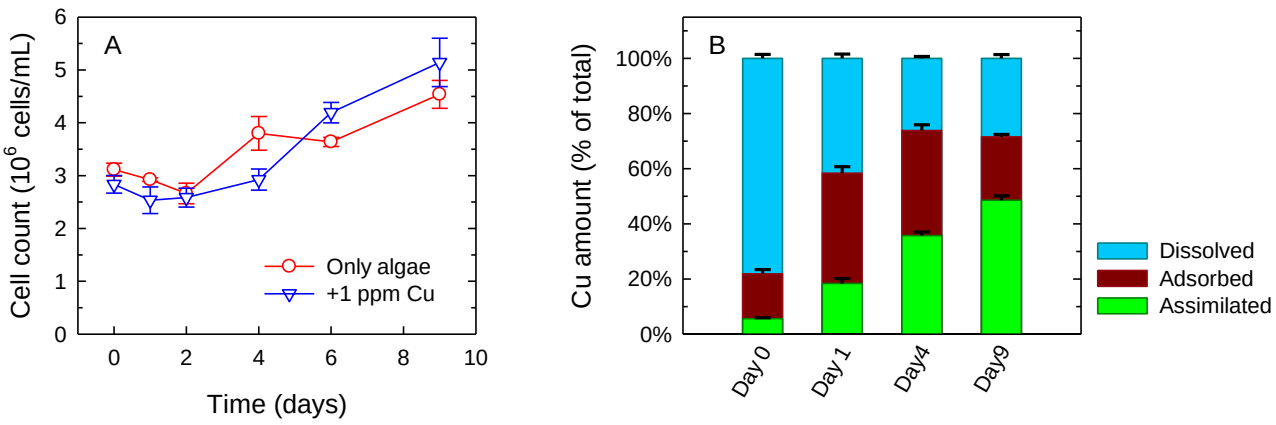


Figure 7.1. Growth of *T. suecica* with or without 1 ppm Cu (A), and Cu removal by this microalgae strain (B).

7.3.2. *De Novo* Transcriptome Assembly and Annotation

To investigate the metabolic changes in *T. suecica* upon copper exposure at the transcriptomic level, samples from each experimental group were collected for RNA extraction on Day 1, as five biological replicates. From RNA samples, mRNA was purified and transcribed into cDNA libraries, which were sequenced on Illumina NextSeq2000. Sequencing was performed with a bigger set of samples (24 libraries) yielding 469 million 760 thousand 098 pairs of reads. After adapter removal and quality trimming, 466 million 682 thousand 420 paired-end reads (99.3%) were utilized for *de novo* transcriptome assembly. Assembly was constructed, and sequences lower than 300 bp were removed, yielding 97670 transcripts with a total length, average length, N50, and longest contig of 148 million 610 thousand 695 bp, 1521.6 bp, 2361 bp, and 22967 bp, respectively. For the annotation of the assembly, two paths were followed: First, similarities were searched against genomes of the green algae in the Phytozome database (in the order specified in Table 7.1), RefSeq plant database of NCBI, non-redundant protein sequence database of NCBI, Swiss-Prot database, and Trembl, hierarchically in the specified order. Annotation was started from the Phytozome database to address green algae genes more accurately. The search was started from *C. reinhardtii* since it is the best-annotated genome in the Phytozome database under the cluster, Chlorophytes. The results of these searches are summarized in Table 7.1. Annotation through omicsbox yielded 10146 transcripts with gene ontology (GO) mapping, and 32080 with GO annotation (the numbers are additive). The species distribution of omicsbox annotations is illustrated in Figure 7.2.

Table 7.1. Annotation of transcripts in *T. suecica* transcriptome

Database	# of Hits *
Chlamydomonas reinhardtii CC-4532 v6.1	35595
Volvox carteri v2.1	2034
Botryococcus braunii v2.1	3002
Micromonas pusilla CCMP1545 v3.0	1772
Micromonas sp RCC299 v3.0	533
Ostreococcus lucimarinus v2.0	243
Dunaliella salina v1.0	410
Chromochloris zofingiensis v5.2.3.2	754
Coccomyxa subellipsoidea C-169 v2.0	476
Total Chlorophyte (Phytozome database)	44819
RefSeq plant	1355
Blast_Nr	2481
Swiss-Prot	40
Trembl	8547
Grand Total	57242

* The numbers are additive. Found hits were separated from the main set of transcripts after each search, and the next search was done with non-annotated sequences only.

After the assembly and annotation of the transcriptome, the high quality reads were individually mapped from five biological replicates of control and copper-exposed sets to the assembly to determine the counts per million (CPM) values for abundance. The CPM values were then log normalized with TMM normalization on edgeR, and transcripts with low expressed reads in both groups were filtered. The resulting dataset is composed of 36354 transcripts. Differential expression analysis was done using the negative binomial distribution model of edgeR, giving 4403 differentially expressed transcripts (DETs) with logarithm (base 2) fold change (logFC) between groups was meaningful (FDR<0.05). Among 4403 DETs, 2235 were upregulated, and 2168 were downregulated significantly (Figure 7.3.A, for volcano plot and p-value frequency plots; see Figure C.4 in Appendix C). Multidimensional scaling analysis was done with logFC data (Figure 7.3.B). The control group and the Cu-exposed group were mainly clustered on the first dimension. The clustered heatmap of all DETs verifies the high correlation within biological replicates (Figure 7.3.C).

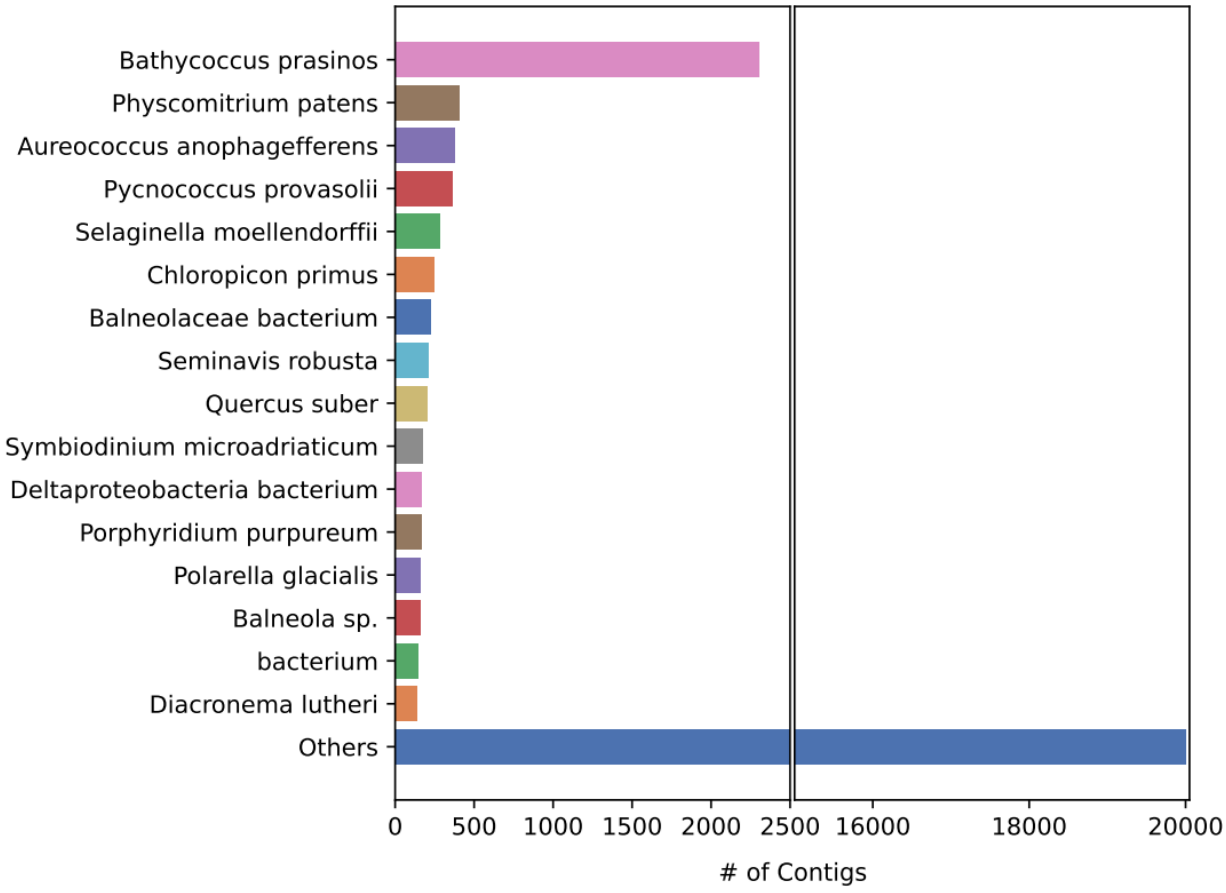


Figure 7.2. Species distribution of *T. suecica* transcriptome derived from the blast on omicsbox.

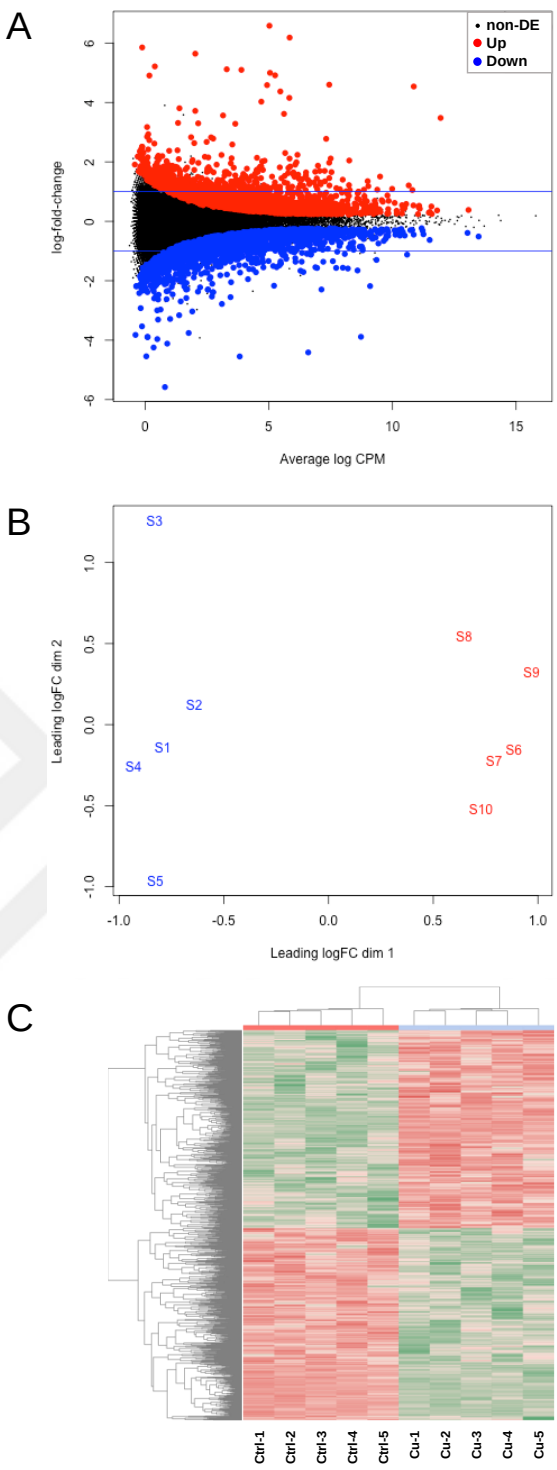


Figure 7.3. Differential expression profile of *T. suecica* between Cu-exposed and control culture. A) Mean difference (MD) plot showing differentially expressed contigs (red dots: upregulated, blue dots: downregulated), B) Multidimensional scaling of groups based on logFC data (S1-S5: control, S6-S10: Cu-exposed), C) Heatmap of differentially expressed genes (left: control group, right: Cu-exposed).

7.3.3. GO and KEGG Gene Set Enrichment Analyses

To elucidate the functional response of *T. suecica* against Cu exposure, transcriptomes of control and copper-exposed sets were classified based on gene ontology (GO) terms. Found GO terms were then analyzed for representation within the Cu-exposed set compared to the whole transcriptome (including both sets). The GO terms overrepresented and underrepresented in the Cu-exposed set are shown in Figure 7.4 and Figure 7.5, respectively. 131 overrepresented GO terms were identified, of which 75 belonged to biological processes (BP), 27 to cellular components (CC), and 29 to molecular functions (MF). 41 GO terms were underrepresented in the Cu-exposed set. Among them, 20 were of BP, 19 were of CC, and 2 were of MF. The top 5 most significantly upregulated GO terms were "rRNA processing" (BP), "rRNA metabolic process" (BP), "nucleolus" (CC), "ribosome biogenesis" (BP), and "ribonucleoprotein complex biogenesis" (BP). The most significant upregulated GO term belonging to MF was "organic cyclic compound binding". Remarkable upregulated GO terms also include "cellular nitrogen compound metabolic process" (BP), "organic substance metabolic process" (BP), "intracellular anatomical structure" (CC), "cellular aromatic compound metabolic process" (BP), "organic acid metabolic process" (BP), "nitrate reductase activity" (MF), "protein transmembrane import into intracellular organelle" (BP), "pentose-phosphate shunt" (BP), and "ATP hydrolysis activity" (MF). The most significantly downregulated GO terms of main GO categories were "cytoplasm" (CC), "cellular process" (BP), and "oxidoreductase activity" (MF). Other remarkable downregulated GO terms include "peptide metabolic process" (BP), "organic substance catabolic process" (BP), "macromolecule biosynthetic process" (BP), "endoplasmic reticulum" (CC), "mitochondrion" (CC), "vacuole" (CC), "transport" (BP), and "plasma membrane" (CC).

A combined pathway analysis was conducted on omicsbox to evaluate the enriched KEGG pathways. 149 pathways from "Metabolism", 22 from "Genetic Information Processing", 38 from "Environmental Information Processing", and 33 pathways from "Cellular Processes" were identified; of which 9, 5, 3, and 2 were enriched, respectively. By normalized enrichment score (NES), the most positively enriched pathways were ribosome (ko03010), aminoacyl t-RNA biosynthesis (ko00970), and photosynthesis - antenna proteins (ko00196). DNA replication (ko03030) and propanoate metabolism (ko00640) had negative NES values. Nitrogen metabolism had the highest rich ratio, and was Fisher significant (FDR: 2.79×10^{-7}). Starch and sucrose metabolism (ko00500), fatty acid metabolism (ko00061), and amino acid metabolisms such as glycine, serine and threonine metabolism (ko00260), cysteine and methionine metabolism (ko00270), lysine biosynthesis (ko00300) and valine, leucine and isoleucine biosynthesis (ko00290) were also enriched (Figure 7.6).

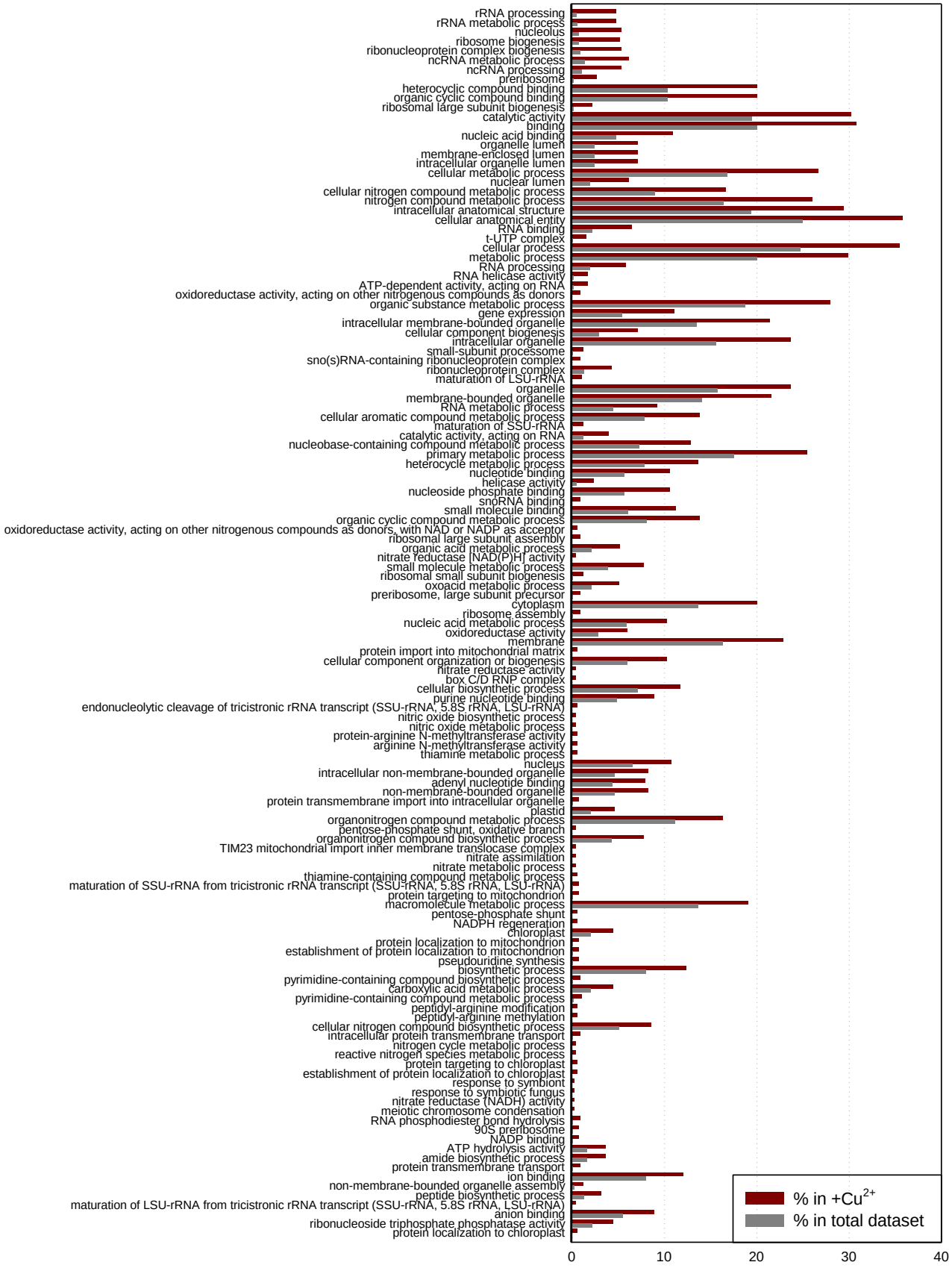


Figure 7.4. Gene ontology terms that were overrepresented in the Cu-exposed set.

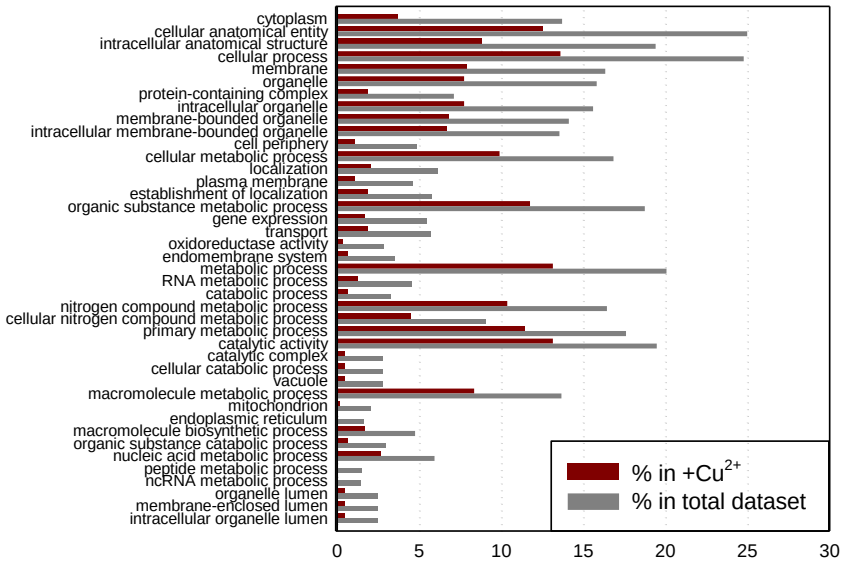


Figure 7.5. Gene ontology terms that were underrepresented in the Cu-exposed set.

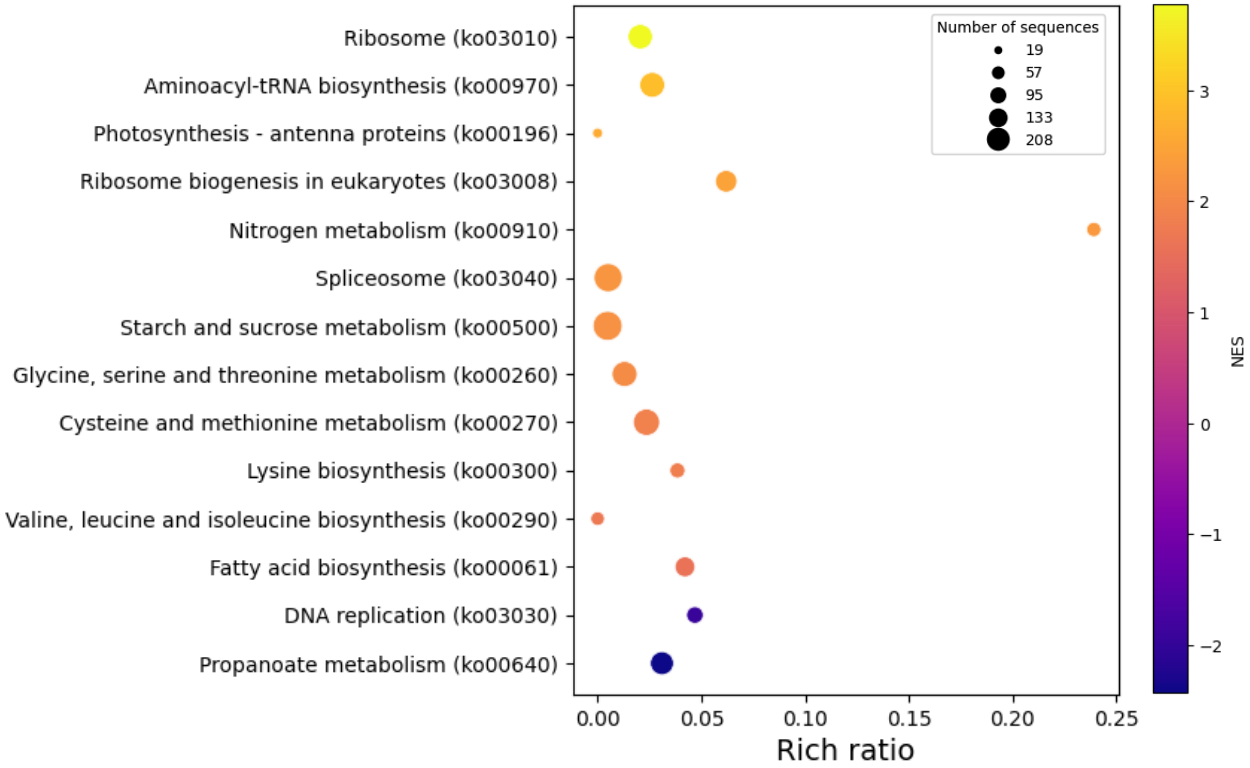


Figure 7.6. KEGG pathways that were enriched in Cu-exposed set. Marker sizes indicate the number of sequences for each pathway (Y-axis), markers are colored by normalized enrichment score (NES).

In the next sections, metabolic changes in *T. suecica* upon Cu exposure were evaluated in more detail. The effects of copper on metal transport channels, oxidative stress metabolism, and main metabolisms related to growth and viability are discussed. Such main metabolisms include energy

generation through the electron transport chain, being both autotrophic and heterotrophic, carbohydrate metabolism, nitrogen metabolism, and lipid metabolism.

7.3.4. Genes Related to Heavy Metal Induced Stress

To counteract and minimize the harmful effects of HMs, plants and algae employ a complex system that regulates metal transport and utilization. Key players include Zrt- and Irt-like proteins (ZIP), copper transporters (Ctr), metal tolerance proteins (MTPs), and P-type ATPases. ZIP and Ctr family proteins are mainly responsible for heavy metal influx into the cytosol from outside or from organelles (Tripathi & Poluri, 2021). MTPs and P-type ATPases (energy-dependent transporters) are mainly responsible for efflux of HMs out of the membrane, or sequestration into vacuoles. P-type ATPases specific to HMs are called heavy metal ATPases (HMAs). Natural resistance-associated macrophage protein family transporters were also utilized by microalgae for heavy metal import in high concentrations of metals (Lu et al., 2019). Another type of transporter enhanced in heavy metal stress is multidrug and toxic compound extrusion (MATE) family efflux proteins, which work as an antiporter with H^+ or Na^+ gradient (Kusakizako, Miyauchi, Ishitani, & Nureki, 2020).

Out of 24 ZIP family proteins, 11 sequences, 3 out of 11 MTP family proteins, 2 out of 2 Ctr family proteins, 2 out of 30 MATE family proteins, 15 out of 41 P-type ATPases, and 10 out of 24 Ca^{2+} channels were identified as differentially expressed under Cu stress (FDR<0.05, Table 7.2). Most of the differentially expressed genes (DEGs) in the ZIP family exhibited overexpression when the algae were exposed to Cu. Previous literature has reported an increase in ZIP transporter expression in microalgae due to prolonged cadmium exposure (Lu et al., 2019), and due to an increase in mercury derivatives in the media (Beauvais-Flück, Slaveykova, & Cosio, 2017). The data presented here is in parallel with the literature. All copper import proteins in the Ctr family were downregulated. In a previous study on *C. reinhardtii* copper accumulation, authors found increased copper uptake during cell division, but the Ctr transporters were suppressed (Deng & Wang, 2023). These data suggest that *T. suecica* may primarily utilize ZIP family metal import channels for copper ion import from the culture media into the cytosol.

All the DEGs in MTP, MATE, and P-type heavy metal transporter families were upregulated, implying an increase in the efflux of copper from the cytosol out of the membrane or into vacuoles. In another transcriptomic study on the effect of copper on *D. salina*, the authors found increased expression of P-type copper transporters, suggesting the importance of active transport for algae to cope with heavy metal stress (Ling et al., 2021). Two out of three HMAs found upregulated in my

study belongs to HMA5 subfamily, which is responsible for the active transport of monovalent copper (Cu^{+}) ions (Blaby-Haas, Padilla-Benavides, Stübe, Argüello, & Merchant, 2014). Although lower in abundance (see LogCPM in Table 7.2), the presence of HMA5 transporters upregulated in the copper-exposed set suggests the reduction of Cu^{2+} ions in microalgae cells.

Excessive amounts of heavy metal ions in the surrounding environment cause oxidative stress on microalgae via induction of ROS (Danouche et al., 2022). This perturbs cellular redox homeostasis, causing damage to cellular components, including lipids, proteins, and nucleic acids. Microalgae produce certain antioxidant chemicals such as carotenoids, flavonoids, GSH, and ascorbate to cope with oxidative stress (Pikula, Zakharenko, Aruoja, Golokhvast, & Tsatsakis, 2019). GSH metabolism is intricately connected to cellular antioxidant defense mechanisms and the handling of ROS. This pathway is important in three aspects: 1) GSH itself is an antioxidant. 2) It participates in detoxifying harmful substances, including xenobiotics and heavy metals. GSH conjugates with toxins, making them more water-soluble and facilitating their excretion from the cytosol. 3) It is involved in the synthesis of PCs that are mainly responsible for heavy metal sequestration in algae.

GSH is a tripeptide composed of glutamate, cysteine, and glycine. Glutathione metabolism begins with the acetylation of L-serine and the subsequent deacetylation of O-acetyl-L-serine, incorporating sulfide ions to produce L-cysteine. The ligation of cysteine and glutamate by glutamate-cysteine ligase forms gamma-Glu-Cys. Glutathione synthase adds a glycine to γ-Glu-Cys, resulting in the formation of GSH. PCS enzyme further contributes to GSH metabolism by ligating another GSH molecule to GSH, while removing one glycine, leading to the formation of PC2. The PCS enzyme continues to add GSH and remove a glycine with each iteration, producing PCn (n=2-11) molecules.

A schematical diagram of glutathione metabolism is shown in Figure 7.7. None of the enzymes here was differentially expressed significantly, in contrast to literature where GSH biosynthesis is associated with high concentrations of HMs (Xing et al., 2021); nonetheless, all enzymes show a non-significant increase in expression in the Cu-exposed set. PCS activation by Cu was shown in the literature before, although not as abundant as the activation by Cd (Ahner, Kong, & Morel, 1995; Inouhe, 2005). Such a change in the PCS expression of *T. suecica* was not observed.

Table 7.2. Transporters that were differentially expressed in Cu treatment of *T. suecica*.

Type	Contig ID	EC code/KO	Description	LogFC	LogCPM
Zrt & Irt -like proteins (Zinc transporters)	TRINITY_DN3815_c1_g2_i2	K14709	Zinc permease family, ZIP2	1.66	7.53
	TRINITY_DN3815_c1_g1_i1	K14709	Zinc permease family, ZIP2	0.61	6.24
	TRINITY_DN5162_c0_g1_i1	K14709	zinc ion transmembrane transporter activity	-1.16	4.71
	TRINITY_DN13313_c0_g1_i1	K14709	Zinc ion transmembrane transporter activity	1.13	1.71
	TRINITY_DN1561_c0_g1_i3	K14709	Zinc ion transmembrane transporter activity	-0.44	6.71
	TRINITY_DN2735_c0_g2_i8	K14709	Zinc ion transmembrane transporter activity	0.86	2.02
	TRINITY_DN13066_c0_g2_i1	K07238	Zinc (Zn^{2+})-Iron (Fe^{2+}) Permease (ZIP) Family.	1.79	0.95
	TRINITY_DN5303_c0_g1_i24	K07238	ZIP family transporter	1.47	0.84
	TRINITY_DN5303_c0_g1_i33	K07238	ZIP family transporter	0.86	2.60
	TRINITY_DN4277_c0_g1_i10	K14714	ZIP Zinc transporter	1.39	3.11
	TRINITY_DN3815_c1_g1_i10	K14714	Zinc (Zn^{2+})-Iron (Fe^{2+}) Permease (ZIP) Family.	1.72	4.50
Metal tolerance proteins (MTP)	TRINITY_DN28091_c0_g1_i3	K14689	Cation efflux family, MTP1	0.85	2.22
	TRINITY_DN28091_c0_g1_i4	K14689	Cation efflux transporter, membrane protein	0.94	2.62
	TRINITY_DN757_c0_g2_i3		Cation efflux family protein, MTP2	0.94	1.65
Ctr family copper transporters	TRINITY_DN5038_c0_g1_i2		Ctr copper transporter family	-0.65	6.43
	TRINITY_DN5038_c0_g2_i1		Copper ion transmembrane transport	-0.76	4.46
MATE efflux family protein	TRINITY_DN2665_c0_g1_i1		MATE efflux family protein	0.47	9.02
	TRINITY_DN14695_c0_g1_i2		MATE efflux family protein	0.71	1.91
P-type heavy metal transporters	TRINITY_DN20272_c0_g1_i2	K01534	Heavy metal ATPase 1, Cd/Zn transporting	0.39	7.27
	TRINITY_DN1425_c0_g1_i15	K17686	Heavy metal ATPase 5, copA	1.28	0.64
	TRINITY_DN1425_c0_g1_i26	K17686	Heavy metal ATPase 5	0.78	0.73
P-type Ca2+ transporters	TRINITY_DN5715_c0_g2_i1	K01537	Calcium-transporting ATPase, ER-type, putative	0.74	1.97
	TRINITY_DN10965_c1_g1_i1	K01537	ER-type Ca^{2+}-ATPase 2	1.21	0.74
	TRINITY_DN8365_c0_g1_i3	K01537	P-type Ca^{2+} transporter, this magnesium-dependent enzyme catalyzes the hydrolysis of ATP coupled with the transport of calcium	0.85	4.72
	TRINITY_DN8365_c0_g1_i14	K01537	Autoinhibited Ca^{2+}-ATPase 9	-0.40	3.66
	TRINITY_DN396_c0_g1_i64	7.2.2.10	Autoinhibited Ca^{2+}-ATPase 9	-0.94	2.83

Table 7.2. Cont.

Type	Contig ID	EC code/KO	Description	LogFC	LogCPM
P-type other transporters	TRINITY_DN11231_c0_g1_i2	K01536	Sodium-exporting ATPase	1.48	3.92
	TRINITY_DN34438_c0_g1_i1	K01536	Belongs to the cation transport ATPase (P-type) (TC 3.A.3) family	1.37	0.69
	TRINITY_DN1437_c0_g1_i9	K14951	haloacid dehalogenase-like hydrolase	-0.78	4.01
	TRINITY_DN1437_c0_g1_i27	K14951	peptidyl-aspartic acid autophosphorylation	-0.70	3.99
	TRINITY_DN648_c0_g1_i76	K14951	peptidyl-aspartic acid autophosphorylation	-1.09	0.97
	TRINITY_DN1437_c0_g1_i1	K14951	haloacid dehalogenase-like hydrolase	-0.66	4.52
	TRINITY_DN15062_c0_g1_i17	K14951	E1-E2 ATPase	-1.63	-0.19
Ca2+ channels	TRINITY_DN796_c1_g1_i12		Transient receptor potential ion channel protein	1.54	0.31
	TRINITY_DN1324_c1_g4_i1		Transient receptor potential ion channel protein	0.30	5.54
	TRINITY_DN8707_c0_g3_i7		Transient receptor potential ion channel protein	0.48	6.28
	TRINITY_DN4445_c0_g1_i4		Transient receptor potential ion channel protein	-0.49	4.09
	TRINITY_DN2925_c0_g1_i2		Putative Ca2+/Na+ exchanger	1.32	0.90
	TRINITY_DN9506_c0_g1_i42		Putative Ca2+/Na+ exchanger	0.75	3.86
	TRINITY_DN188_c0_g1_i23		Calcium ion transmembrane transport	-0.89	1.15
	TRINITY_DN19643_c0_g1_i6		Belongs to the Ca(2) cation antiporter (CaCA) (TC 2.A.19) family	-0.65	2.42
	TRINITY_DN14139_c0_g1_i1		Two pore calcium channel protein, TPC1	-0.29	7.87
	TRINITY_DN4753_c0_g1_i1		Putative Mn and/or Ca transporter	1.01	1.76

There are also enzymes upregulated by algae upon oxidative stress, which catalyze reactions for oxidative stress relief via converting ROS into less harmful compounds like oxygen. Such enzymes include superoxide dismutase (SOD), catalase, catalase/peroxidase (katG), ascorbate peroxidase (APX), glutathione peroxidase (GPX), cytochrome-c peroxidase (CCP), peroxiredoxin, glutaredoxin (GRX), and thioredoxin (TRX). SOD catalyzes the reaction of dismutation of superoxide radical into H_2O_2, which is then turned into oxygen by various peroxidases. GRX and TRX, along with the GSH pool, help maintain the reduced state of proteins in the presence of imbalance caused by ROS (Ströher & Millar, 2012).

Most of the contigs related to antioxidant enzymes in *T. suecica* showed insignificant differences in expression upon exposure to Cu (refer to Table D.1 in Appendix D). Specifically, six contigs were upregulated, and three were downregulated. Among the downregulated contigs, two were annotated as APX. The upregulated contigs were annotated as follows (with the number of contigs in brackets):

katG (1), CCP (1), putative haloperoxidase (1), TRX (2), and thioredoxin-dependent peroxiredoxin (1). Notably, contigs associated with SOD exhibited no significant change in expression between the control and Cu-exposed sets. The dismutation of superoxide to O_2 by divalent copper ions, following the reduction of Cu^{2+} into Cu^{+} is a known phenomenon (Nowicka, 2022), and could explain both the non-differential expression of SOD and the upregulation of the monovalent copper ion transporter, HMA5.

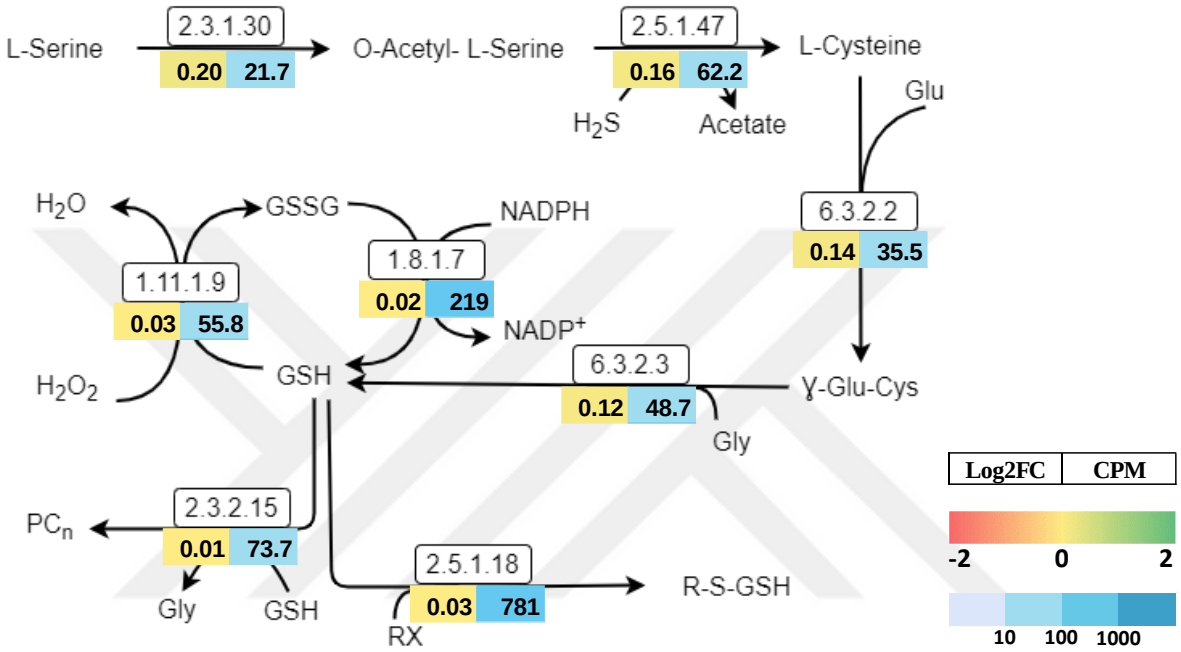


Figure 7.7. Glutathione metabolism. Enzymes include: serine O-acetyltransferase (EC: 2.3.1.30), cysteine synthase (EC: 2.5.1.47), glutamate--cysteine ligase (EC: 6.3.2.2), glutathione peroxidase (EC: 1.11.1.9), glutathione-disulfide reductase (EC: 1.8.1.7), glutathione synthase (EC: 6.3.2.3), glutathione S-transferase (2.5.1.18), phytochelatin synthase (EC: 2.3.2.15). Under each enzyme log2 fold change (Log2FC, left) and counts per million (CPM) are shown. Expressions and abundances were color-coded. The red to green bar represents the color code for Log2FC, bar with shades of blue represents CPM values. Significant differential expressions were marked with red asterisks ("*" for $p<0.05$ or "**" for $p<0.01$, none in this figure).

7.3.5. Nitrogen Assimilation

While nitrogen is abundant on Earth, it is primarily found as dinitrogen (N_2), which is not readily available for many organisms. Photoautotrophic organisms efficiently assimilate ammonium and nitrate, using fixed carbon to generate carbon skeletons for nitrogen assimilation. Amino acids derived from proteins are the primary nitrogen source for organisms. Nitrogen, along with phosphorus, is a major limiting nutrient for plant and algal growth in ecosystems. The increased

demand for food has led to extensive use of ammonium-containing fertilizers, generated through the energy-intensive Haber-Bosch process (Boerner, 2019). Unfortunately, a significant percentage of applied nitrogen fertilizers are lost to the atmosphere and aquatic systems, contributing to environmental issues such as greenhouse gas emissions and eutrophication. Algal and cyanobacterial blooms resulting from nitrogen influx influence ecosystems and human health. There is a growing interest in understanding microbial and plant nitrogen utilization, with a particular focus to enhance nitrogen use efficiency in crop plants for sustainable agriculture (Calatrava, Tejada-Jimenez, Sanz-Luque, Fernandez, & Galvan, 2023).

In the presence of Cu, a significant increase was observed in the expression of nitrate/nitrite transporters that import NO_3^-/NO_2^- into the cytosol. Additionally, nitrate reductase, ferredoxin-nitrite reductase, cyanase, and the genes related to the conversion of ammonia to glutamate as glutamine synthetase and glutamate synthetase were significantly upregulated (Figure 7.8). Cyanase ligates bicarbonate and cyanate into carbamate, which spontaneously turns into ammonia and CO_2.

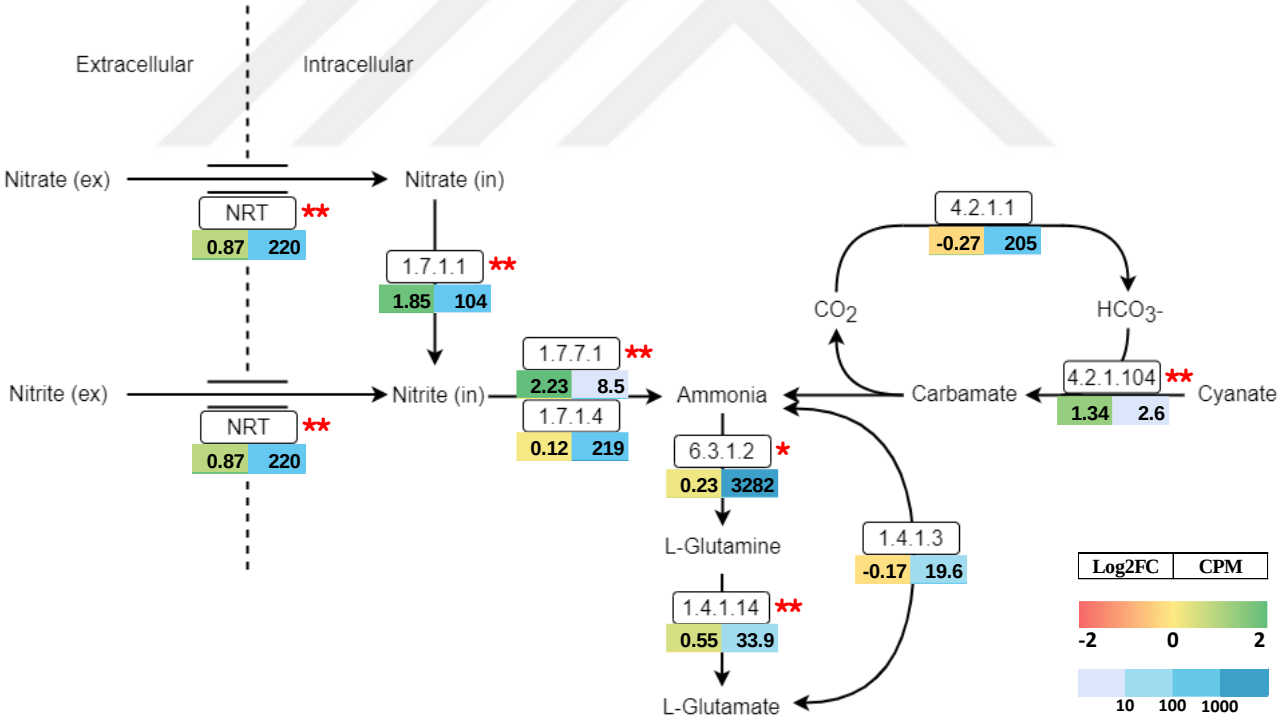


Figure 7.8. Nitrogen assimilation. Enzymes include: nitrate/nitrite transporter (NRT), nitrate reductase (EC: 1.7.1.1), ferredoxin-nitrite reductase (EC: 1.7.7.1), NAD(P)H-dependent nitrite reductase (EC: 1.7.1.4), glutamine synthetase (EC: 6.3.1.2), glutamate synthase (EC: 1.4.1.14), glutamate dehydrogenase (EC: 1.4.1.3), carbonic anhydrase (EC: 4.2.1.1), cyanase (EC: 4.2.1.104). Please refer to Figure 7.7 for figure legends and marks.

7.3.6. Oxidative Phosphorylation and Photosynthesis

Oxidative phosphorylation and photosynthesis are fundamental processes in the energy metabolism of algae, contributing to the generation and utilization of cellular energy. These processes play pivotal roles in the overall energy balance of algae, sustaining their growth, development, and cellular functions. Oxidative phosphorylation is a key component of cellular respiration, occurring in the mitochondria of eukaryotic algae. It involves the transfer of electrons through a series of protein complexes in the electron transport chain (ETC), coupled with the pumping of protons across the inner mitochondrial membrane. This creates a proton gradient, and the subsequent flow of protons back into the mitochondrial matrix through ATP synthase drives the phosphorylation of ADP to ATP.

In the present RNA-seq data, there were 23 subunits of NADH dehydrogenase (EC: 7.1.1.2), 4 subunits of succinate dehydrogenase (EC: 1.3.5.1), 6 subunits of cytochrome c reductase (EC: 7.1.1.8), 8 subunits of cytochrome c oxidase (EC: 7.1.1.9), and 9 and 13 subunits of F-type and V-type ATPases (EC: 7.1.2.2), respectively (Figure 7.9). Among oxidative phosphorylation genes, the alpha/beta subcomplex of NADH dehydrogenase (NDUFAB), gamma subunit of eukaryotic F-type ATPase (ATPeF1G), V-type ATPase subunit C, H, F (ATPeV1C, ATPeV1H, ATPeV1F), and 21 kDa proteolipid subunit (ATPeV0B), along with inorganic pyrophosphatase (EC: 3.6.1.1) and proton-translocating P-type ATPase (EC: 7.1.2.1) were strongly upregulated ($p<0.01$). 7 other proteins were differentially expressed ($p<0.05$). A polyphosphate kinase (EC: 2.7.4.1) was also identified, which is not found in green algae according to the KEGG database but occurs only in *Ostreococcus lucimarinus* among green algae according to the Phytozome database.

In photosynthetic algae, the light reactions of photosynthesis take place in the chloroplasts. During these reactions, light energy is absorbed by chlorophyll and other pigments, initiating the conversion of solar energy into chemical energy (Leegood, 2013). Water molecules are the electron donors of the photosynthetic ETC, which leads to the establishment of a proton gradient across the thylakoid membrane. Similar to oxidative phosphorylation, this proton gradient powers ATP synthesis through ATP synthase. Additionally, the light reactions produce reduced nicotinamide adenine dinucleotide phosphate (NADPH), another crucial coenzyme involved in various biosynthetic pathways.

Upon copper exposure, *T. suecica* exhibited a strong induction of photosystem II subunits PsbQ and Psb28, accompanied by a significant downregulation of cytochrome c6 (PetJ, Figure 7.10). The PsaH subunit of photosystem I and the gamma subunit of F-type ATPase (ATPF1G) were

significantly upregulated ($p<0.05$). Among the 11 light harvesting enzymes found (Figure 7.11), only one of them showed significance ($p<0.05$). Different transcriptomic findings exist regarding the copper effect on the photosynthesis of microalgae and cyanobacteria. In a study conducted with *D. salina*, which was exposed to 30 µM (1.9 mg-Cu/L) $CuCl_2$ for 72 h, most of the DEGs related to photosynthesis were upregulated (Ling et al., 2021). A study on the effect of 10 µM (0.64 mg-Cu/L) copper on seaweed *Ulva compressa* after 24 h exposure found similar results (Rodríguez et al., 2018). In another study, photosynthesis-related DEGs of freshwater cyanobacteria, *M. aeruginosa*, exposed to 0.5 mg-Cu/L (~7.9 µM) $CuSO_4$ were all downregulated (T. Wang et al., 2020). Cyanobacteria were more sensitive to copper than green algae as indicated in Chapter 4. Other studies in the literature also report either upregulation or no change in the photosynthetic activity of microalgae upon acute copper exposure (Olsson et al., 2015).

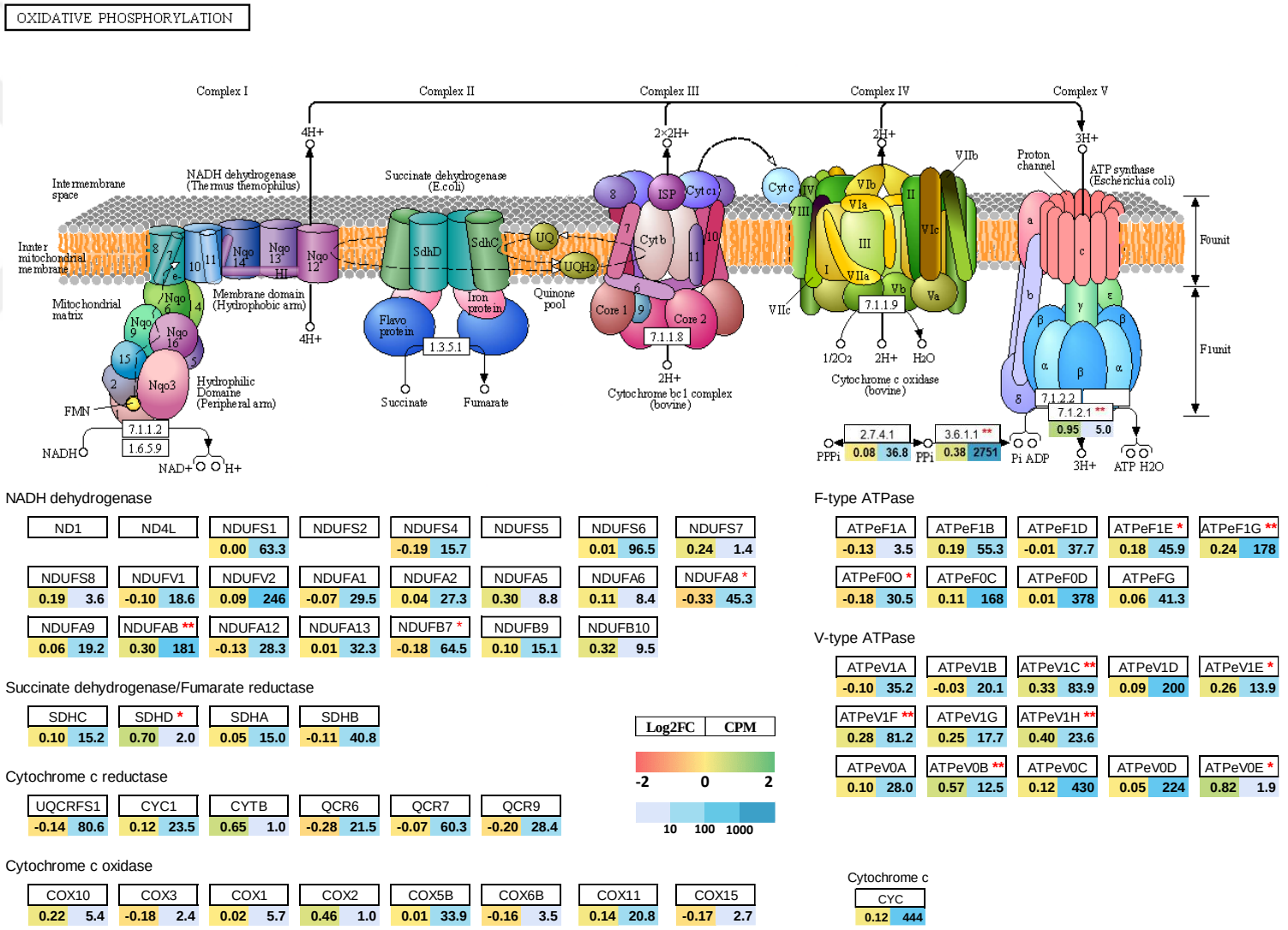
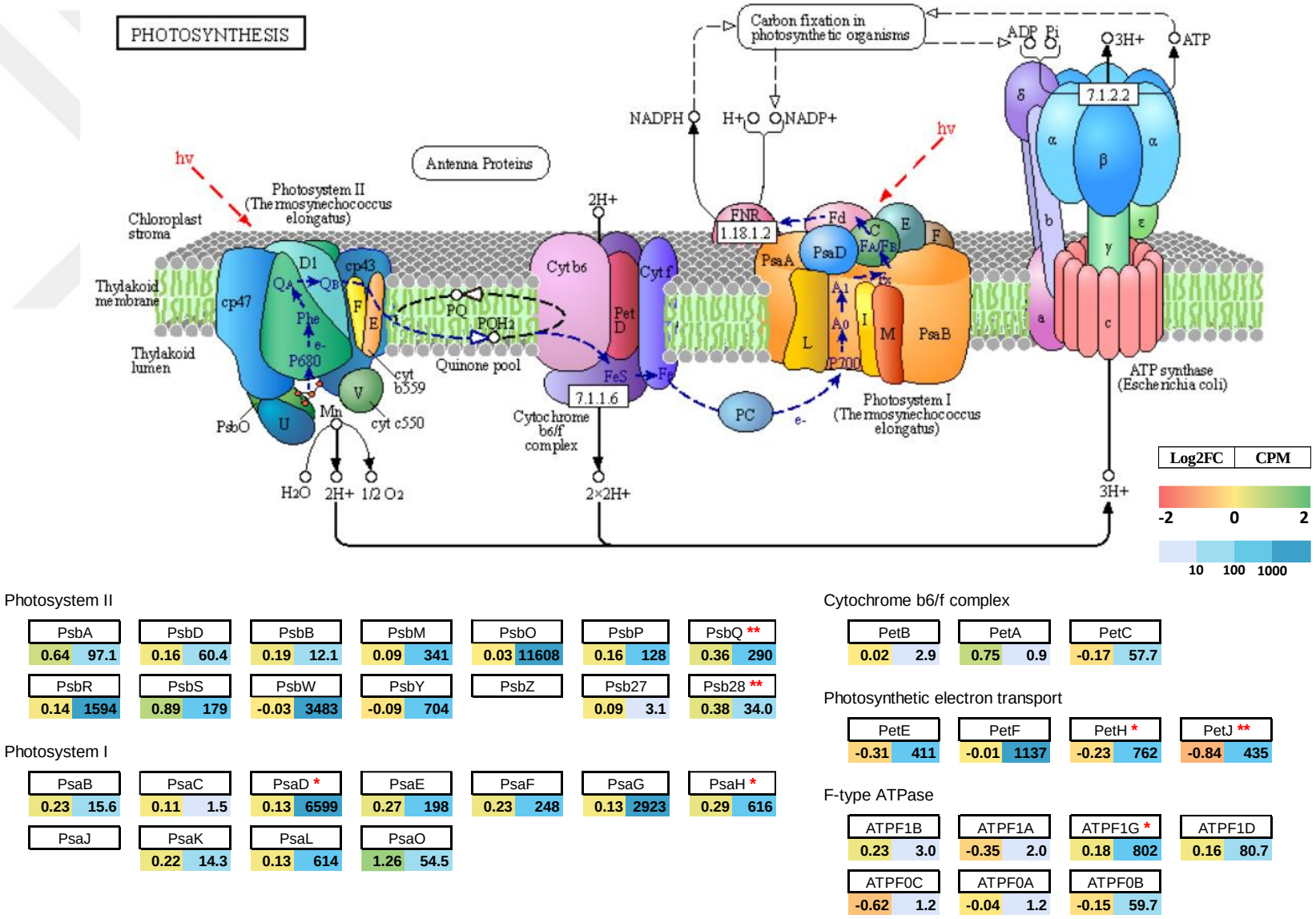


Figure 7.9. The effect of copper on oxidative phosphorylation. The listed subunits of enzyme complexes were only the ones found via annotation of the assembly. Because edgeR filters the contigs that have low matched read counts, some annotated genes have no expression data. Please refer to Figure 7.7 for figure legends and marks.



Photosystem II

PsbA	PsbD	PsbB	PsbM	PsbO	PsbP	PsbQ **
0.64 97.1	0.16 60.4	0.19 12.1	0.09 341	0.03 11608	0.16 128	0.36 290

PsbR	PsbS	PsbW	PsbY	PsbZ	Psb27	Psb28 **
0.14 1594	0.89 179	-0.03 3483	-0.09 704		0.09 3.1	0.38 34.0

Photosystem I

PsaB	PsaC	PsaD *	PsaE	PsaF	PsaG	PsaH *
0.23 15.6	0.11 1.5	0.13 6599	0.27 198	0.23 248	0.13 2923	0.29 616

PsaJ	PsaK	PsaL	PsaO
	0.22 14.3	0.13 614	1.26 54.5

Cytochrome b6/f complex

PetB	PetA	PetC
0.02 2.9	0.75 0.9	-0.17 57.7

Photosynthetic electron transport

PetE	PetF	PetH *	PetJ **
-0.31 411	-0.01 1137	-0.23 762	-0.84 435

F-type ATPase

ATPF1B	ATPF1A	ATPF1G *	ATPF1D
0.23 3.0	-0.35 2.0	0.18 802	0.16 80.7

ATPF0C	ATPF0A	ATPF0B
-0.62 1.2	-0.04 1.2	-0.15 59.7

Figure 7.10. The effect of copper on photosynthesis. The listed subunits of photosynthesis enzyme complexes were only the ones found via annotation of the assembly. Please refer to Figure 7.7 and Figure 7.9 for figure legends and marks.

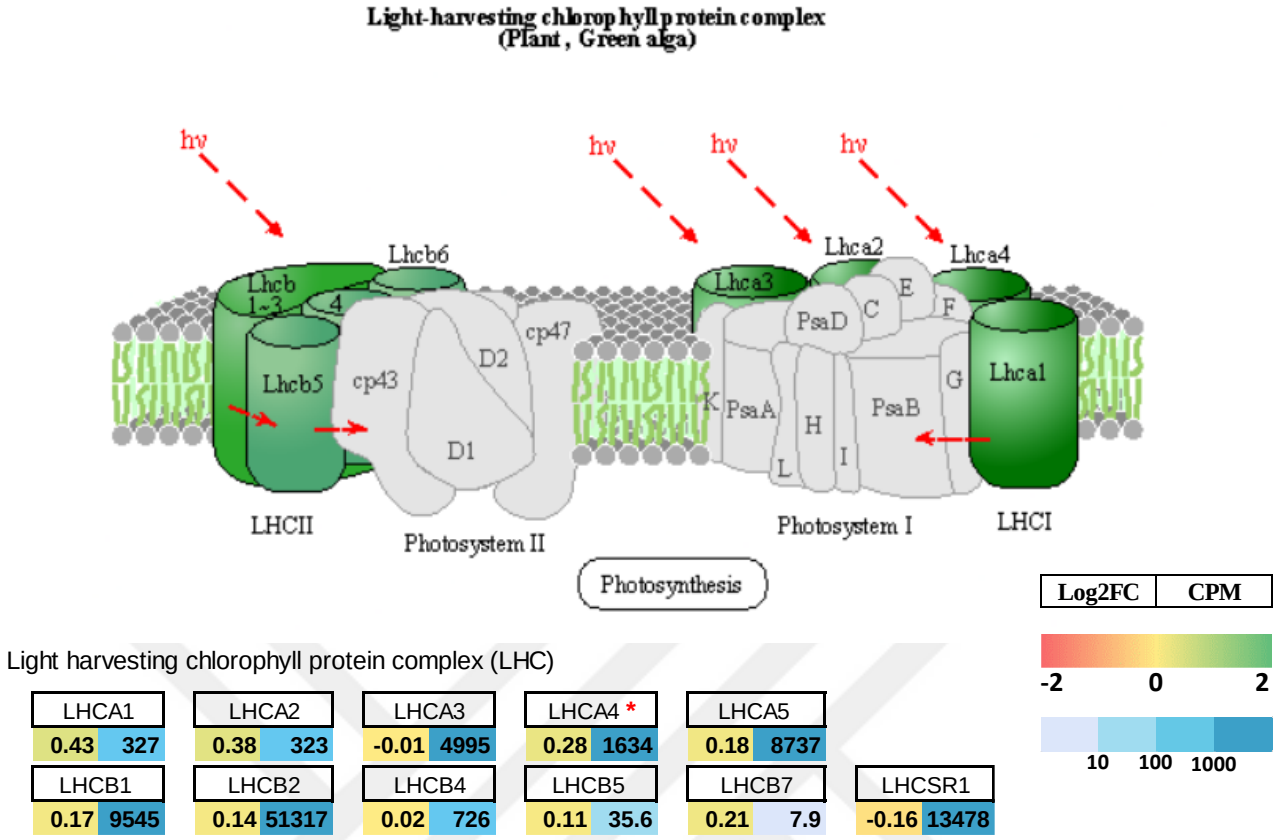


Figure 7.11. The effect of copper on light harvesting proteins. The listed subunits of light harvesting chlorophyll-protein complexes were only the ones found via annotation of the assembly. Please refer to Figure 7.7 for figure legends and marks.

7.3.7. Carbohydrate Metabolism

As photosynthetic organisms, microalgae utilize energy derived from the photosynthetic ETC for the assimilation of CO_2 into glucose. Produced glucose is used in starch generation for storage, or broken down into pyruvate for ATP synthesis. There are key molecules, which play pivotal roles in the metabolism of energy vessels like ATP, NAD^+/NADH, $NADP^+$/NADPH, or act as intermediates in the anabolic and catabolic reactions. Such molecules include 3-phosphoglycerate (3PGA), glyceraldehyde-3-phosphate (G3P), glycerone phosphate (DHAP), 2-oxoglutarate, oxaloacetate (OAA), phosphoenolpyruvate, pyruvate, and acetyl CoA (Behrenfeld, Halsey, & Milligan, 2008; Behrenfeld, Prasil, Babin, & Bruyant, 2004).

Three DEGs were identified in the Calvin cycle: fructose-bisphosphate aldolase (EC: 4.1.2.13), fructose-bisphosphatase (EC: 3.1.3.11), and sedoheptulose-bisphosphatase (EC: 3.1.3.37), first two were downregulated and the latter upregulated (Figure 7.12). The expression of ribulose-bisphosphate carboxylase (RuBisCO) was not significantly altered, suggesting no substantial change in CO_2

assimilation upon copper exposure. Triosephosphate isomerase (EC: 5.3.1.1) expression was not significantly changed. This enzyme converts G3P into DHAP, which can be converted by glycerol-3-phosphate dehydrogenase into glycerol-3-phosphate in triacylglycerol (TAG) production (see Figure 7.15).

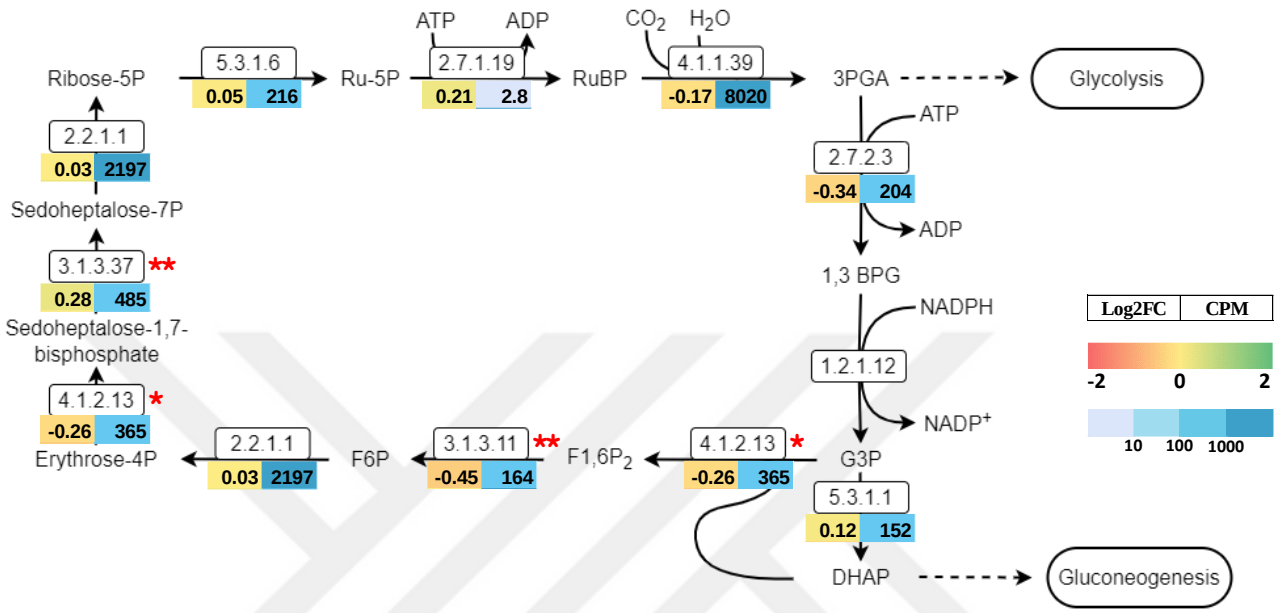


Figure 7.12. Calvin cycle (CO_2 assimilation). Enzymes include: phosphoglycerate kinase (EC: 2.7.2.3), glyceraldehyde-3-phosphate dehydrogenase (EC: 1.2.1.12), triosephosphate isomerase (EC: 5.3.1.1), fructose-bisphosphate aldolase (EC: 4.1.2.13), fructose-bisphosphatase (EC: 3.1.3.11), transketolase (EC: 2.2.1.1), sedoheptulose-bisphosphatase (EC: 3.1.3.37), ribose-5-phosphate isomerase (EC: 5.3.1.6), phosphoribulokinase (EC: 2.7.1.19), ribulose-bisphosphate carboxylase (RuBisCO, EC: 4.1.1.39). Please refer to Figure 7.7 and Figure 7.9 for figure legends and marks.

Strong induction of glucose-6-P-isomerase (EC: 5.3.1.9), phosphoglycerate mutase (EC: 5.4.2.12), phosphopyruvate hydratase (EC: 4.2.1.11), pyruvate kinase (EC: 2.7.1.40), pyruvate dehydrogenase E2 component (EC: 2.3.1.12), and substantial reduction of fructose-bisphosphatase (EC: 3.1.3.11) and phosphoenolpyruvate (ATP) carboxykinase (EC: 4.1.1.49) in glycolysis pathway were observed (Figure 7.13). The upregulated ones are related to glycolysis, and the downregulated ones are the enzymes responsible in gluconeogenesis-like metabolism. The E2 component of pyruvate dehydrogenase (EC: 2.3.1.12) acts in the second step of the conversion of pyruvate to acetyl CoA. Acetyl CoA is the first precursor of fatty acid biosynthesis and also enters the citrate cycle along with OAA (Figure 7.14). Data also suggests that all the enzymes in the metabolic chain from 3PGA to pyruvate are strongly induced. That is important as pyruvate from glycolysis along with G3P from the Calvin cycle are substrates of DOXP synthase enzyme which catalyzes the synthesis of 1-deoxy-D-xylulose 5-phosphate, which is the initial substrate of MEP/DOXP pathway in terpenoid backbone

biosynthesis (Borhan et al., 2022). There was strong upregulation observed, of the enzymes starch phosphorylase (EC: 2.4.1.1), alpha-amylase (EC: 3.2.1.1), beta-amylase (EC: 3.2.1.2), 4-alpha-glucanotransferase (EC: 2.4.1.25), and alpha-glucosidase (EC: 3.2.1.20), which are all related to carbohydrate catabolism (Figure 7.15).

There was a substantial upregulation of the enzyme isocitrate dehydrogenase (EC: 1.1.1.42), which converts isocitrate into 2-oxoglutarate via two consecutive steps using $NADP^+$ as e^- acceptor (Figure 7.14). 2-oxoglutarate is important because it acts in ammonia assimilation into glutamine and glutamate. There is a phenomenon in phytoplankton that when amino acid synthesis is metabolically activated, carbohydrate storage is repressed (Behrenfeld et al., 2008). A somewhat similar pattern can be discussed in *T. suecica* exposed to copper, as strong enrichment in nitrogen assimilation, downregulation in the gluconeogenesis-like pathway, and upregulation in enzymatic production of 2-oxoglutarate.

There was a substantial decrease in the expression of trehalose phosphate synthase (TPS, EC: 2.4.1.15), which is one of the two enzymes responsible for the 2-step conversion of UDP-glucose to trehalose (Figure 7.15). Trehalose is a known stress protector of yeasts, fungi, and plants, working by the stabilization of proteins and cell membrane (Duman, Aksoy, Aydin, & Temizgul, 2011). Its increased expression in yeast favors heavy metal tolerance of the organism (T. Liu et al., 2017). That is not what was observed in the present case. That might be explained by the strong upregulation of UDP glucose pyrophosphorylase (EC: 2.7.7.9), which converts glucose-1-phosphate into UDP-glucose whose level might have got so high that TPS expression decreased although trehalose production still increased. Actual trehalose concentration was not measured in Cu-exposed cells; this one should be examined in later studies for verification.

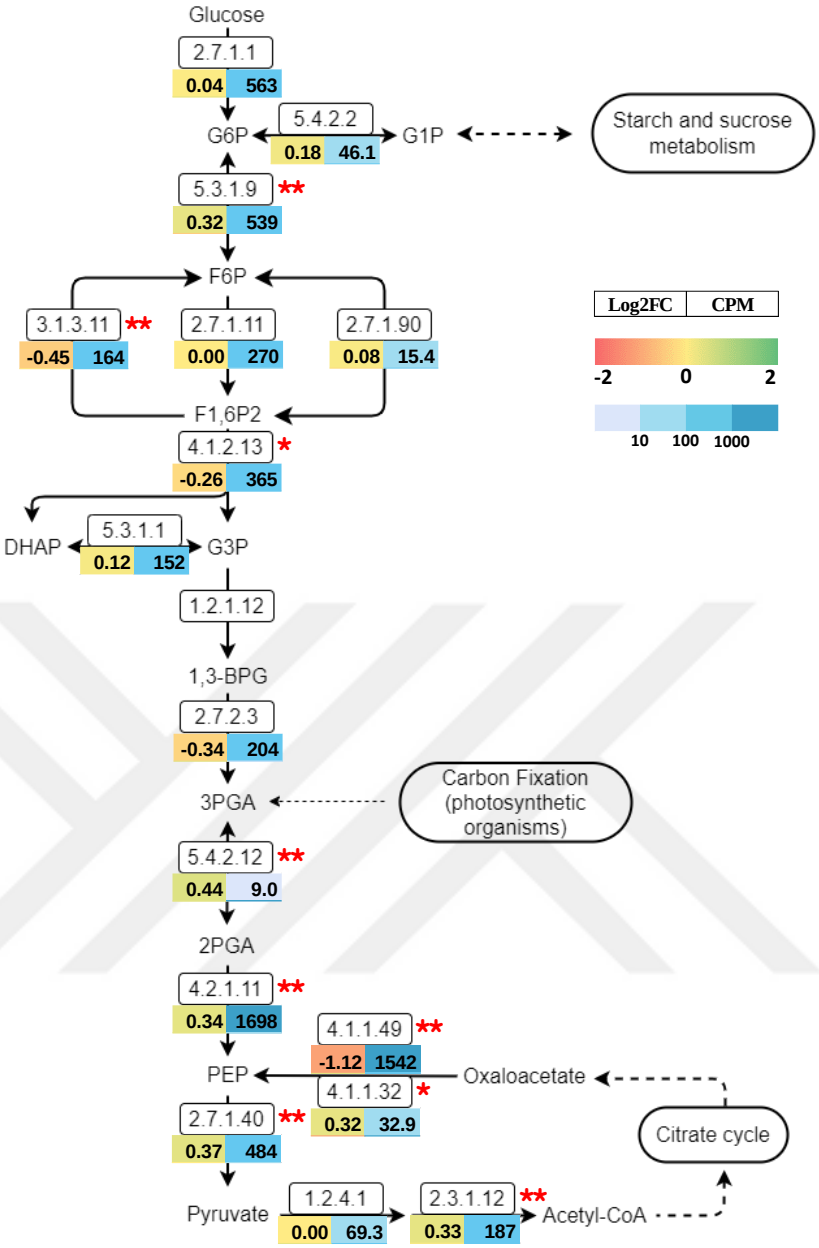


Figure 7.13. Glycolysis. Enzymes include: hexokinase (EC: 2.7.1.1), phosphoglucomutase (EC: 5.4.2.2), glucose-6-phosphate isomerase (EC: 5.3.1.9), fructose-bisphosphatase (EC: 3.1.3.11), 6-phosphofructokinase (EC: 2.7.1.11), diphosphate-dependent phosphofructokinase (EC: 2.7.1.90), fructose-bisphosphate aldolase (EC: 4.1.2.13), triosephosphate isomerase (EC: 5.3.1.1), phosphoglyceraldehyde dehydrogenase (EC: 1.2.1.12), phosphoglycerate kinase (EC: 2.7.2.3), phosphoglycerate mutase (EC: 5.4.2.12), phosphopyruvate hydratase (EC: 4.2.1.11), pyruvate kinase (EC: 2.7.1.40), pyruvate dehydrogenase E1 component (EC: 1.2.4.1), pyruvate dehydrogenase E2 component (EC: 2.3.1.12), phosphoenolpyruvate (ATP) carboxykinase (EC: 4.1.1.49), phosphoenolpyruvate (GTP) carboxykinase (EC: 4.1.1.32). Please refer to Figure 7.7 and Figure 7.9 for figure legends and marks.

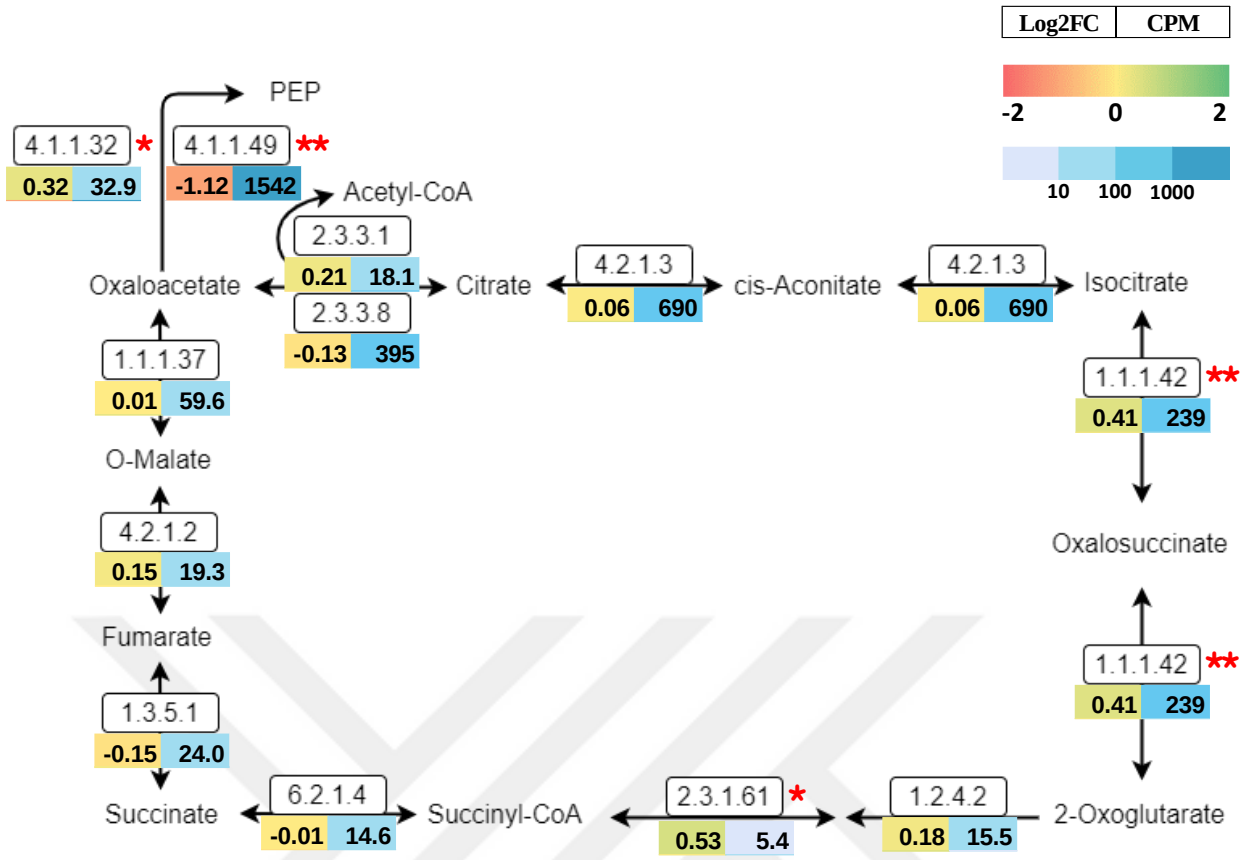


Figure 7.14. Citric acid cycle (TCA cycle). Enzymes include: phosphoenolpyruvate (GTP) carboxykinase (EC: 4.1.1.32), phosphoenolpyruvate (ATP) carboxykinase (EC: 4.1.1.49), malate dehydrogenase (EC: 1.1.1.37), fumarate hydratase (EC: 4.2.1.2), succinate dehydrogenase (EC: 1.3.5.1), succinyl-CoA synthetase (EC: 6.2.1.4), 2-oxoglutarate dehydrogenase E2 component (dihydrolipoamide succinyltransferase, EC: 2.3.1.61), 2-oxoglutarate dehydrogenase E1 component (EC: 1.2.4.2), isocitrate dehydrogenase (EC: 1.1.1.42), aconitate hydratase (EC: 4.2.1.3), citrate (Si)-synthase (EC: 2.3.3.1), ATP-citric lyase (EC: 2.3.3.8). Please refer to Figure 7.7 for figure legends and marks.

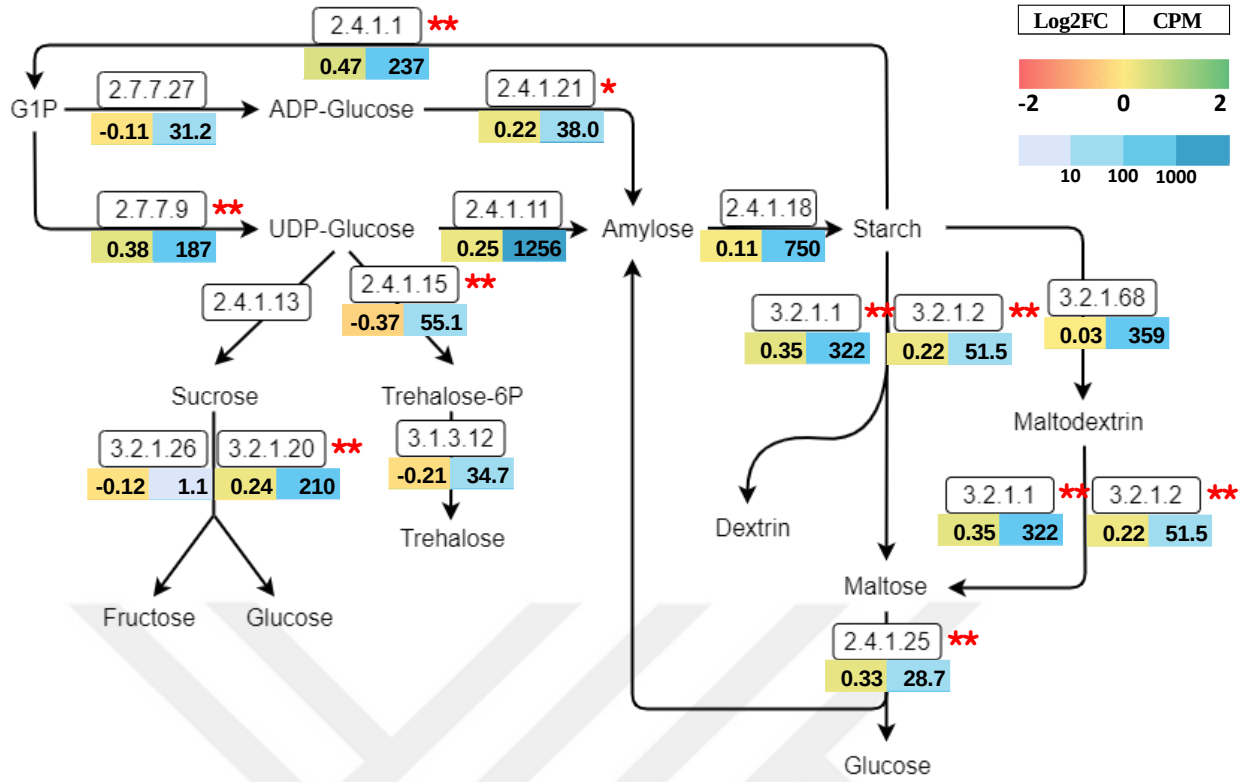


Figure 7.15. Starch and sucrose metabolism. Enzymes include: starch phosphorylase (EC: 2.4.1.1), glucose-1-phosphate adenylyltransferase (EC: 2.7.7.27), starch synthase (EC: 2.4.1.21), UDP glucose pyrophosphorylase (EC: 2.7.7.9), glycogen (starch) synthase (EC: 2.4.1.11), 1,4-alpha-glucan branching enzyme (EC: 2.4.1.18), alpha-amylase (EC: 3.2.1.1), beta-amylase (EC: 3.2.1.2), 4-alpha-glucanotransferase (EC: 2.4.1.25), isoamylase (EC: 3.2.1.68), sucrose synthase (EC: 2.4.1.13), alpha-glucosidase (EC: 3.2.1.20), beta-fructofuranosidase (EC: 3.2.1.26), trehalose phosphate synthase (EC: 2.4.1.15), trehalose-phosphatase (EC: 3.1.3.12). Please refer to Figure 7.7 and Figure 7.9 for figure legends and marks.

7.3.8. Lipid Metabolism

Fatty acid (FA) biosynthesis starts with the conversion of acetyl CoA to malonyl CoA by acetyl-CoA carboxylase (EC: 6.4.1.2). The required acetyl CoA pool could be supplied either from glycolysis through conversion of pyruvate (enzymes: 1.2.4.1 and 2.3.1.12) or from citrate (enzyme: 2.3.3.8) in citrate cycle (refer to Figure 7.13 and Figure 7.14, see relevant text) (de Jaeger et al., 2018). Malonyl CoA is then converted to malonyl-ACP by malonyl-CoA:ACP transacylase (EC: 2.3.1.39). Subsequently, a cycle of four enzymatic reactions starts (Borhan et al., 2022). Malonyl-ACP is added to an acyl-ACP enzymatically by beta-ketoacyl-ACP synthase I (EC: 2.3.1.41) forming a beta-ketoacyl-ACP. Then this compound undergoes a reduction by 3-oxoacyl-ACP reductase (EC: 1.1.1.100), turning into a beta-hydroxyacyl-ACP. The formed compound is dehydrated by 3-

hydroxyacyl-ACP dehydratase (EC: 4.2.1.59) to a trans-2-enoyl-ACP. Finally, a cycle ends via the reduction of trans-2-enoyl-ACP by the breakup of the C-C double bond into an acyl-ACP, done by enoyl-ACP reductase (EC: 1.3.1.9). Each cycle adds 2 carbon to acyl-ACP. After 6 cycles hexadecanoyl-ACP is formed. An additional cycle starts with hexadecanoyl-ACP, where beta-ketoacyl-ACP synthase II (EC: 2.3.1.179) instead of 2.3.1.41 is responsible for the first enzymatic reaction of the cycle, ultimately ending with the formation of octadecanoyl-ACP (Blasio & Balzano, 2021). A substantial upregulation was observed in three fatty acid biosynthesis enzymes upon Cu exposure (Figure 7.16): 6.4.1.2, 1.1.1.100, and 1.3.1.9. Copper deficiency may increase fatty acid biosynthesis (Castruita et al., 2011). Present transcriptomic findings suggest that copper excess might also increase the production of fatty acids. Additionally, there was a very significant upregulation ($p<10^{-6}$) in the expression of long-chain-fatty-acid---CoA ligase (EC: 6.2.1.3), responsible for converting a fatty acid into an acyl CoA. This acyl CoA can then either enter glycerolipid metabolism for triacylglycerol (TAG) production or undergo beta-oxidation. Two of the TAG biosynthesis enzymes, glycerol-3-phosphate 1-O-acyltransferase (EC: 2.3.1.15) and 1-acylglycerol-3-phosphate O-acyltransferase (EC: 2.3.1.51) were upregulated in the Cu-exposed set.

There was a strong induction ($p<10^{-5}$) of the enzyme enoyl-CoA hydratase (EC: 4.2.1.17) observed upon copper exposure to *T. suecica*. This enzyme is present in both fatty acid elongation and fatty acid beta-oxidation pathways (Figure 7.17.A, Figure 7.18), responsible for opposite reactions. It is virtually impossible to determine in which direction the reaction enhanced from *de novo* transcriptomic data. In the beta-oxidation pathway the enzyme acyl-CoA oxidase (EC: 1.3.3.6), which is absent in fatty acid elongation, was substantially downregulated ($p<0.001$). Additionally, other enzymes in fatty acid degradation (except for 4.2.1.17) were all downregulated, although not all of them were significant. One might consider that there was a cumulative downregulation of beta-oxidation, and the acyl CoA synthesized by the enzyme 6.2.1.3 mostly underwent TAG biosynthesis. Several studies in the literature have associated HM exposure with an increase in TAG biosynthesis in microalgae (Lu et al., 2019; Tripathi, Kairamkonda, Gupta, & Poluri, 2023). All the enzymes that I annotated to the long-chain fatty acid elongation pathway were upregulated (Figure 7.16.B), of which the induction of very-long-chain 3-ketoacyl-CoA synthase (EC: 2.3.1.199) and very-long-chain enoyl-CoA reductase (EC: 1.3.1.93) were very strong ($p<10^{-5}$).

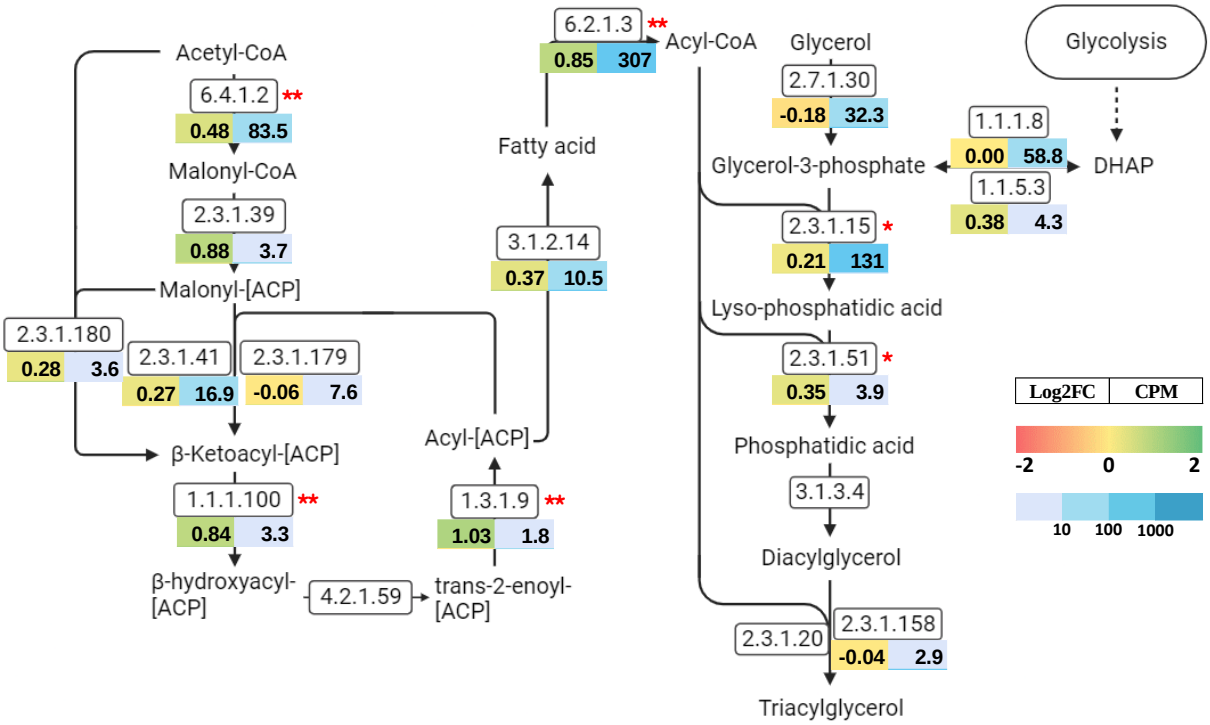


Figure 7.16. Fatty acid biosynthesis and TAG production. Enzymes include: Beta-ketoacyl-(acyl-carrier-protein) synthase I (EC: 2.3.1.41), beta-ketoacyl-(acyl-carrier-protein) synthase II (EC: 2.3.1.179), beta-ketoacyl-(acyl-carrier-protein) synthase III (EC: 2.3.1.180), 3-oxoacyl-(acyl-carrier-protein) reductase (EC: 1.1.1.100), acetyl-CoA carboxylase (EC: 6.4.1.2), malonyl-CoA:ACP transacylase (EC: 2.3.1.39), 3-hydroxyacyl-(acyl-carrier-protein) dehydratase (EC: 4.2.1.59), enoyl-(acyl-carrier-protein) reductase (EC: 1.3.1.9), oleoyl-(acyl-carrier-protein) hydrolase (EC: 3.1.2.14), long-chain-fatty-acid---CoA ligase (EC: 6.2.1.3), glycerol kinase (EC: 2.7.1.30), glycerol-3-phosphate 1-O-acyltransferase (EC: 2.3.1.15), 1-acylglycerol-3-phosphate O-acyltransferase (EC: 2.3.1.51), phosphatidate phosphatase (EC: 3.1.3.4), diacylglycerol O-acyltransferase (EC: 2.3.1.20), phospholipid:diacylglycerol acyltransferase (EC: 2.3.1.158), glycerol-3-phosphate dehydrogenase (NAD+) (EC: 1.1.1.8), FAD-dependent glycerol-3-phosphate dehydrogenase (EC: 1.1.5.3). Please refer to Figure 7.7 and Figure 7.9 for figure legends and marks.

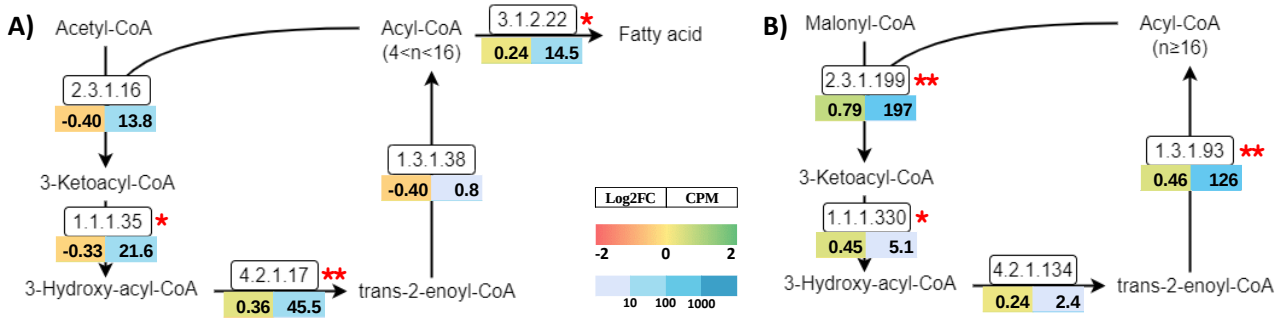


Figure 7.17. Fatty acid elongation for the molecules up to C16 (A), and very long chain fatty acids (B). Enzymes include: acetyl-CoA C-acyltransferase (EC: 2.3.1.16), 3-hydroxyacyl-CoA dehydrogenase (EC: 1.1.1.35), enoyl-CoA hydratase (EC: 4.2.1.17), NADPH-dependent trans-2-enoyl-CoA reductase (EC: 1.3.1.38), palmitoyl-protein hydrolase (EC: 3.1.2.22), very-long-chain 3-ketoacyl-CoA synthase (EC: 2.3.1.199), very-long-chain 3-ketoacyl-CoA reductase (EC: 1.1.1.330), very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase (EC: 4.2.1.134), very-long-chain enoyl-CoA reductase (EC: 1.3.1.93). Please refer to Figure 7.7 for figure legends and marks.

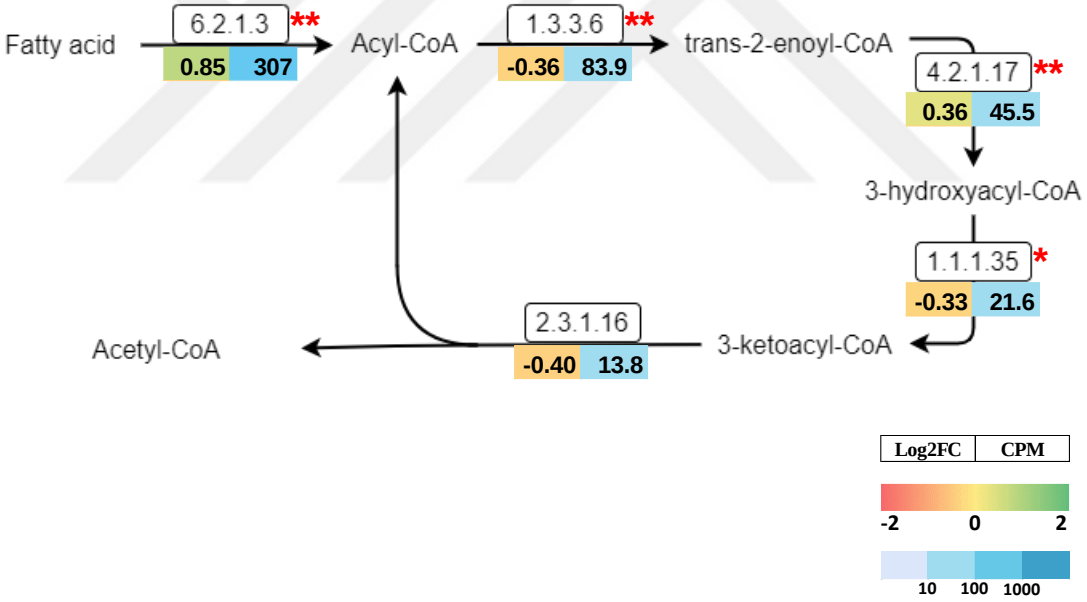


Figure 7.18. Fatty acid beta-oxidation. Enzymes include: long-chain acyl-CoA synthetase (EC: 6.2.1.3), acyl-CoA oxidase (EC: 1.3.3.6), enoyl-CoA hydratase (EC: 4.2.1.17), 3-hydroxyacyl-CoA dehydrogenase (EC: 1.1.1.35), acetyl-CoA C-acyltransferase (EC: 2.3.1.16). Please refer to Figure 7.7 for figure legends and marks.

7.3.9. Mechanisms of the Consortium as Revealed in This Study

The results of Chapter 6 indicate a symbiotic interaction within the consortium in the presence of BAC and Cu. In light of the results of Chapter 7, a reciprocal toxicity reduction mechanism is

proposed as follows: The presence of BAC inhibits the growth of *T. suecica*. *P. sessilinigenes* BIOMIG1 mitigates this toxicity through the biodegradation of BAC. When copper is also present, the BAC-degrading activity of BIOMIG1 is hindered. *T. suecica* removes copper through adsorption and assimilation, with assimilation being the predominant mechanism. *T. suecica* cells primarily uptake copper through ZIP transporters, not Ctr channels. Copper is toxic to *T. suecica*, and the algae overcome Cu toxicity by upregulating the efflux pumps belonging to the MATE, MTP, and HMA transporter families. These pumps remove copper from the cytosol into vacuoles or outside of the cells. These mechanisms are summarized in Figure 7.19.

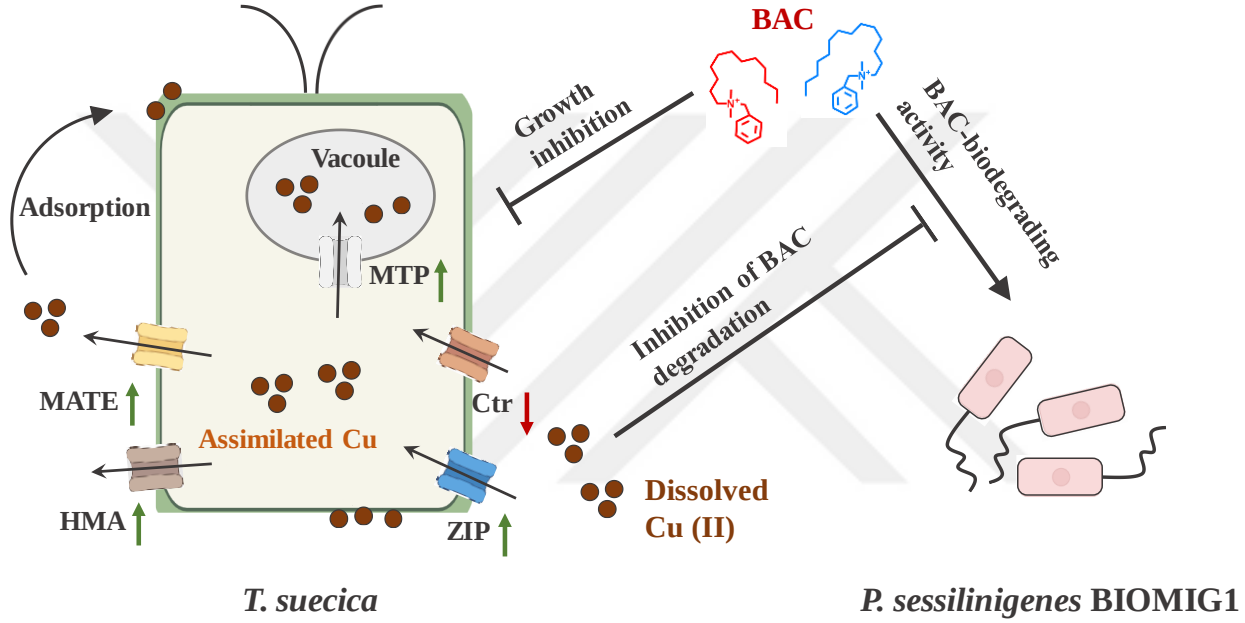


Figure 7.19. The mechanisms within the consortium against BAC and Cu, elucidated in this study

7.4. Summary

In the preceding chapters, the HM tolerance of *Tetraselmis suecica* was shown to be higher than that of the other microalgae studied in this dissertation. Additionally, the removal of copper by *T. suecica* supported the survival of the bacteria *P. sessilinigenes* BIOMIG1, as demonstrated in Chapter 6. This chapter aimed to elucidate the copper tolerance and removal mechanisms of *T. suecica* at the transcriptomic level. *T. suecica* was cultivated in the presence of 1 mg Cu/L, and the growth and copper accumulation were monitored. Transcriptome analysis was conducted using RNA samples extracted on Day 1.

36354 transcripts were identified with count data in either the control or Cu-exposed groups. Among these, 4403 were deemed significant with an FDR below 0.05. The differentially expressed

transcripts (DETs) that belong to the ZIP family of heavy metal importers were predominantly upregulated. Two DETs belonging to the copper import family (Ctr) were identified, both of which were downregulated. The DETs related to metal efflux from the cytoplasm, specifically MATE, MTP, and HMA family proteins, were upregulated. No significant alterations were observed in oxidative stress-related metabolisms, such as glutathione metabolism, and the expression of antioxidants remained largely unchanged.

Upon exposure to copper, increased expressions were observed in pathways related to carbohydrate catabolism, nitrogen assimilation, fatty acid biosynthesis, and long-chain fatty acid elongation. Some F- and V-type ATPases of oxidative phosphorylation, along with certain components of photosystem I and photosystem II were upregulated. Cytochrome c6, however, was downregulated. These findings provide insight into how energy generation in microalgae is affected by heavy metal exposure, specifically copper ions, and how the energy is stored or utilized in the form of organic carbon.

8. CONCLUSIONS

MPs are ubiquitous in wastewater all around the world. Discharge of MPs mistreated in wastewater into receiving environments may lead to environmental and human health hazards. Economically viable MP treatment options are necessary to achieve and maintain sustainable development. Due to the diverse chemistry of MPs and their presence together in wastewater, treatment become a more complicated issue and conventional biological treatment is ineffective in solving the problem. In this dissertation, a biological treatment option involving algae and bacteria in a consortium for application to "hard to treat" wastewaters containing HMs and ECs together was proposed. For this main objective, specific microalgae and bacterial species were selected, the viability and activity of their co-culture were determined, and the treatment of selected MPs by their consortium was evaluated. Metabolic interactions during treatment were also investigated. Hazardous HMs arsenic, cadmium, and lead, HMs commonly found in wastewaters, copper and zinc, and a very common surfactant used as an ingredient in consumer products for hygiene purposes, BAC, were selected as the contaminants of interest.

Firstly, the tolerance of microalgae from various taxa against HMs and BAC was evaluated. IC_{50} values showed that the best challenger against cadmium, copper, zinc, and BAC was the marine microalgae, *Tetraselmis suecica*. This strain also showed a good performance for the removal of cadmium and copper from a synthetic media containing a mixture of the salts of those 5 HMs. Second, a bacterial strain that could degrade BAC and which could tolerate HMs was identified. A BAC-degrader strain, *P. sessilinigenes* BIOMIG1, was challenged against HMs for the inhibition of growth and BAC degradation activity. Arsenic and cadmium inhibited both growth and BAC degradation above certain levels. BIOMIG1 was resilient against increasing concentrations of lead and zinc. Copper did not affect growth, but inhibited BAC degrading activity.

A consortium of *T. suecica* and *P. sessilinigenes* BIOMIG1 was constructed in an optimized 8‰ F/2 culture medium on an organic substrate, BDMA, which is a biotransformation product of BAC, and can be degraded by BIOMIG1. Without bacteria, *T. suecica* ceased to grow and culture density decreased in the presence of BDMA. When BIOMIG1 was introduced, a slight growth of *T. suecica* was observed in the reactors started with low initial algae density. No algae growth was observed but algae maintained their health for a longer period in the consortium reactors started with higher initial algae density. The dynamic relationship between algae and bacteria indeed depended on the inoculum ratio. The removal rate of BDMA was similar in sole bacteria and consortium sets. A further research

topic could be how consortium members interchange O_2 and CO_2 during the elimination of an EC.

The results of this study revealed that BAC removal could occur in the absence of bacteria through adsorption onto microalgae biomass or biotransformation by microalgae. A key determinant for the removal mechanism was the concentration of BAC in the media. BAC was toxic to microalgae, and adsorption was the prevalent mechanism at higher BAC concentrations. Adsorption was also more pronounced in the presence of copper. The genetic background of BAC biotransformation by *T. suecica* was different from the mechanism of BAC degradation by *P. sessilinigenes* BIOMIG1. BAC biotransformation by *T. suecica* was also slower, making it less viable for the treatment of BAC using only algae. Additionally, outcomes of this study unveiled that the treatment of a synthetic media containing BAC and copper could be achieved using a consortium of *T. suecica* and *P. sessilinigenes* BIOMIG1 at 20 ppm BAC and up to 1 ppm Cu. The removal mechanisms for BAC included bacterial biotransformation and adsorption onto microalgae biomass. Copper removal primarily occurred through adsorption onto and assimilation into microalgae biomass.

In this study, the mechanisms behind the mutually beneficial interactions between consortium members were also investigated. The results showed that the presence of bacteria remediated the growth inhibition as well as the reduction in fluorescence intensity, which is an indicator of the chlorophyll content of the algae. The outcomes of this study also demonstrated that the presence of microalgae favored the bacteria to recover inhibition of growth and BAC-degrading activity against increasing concentrations of copper. The effect of copper exposure on *T. suecica* was also investigated to elucidate the mechanisms within algae cells while treating copper from the media. The transcriptomic study unveiled expressional changes in distinct fundamental pathways such as energy, carbohydrate, fatty acid, nitrogen, and various amino acid metabolisms. The results suggested an increase in carbohydrate catabolism coupled with increased fatty acid synthesis and elevated nitrogen assimilation. These results might give insights into how the energy generated through photosynthesis and oxidative phosphorylation was utilized in different forms of fixed carbon upon exposure to HMs. The evidence from transcriptional analysis also suggested the increased expression of ZIP, MTP, and MATE family transporters, on the other hand, no major expressional changes in oxidative stress-related metabolisms. These results offer clues regarding how microalgae cope with copper in the media and elaborate copper assimilation mechanisms. The results of this study have also raised new questions regarding the cellular compartments for copper decomposition in *T. suecica*, the fatty acid production potential and characterization of produced fatty acids, and the sugars that might be generated through carbohydrate catabolism upon copper exposure.

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APPENDIX A: PSC GENE-BEARING MICROALGAE AND CYANOBACTERIA

Table A.1. Algae that express genes translated into PCS

Type	Classification	Species name	Strain ID	# of hits	PCS NCBI ID
Green algae	Chlorodendrophyceae	*Tetraselmis sp.*	GSL018	5	Jinkerson et al., 2014 5 predicted (2 partial), JAC79773.1
Green algae	Chlorophyceae	*Monoraphidium neglectum*		2	Bogen et al., 2013 1 predicted, 1 hypothetical, KIZ05459.1
Green algae	Chlorophyceae	*Tetrabaena socialis*	NIES-571	2	Featherston et al., 2017 1 predicted, 1 hypothetical, PNH12509.1
Green algae	Chlorophyceae	*Chlamydomonas eustigma*		2	Hirooka et al., 2017; hypothetical, GAX77974.1
Green algae	Chlorophyceae	*Chlamydomonas reinhardtii*		2	Merchant et al., 2007 hypothetical, PNW73374.1
Green algae	Chlorophyceae	*Gonium pectorale*		2	Hanschen et al., 2017 hypothetical, KXZ56469.1
Green algae	Chlorophyceae	*Volvox carteri f. nagariensis*		2	Prochnik et al., 2010; hypothetical, EFJ45552.1
Green algae	Klebsormidiophyceae	*Klebsormidium nitens*		2	Hori et al., 2014, predicted, GAQ78081.1
Green algae	Mamiellophyceae	*Micromonas pusilla*	CCMP1545	1	Worden et al., 2009 predicted protein, partial, EEH55879.1
Green algae	Mamiellophyceae	*Micromonas commoda*	RCC299 / NOUM17 / CCMP2709	1	Worden et al., 2009 hypothetical, ACO65712.1
Green algae	Trebouxiophyceae	*Auxenochlorella protothecoides*		3	Gao et al., 2014; predicted, KFM27944.1 Heller, 2015; hypothetical, JAT68536.1
Green algae	Trebouxiophyceae	*Chlorella variabilis*		1	Blanc et al., 2010, hypothetical, EFN53566.1
Green algae	Trebouxiophyceae	*Chlorella sorokiniana*		1	Arriola et al., 2018, predicted, PRW61025.1
Green algae	Trebouxiophyceae	*Coccomyxa subellipsoidea*	C-169	1	Blanc et al., 2010 hypothetical, partial, XP_005643662.1
Green algae	Trebouxiophyceae	*Helicosporidium sp.*	ATCC 50920	1	Pombert et al., 2014 hypothetical, partial, KDD74482.1
Green algae	Trebouxiophyceae	*Micractinium conductrix*		1	Arriola et al., 2018, predicted, GBG30561.1
Red algae	Cyanidiophyceae	*Cyanidioschyzon merolae*	10D	1	Matsuzaki et al., 2004 probable PCS, BAM80001.1
Red algae	Porphyridiophyceae	*Porphyridium purpureum*		1	Tong,S, 2016, ARS02979.1
Diatoms	Bacillariophyceae	*Amphora acutiuscula*		1	Nguyen-Deroche et al., 2012 predicted, partial, CBP94208.1
Diatoms	Bacillariophyceae	*Amphora coffeiformis*		1	Nguyen-Deroche et al., 2012 predicted, partial, CBP94208.1
Diatoms	Bacillariophyceae	*Nitzschia palea*		1	Nguyen-Deroche et al., 2012 predicted, partial, CBP94208.1

Diatoms	Bacillariophyceae	*Entomoneis paludosa*		2	Nguyen-Deroche et al., 2012 predicted, partial, CBP94208.1
Diatoms	Bacillariophyceae	*Fistulifera solaris*		6	Tanaka et al., 2015 2 predicted, 4 hypothetical, GAX21495.1
Diatoms	Bacillariophyceae	*Phaeodactylum tricornutum*	CCAP 1055/1	3	Bowler et al., 2008 predicted protein, EEC45818.1
Diatoms	Bacillariophyceae	*Fragilariopsis cylindrus*	CCMP1102	3	Mock et al., 2017 2 predicted, 1 hypothetical, OEU14151.1
Diatoms	Mediophyceae	*Thalassiosira pseudonana*	CCMP1335	6	Gupton-Campolongo et al., 2013 1 predicted, 2 PCS-like, AET85547.1 Armbrust et al., 2004; 1 PCS-like, 2 hypothetical, all partial, ACI64846.1
Diatoms	Mediophyceae	*Thalassiosira oceanica*		2	Lommer et al., 2012; hypothetical, EJK76788.1
Cyanobacteria	Nostocophycidae	*Anabaena (Trichormus) variabilis*	1) NIES-23 2) ATCC 29413 / PCC 7937	3	1) Hirose et al., 2017; hypothetical, BAY71948.1 2) Copeland et al., 2005 1 predicted, 1 hypothetical, ABA22569.1
Cyanobacteria	Nostocophycidae	*Anabaena sp.*	90	1	Wang et al., 2012; predicted, AFW93374.1
Cyanobacteria	Nostocophycidae	*Anabaenopsis circularis*	NIES-21	3	Hirose et al., 2017; 1 predicted, 1 plasmid, 1 hypothetical, BAY18879.1
Cyanobacteria	Nostocophycidae	*Aulosira laxa*	NIES-50	2	Hirose et al., 2017; 1 plasmid, 1 hypothetical, BAZ78561.1
Cyanobacteria	Nostocophycidae	*Calothrix brevissima*	NIES-22	2	Hirose et al., 2017; hypothetical, BAY65935.1
Cyanobacteria	Nostocophycidae	*Calothrix sp.*	336/3	1	Isojarvi et al., 2015; predicted, AKG24506.1
Cyanobacteria	Nostocophycidae	*Calothrix sp.*	PCC 6303	1	Gugger et al., 2012; predicted, AFZ04119.1
Cyanobacteria	Nostocophycidae	*Calothrix sp.*	NIES-4101	1	Hirose et al., 2017; predicted, BAZ37535.1
Cyanobacteria	Nostocophycidae	*Calothrix sp.*	1) NIES-2098 2) NIES-2100 3) NIES-4071 4) NIES-4105	4	Hirose et al., 2017 4 hypothetical, BAY10531.1, BAY24677.1, BAZ09562.1, BAZ55710.1
Cyanobacteria	Nostocophycidae	*Cylindrospermum sp.*	NIES-4074	1	Hirose et al., 2017; predicted, BAZ31631.1
Cyanobacteria	Nostocophycidae	*Cylindrospermum stagnale*	PCC 7417	1	Gugger et al., 2012; predicted, AFZ23969.1
Cyanobacteria	Nostocophycidae	*Dolichospermum compactum*	NIES-806	1	Hirose et al., 2017; predicted, BAZ83962.1
Cyanobacteria	Nostocophycidae	*Fischerella muscicola*	CCMEE 5323	1	Miller, 2017; predicted, PLZ88465.1
Cyanobacteria	Nostocophycidae	*Fischerella sp.*	NIES-4106	1	Hirose et al., 2017; hypothetical, BAZ65889.1
Cyanobacteria	Nostocophycidae	*Fischerella thermalis*	CCMEE 5201	2	Miller, 2017; predicted, PMB51113.1
Cyanobacteria	Nostocophycidae	*Fremyella diplosiphon*	NIES-3275	1	Hirose et al., 2017; hypothetical, BAY93481.1
Cyanobacteria	Nostocophycidae	*Hapalosiphon sp.*	MRB220	1	Tan et al., 2015; predicted, KOP24831.1

Cyanobacteria	Nostocophycidae	*Hassallia byssoidea*	VB512170	1	Singh et al., 2015; predicted, KIF36727.1
Cyanobacteria	Nostocophycidae	*Mastigocladus laminosus*	UU774	1	Tripathy et al., 2015; predicted, KIY11345.1
Cyanobacteria	Nostocophycidae	*Nodularia sp.*	NIES-3585	1	Hirose et al., 2017; hypothetical, GAX37371.1
Cyanobacteria	Nostocophycidae	*Nostoc carneum*	NIES-2107	1	Hirose et al., 2017; hypothetical, BAY32502.1
Cyanobacteria	Nostocophycidae	*Nostoc cycadae*	WK-1	2	Kanesaki et al., 2016; predicted, GBE95157.1
Cyanobacteria	Nostocophycidae	*Nostoc flagelliforme*	CCNUN1	1	Shang, 2017; predicted, AUB34292.1
Cyanobacteria	Nostocophycidae	*Nostoc linckia*	NIES-25	1	Hirose et al., 2017; hypothetical, BAY73860.1
Cyanobacteria	Nostocophycidae	*Nostoc piscinale*	CENA21	1	Leao et al., 2016; predicted, ALF51813.1
Cyanobacteria	Nostocophycidae	*Nostoc punctiforme*	ATCC 29133 / PCC 73102	1	Ekman et al., 2013; predicted, ACC81212.1
Cyanobacteria	Nostocophycidae	*Nostoc sp.*	1) ATCC 29411 / PCC 7524 2) PCC 7107	3	Gugger et al., 2012 3 predicted, AFY46544.1, AFY42625.1
Cyanobacteria	Nostocophycidae	*Nostoc sp.*	PCC 7120 / SAG 25.82 / UTEX 2576	1	Tsuji et al., 2004; primitive PCS, BAD10973.1
Cyanobacteria	Nostocophycidae	*Nostoc sp.*	1) 106C 2) T09 3) RF31Y	3	Cruz-Morales et al., 2017 3 predicted, OUL31578.1, OUL37007.1, OUL26371.1
Cyanobacteria	Nostocophycidae	*Nostoc sp.*	CENA543	2	Shishido et al., 2017; predicted, AUS99426.1
Cyanobacteria	Nostocophycidae	*Nostoc sp.*	KVJ20	1	Altermark et al., 2016; predicted, ODG99424.1
Cyanobacteria	Nostocophycidae	*Nostoc sp.*	MBR 210	2	Te et al., 2016; predicted, OCQ94811.1
Cyanobacteria	Nostocophycidae	*Nostoc sp.*	1) NIES-2111 2) NIES-3756 3) NIES-4103	6	Hirose et al., 2017 1) 2 hypothetical, BAY37586.1 2) 1 putative, 1 hypothetical, BAT53535.1 3) 1 predicted, 1 putative BAZ52398.1
Cyanobacteria	Nostocophycidae	*Nostoc sp.* '*Lobaria pulmonaria* (5183) cyanobiont'	5183	1	Gagunashvili, 2018; predicted, AVH69366.1
Cyanobacteria	Nostocophycidae	*Nostoc sp.* '*Peltigera membranacea* cyanobiont'	210A	1	Gagunashvili and Andresson, 2017 hypothetical, OYD93044.1
Cyanobacteria	Nostocophycidae	*Nostocales cyanobacterium*	HT-58-2	1	Hughes et al., 2017; predicted, ARV58729.1
Cyanobacteria	Nostocophycidae	*Scytonema millei*	VB511283	1	Sen et al., 2015; predicted, ODN56621.1
Cyanobacteria	Nostocophycidae	*Scytonema sp.*	NIES-4073	3	Hirose et al., 2017 2 predicted, 1 plasmid, BAZ20094.1
Cyanobacteria	Nostocophycidae	*Scytonema sp.*	HK-05	1	Hirose et al., 2017; hypothetical, BAY43071.1
Cyanobacteria	Nostocophycidae	*Scytonema tolypothrichoides*	VB-61278	1	Das et al., 2015; predicted, KIJ84765.1

Cyanobacteria	Nostocophycidae	*Tolypothrix campylonemoides*	VB511288	1	Das et al., 2015; predicted, KIJ79018.1
Cyanobacteria	Nostocophycidae	*Tolypothrix sp.*	NIES-4075	1	Hirose et al., 2017; predicted, GAX41154.1
Cyanobacteria	Nostocophycidae	*Tolypothrix sp.*	PCC 7601	1	Yerrapragada et al., 2015; putative, EKE99458.1
Cyanobacteria	Nostocophycidae	*Tolypothrix tenuis*	PCC 7101	2	Hirose et al., 2017 1 plasmid, 1 hypothetical, BAZ03166.1
Cyanobacteria	Oscillatoriophycidae	*Chroococcidiopsis thermalis*	PCC 7203	1	Gugger et al., 2012; predicted, AFY87892.1
Cyanobacteria	Oscillatoriophycidae	*Coleofasciculus chthonoplastes*	PCC 7420	2	Tandeau et al., 2008; PSC family, EDX74565.1
Cyanobacteria	Oscillatoriophycidae	*Crinalium epipsammum*	PCC 9333	2	Gugger et al., 2012; 2 predicted, AFZ11668.1
Cyanobacteria	Oscillatoriophycidae	*cyanobacterium TDX16*		1	Dong and Xing, 2018; predicted, OWY66217.1
Cyanobacteria	Oscillatoriophycidae	*Cyanothece sp.*	1) PCC 7425 / ATCC 29141 2) PCC 7822 3) CCY0110 4) ATCC 51142	4	1) Lucas et al., 2009; predicted, ACL46092.1 2) Lucas et al., 2010; predicted, ADN15573.1 3) Stal et al., 2007; predicted, EAZ91501.1 4) Welsh et al., 2008; putative, ACB51246.1
Cyanobacteria	Oscillatoriophycidae	*Geitlerinema sp.*	PCC 7407	1	Gugger et al., 2012; predicted, AFY66209.1
Cyanobacteria	Oscillatoriophycidae	*Hydrocoleum sp.*	CS-953	1	Willis et al., 2015; predicted, OZH53057.1
Cyanobacteria	Oscillatoriophycidae	*Limnoraphis robusta*	CS-951	2	Willis et al., 2015 2 predicted (1 partial), KKD35656.1
Cyanobacteria	Oscillatoriophycidae	*Lyngbya aestuarii*	BL J	1	Kothari et al., 2013 PCS family protein, ERT05621.1
Cyanobacteria	Oscillatoriophycidae	*Lyngbya sp.*	PCC 8106	1	Stal et al., 2006; hypothetical, EAW34439.1
Cyanobacteria	Oscillatoriophycidae	*Microcoleus vaginatus*	FGP-2	2	Starkenburg et al., 2011; predicted, EGK88687.1
Cyanobacteria	Oscillatoriophycidae	*Moorea bouillonii*	PNG	1	Leao et al., 2017; predicted, OLT62216.1
Cyanobacteria	Oscillatoriophycidae	*Moorea producens*	1) 3L 2) PAL-8-15-08-1 3) JHB	3	1) Jones et al., 2011; predicted, EGJ30718.1 2,3) Leao et al., 2017 2 predicted, AOX03097.1, AOY83539.1
Cyanobacteria	Oscillatoriophycidae	*Oscillatoria nigro-viridis*	PCC 7112	1	Gugger et al., 2012; predicted, AFZ07216.1
Cyanobacteria	Oscillatoriophycidae	*Phormidium ambiguum*	IAM M-71	1	Zhu et al., 2017; predicted, OKH37366.1
Cyanobacteria	Oscillatoriophycidae	*Phormidium tenue*	NIES-30	1	Zhu et al., 2017; predicted, OKH51158.1
Cyanobacteria	Oscillatoriophycidae	*Planktothrix paucivesiculata*	PCC 9631	2	Regsiter and William, 2015 predicted, CUR11672.1
Cyanobacteria	Oscillatoriophycidae	*Roseofilum reptotaenium*	AO1-A	1	Buerger et al., 2016; predicted, OJJ24789.1
Cyanobacteria	Oscillatoriophycidae	*Rubidibacter lacunae*	KORDI 51-2	1	Choi et al., 2013; predicted, ERN41197.1
Cyanobacteria	Oscillatoriophycidae	*Trichodesmium erythraeum*	IMS101	1	Copeland et al., 2006; PCS-like, ABG51682.1

Cyanobacteria	Synechococcophycidae	*Chamaesiphon minutus*	ATCC 27169 / PCC 6605	1	Gugger et al., 2012; predicted, AFY91394.1
Cyanobacteria	Synechococcophycidae	*Cyanobium gracile*	ATCC 27147 / PCC 6307	1	Gugger et al., 2012; predicted, AFY29492.1
Cyanobacteria	Synechococcophycidae	*Cyanobium sp.*	CACIAM 14	1	Lima, 2014; predicted, KEF42411.1
Cyanobacteria	Synechococcophycidae	*Leptolyngbya sp.*	1) PCC 7376 2) 'hensonii'	2	1) Gugger et al., 2012; predicted, AFY37132.1 2) Hamilton et al., 2012; predicted, OLP16647.1
Cyanobacteria	Synechococcophycidae	*Prochlorococcus marinus*	1) MIT 1323 2) MIT 1320 3) MIT 1318, MIT 1313 4) MIT 1342 5) MIT 1327, MIT 1312	5	Cubillos-Ruiz et al., 2016 predicted, KZR78235.1, KZR77596.1, KZR70669.1, KZR73177.1, KZR82948.1, KZR83423.1, KZR66486.1
Cyanobacteria	Synechococcophycidae	*Prochlorococcus marinus*	1) MIT 9313 2) MIT 9303	2	1) Kettler et al., 2007; putative, ABM77828.1 2) Rocap et al., 2003; putative, CAE21188.1
Cyanobacteria	Synechococcophycidae	*Prochlorococcus sp.*	1) MIT 1303 2) MIT 1306 3) MIT 0701, MIT 0702, MIT 0703	3	1,2) Cubillos-Ruiz et al., 2016 predicted, KZR68122.1, KZR65582.1 3) Biller et al., 2014 predicted, KGG28100.1, KGG27537.1, KGG32823.1
Cyanobacteria	Synechococcophycidae	*Pseudanabaena sp.*	SR411	1	Stowe et al., 2017; predicted, OYQ63741.1
Cyanobacteria	Synechococcophycidae	*Synechococcus sp.*	1) ATCC 27167 / PCC 6312 2) WH 5701	2	1) Gugger et al., 2012; predicted, AFY62739.1 2) Scanlan et al., 2006; predicted, EAQ74049.1
Alveolate	Chromerida	*Vitrella brassicaformis*	CCMP3155	3	Otto et al., 2014 hypothetical, CEM25435.1
Alveolate	Chromerida	*Chromera velia*	CCMP2878	4	No NCBI entry, hypothetical, CEM41364.1
Cryptomonad	Cryptophyceae	*Guillardia theta*	CCMP2712	2	Curtis et al., 2012 hypothetical, EKX38302.1
Dinoflagellate	Dinophyceae	*Symbiodinium microadriaticum*		5	Aranda et al., 2016 predicted, OLP84719.1
Haptophyte	Coccolithophyceae	*Emiliania huxleyi*		9	Kuo et al., 2012 hypothetical, EOD07692.1
Haptophyte	Coccolithophyceae	*Chrysochromulina sp.*	CCMP291	1	Hovde et al., 2014 predicted, KOO34056.1
Heterokont	Pelagophyceae	*Aureococcus anophagefferens*		3	Gobler et al., 2011 hypothetical, EGB07651.1
Heterokont	Eustigmatophyceae	*Nannochloropsis gaditana*	1) CCMP526 2) B-31	3	1) Radakovits et al., 2012 predicted, partial, EKU21433.1 2) Corteggiani Carpinelli et al., 2014 predicted, EWM22234.1

The PCS data is gathered from UniProt (https://www.uniprot.org)

APPENDIX B: QUALITY CONTROL FOR RNA SAMPLES

Table B.1. RIN values of the libraries used in RNA-seq

	RINe	28S/18S (Area)	Conc. [pg/µl]
Ctrl-1	7.7	1.2	4770
Ctrl-2	7.8	1.2	4610
Ctrl-3	7.6	1.1	3490
Ctrl-4	7.7	1.2	4770
Ctrl-5	8	1.2	5560
Cu-1	8.1	1.3	5730
Cu-2	8	1.2	5660
Cu-3	7.9	1.3	3080
Cu-4	8	1.2	6520
Cu-5	8	1.2	5970

APPENDIX C: QUALITY CONTROL OF THE DATA OF RNA-SEQ LIBRARIES

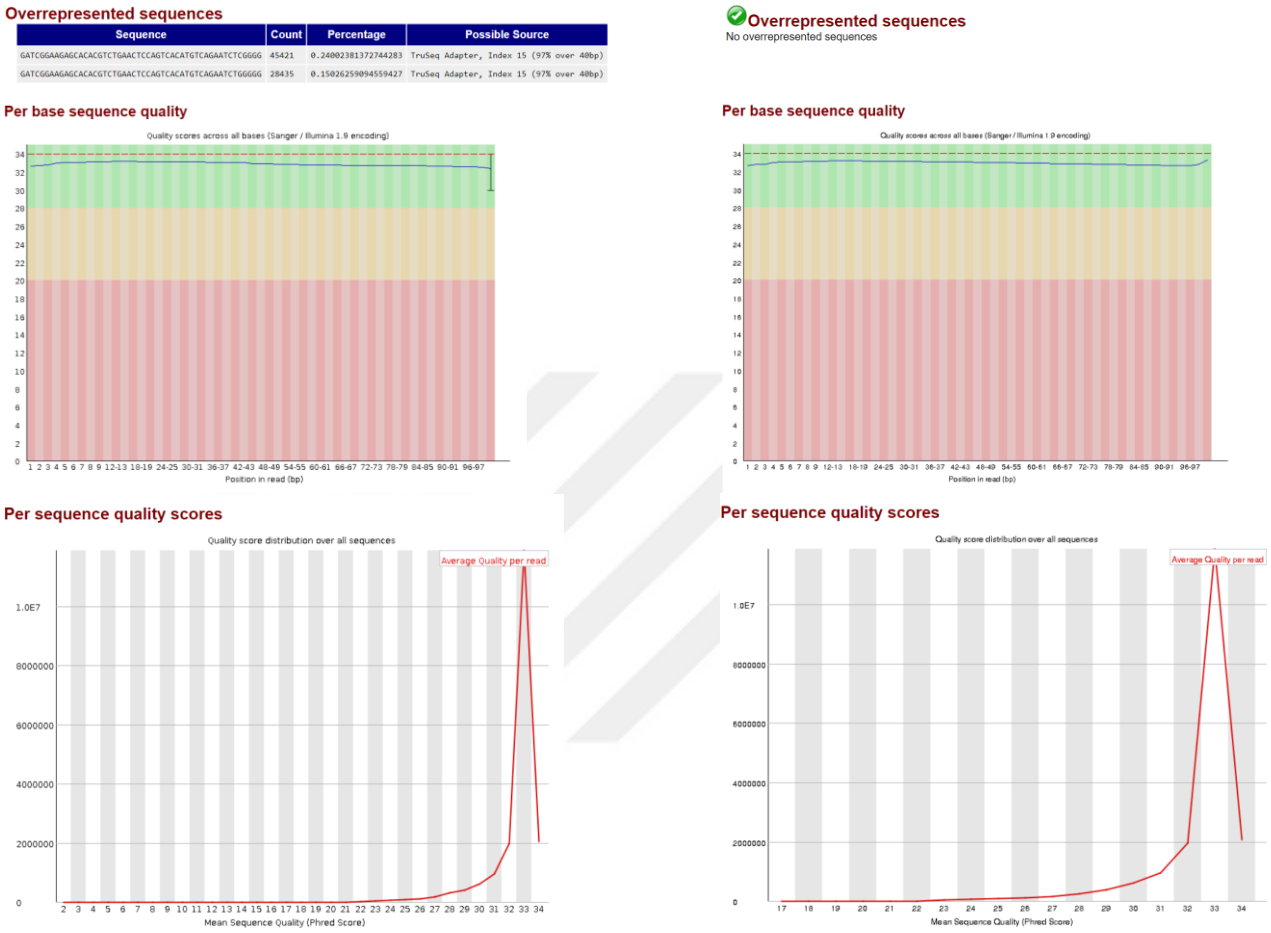


Figure C.1. FastQC reports of a selected library before (left) and after (right) the adaptor removal, trimming, and quality filtering.

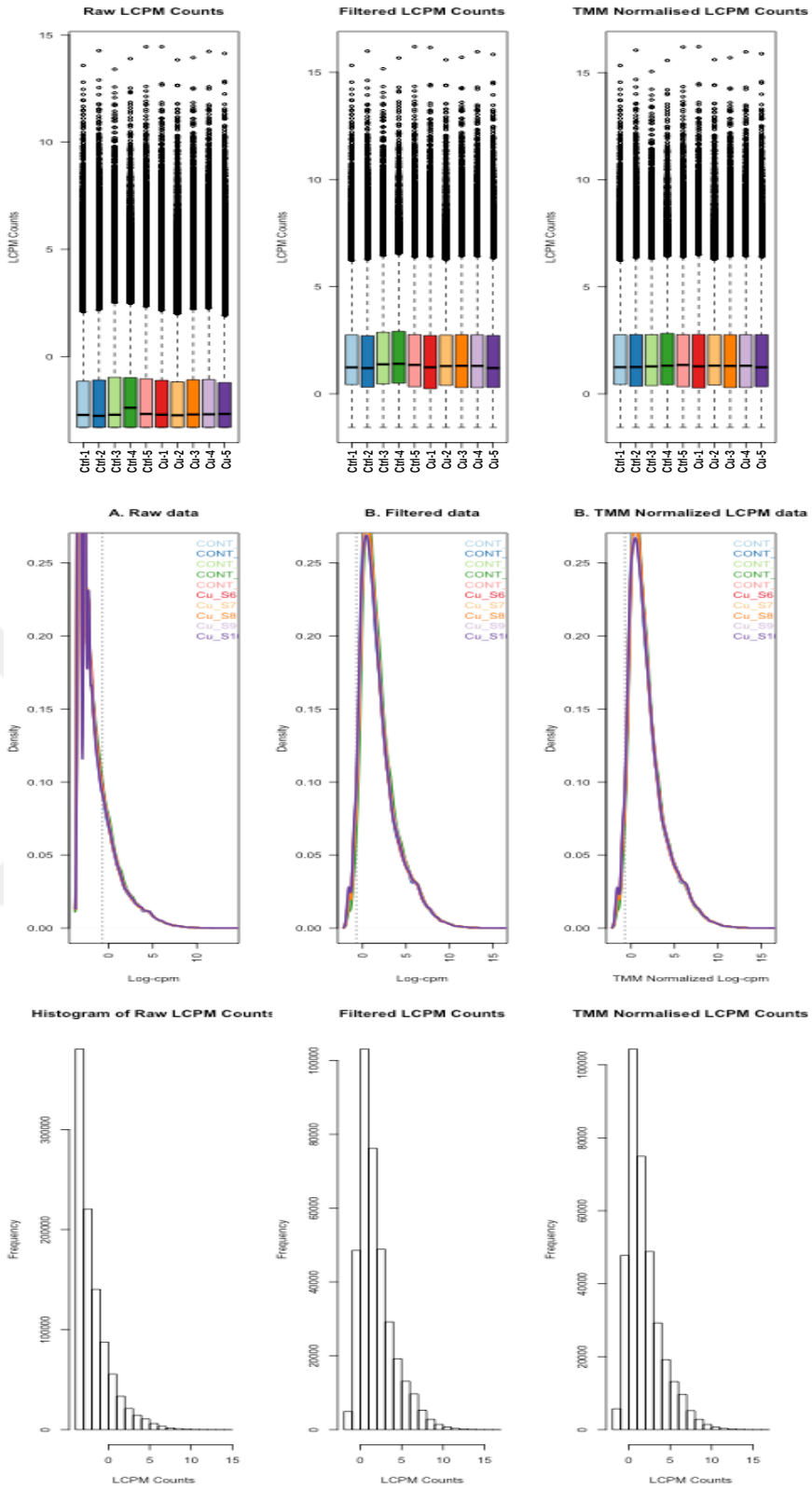


Figure C.2. Filtering and TMM normalization on edgeR. Upper graph, logCPM counts distribution in each library, middle graph, density of logCPM values, lower graph, frequencies of logCPM values. In each graph, left is the raw data, middle is filtered for low count reads, right is TMM normalized data after filtering. Reads were filtered for a contig if a contig does not get worthwhile expression in at least 4 samples.

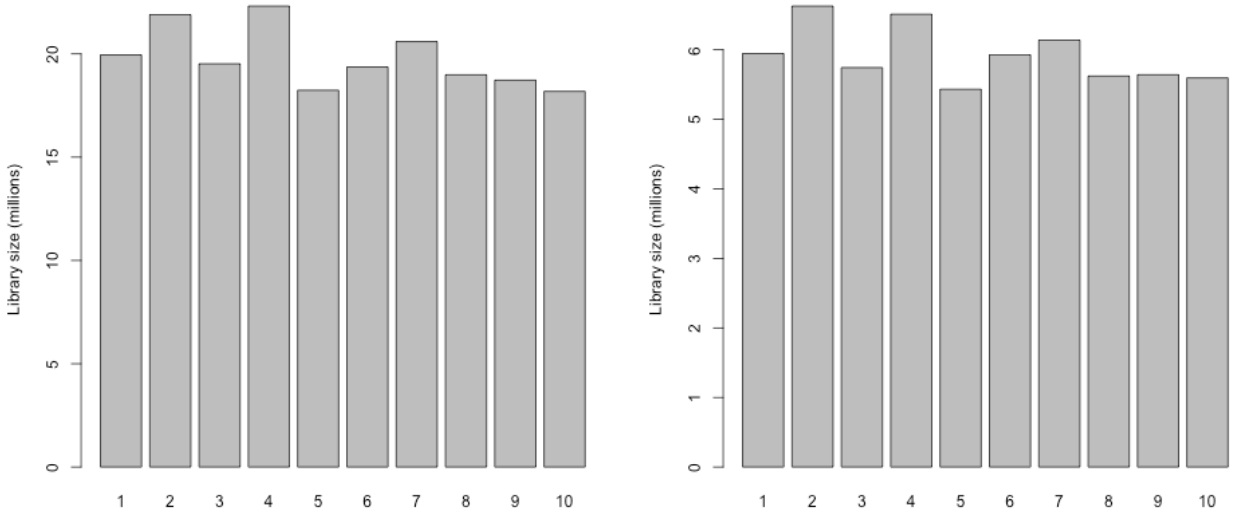


Figure C.3. Read counts before (Left) and after (Right) filtering the libraries through edgeR. For details of filtering, please refer to Figure C2 and text in the relevant section.

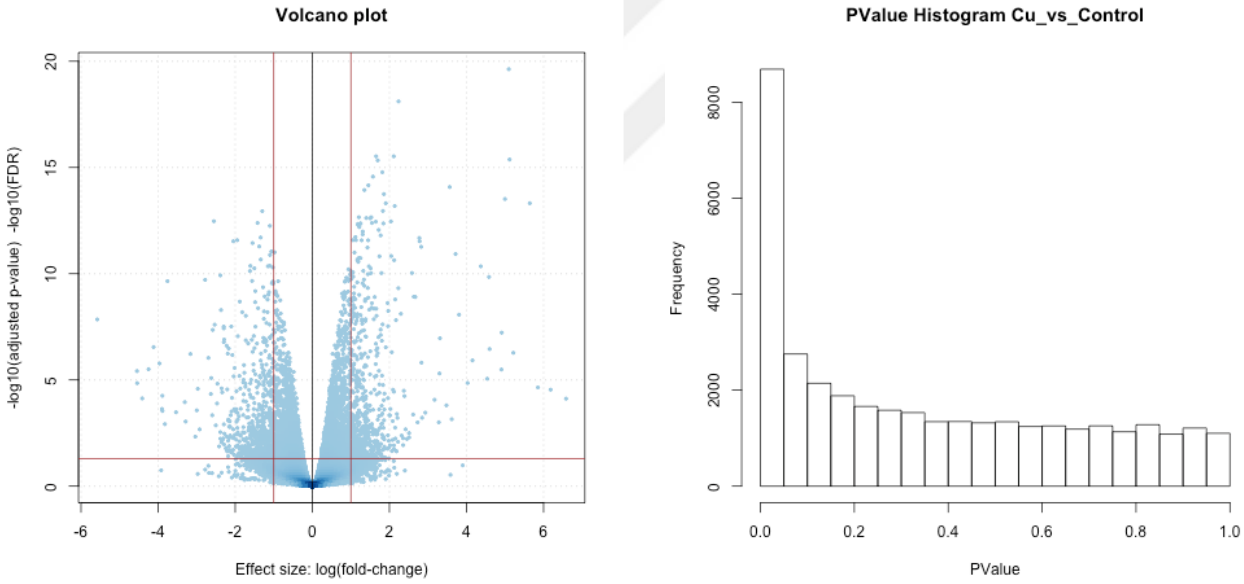


Figure C.4. Volcano plot (left) and p-value histogram of RNA-seq data (right).

APPENDIX D: ANTIOXIDANT GENES FOUND IN TRANSCRIPTOME ANALYSIS OF *Tetraselmis suecica*

Table D.1. Antioxidant genes, their differential expression (DE) under copper stress, and significance of DE

Contig ID	EC code/KO	Description	LogFC	LogCPM	FDR	p-value
TRINITY_DN7213_c0_g1_i50		Nickel-containing superoxide dismutase	0.36	2.53	0.266	0.074
TRINITY_DN7213_c0_g1_i26		Nickel-containing superoxide dismutase	0.69	0.52	0.345	0.115
TRINITY_DN7213_c0_g1_i35		Nickel-containing superoxide dismutase	0.13	2.93	0.733	0.476
TRINITY_DN2928_c0_g1_i43	1.15.1.1	Mn superoxide dismutase	1.20	-0.03	0.167	0.035
TRINITY_DN2928_c0_g1_i24	1.15.1.1	Mn superoxide dismutase	-0.33	2.42	0.343	0.114
TRINITY_DN2928_c0_g1_i8	1.15.1.1	Mn superoxide dismutase	-3.92	2.20	0.180	0.040
TRINITY_DN2063_c0_g1_i5	1.11.1.21	Catalase/peroxidase (katG)	0.95	1.72	**0.018**	0.002
TRINITY_DN14607_c1_g2_i3	1.11.1.21	Catalase/peroxidase (katG)	-1.14	0.13	0.112	0.019
TRINITY_DN14607_c1_g1_i4	1.11.1.21	Catalase/peroxidase (katG)	0.86	0.47	0.209	0.050
TRINITY_DN1107_c0_g1_i7	1.11.1.21	Catalase/peroxidase (katG)	-0.42	4.30	0.477	0.206
TRINITY_DN2063_c0_g1_i6	1.11.1.21	Catalase/peroxidase (katG)	0.42	0.55	0.576	0.294
TRINITY_DN1107_c0_g1_i9	1.11.1.21	Catalase/peroxidase (katG)	0.15	4.10	0.842	0.651
TRINITY_DN7997_c0_g1_i1	1.11.1.21	Catalase/peroxidase (katG)	0.17	0.38	0.859	0.680
TRINITY_DN10468_c1_g1_i3	1.11.1.5	Cytochrome-c peroxidase	2.82	2.66	**0.000**	0.000
TRINITY_DN819_c0_g1_i2	1.11.1.5	Cytochrome-c peroxidase	-0.18	3.18	0.628	0.349
TRINITY_DN819_c0_g1_i1	1.11.1.5	Cytochrome-c peroxidase	-0.02	1.55	0.978	0.945
TRINITY_DN3471_c0_g2_i9	1.11.1.11	Ascorbate peroxidase, APX1	-0.50	3.59	**0.036**	0.004
TRINITY_DN12089_c0_g1_i8	1.11.1.11	Ascorbate peroxidase, APX1	0.21	4.25	0.605	0.323
TRINITY_DN12089_c0_g1_i5	1.11.1.11	Ascorbate peroxidase, APX1	-0.10	5.16	0.703	0.437
TRINITY_DN5477_c0_g1_i3	1.11.1.11	Ascorbate peroxidase-like	-0.17	8.03	0.258	0.070
TRINITY_DN1639_c0_g1_i8	1.11.1.11	Ascorbate peroxidase-related	-0.61	6.64	**0.046**	0.005
TRINITY_DN1639_c0_g1_i10	1.11.1.11	Ascorbate peroxidase-related	-0.87	0.93	0.080	0.012
TRINITY_DN1988_c1_g2_i7	1.11.1.11	Ascorbate peroxidase-related	0.38	1.43	0.446	0.182
TRINITY_DN1988_c1_g2_i6	1.11.1.11	Ascorbate peroxidase-related	0.27	0.73	0.729	0.471
TRINITY_DN4176_c0_g1_i2	1.11.1	Acting on a peroxide as acceptor	-0.41	4.82	**0.036**	0.004
TRINITY_DN10772_c0_g1_i1	1.11.1	Putative haloperoxidase	2.11	5.83	**0.000**	0.000
TRINITY_DN6550_c0_g1_i2	K03676	Glutaredoxin	-2.01	0.94	0.284	0.083
TRINITY_DN6550_c0_g1_i3	K03676	Glutaredoxin	-2.02	1.18	0.314	0.098
TRINITY_DN6498_c0_g1_i2	K03676	Glutaredoxin	-2.43	2.50	0.341	0.113
TRINITY_DN18286_c0_g1_i18	K03676	Glutaredoxin	-0.23	2.64	0.475	0.204
TRINITY_DN11104_c0_g1_i1	K03676	Glutaredoxin	0.11	6.30	0.523	0.244
TRINITY_DN9625_c0_g1_i3		Glutaredoxin-like protein	0.21	3.46	0.932	0.838
TRINITY_DN3106_c3_g1_i3	K07390	Mono thiol glutaredoxin	-0.34	0.20	0.734	0.478
TRINITY_DN3106_c3_g1_i2	K07390	Mono thiol glutaredoxin	-0.08	5.61	0.757	0.513
TRINITY_DN4594_c0_g1_i2	K03671	Belongs to the thioredoxin family	0.64	1.31	0.161	0.033
TRINITY_DN1384_c1_g1_i1	K03671	Belongs to the thioredoxin family, Trx3	0.09	5.35	0.647	0.370
TRINITY_DN4151_c0_g1_i5	K03671	Belongs to the thioredoxin family, Trx3	0.17	2.44	0.666	0.392

TRINITY_DN3977_c0_g1_i2	K03671	Belongs to the thioredoxin family	0.04	4.66	0.894	0.754
TRINITY_DN5382_c0_g1_i2	K03671	Thioredoxin	0.45	4.87	**0.003**	0.000
TRINITY_DN1265_c0_g2_i1	K03671	Thioredoxin	0.27	5.54	**0.038**	0.004
TRINITY_DN5622_c0_g1_i5	K03671	Thioredoxin	0.49	2.03	0.195	0.045
TRINITY_DN1413_c0_g1_i28		Thioredoxin	0.29	3.37	0.246	0.065
TRINITY_DN5622_c0_g1_i4	K03671	Thioredoxin	-0.84	-0.16	0.385	0.140
TRINITY_DN5462_c0_g1_i55		Thioredoxin, calredoxin, CRX1	1.90	1.07	0.459	0.191
TRINITY_DN266_c0_g2_i20	K03671	Thioredoxin	0.36	1.22	0.553	0.271
TRINITY_DN8730_c0_g1_i5	K03671	Thioredoxin	-0.20	0.32	0.845	0.656
TRINITY_DN7788_c0_g1_i2	K03671	Thioredoxin	0.06	2.91	0.873	0.709
TRINITY_DN276_c0_g2_i2	K03671	Thioredoxin	0.12	0.40	0.914	0.792
TRINITY_DN23754_c0_g2_i5	K03671	Thioredoxin-like 2-1, chloroplastic	0.70	0.40	0.373	0.133
TRINITY_DN1486_c0_g1_i21	K03671	Thioredoxin-like domain	-0.23	4.14	0.312	0.097
TRINITY_DN9627_c0_g2_i1	K03671	thioredoxin-like protein	0.08	5.87	0.659	0.384
TRINITY_DN5605_c1_g1_i10	1.11.1.24	Thioredoxin-dependent peroxiredoxin	0.70	2.19	**0.023**	0.002
TRINITY_DN4843_c0_g1_i1	1.11.1.24	Thioredoxin-dependent peroxiredoxin	-0.23	3.14	0.453	0.187
TRINITY_DN4887_c0_g1_i1	1.11.1.24	Thioredoxin-dependent peroxiredoxin	-0.02	7.14	0.936	0.848
TRINITY_DN4954_c1_g1_i1	K03386	2-cys Peroxiredoxin, PRX1	0.22	8.26	0.102	0.017
TRINITY_DN43424_c0_g1_i1	K03386	2-cys Peroxiredoxin, PRX2	0.06	5.81	0.727	0.468
TRINITY_DN2748_c0_g2_i1	1.10.3.11	Plastid terminal oxidase, PTOX1	0.22	5.13	0.290	0.086
TRINITY_DN164_c0_g1_i25	1.10.3.11	Plastid terminal oxidase, PTOX1	0.24	3.43	0.398	0.148
TRINITY_DN2984_c0_g1_i18	1.10.3.11	Plastid terminal oxidase, PTOX1	-0.28	5.56	0.522	0.243
TRINITY_DN2984_c0_g1_i13	1.10.3.11	Plastid terminal oxidase, PTOX1	-0.18	6.78	0.728	0.469
TRINITY_DN449_c0_g1_i13	1.10.3.11	Alternative oxidase, mitochondrial	-0.20	1.85	0.773	0.538
TRINITY_DN3302_c1_g1_i1	1.10.3.11	Plastid terminal oxidase, PTOX2	-0.20	0.32	0.835	0.641
TRINITY_DN164_c0_g1_i17	1.10.3.11	Plastid terminal oxidase, PTOX1	0.12	1.15	0.861	0.683
TRINITY_DN2984_c0_g1_i5	1.10.3.11	Plastid terminal oxidase, PTOX1	-0.10	6.72	0.869	0.700
TRINITY_DN3302_c1_g1_i3	1.10.3.11	Plastid terminal oxidase, PTOX1	-0.02	7.59	0.982	0.957
TRINITY_DN4746_c0_g1_i4		AhpC/TSA antioxidant enzyme	-0.28	1.32	0.619	0.340
TRINITY_DN5457_c0_g1_i11		AhpC/TSA antioxidant enzyme	-0.11	4.18	0.630	0.352
TRINITY_DN5457_c0_g1_i7		AhpC/TSA antioxidant enzyme	-0.02	5.15	0.951	0.878
TRINITY_DN4746_c0_g1_i7		AhpC/TSA antioxidant enzyme	-0.01	3.67	0.980	0.950

EC: enzyme commission, KO: KEGG orthology, LogFC: log-2 fold change, LogCPM: log-2 counts per million, FDR: false discovery rate. Glutathione-S-transferase (GST), glutathione reductase (GR), and glutathione peroxidase (GPX) are not included here; they were discussed in glutathione metabolism.

APPENDIX E: DETERMINATION OF THE GROWTH METRICS OF THE ALGAE STRAINS

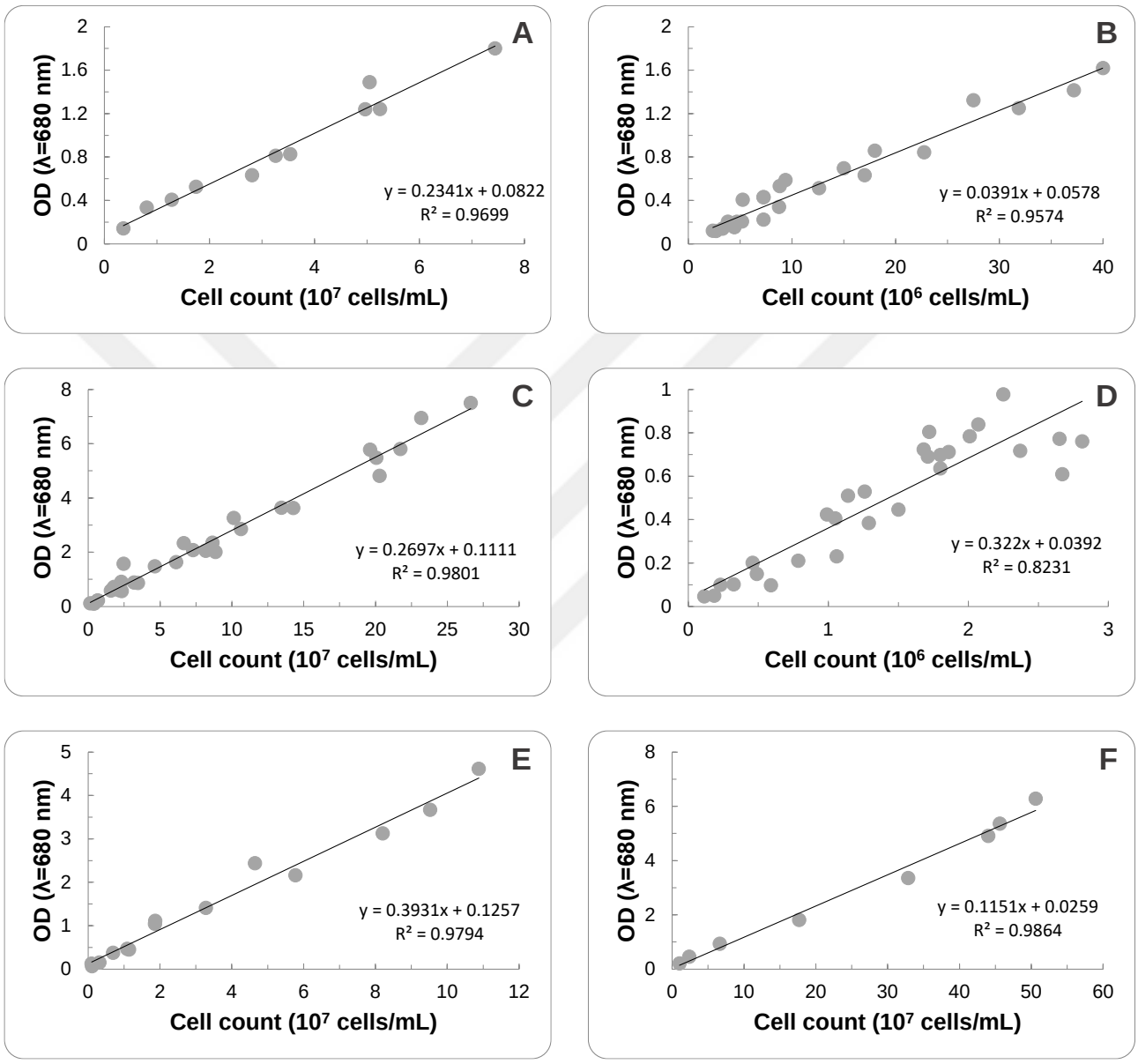


Figure E.1. OD-cell count correlation curves of the microalgae utilized in this dissertation. A) *Chlorella vulgaris*, B) *Coccomyxa subellipsoidea*, C) *Ettlia oleoabundans*, D) *Tetraselmis suecica*, E) *Anabaena flos-aquae*, F) *Cyanobacterium sp.* AV1403.

APPENDIX F: QUALITY CONTROL FOR RNA EXTRACTION

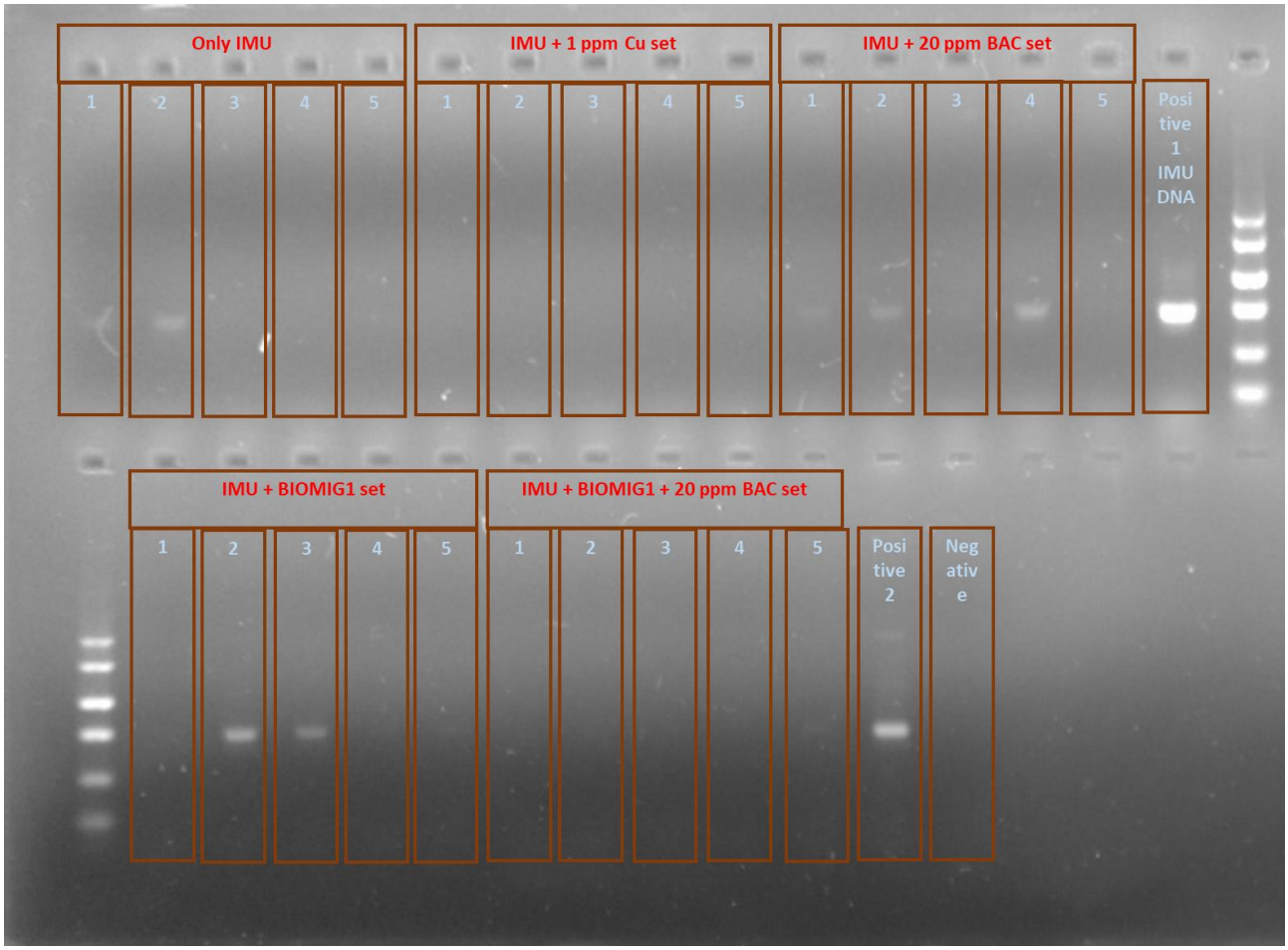


Figure F.1. Eukaryotic DNA contamination checks for RNA samples on agarose gel electrophoresis. DNA contaminated RNA samples underwent an additional DNAse treatment.

APPENDIX G: ALGAE GROWTH UNDER BAC STRESS

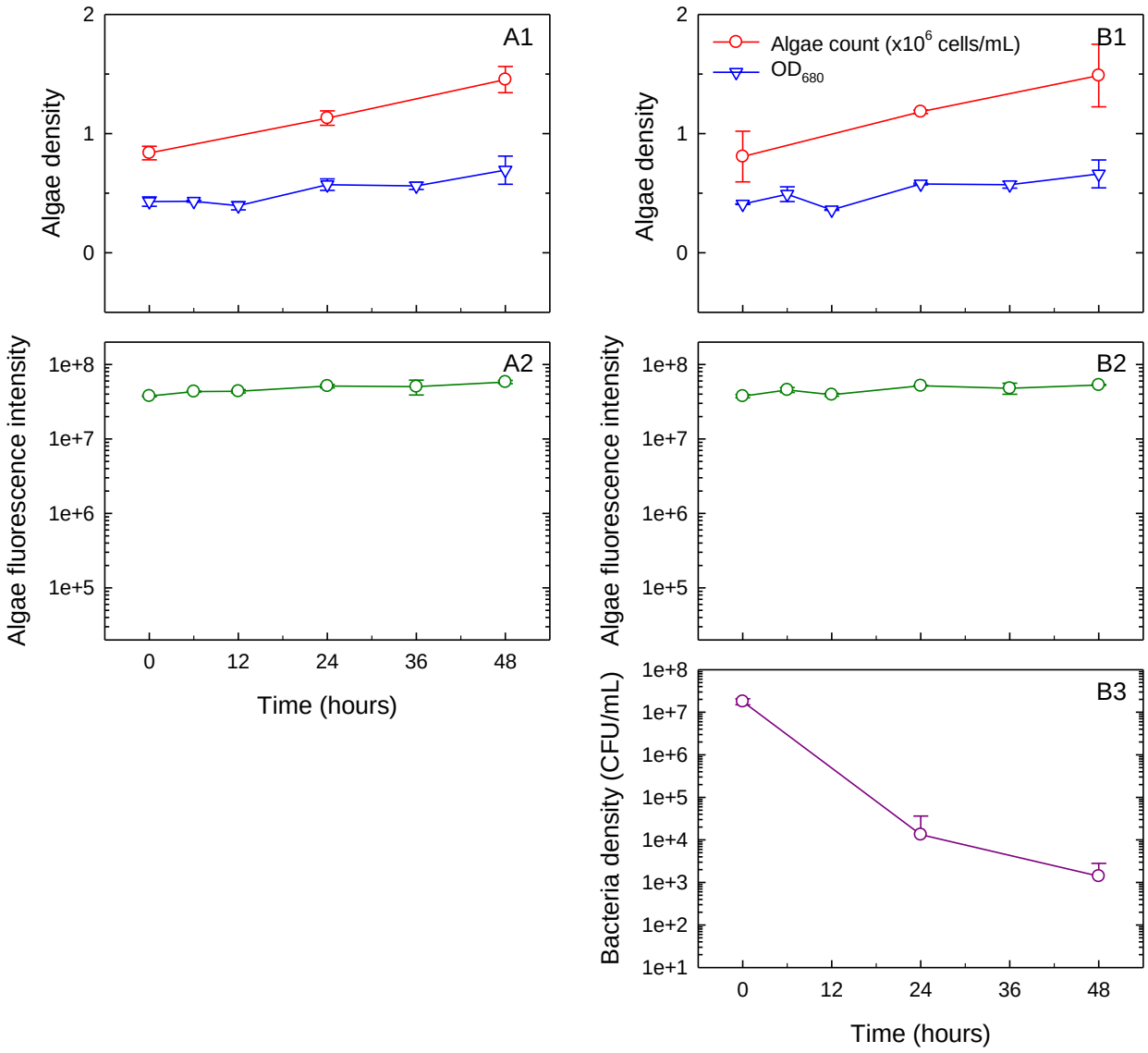


Figure G.1. Algae not exposed to BAC as a control group in the absence (A) or presence (B) of bacteria. Algae density (A1, B1), algae fluorescence intensity (A2, B2) and bacteria density (B3).

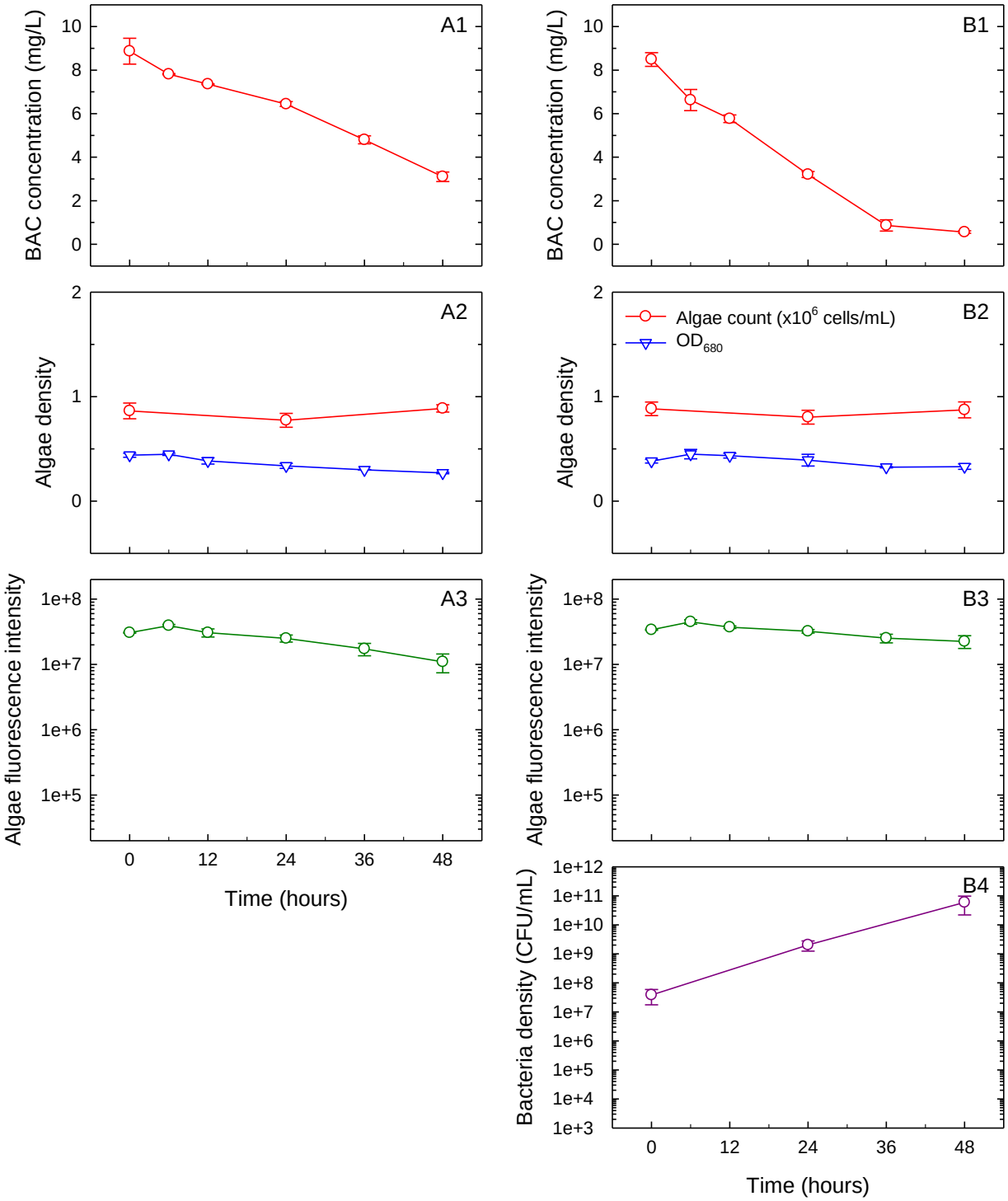


Figure G.2. Algae exposed to 10 ppm BAC in the absence (A) or presence (B) of bacteria. BAC concentration in the media (A1, B1), algae density (A2, B2), algae fluorescence intensity (A3, B3), and bacteria density (B4).

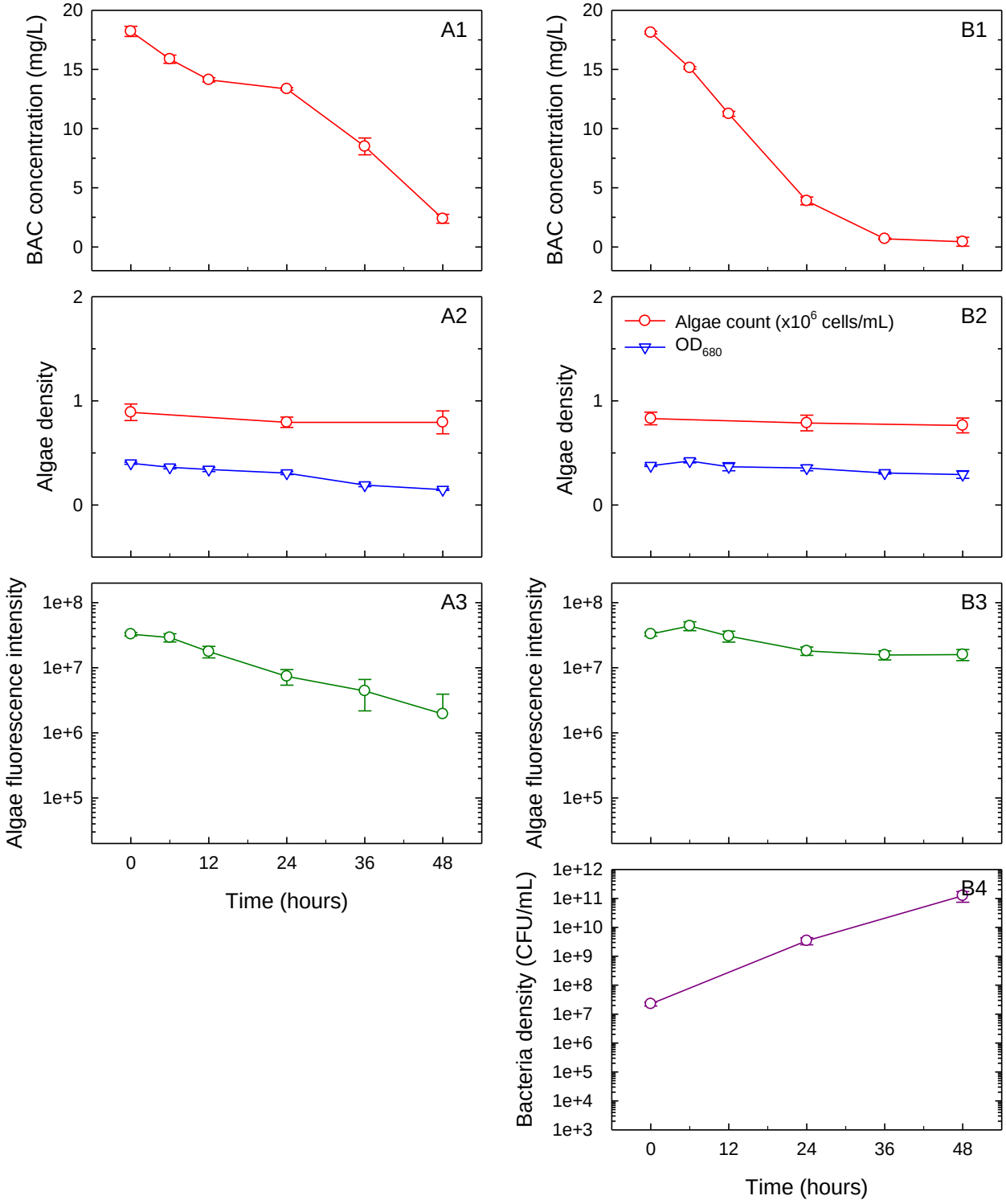


Figure G.3. Algae exposed to 20 ppm BAC in the absence (A) or presence (B) of bacteria. BAC concentration in the media (A1, B1), algae density (A2, B2), algae fluorescence intensity (A3, B3), and bacteria density (B4).