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Chlorella vulgaris as a potential bioremediation agent of aquaculture waste from Marine Science Techno Park, Diponegoro University

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A B S T R A C T

Aquaculture activities that produce liquid waste from leftover feed can pollute aquatic ecosystems because this waste contains phosphate and nitrogen. One alternative that can be an asset is to employ *Chlorella vulgaris* as a bioremediation agent. The purpose of this research is to evaluate the potential ability of *C. vulgaris* in bioremediation of aquaculture waste based on the *C. vulgaris* cell density growth and the water parameter tests, including nitrate and nitrite. This research design uses a Completely Randomized Design (CRD) with six treatments with different aquaculture waste concentrations: K (0 mg/L), P1 (10 mg/L), P2 (20 mg/L), P3 (30 mg/L), P4 (40 mg/L), and P5 (50 mg/L). The research data results include *C. vulgaris* cell density, measurement, and test parameters of water quality, such as temperature (°C), pH, salinity (ppt), nitrate (mg/L), and nitrite (mg/L), which were carried out by descriptive analysis. The concentration of aquaculture waste at 20 mg/L on day 5 had the highest *C. vulgaris* cell density of 717 × 10^4 cells, indicating the exponential phase; meanwhile, the lowest *C. vulgaris* cell density was 32 × 10^4 cells at 30 mg/L on day 6, indicating the senescence phase. The *C. vulgaris* potential in bioremediating the aquaculture waste at a concentration of 10 mg/L of nitrate was 27.27%; meanwhile, nitrite was 50%. Moderate wastewater concentrations enhanced microalgal growth and nutrient uptake, whereas higher concentrations inhibited cell proliferation, likely due to nitrogen-related toxic stress. These findings demonstrate the potential application of *C. vulgaris* for wastewater polishing in sustainable aquaculture systems, particularly Recirculating Aquaculture Systems (RAS) and Integrated Multi-Trophic Aquaculture (IMTA).

Keywords: Aquaculture waste; Bioremediation; *Chlorella vulgaris*

1. INTRODUCTION

Aquaculture refers to artificial ponds created in various locations, such as freshwater and saltwater areas (Rocha et al., 2022). Aquaculture activities aim to cultivate aquatic organisms, such as fish, shellfish, and shrimp (Sarwana et al., 2019) and aquatic plants (Food and Agriculture Organization of the United States, 2022). Aquaculture productivity serves to increase the economic income of coastal communities. However, these activities are anthropogenic in nature because they have a negative impact on the environment, one example being waste from aquaculture activities (Choeronowati et al., 2019). The increasing amount of aquaculture waste is due to the increasing productivity of aquaculture (Prasetiyono et al., 2023).

The Marine Science Techno Park (MSTP) at Diponegoro University is an example of an area used for aquaculture activities and mangrove forest ecosystem conservation. MSTP, Diponegoro University focuses on aquaculture activities, such as vaname shrimp (*Litopenaeus vannamei*), milkfish (*Chanos chanos*), and tilapia (*Oreochromis niloticus*) (Putra *et al*., 2022). According to Krisyanto *et al*. (2021), MSTP, Diponegoro University has one plot for storing waste from aquaculture activities. Waste management generally utilizes microorganisms.

The type of waste from aquaculture activities is generally liquid waste originating from the metabolic residues of marine organisms and leftover feed residues. This waste contains both inorganic and organic materials, such as ammonia (NH_2), phosphorus (P), potassium (K), carbohydrates, fats, proteins, nitrogen (N_2), and urea ($(NH_2)_2CO$). Liquid waste can affect aquaculture water quality and pollute ecosystems, potentially causing significant adverse effects on aquatic organisms (Febrianto et al., 2016). The high protein content in uneaten feed residues, approximately 40%, can produce toxic nitrogen compounds if not consumed or undergo decomposition. Additionally, high nitrogen concentrations in water can accumulate with phosphorus, leading to eutrophication, which plays a role in reducing Biological Oxygen Demand (BOD) (Suwoyo et al., 2015).

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One approach to addressing aquaculture waste issues is to use living organisms for bioremediation. Aquaculture waste inherently contains very high levels of phosphorus and nitrogen. Living organisms, such as microalgae, have the ability to utilize waste containing phosphorus and nitrogen as a source of energy and nutrients by enzymatically degrading the waste and bioaccumulating it (Djide et al., 2018; Anggraini et al., 2022). One example of microalgae, *C. vulgaris*, can be used as a bioremediation agent for aquaculture waste. This is because microalgae can utilize inorganic materials, such as ammonia, for photosynthesis to produce biomass and can adapt to water ecosystems contaminated with waste due to their content of polymines and phytohormones (Syaichudin et al., 2021).

Previous research by Wang et al. (2022) reported that *C. vulgaris* can be used for waste management and to reduce phosphorus, nitrogen, and Chemical Oxygen Demand (COD) levels. The study by Carvajal *et al*. (2017) tested that *C. vulgaris* can perform bioremediation of aquaculture waste by reducing phosphate (PO_4) and nitrogen levels by approximately 99%. Recent studies reported that microalgae-based wastewater treatment provides efficient nutrient recovery and improves environmental sustainability in aquaculture systems (Xin et al., 2021; Zhao et al., 2024). *C. vulgaris* has also demonstrated high adaptability and nitrogen-removal performance in nutrient-rich wastewater environments (Cho et al., 2022). Recent studies have also reported that microalgae-based wastewater treatment offers high nutrient removal efficiency and supports sustainable aquaculture management systems (Díaz et al., 2022; Gonçalves et al., 2023).

Studies in algal research and aquaculture reports further demonstrated that *C. vulgaris* effectively removes dissolved nitrogen compounds and improves wastewater quality under aquaculture cultivation systems (Markou et al., 2016; Saleh et al., 2023). Microalgae such as *C. vulgaris* can assimilate dissolved nitrogen and phosphorus into biomass while simultaneously improving water quality through nutrient uptake and bioaccumulation mechanisms (Abdelfattah et al., 2022). Several studies have evaluated the application of *C. vulgaris* in wastewater bioremediation under different environmental conditions (Markou et al., 2016).

However, no study has quantified how incremental increases in waste concentration from aquaculture facilities affect C. vulgaris growth kinetics and nutrient removal efficiency under controlled conditions, particularly using aquaculture wastewater from the Marine Science Techno Park (MSTP), Diponegoro University. This study aimed to evaluate the potential of *C. vulgaris* as a bioremediation agent for aquaculture wastewater from MSTP, Diponegoro University, based on cell density growth and nitrate-nitrite removal performance across different wastewater concentrations. This study hypothesized that low-to-moderate waste concentrations would increase C. vulgaris growth and nitrate-nitrite removal efficiency, whereas high concentrations would inhibit cell proliferation due to nutrient toxicity and metabolic stress.

2. Material and Methods

2.1 Research location

Aquaculture waste from MSTP, Diponegoro University, was collected from the Wastewater Treatment Plant. Research and testing were conducted at the Ecology Laboratory, Biology Study Program, Faculty of Science and Mathematics, and the Fisheries Laboratory, Marine Science Study Program, Faculty of Marine Science and Fisheries, Diponegoro University. The research was conducted over approximately two months, from May to June 2024. Figure 1. shows the research location map.

2.2 Research design

This study used a completely randomized design (CRD) with six waste concentration treatments: K (0 mg/L), P1 (10 mg/L), P2 (20 mg/L), P3 (30 mg/L), P4 (40 mg/L), and P5 (50 mg/L).

2.3 Inoculation and dilution of *Chlorella vulgaris*

The desired density of *C. vulgaris* in this study was 105 cells with a culture medium volume of 2000 mL. Before determining the density, a dilution was prepared using the following formula.

$$V1\ x\ N1 = V2\ x\ N2 \quad (1)$$

Description:
V1: Desired *C. vulgaris* inoculum volume (mL)
N1: Initial *C. vulgaris* cell density (cells/mL)
V2: Required *C. vulgaris* inoculum volume (mL)
N2: Desired *C. vulgaris* cell density (cells/mL)

Aquaculture waste was added to K at 0 mL, to P1 at 200 mL, to P2 at 400 mL, to P3 at 600 mL, to P4 at 700 mL, and to P5 at 1000 mL. *C. vulgaris* was inoculated at 400 mL in each treatment. A 5000 mL glass jar was used as a container for cultivation. The medium was aerated using an aerator for 1 day, and 3 mL of walnut fertilizer was added to support the growth and development of *C. vulgaris* (Buwono & Nurhasanah, 2018).

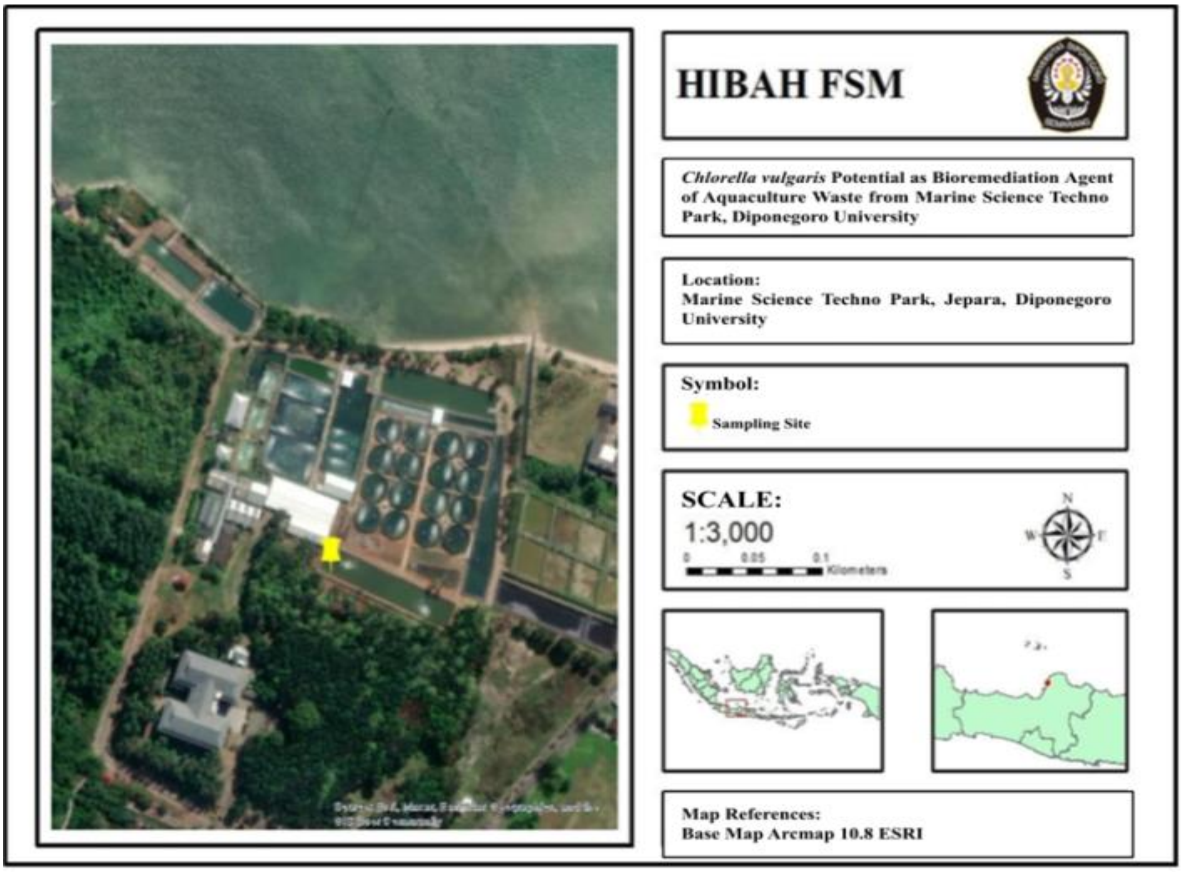

Figure 1. Research location map

2.4 Calculation of *Chlorella vulgaris* density

The observation and calculation of the density of *C. vulgaris* will use the formula following Novianti *et al*. (2017) as follows. The observation and calculation are assisted by a light microscope and hemocytometer. The calculation of the density of *C. vulgaris* cells using a hemocytometer is carried out with four repetitions of the field of view.

$$N = \frac{n1+n2+n3+n4}{4} \, x \, 10^4 \quad (2)$$

Description:
N: Density of *C. vulgaris* (cells/mL)
n1: Number of *C. vulgaris* in field of view 1
n2: Number of *C. vulgaris* in field of view 2
n3: Number of *C. vulgaris* in field of view 3

n4: Number of *C. vulgaris* in field of view 4

Calculation of *C. vulgaris* cell density growth, measurement of water quality parameters including temperature (°C), pH, and salinity (ppt) using a Total Dissolved Solid (TDS) measuring device, as well as testing of water quality parameters including nitrate (mg/L) and nitrite (mg/L) were conducted over 8 days, with day 1 (first) being the measurement before bioremediation and day 8 (last) being the measurement after bioremediation.

2.5 Data analysis

The research data were analyzed descriptively. According to Budiastuti & Bandur (2018), the main function of descriptive analysis in a study is to obtain information from the results of data analysis based on the influence of variables to understand the characteristics of the data, which can then be used to determine the type of statistical test from the research results.

3. RESULTS DAN DISCUSSION

3.1 Growth phase of *Chlorella vulgaris*

C. vulgaris that underwent growth and death after being cultured for 8 days showed differences in bioremediation ability in degrading aquaculture waste. According to Caprio *et al*. (2016), the presence of pollutants, namely aquaculture waste, which experienced a decrease in pollutant levels, could be due to degradation by *C. vulgaris*. Figure 2. shows the results of observations of the growth pattern of *C. vulgaris* over 8 days, including the lag phase, exponential phase, stationary phase, and death phase in the growth of *C. vulgaris*.

Descriptive analysis showed that the growth performance of *C. vulgaris* differed among treatments. Treatment P2 (20 mg L^{-1}) yielded the highest average cell density (269.13 ± 228.34 cells mL^{-1}), while treatment P3 (30 mg L^{-1}) showed the lowest average cell density (83.50 ± 51.02 cells mL^{-1}). The relatively large standard deviation observed in treatment P2 indicates greater biological variability among replicates during the exponential growth phase. A one-way ANOVA analysis showed that wastewater concentration significantly affected the growth of *C. vulgaris* ($F = 3.461$, $p = 0.010$). The calculated effect size ($\eta^2 = 0.292$) indicated that wastewater concentration accounted for approximately 29.2% of the total variation in microalgal growth, suggesting a substantial treatment effect. Tukey's HSD multiple comparison test further revealed that treatment P2 differed significantly from treatment P3 ($p = 0.017$) and the control treatment ($p = 0.036$), while no significant differences were detected among the other treatments.

Table 1. Summary of one-way ANOVA and Tukey's HSD analysis for the growth of *C. vulgaris*.

Parameter	Value
F-value	3.461
p-value	0.010*
Effect size (η^2)	0.292
Significant pairwise comparisons	P2 vs P3 (p = 0.017); P2 vs Control (p = 0.036)

Note: significant at $p < 0.05$.

The lag phase, also known as the adaptation phase, occurs from day 1 to day 3. *C. vulgaris* during this period is adapting to the environment and nutrients from walnut fertilizer and aquaculture waste. According to Febrianto et al. (2016), liquid aquaculture waste contains phosphorus and nitrogen, which can serve as nutrient sources for

C. vulgaris. The observation results showed that the lag phase in all treatments exhibited relatively similar increases. The exponential phase, or logarithmic phase, is characterized by an increase in the density of *C. vulgaris* cells. Each treatment has a different exponential phase. The observation results showed that P2, the treatment with aquaculture waste at a concentration of 20 mg/L, had the highest exponential phase rate. According to Selvika et al. (2016), the changes that occur during the exponential phase are due to the utilization of nutrients for growth and reproduction.

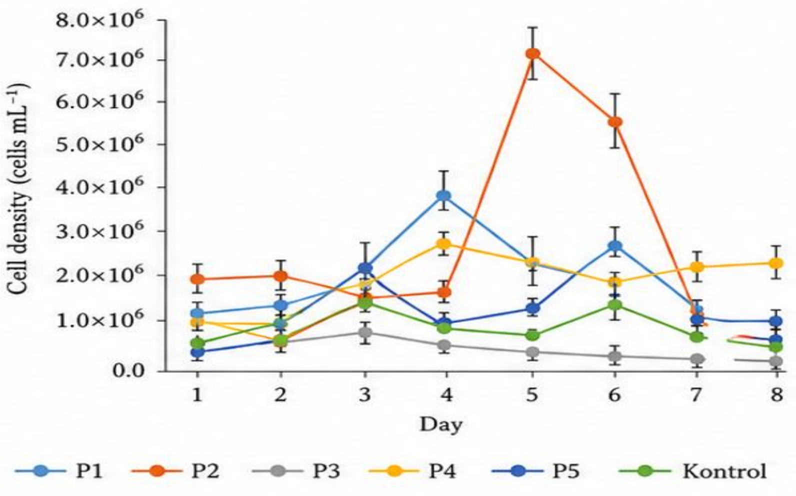

Figure 2. Line chart of growth rate *Chlorella vulgaris* at different concentration of aquaculture waste for 8 days

The growth of *C. vulgaris* in all treatments began to enter the stationary phase on days 6 and 7. The stationary phase is characterized by a decrease or increase in the number of *C. vulgaris* cells. This is because the stationary phase is the phase that precedes the death phase. According to Novianti et al. (2017), the stationary phase typically occurs on day 6, which serves as a marker for harvesting *C. vulgaris*. The stationary phase also indicates that *C. vulgaris* produces various types of secondary metabolites, such as alkaloids, phenols, and carotenoids. *C. vulgaris* begins to enter the death phase, marked by a higher rate of cell death compared to growth. The observation results show that all treatments experienced the death phase on day 8, except for P4. According to Selvika et al. (2016), *C. vulgaris* entering the death phase can be influenced by several factors, such as reduced nutrient content from organic and inorganic materials in the water. Additionally, when performing bioremediation using aquaculture waste, the concentration of aquaculture waste significantly affects the growth rate of *C. vulgaris*.

3.2 Growth density of *Chlorella vulgaris*

The *C. vulgaris* culture conducted over 8 days showed varying cell density growth in each treatment. This is demonstrated by the observation results shown in Table 1. which indicates that the cell density growth of *C. vulgaris* in each treatment experienced both increases and decreases. The highest growth of *C. vulgaris* was observed in P2, which was the aquaculture waste treatment at a concentration of 20 mg/L on day 5, where the average cell density of *C. vulgaris* was 717 x 10^4 cells. The very high cell density indicates that *C. vulgaris* is in the exponential phase and will enter the stationary phase.

Table 2. *C. vulgaris* cell density growth cultured for 8 days

Day of culture	***C. vulgaris* cell density (x10^4 cells)**					
	K	**P1**	**P2**	**P3**	**P4**	**P5**
1	73	156	228	159	129	51
2	108	153	198	93	85	93
3	168	137	169	153	92	11
4	78	367	176	99	85	52
5	92	130	717	48	25	29
6	157	265	519	32	23	97
7	78	123	104	43	20	73
8	40	126	52	41	57	50

Unlike P3, which was treated with an aquaculture waste concentration of 30 mg/L on day 6, the results showed that the growth density of *C. vulgaris* was the lowest among all treatments, averaging 32 x 10^4 cells. According to Anggraini et al. (2023), the low and high growth densities of *C. vulgaris* are due to *its utilization of both inorganic and organic materials from aquaculture waste and the* nutrients contained in the water medium. Variations in *C. vulgaris* cell density growth can also be influenced by aquaculture waste concentration. This is because in some treatments, such as P3, P4, and P5, the high waste concentration inhibits *C. vulgaris* growth.

Good growth indicates that *C. vulgaris* cells can perform optimal bioremediation of aquaculture waste. A higher growth rate indicates that microalgae cell division occurs more rapidly. This results in higher *C. vulgaris* cell density in some treatments. Hartami et al. (2022) stated that the growth rate of microalgae, as indicated by the highest increase in cell numbers, is influenced by macronutrient content, such as phosphorus and nitrogen, in the culture medium.

This is demonstrated by the various treatments of aquaculture waste concentrations in this study, in which K, P1, and P2 had higher *C. vulgaris* cell densities than P3, P4, and P5. High waste concentrations can also affect the growth and division of microalgal cells. There are several other factors that contribute to differences in *C. vulgaris* cell density. This is supported by Markou et al. (2016), who found that high concentrations of pollutants, such as ammonia, can inhibit the growth and metabolism of *C. vulgaris*.

3.3 Measurement and testing of water quality parameters

3.4.1 Temperature

Temperature measurements for all treatments on day 1 ranged from 27.30 to 28.50°C, and on day 8, from 27.40 to 28.10°C. The temperature measurements obtained can be said to support the growth of *C. vulgaris* during the bioremediation of aquaculture waste over 8 days. The research by Nurlaili et al. (2015) states that the optimal growth of *C. vulgaris* occurs at temperatures between 25-35°C.

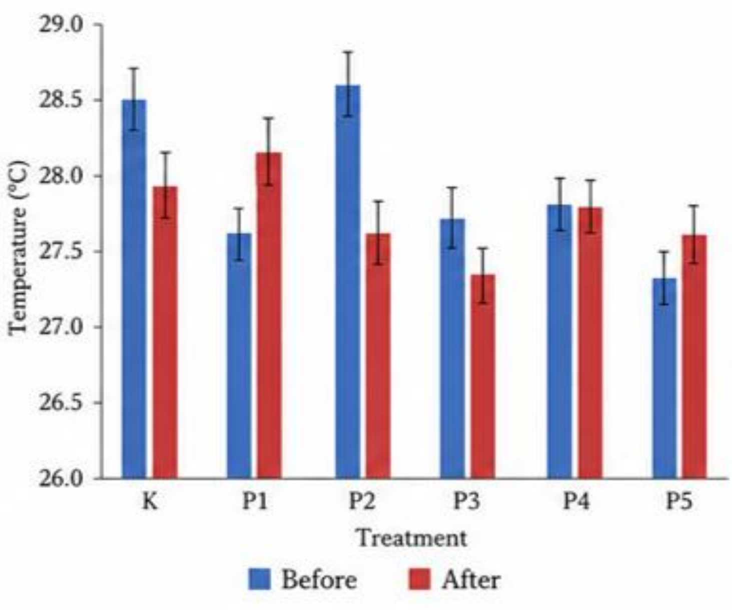

Figure 3. Temperature before and after bioremediation

3.4.2 pH

pH measurements on all treatments on day 1 ranged from 7.72 to 8.01, while on day 8 they ranged from 7.97 to 8.38. These pH ranges support the growth of *C. vulgaris* during the bioremediation of aquaculture waste over 8 days. According to Alvateha et al. (2020), *C. vulgaris* grows optimally in water with a pH value of approximately 7.90–9.10. The increase in pH value during the 8-day culture may be influenced by the density of *C. vulgaris* cells. According to Alvateha et al. (2020), the increase in pH may be caused by the photosynthesis process carried out by *C. vulgaris*. The presence of carbon dioxide gas in the water decreases, leading to an increase the pH.

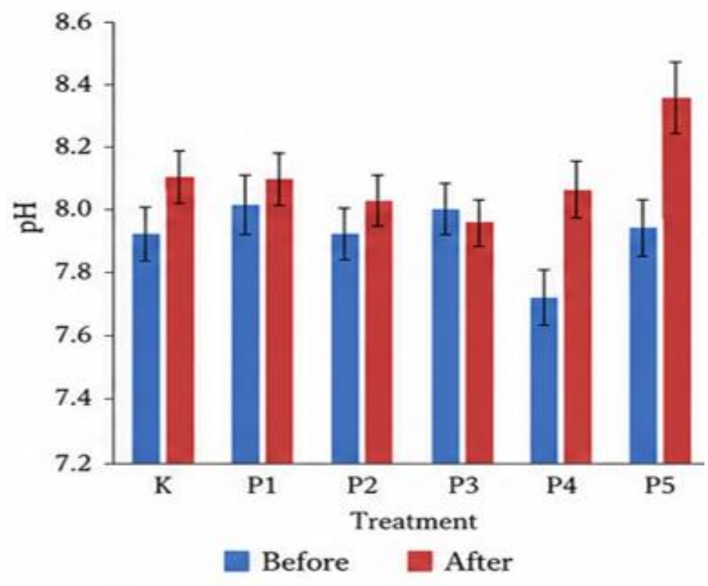

Figure 4. pH before and after bioremediation

3.4.3 Salinity

Salinity measurements on all treatments on day 1 had a value of 30 ppt, while on day 8 the values ranged from 30 to 33.33 ppt. The salinity measurement results support the growth of *C. vulgaris* for bioremediation of aquaculture waste within 8 days. According to Alvateha et al. (2020), *C. vulgaris* can survive and grow at around

30 ppt, but can tolerate salinity levels of 33-40 ppt. The increase in salinity during the 8-day culture was driven by *C. vulgaris* excretion. According to Widayat et al. (2018), the increase in salinity values is significantly influenced by the evaporation process resulting from energy production. The energy produced comes from the carbon metabolism of *C. vulgaris*.

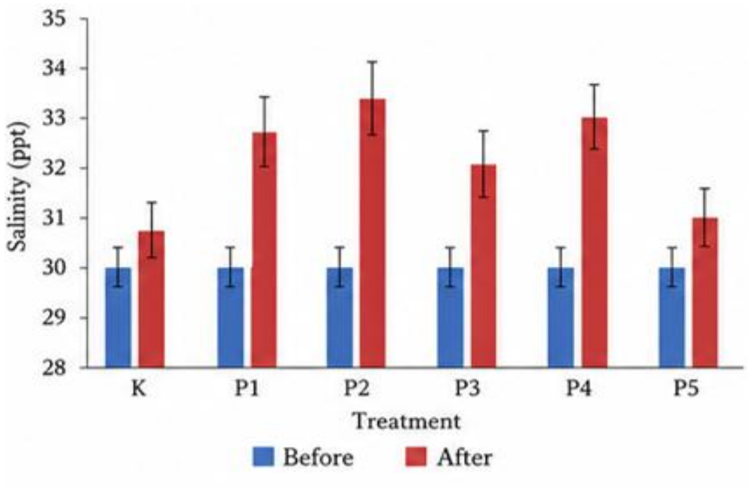

Figure 5. Salinity before and after bioremediation

3.4.4 Nitrate

Nitrate Nitrate concentrations before cultivation ranged from 1.8 to 3.3 mg L^{-1}, while after eight days of cultivation, they ranged from 2.1 to 10.4 mg L^{-1} (Figure 6.). Nitrate removal was observed only in the control treatment and the P1 treatment (10 mg L^{-1}), with removal efficiencies of 22.58% and 27.27%, respectively. Among all treatments, P1 showed the highest nitrate removal efficiency, indicating that low wastewater concentrations provide the most favorable conditions for nutrient assimilation by *C. vulgaris*.

Nitrogen removal by *C. vulgaris* primarily occurs through the assimilatory nitrate reduction pathway. In this process, nitrate (NO_3^-) is actively transported into the cell and reduced to nitrite (NO_2^-) by the enzyme nitrate reductase. Nitrite is then converted to ammonium (NH_4^+) by nitrite reductase before being incorporated into amino acids, proteins, chlorophyll, and other nitrogen-containing cellular components. Therefore, nitrate removal is closely related to microalgal growth because the assimilated nitrogen is directly utilized for biomass production. The efficiency of assimilative nitrate reduction is influenced by environmental conditions, particularly pH. The pH values recorded in this study (7.72–8.38) fall within the optimal range for the growth of *C. vulgaris* and support nitrate reductase activity. Slightly alkaline conditions promote nitrate assimilation by increasing enzyme activity and photosynthetic carbon fixation, whereas unfavorable pH conditions reduce nitrate uptake and metabolic efficiency.

Although nitrate was removed in this study, the removal efficiency was lower than that reported in previous international studies. Saleh *et al*. (2023) reported nitrate removal efficiencies exceeding 50%, while other studies have shown nitrogen removal efficiencies ranging from 50% to 90% under optimized cultivation conditions. The lower removal efficiency observed in this study may be due to the short cultivation period, fluctuating nutrient concentrations, and relatively high nitrogen loads in some treatments, particularly P3, P4, and P5. Excessive nitrogen availability can reduce nutrient assimilation efficiency as cellular metabolism shifts from biomass production to adaptation to physiological stress.

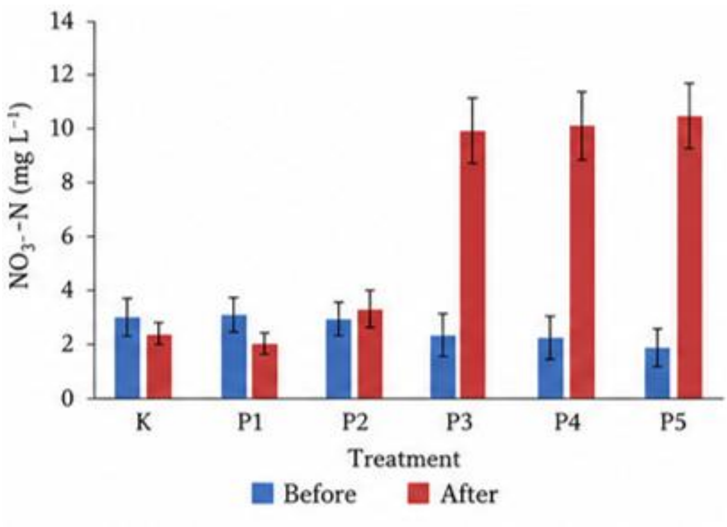

Figure 6. Nitrate concentration before and after bioremediation

3.4.5 Nitrite

Nitrite concentrations prior to cultivation ranged from 0.01 to 0.02 mg L^{-1} and increased to 0.01–0.20 mg L^{-1} after cultivation (Figure 7.). The greatest decrease in nitrite concentration was observed in treatment P1, with a removal efficiency of 50%, while nitrite accumulation occurred in treatments P3, P4, and P5. Nitrite is an intermediate product formed during the assimilatory reduction of nitrate. Consequently, a temporary accumulation of nitrite can occur when nitrate is reduced faster than the rate at which nitrite is converted to ammonium. This condition indicates that nitrogen assimilation is not yet complete. Taziki *et al*. (2015) reported that nitrate reductase catalyzes the reduction of nitrate to nitrite, while nitrite reductase subsequently converts nitrite to ammonium for cellular metabolism.

The increase in nitrite concentration observed in treatments P3, P4, and P5 may also be related to partial nitrification and intracellular redox imbalance due to excessive nutrient loading. High nitrogen concentrations can inhibit nitrite reductase activity, thereby slowing the conversion of nitrite to ammonium and causing nitrite accumulation in the culture medium. Furthermore, an excessive nitrogen load can increase ammonia concentrations, leading to ammonia toxicity. Markou *et al*. (2016) demonstrated that ammonia toxicity suppresses photosystem II activity, inhibits chlorophyll fluorescence, and reduces photosynthetic electron transport. Another mechanism that may explain the observed response is excess uptake. Under conditions of moderate nutrient availability, *C. vulgaris* rapidly assimilates nitrogen and phosphorus in excess of its current metabolic needs and stores these nutrients within the cells as a temporary reserve. This mechanism allows the microalgae to maintain its growth when external nutrient concentrations decline. However, under conditions of excessive nutrient loading, the cells' storage capacity can become saturated, resulting in reduced nutrient assimilation efficiency as well as the accumulation of residual nitrates and nitrites in the culture medium.

Compared to previous international studies, the nitrogen removal efficiency observed in this study was relatively lower than the 50–90% removal efficiency generally reported for *C. vulgaris* cultured under optimized laboratory conditions. Differences in wastewater characteristics, initial nutrient concentrations, cultivation duration, environmental conditions, and light availability may explain this discrepancy. Nevertheless, this study demonstrates that *C. vulgaris* effectively reduces nitrogen compounds at low to moderate wastewater concentrations and provides valuable baseline information for the application of microalgae in tropical aquaculture wastewater treatment systems.

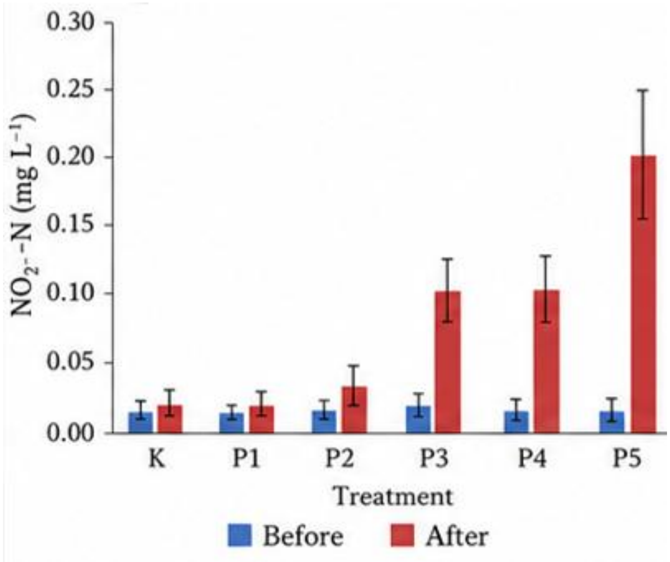

Figure 7. Nitrite concentration before and after bioremediation

4. Conclusion

C. vulgaris demonstrated the considerable potential for the bioremediation of aquaculture wastewater from the Marine Science Techno Park (MSTP), Diponegoro University. Treatment P2 (20 mg L^{-1}) produced the highest cell density (7.17×10^6 cells mL^{-1}), whereas treatment P1 (10 mg L^{-1}) achieved the highest nitrate and nitrite removal efficiencies of 27.27% and 50%, respectively. These findings indicate that moderate wastewater concentrations (≤20 mg L^{-1}) enhance the growth and bioremediation performance of *C. vulgaris*, whereas higher concentrations inhibit growth, likely due to ammonia and nitrite toxic stress. This study was limited by the absence of ammonia and phosphate measurements and by the lack of evaluation of nitrogen degradation kinetics. Future research should investigate the application of *C. vulgaris* in real aquaculture systems, such as IMTA and RAS, evaluate microalgae–bacteria consortia, and develop nitrogen degradation kinetic models to improve wastewater treatment efficiency.

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