Effectiveness of micro-nanobubble aeration and phytoremediation in treating filtered palm oil mill effluent on bacteria diversity and water properties

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Abstract. *Karno R, Arisoesilaningsih E, Mustafa I, Siswanto D. 2024. Effectiveness of micro-nanobubble aeration and phytoremediation in treating filtered palm oil mill effluent on bacteria diversity and water properties. Biodiversitas 25: 4340-4349.* Palm oil waste treatment has gained much significance in recent years, as the palm oil industry has been on the rise. In addition, the biological removal of open treatment ponds and the subsequent activity of microorganisms, mainly bacteria, have been given special consideration. Therefore, this study aimed to evaluate the effectiveness of phytoremediation (Phyt) and micro-nanobubble (MnB) aeration technologies on the improvement of physicochemical properties of filtered palm oil mill secondary effluent (POMSE). The effect of the technologies on bacteria density and composition using a laboratory-scale reactor with 24-hour incubation was also explored. Metagenomic analysis using Next Generation Sequencing (NGS) and traditional methods, such as Total Plate Count (TPC) was used to evaluate bacteria density and composition. Physicochemical analyses were then conducted using an Indonesian standard method (SNI). The results showed that the MnB+Phyt reactor was more effective in reducing waste pollutants and meeting waste standards than control, MnB, or Phyt reactors after 24 hours. In addition, TPC counts showed that the highest bacteria density occurred at the 6-hour mark in the MnB+Phyt, Phyt, or MnB reactors compared to the control. Despite variations in bacteria composition among reactors, the dominant phylum and family were Pseudomonadota, Comamonadaceae, Zoogloeaceae, and Alcaligenaceae. The results also showed that MnB treatment significantly increased alpha diversity and altered genera composition. In conclusion, MnB aeration and phytoremediation technologies effectively reduced filtered POMSE pollutants with a removal percentage ranging from 28.37% to 56.69% under 24-hour treatment.

Keywords: Bacteria, NGS, micro-nanobubble, POMSE, phytoremediation

Abbreviations: POMSE: Palm Oil Mill Secondary Effluent; MnB: Micro-nanobubble; NGS: Next Generation Sequencing; OUT: Operational Taxonomic Unit; COD: Chemical Oxygen Demand; BOD: Biological Oxygen Demand; TSS: Total Suspended Solid; TPC: Total Plate Count

INTRODUCTION

Palm Oil Mill Effluent (POME) has a negative impact on the environment of water bodies because it contains high levels of organic pollutants. These pollutants include Chemical Oxygen Demand (COD), Biological Oxygen Demand (BOD), Total Suspended Solid (TSS), and total nitrogen, which harm aquatic life. Research shows that POME has high organic content, with average BOD, COD, and SS of >25.000 mg/L, 55.250 mg/L, and 19.610 mg/L, respectively (Abdurahman et al. 2013). In addition, POME effluent also smells bad, makes waterbodies less fertile, affects nearby active organisms, and causes serious environmental pollution if not handled (Dashti et al. 2022). Recent studies have reported the characteristics of Palm Oil Mill Secondary Effluent (POMSE), often found in final treatment ponds and contains lower COD, BOD, and TSS compared to POME. The results showed that the COD, BOD, and TSS of POMSE were reduced to 750 mg/L, 350

mg/L, and 790 mg/L, respectively (Darajeh et al. 2016). Bacteria-based biological treatment to break down organic and contaminant compounds has an impact on reducing POME's harm to the environment. Research shows that various bacteria species contribute to different treatment stages. Lactobacillaceae, for example, dominate early treatment by creating high amounts of lactic acid. Methanosaetaceae, on the other hand, plays a key role in degrading acetic acid and producing methane in open and closed ponds (Mohd-Nor et al. 2019). A study by Karno et al. (2024) also found the genus *Allochromarium* as a main group in POMSE. Even with current research on bacteria treatment of POME, the process still takes a lot of time. As a result, researchers have looked into various ways to treat this waste, including using activated sludge to break down waste in ponds without oxygen (Zahrim et al. 2014), micro-bubbles generated from Polyaluminium Chloride (PAC) in anaerobic treatment (Poh et al. 2014), photocatalytic membrane POMSE treatment reactor (Alhaji et al. 2016;

Zainuri et al. 2018; Sidik et al. 2019; Sidik et al. 2020), optimization of electrocoagulation in purifying POME (Rusdianasari et al. 2017), Ultrasonic Membrane Anaerobic System (UMAS) technology (Abdurahman and Azhari 2018), biofilm reactor (Soo et al. 2022), and anaerobic-aerobic Down-Flow Hanging Sponge (DHS) technology (Natalia et al. 2023). These methods have made big steps in reducing pollutants in wastewater. However, the sole focus has been on POME, leaving the treatment of POMSE needing of further optimization.

Several previous findings have identified microorganisms as significant in the anaerobic, aerobic, and facultative processes for wastewater treatment within POME reactors. Thus, the presence and development of microbes are preconditions for a successful breakdown of pollutants in wastes. Another main cause of aiding aerobe bacteria growth is the provision of adequate oxygen, which can be done by applying the aeration systems through air bubbles. Moreover, combining aeration with phytoremediation systems seems to be another possibility for increasing these engineered systems' treatment capacity. Several past research studies have revealed that nanobubble aeration technology could prove quite effective in improving aerobic biofilm systems and enriching the capability of the removal of organic compounds and pollutants in wastewater. The present investigation on biofilm growth indicates that nanobubble aeration has the potential of being a quick, effective, economical initiation method (Xiao and Xu 2020). In addition, phytoremediation can effectively address organic matter and pollutants in POME. For example, vetiver plants can reduce BOD by 60% in highly concentrated POME, compared to a 15% reduction without plant intervention (Darajeh et al. 2014). *Eichhornia crassipes* plants have also been shown to reduce BOD by 92% within 21 days (Wei et al. 2019). *Salvinia×molesta* was reported to efficiently remove 39% of COD and 95% of phosphate (Ng and Chan 2017), while *Azolla pinnata* efficiently absorbed and accumulated organic carbon (COD), phosphate nutrients (PO_4^{3-}), nitrate nitrogen (NO_3-N), and ammonia nitrogen (NH_3-N) (Al-Baldawi et al. 2023). Phytoremediation using *Scirpus grossus* also has great potential in nutrient removal (Sa'At et al. 2022). Thus, the use of phytoremediation (Phyt) technology in conjunction with a novel, efficient micro-nanobubble (MnB) aeration technology is advised as the most suitable and effective method for treating POME. Therefore, the goal of the present work was to enhance the physicochemical characteristics of the filtered POMSE employing Phyt and MnB treatments.

MATERIALS AND METHODS

Sample collection and preservation

Raw POMSE water samples were collected aseptically from the Sei Rokan palm oil mill, which used a pond system. POMSE effluent was obtained from the secondary pond of the palm oil mill. Samples were collected from 3 sampling points compositely, stored in bottles, and refrigerated until further analysis (Karno et al. 2024).

Micro-nanobubble and phytoremediation reactors

The reactor system consisted of 4 reactor basins used for 4 treatments, namely basin 1 (control), basin 2 (Phyt), 3 (MnB), and 4 (MnB+Phyt). In this study, the phytoremediation system used 4 types of hydromacrophytes that were selected based on their effectiveness in removing pollutants and their presence in the environment surrounding POME treatment ponds. The selected hydromacrophytes included *Hydrilla verticillata* (submerged), *Commelina communis* (emergent), *Limnobium laevigatum* (floating leaf), and *Lemna minor* (floating leaf/emergent).

Water tanks were used to fill each basin with a total volume of 120 L. This tank was sized according to the specific needs of each reactor basin and was filled with 20 L of effluent in reactor basins with a volume of 40 L (Figure 1). The methods used in this study were adapted and modified from a previous design by Oktorina et al. (2019). This relatively large basin volume made it possible to accommodate larger plants or a greater number of plants, hence minimizing the impact of daily fluctuations in water quality.
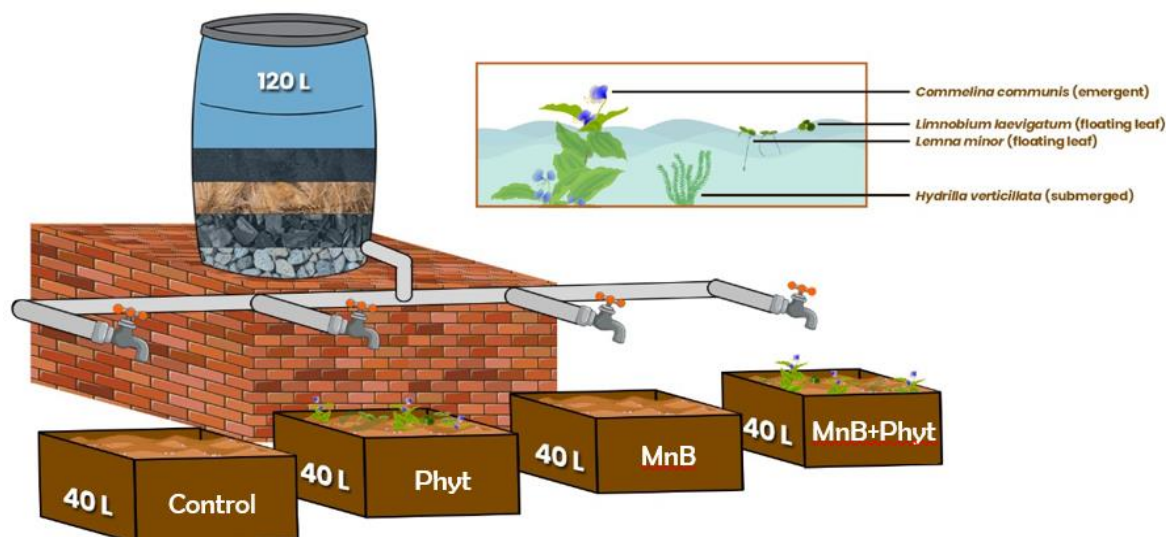

Figure 1. Reactor of micronanobubble air and phytoremediation systems (Phyt: phytoremediation, MnB: micro-nano bubble)

Bacteria counting methods

Number of bacteria by total plate count

TPC was performed using serial dilution and pour plate methods. Dilution was conducted using buffered peptone water with a sample ratio of 1:9. Bacteria plating was performed using Plate Count Agar (PCA) media and incubation was carried out at a temperature of 34 to 36°C for 48 hours. The number of bacteria was calculated using the following formula (Cundell 2015).

$$Bacteria\ number = colony\ number \times \frac{1}{dilution\ factor}$$

Bacterial metagenomic diversity

The metagenomic diversity analysis of the bacterial community was performed by using the Next Generation Sequencing (NGS) according to the marker of the whole 16S rRNA gene. The analysis covered the V1 to V9 regions of the gene using the Oxford Nanopore Technology (ONT) platform. Bacterial DNA was isolated with the Quick-DNA Magbead Plus Kit (Zymo Research, D4082). Based on DNA isolation, Polymerase Chain Reaction (PCR) was performed with primers 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′). The resulting amplicons were prepared for library construction. Sequencing was conducted, and the data were analyzed using an appropriate bioinformatics pipeline. Quality control was performed at each step using genomic DNA quantification techniques with a Nanodrop and Qubit Fluorometer (Yip et al. 2021; Adzdzakiy et al. 2024). Furthermore, the bioinformatic analytic pipeline included the sequencing process was performed using MinKNOW (v23.05.5)3. According to a study by Wick et al. (2019), the base-calling process used Guppy (v6.5.7) with a high-accuracy model. Quality control of raw FASTQ data was visualized by NanoPlot and filtered with NanoFilt (de Coster et al. 2018). The read data is classified by Centrifuge classifier (Kim et al. 2016). based on the NCBI 16S Refseq database. Advanced analysis and visualization were performed with Pavian, Krona, and RStudio (R v4.2.3).

Physicochemical evaluation of samples

Observing the physicochemical parameters of POMSE was essential for evaluating environmental quality, particularly in wastewater management. In Indonesia, the minimum concentration limits for palm oil industry effluents were followed by the Regulation of the Minister of Environment of the Republic of Indonesia No. 5 of 2014 (Karno et al. 2024). Temperature (°C) and pH were measured on-site using a multi-portable meter (Eutech Expert pH, Thermo Scientific, Sweden). Upon arrival at the laboratory, COD was assessed using a COD reactor (HACH 45600) following the HACH 8000 method. The 5-day BOD5 was measured following the COD test, according to APHA 5210-B (APHA 2005). Water acidity was determined with a pH meter, while TSS was measured using the titrimetric method (SNI 6989.3-2019). Furthermore, oil and lipid content were quantified by the gravimetric method (Indonesian Standard: 6989.10-2011), and total nitrogen was tested using the Kjeldahl method as outlined by Bremner and Mulvaney (1982).

Data analysis

The number of bacteria (log CFU) data was prepared descriptively and presented in the form of figures and tables. The relationship between independent and dependent variables was determined using regression analysis. The statistical test was carried out using MANOVA.

RESULTS AND DISCUSSION

Significant bacterial growth improvement under nanobubble and phytoremediation

The best value of bacterial TPC was observed in the MnB+Phyt treatment after 6 hours of treatment with the number of bacteria being 6.7×10^6 log CFU/ml and significantly different from the treatment and hours 0, 12, 18, and 24. However, there was a less significant difference between from MnB hours 6 and 12 and Phyt at hour 6. The number of bacteria then dropped after 6 hours of incubation and continued to decrease until the incubation period of 24 hours. The notations (a-f) presented in Figure 2 show that there were no significant differences in bacterial growth between treatments at each hour, but there were significant differences at hours 6 and 24, especially in the Mnb, Phyt, and MnB+Phyt treatments.

Diversity and structure of bacteria under micro-nanobubble technology and phytoremediation

Bacteria relative abundances were explored in 5 sample groups. At the phylum level, Pseudomonadota consistently dominated the communities in all 5 samples, followed by Bacillota and Bacteroidota. At the family level, Zoogloeaceae, Comamonadaceae, and Alcaligenaceae were generally the most abundant family collectively, in order. However, in the MnB, Zoogloeaceae abundance level was different from the other group, which was less than Burkholderiaceae. This similar data trend could also be observed at the genus level, while control, phytoremediation, and MnB+phytoremediation exhibited *Thauera, Hydrogenophaga* and *Zeimonas* as the most abundant taxa consecutively. Group 4 had *Zeimonas, Hydrogenophaga,* and *Thauera* as the most abundant taxa consecutively (Figure 3).

At the qualitative genera level, bacterial community composition and relative abundance of each taxon were quite similar between all sample groups. The dominating genera in these 4 treatments were groups from the genus *Thauera* (7% in control; 9% Phyt; 2% MnB; and 12% MnB + Phyt) and *Zeimonas* (3% control; 3% Phyt; 5% MnB; and 2% MnB + Phyt). The phylogenetic relationships between all taxa found from each sample group are presented in Figure 3. In addition, the Sankey Diagram visualized how abundant each taxon found in the corresponding sample group was. The thicker the horizontal line, the more abundant a taxon was compared to other taxa in its taxonomic hierarchy. The visualized phylogenetic tree was based on the number of 'clade reads' of each taxon. In the control, bacterial kingdom had 904,000 reads, while the phylum Pseudomonadota, Bacillota, and Bacteroidota had 472,000, 161,000, and 107,000 reads, respectively. In Phyt, bacteria kingdom had 905,000 readings, while the phylum Bacillota,

Pseudomonadota, and Bacteroidota, had 465,000, 167,000, and 113,000 readings respectively. In Group 4 (MnB), bacterial kingdom had 894,000 readings, while the phylum Pseudomonadota had 470,000 readings, Bacillota had 140,000 readings, and Bacteroidota had 111,000 readings. Finally, in Group 5 (MnB+Phyt), bacteria kingdom had 914,000 readings, while the Pseudomonadota phylum had 476,000 readings, Bacillota had 173,000 readings, and Bacteroidota had 113,000 readings (Figure 4).

Alpha diversity could be used to measure the internal bacteria diversity index in a sample. The Chao1 and ACE indexes represented the abundance of a microbial community, while the Shannon and Simpson indexes represented its diversity. All indexes showed that the most diverse bacteria community was in MnB on all biodiversity indices. Consequently, the next most diverse sample group was the number control, Phyt, and MnB+Phyt (Figure 5).

The ratio of relative abundance of a particular taxon from various sample groups was visualized in the heatmap (Figure 6). This visualization was used to identify when there was any pattern in the recorded data. The more relatively abundant a taxon was, the more the reddish the color appearance in the heatmap. Meanwhile, when a taxon had a relatively low abundance, it could appear more blackened. In this study, the data generally had similar characteristics. However, the genera *Allocrhomatium*, *Macromonas*,

Tepidibaculum, and *Rhodocyclus* appeared to have slightly varying relative abundances among all sample groups, specifically in the MnB sample group.
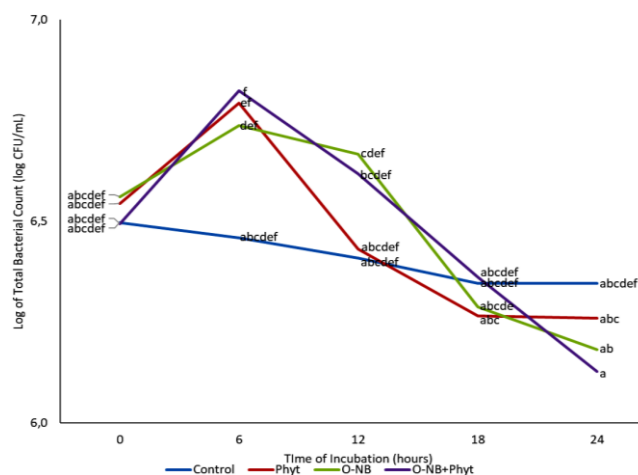

Figure 2. Bacterial abundance of POMSE after treatments by the TPC method. Phyt: phytoremediation reactor; MnB: micro-nanobubble reactor. Different statistical notations (a, b, c, d, e, f) in the lines indicate the significant differences ($p < 0.05$)
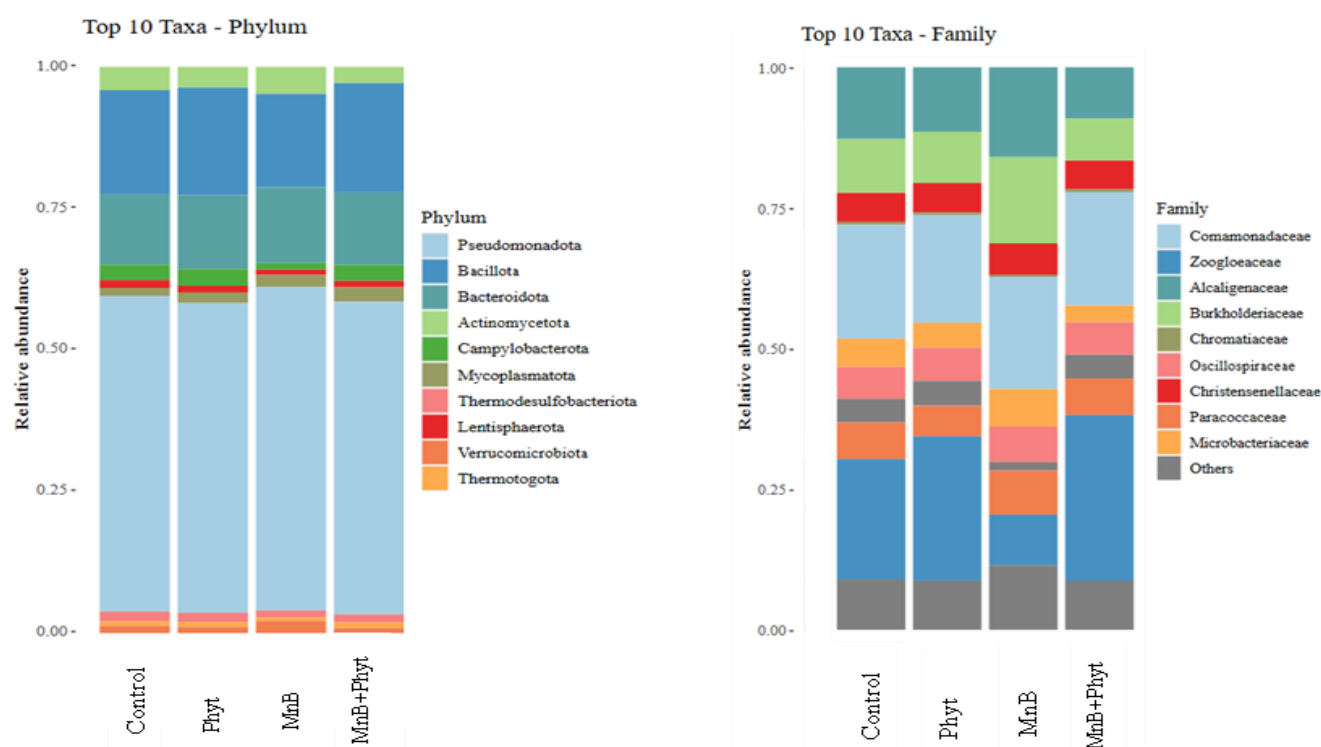

Figure 3. Top 10 of relative abundance phylum and family level. Phyt: phytoremediation, MnB: micro-nanobubble
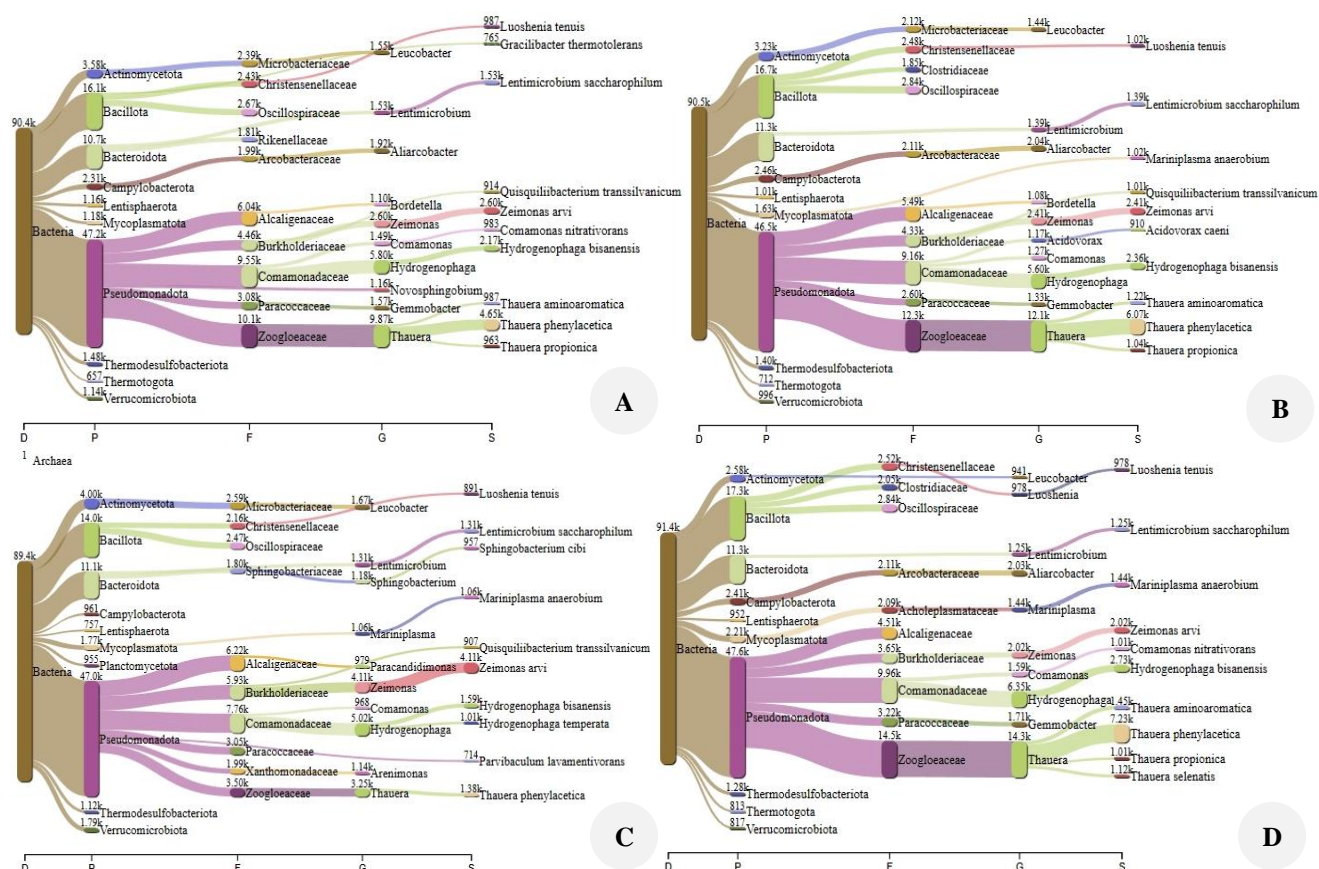
Figure 4. Sankey diagram shows the phylogenetic trees of the taxa from each sample group, including its relative abundance data. A: control; B: phyt; C: MnB; D: MnB+Phyt
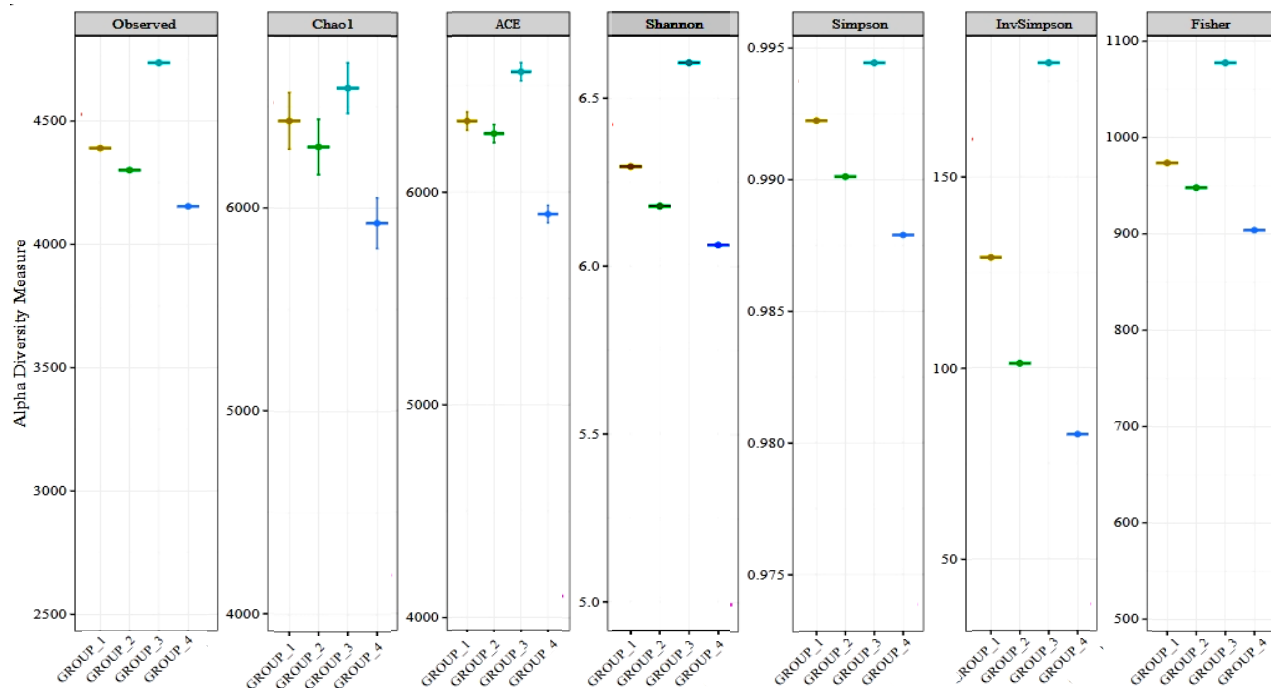

Figure 5. Alpha diversity indices visualization. Group_1: Control; group_2: phytoremediation result; group_3: micro-nanobubble; and group_4: micro-nanobubble+phyteremediation

Physicochemistry of POMSE after treatment by micro-nanobubble technology and phytoremediation

The enhancement of bacteria growth significantly impacted the physicochemical properties of POMSE. In addition, the results showed that both MnB aeration and phytoremediation technologies were effective in removing pollutants from the effluent. These methods effectively reduced the organic pollutants in POMSE. However, even untreated POMSE showed a decrease in its physicochemical properties over time due to the incubation period. Both phytoremediation and MnB aeration technologies succeeded in reducing pollutant levels to meet quality standards. In this study, there was a percentage removal of physicochemical properties for all samples. The BOD_5 value experienced the highest removal in the MnB+Phyt reactor with a percentage of 56.8%. There were significant differences between control and other treatments for BOD and COD based on the notation (a-b) (Table 1).

Bacterial abundance in the control tended to decrease from hour 0 to hour 24, while the Ft, O-NB, and O-NB+Ft treatments experienced an increase in numbers at hours 6 and 12, which then decreased until hour 24. Fluctuations in the increase and decrease in bacteria abundance impacted the downward trend in BOD and COD content of the effluent. The downward trend in BOD and COD values was slimmer compared to the Ft, O-NB, and O-NB+Ft values, which experienced a greater decrease. This provided information that an increase in bacteria population accelerated the removal of BOD and COD values (Figure 7).

Table 1. Efficiency of phytoremediation, micronanobubble, and their combination technologies on the physicochemical properties of POMSE effluent after 24 hours of incubation

Condition	Removal efficiency of physicochemical properties (%)				
	BOD	**COD**	**TSS**	**Total N**	**Oil & grease**
Control	15.34±13.3a	15.05±13.7a	12.37±6.8a	5.77±2.8a	25.07±10.5a
Phyt	45.38±17.8b	32.54±8.2ab	32.39±15.9a	21.87±15.5a	36.03±11.8a
MnB	30,30±2.6ab	31.30±1.5ab	26.88±8.7a	16.59±8.4a	37.08±6.4a
MnB+Phyt	56.69±2.2b	38.14±5.0b	34.89±7.1a	28.37±11.2a	41.16±10.2a

Notes: Different statistical notations (a, b) in the same column indicate the significant differences (p<0.05)
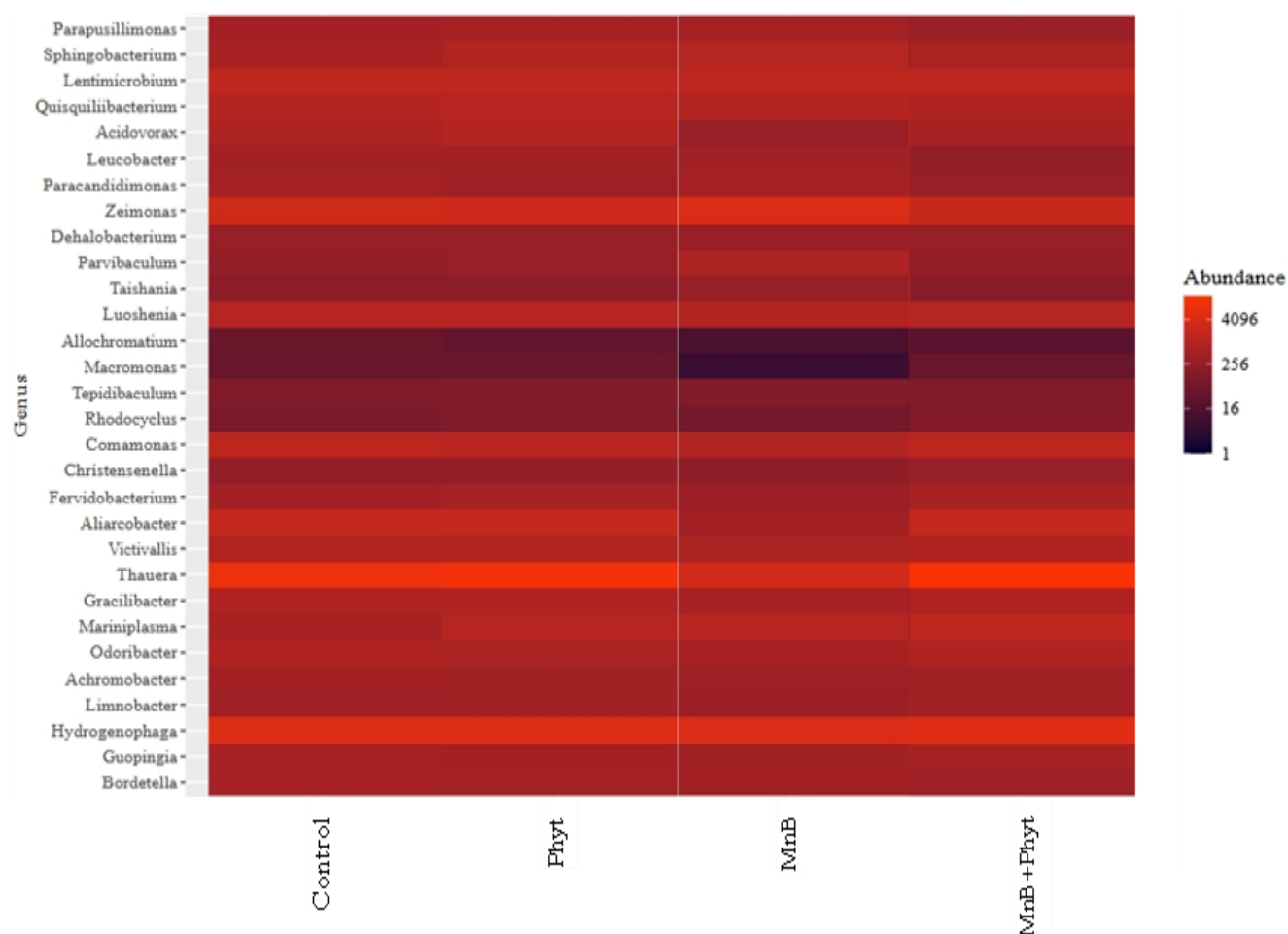

Figure 6. Heatmap analysis on genera level. Phyt: phytoremediation, MnB: micro-nanobubble, MnB+Phyt: micro-nanobubble+ phytoremediation
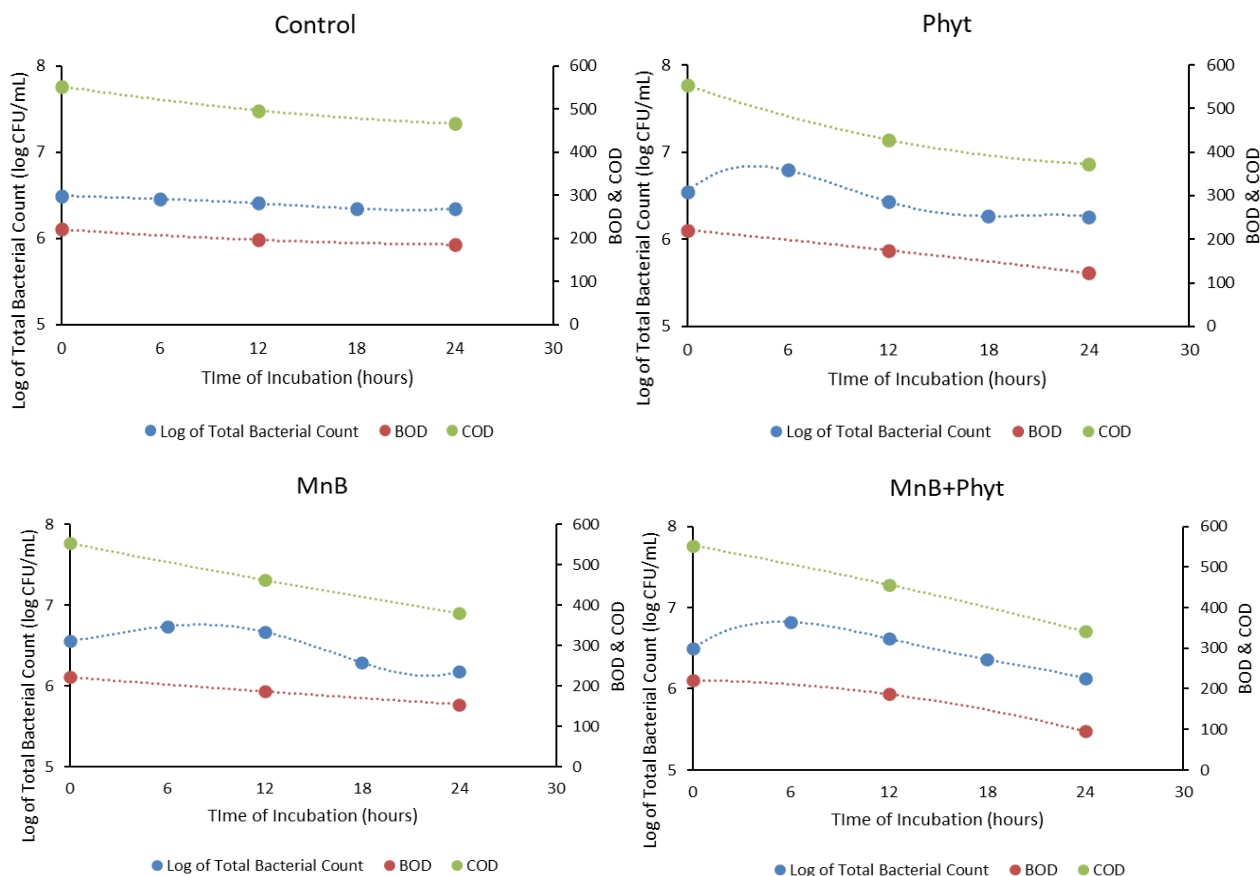
Figure 7. Interaction of POMSE bacteria population increases after Micro-nanobubble technology and phytoremediation on BOD and COD content. Phyt: Phytoremediation; MnB: Micro-nanobubble

Discussion

Bacteria abundance

The bacterial growth experienced several phases including lag, log, stationary, and death phases. Data on the abundance of bacteria using the TPC method in this study showed that bacteria experienced a stationary phase after 6 hours of incubation. This was shown due to the reduction of nutrients in the waste due to rapid bacteria development. The results also showed that MnB aeration could increase and sustain bacteria abundance for 12 hours. Integration of MnB aeration and phytoremediation is more optimized to increase the growth and sustain the number of bacteria. The rapid development was influenced by the effective MnB+Phyt in the treatment. Nanobubble aerators significantly increased the abundance of bacteria in the pond (Xu et al. 2022). The application of nanobubble effectively provided extra oxygen, hence increasing microbial aggregates (Xiao et al. 2021). In addition, the growth of bacteria was heavily influenced by certain environmental conditions, including the availability of nutrients and their mutualism symbiosis with some plants. Bacteria that had a natural habitat in water form consortia with bacteria attached to plants, which had a significant impact on the ability to treat waste in phytoremediation technology. Several strains of rhizosphere bacteria and endophytes have proven effective in various phytoremediation schemes (Kong and Glick 2017).

Diversity and taxonomic of bacteria after treatment

Diverse microbes could be found in industrial wastewater, including in oil palm wastewater. Diverse microorganism communities were known to help the effectiveness of the biodegradation and bioremediation process of wastewater. The presence of microorganisms in the wastewater could be because oil palm wastewater contains a lot of organic compounds including carbohydrates, proteins, lipids, minerals, nitrogenous compounds, cellulose, hemicelluloses, and lignin. Some microbial species found in oil palm wastewater in Malaysia had the prospective to degrade carbon and oil (lipid) as the carbon sources by hydrolyse oil and producing the lipase enzyme. Some of these bioactivities was observed on *Micrococcus luteus* 101PB, *Stenotrophomonas maltophilia* 102PB, *Bacillus subtilis* 106PB, and *Bacillus cereus* 103PB (Bala et al. 2014). Other sources claimed that the microbes were comparable to microorganisms that could be found in wastewater-polluted areas around the oil palm industry (Bala et al. 2015). These microorganisms were used to reduce the COD in wastewater, by degrading oil and cellulose into water and carbon dioxide (Singh et al. 2010).

At the genus level qualitatively, bacteria community composition and each of its taxa relative abundance was similar between all the group samples. However, when compared to the bacterial community in the initial effluent before the experiment (inlet of the POMSE), bacteria

composition at the species level in this study had a significant difference. Bacteria species at the inlet of POMSE were dominated by a group of bacteria from the genus *Allochromatium* (Karno et al. 2024), while the genus that dominated in these 4 treatments were groups from *Thauera* and *Zeimonas*. Unfortunately, there was not much information on *Thaeura* and *Zeimonas* in palm oil mill waste, although this genus of bacteria was often found in anaerobic and facultative environments and was studied for its potential for environmental improvement. The genus *Thauera* was known as carbon and nitrate-sequestering bacteria and nitrate-reducing bacteria (Mao et al. 2014). In addition to nitrate reduction, it also produces N_2 and NH_3 that could enter the cell for organic N biosynthesis (Zhang et al. 2019). *Thauera* was a type of bacteria in denitrifying bacteria, which were functional taxa in terms of nitrogen and phosphorus removal and was the most abundant bacteria species found in microalgal-bacteria granules (Ji et al. 2019; Ji et al. 2020).

The genus *Thauera* was a useful heterotrophic bacteria in the process of absorbing organic substrates in wastewater (Thomsen et al. 2007). Meanwhile, *Zeimonas* was a genus of bacteria widely found in several environments, including wastewater. Bacteria of *Zeimonas* could degrade organic compounds and participate in nutrient cycling. In wastewater treatment, microbial communities, including bacteria such as *Zeimonas*, played an important role in breaking down organic matter and pollutants, hence contributing to the purification of wastewater before it was released back into the environment or reused. These bacteria were part of a complex ecosystem of microorganisms that collaborated in the wastewater treatment process. *Zeimonas* showed its potential role in treating and remediating wastewater by breaking down pollutants such as biphenyls and phenolic acids. *Zeimonas arvi* could be useful in environmental remediation processes, specifically in wastewater treatment plants, due to its ability to metabolize xenobiotics and allelochemicals. *Zeimonas* were positive for catalase and oxidase activity and were able to reduce nitrate to nitrite (Lin et al. 2021).

The capabilities of MnB and phytoremediation to improve bacteria growth could reduce organic pollutants, specifically BOD and COD. The increase in the total number of bacteria in the Phyt, MnB, and MnB+Phyt reactors optimized the removal of BOD and COD, even in the MnB+Phyt reactor could remove BOD and COD to meet the quality standards of palm oil industry waste. Compared to the control reactor, the increase in the number of bacteria in phytoremediation, MnB, and micro-nanobubble+phytoremediation treatments showed an increase in the number of bacteria specifically at hour 6, and was able to last up to 12 hours, then decreased in the following hours. The decrease in the number of bacteria was due to the organic levels of POMSE waste, specifically BOD and COD, whose levels dropped at relatively the same hours. This was caused by bacteria contained in the waste that used organic matter as the primary source of energy to ensure that a decrease in organic content could also have an impact on the decrease in the population of bacteria. In wastewater, high BOD and COD values showed more

substrates that could be used by bacteria, specifically aerobic bacteria (Jouanneau et al. 2014). A high BOD value showed a larger bacteria population. The increased BOD and COD levels tended to increase the number of bacteria populations (Akan et al. 2009). The bacterial community affected the level of pollutants, while the pollutants contained in the effluent were factors that could change the composition of bacterial community (Zhang et al. 2023). Microbial activity affected the temperature of the sewage treatment system (Tchobanoglous et al. 2014), altered the pH of the effluent (Gerardi 2003), contributed to increased turbidity and suspended solid (Bitton 2010), reduced DO content, and increased BOD (Madigan et al. 2021).

In conclusion, MnB aeration and phytoremediation technologies significantly reduced filtered POMSE pollutants in 24 hours with a removal percentage ranging from 28.37% to 56.69%. The abundance of bacteria increased and achieved the highest after 6 hours of incubation in the MnB+Phyt treatment. In addition, bacterial communities showed similar bacteria composition for all treatments, with Pseudomonadota as the dominant phylum, Zoogloeaceae, Comamonadaceae, and Alcaligenaceae as the dominant families, and *Thauera* and *Zeimonas* as the two dominant genera. The highest alpha diversity was found in MnB and the lowest in MnB+Phyt.

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