CHAPTER 5

DISCUSSION

5.1 Metagenome Sequence Reveals The Presence of Beneficial and Bioremediation Bacteria Related to The Mangrove Environment

5.1.1 How Does Carbon Fixation Metabolism Occur in Mangrove Soil in Sungai Lukut?

"Carbon-fixing" autotrophic organisms, such as photosynthesising plants and the photo- and chemoautotrophic microorganisms that convert atmospheric carbon dioxide (CO_2) into organic material, are responsible for the transport of carbon from the atmosphere to soil (Gougoulias et al., 2014). Bacteria play a crucial role in recycling dead matter by decomposing it and changing it into substances that can be utilised again by other organisms. The cycle of carbon cannot function without the help of other elements necessary for carbon metabolism (Gougoulias et al., 2014). According to the KEGG carbon fixation pathway, the TCA cycle is interconnected with other metabolic processes, including glycolysis and the Calvin cycle. These pathways play a crucial role in turning inorganic carbon into organic forms that can be utilised for growth. Although it is widely established that some prokaryotes may convert CO_2 into organic carbon (Lynn et al.,

2016), the roles of prokaryotes in carbon fixation in mangrove ecosystems remain largely unexplored. In this study, genus *Pseudolabrys* in soil 1 exhibited high contributions in CO_2 fixation, while *Methyloceanibacter* in soil 2 and *Nocardioides* in soil 3. *Methyloceanibacter*'s capability to utilise and fix carbon from methane exemplifies a unique aspect of carbon fixation in microbial ecosystems. In contrast, the 3-hydroxypropionate bi-cycle is not limited distinctive to the phylum *Chloroflexota*, as was previously believed, and the genomic potential for carbon fixation is revealed in extensively unrecognised archaeal and bacterial phyla (i.e. *Thermoplasmatota* and *Elusimicrobiota*).

The findings of this study further showed that mangrove soil in Sungai Lukut exhibited the highest abundances of genes involved in carbon-related pathways including genes PDH, MDH, IDH, ACL, *fumABC, sdhABCD, frdABCD, tfrAB, sucCD, ccsAB, porACD, ppddK* and *ppc*. Both an ATP-dependent citrate lyase (ACL) and a sequential reaction between an ATP-dependent citryl-CoA synthetase (CCS) and a citryl-CoA lyase (CCL) are capable of catalysing the citrate cleavage (Garritano et al., 2022). Different from the ATP-dependent cleavage of citrate to OAA and acetyl-CoA catalysed by ACL and CCS/CCL, the action of citrate lyase, which cleaves citrate into acetate and oxaloacetate (OAA), is separate (Garritano et al., 2022). Two distinct genes, *aclA* and *aclB*, code for ATP citrate lyase. According to Hynes and Murray, (2010), the presence of *aclBA* and the high levels of citrate cleavage activities provide compelling evidence that *Desulfurobacteriaceae* make use of ATP citrate lyase in order to complete the citrate cleavage process. Only prokaryotes, specifically *Proteobacteria*, which use the reductive

TCA cycle for autotrophic carbon fixation, have been found to possess ATP citrate lyase genes (Correa et al., 2023). According to Tang et al. (2011), some anaerobic chemotrophic bacteria catalyse a process that results in the synthesis of citrate for the oxidation of acetate. Citrate cleavage via CCS and CCL has recently been discovered in the obligate chemolithoautotrophic bacterium *Hydrogenobacter thermophilus* (Arai et al., 2010).

According to the result from KEGG database in this study *frdABCD* and *sdhABCD* are abundant in soil 2 which responsible in the conversion of succinate to fumarate which can be a transition step in energy production in the form of NADH. While *porACD* are participate in the conversion of α-ketoglutarate to succinyl-CoA, an essential step in the reductive citric acid cycle and are found in all soils (soil 1, 2 and 3). This study findings are comparable to Cucio et al., (2018) where genes for two carbon fixation pathways were identified including *frdA, sdhA, sdhC,* and *porAB* from rTCA cycle. In addition, Mueller et al. (2020) identified that *Nitrospinae* contributed to *porA, porB, porD,* and *por*. More than 50% of the gene *ccsB* belonging to carbon fixation in prokaryotes. The gene *ccsB* acts on the reduction of the citrate cycle (Arnon-Buchanan cycle) where the CO_2 is fixated into the microbial cells (Garritano et al., 2022). Similarly, to the outcome to this study where the citrate is reduced into oxaloacetate and acetyl-CoA.

Numerous bacteria species found in Sungai Lukut's mangrove including *Woeseia oceani, Methyloceanibacter superfactus, Thiohalobacter thiocyanaticus, Phycicoccus jejuensis, Solirubrobacter soli, Solirubrobacter sp*. URHD0082, and *Pseudolabrys*

taiwanensis. In the context of carbon fixation. *Thiohalobacter thiocyanaticus* has a role in the degradation of thiocyanate, which contains carbon and nitrogen atoms, and so indirectly affects carbon cycling (Oshiki et al., 2019). It is also important to note that the relationship between carbon fixation and the growth of plant is regulated by other macronutrients, such as nitrogen and phosphorus, which are necessary for a variety of metabolic processes. Carbon dioxide and nitrogen molecules are produced as thiocyanate decomposes. In addition to fixing CO_2 through the Calvin cycle and CO oxidation, several aerobic CO-oxidizing bacteria, such as *Bradyrhizobium japonicum*, *Mycobacterium sp*. JC1, and *Burkholderia sp.*, possess the *cbbL* gene (Lynn et al., 2016). The TCA cycle produces intermediates that are essential for the synthesis of amino acids, which play a critical role in the assimilation of nitrogen. Tian et al. (2021) investigated the methods by which rhizosphere microbes detoxifies ammonium and found that carbon and nitrogen metabolism are closely interconnected, especially when exposed to ammonium stress. Overall, this study suggested that mangrove soil in Sungai Lukut exhibited a conversion of organic matter between plants and microbes owing to high carbon utilization and transportation. Microbial carbon fixation contributed to carbon sequestration, whereas the degraded small organic molecules could be conducive to the mangrove's growth.

5.1.2 How Does Methane Metabolism Occur in Mangrove Soil in Sungai Lukut?

Methane (CH_4) is a byproduct of the microbial breakdown of organic matter that occurs in anaerobic conditions (Begmatov et al., 2021). The microbial community responsible for methane metabolism in mangrove soil is diverse and includes both methylotrophs and methanotrophs. Methanotrophs are organisms that consume the methane found in soil on both the seafloor and the land (Khider et al., 2021). All mangrove soil contains methanotrophs, which are actively engaged in the methanogenesis process (Salvador et al., 2021). Previous research showed that *Methylocaldum, Methyloceanibacter, Methylococcus, Methylobacter* and *Crenothrix* identified genes correspond to methanotrophic metabolism pathways (Campbell et al., 2011; Kalyuzhnaya et al., 2015; Skennerton et al., 2015; Oswald et al., 2017; Luangthongkam, 2018), while the result from this study showed *Methylosarcina, Methylocaldum, Methylobacter, Methylibium, Methylonera, Methyloversatilis, Methylocystis* and *Methyloceanibacter* involved in methane pathway from soil 1, 2 and 3. MOB have a wide range of genetic diversity (Smith and Wrighton, 2019) and are known to rapidly metabolize significant volumes of CH_4 in the presence of oxygen (Bastviken et al., 2023).

The methane metabolism pathway encompasses crucial enzymes, including methyl-coenzyme M reductase, which facilitates the ultimate stage of methane generation in methanogenic archaea, and methane monooxygenase (MMO), which triggers methane oxidation in methanotrophic bacteria. The growth of plants can be influenced indirectly by these processes, as they can cause changes in the structure of the microbial community and the availability of nutrients in soil (Wang et al., 2021). In their study, Wang et al.

(2021) showed that submerged plants had a substantial impact on the functional capabilities of microbial communities in wetland sediments, particularly in relation to methane metabolism. There are two stages to the methanotrophic process: the oxidation of CH_4 to formaldehyde and the assimilation of formaldehyde (Wang et al., 2023). Both soluble and particulate methane monooxygenase (sMMO and pMMO, respectively) are expressed by methanotrophs (Dalton, 2005). All methanotrophs contain methane monooxygenase (MMO) are found to be the most abundant in soil 3. Whereas, MMO is restricted to species belonging to the genera *Methylocystis* in soil 2. The sMMO enzyme complex is composed of up of a regulatory protein encoded by *mmoB*, a hydroxylase component encoded by *mmoXYZ*, a reductase component encoded by *mmoC* (Nakamura et al., 2007). According to reports of Merkx and Lippard, (2002), *mmoD* is involved in the assembly of the sMMO and the hydroxylase-component di-iron center. MMO enables aerobic methanotrophs to utilize methane as a sole carbon and energy source (Khider et al., 2021) and act as a filter and reduce methane emissions (Knief, 2019). The MMO enzyme complex catalyses the cleavage of two important chemical bonds; the dioxygen bond and the carbon-hydrogen bond of methane (Tinberg and Lippard, 2011), allowing methane oxidation in methanotrophs (Ross and Rosenzweig, 2017). As previously indicated, MMO is encoded by a complex operon containing multiple genes, some of which have secondary roles in maintaining the health of the plant (Khider et al., 2021). Methylotrophs, on the other hand, possess the enzyme methanol dehydrogenase (MDH), which is involved in the breakdown of methanol, leading to the production of formaldehyde (Sarwar and Lee, 2023). These organisms can utilize methanol without

external help, making them important players in the carbon and energy cycling of mangrove soil.

Methane-oxidizing bacteria can stimulate nitrogen fixation by supplying carbon to diazotrophs, hence boosting the growth of nitrogen-dependent plants. The results from this study showed that all of the methanotrophs are *Methylocystis sp., Meythlopila sp., Methylosarcina lacus, Methyloceanibacter methanicus)* and those of methylotroph are *Methylobacterium nodulans, Methylobacterium sp. 17SD2-17, Methylibium sp., Methylotenera sp. 24-45-7, Methylocaldum sp., Methyloversatilis universalis, Methyloceanibacter caenitepidi, Methyloceanibacter marginalis, Methyloceanibacter sp. wino2, Methyloceanibacter superfactus* and *Hyphomicrobium sp.* Methylotrophs (*Hyphomicrobiaceae*) frequently coexist with methanotrophs (methane-eating bacteria) and can aid in the oxidation of methane. Particularly, methane was shown to be the sole carbon and energy source for the *Methyloceanibacter* strain R67174 (Vekeman et al., 2016). Additionally, *Methyloceanibacter caenitepidi* is a facultative methylotroph since it can utilise methanol, methylamine, trimethylamine, and other multi-carbon molecules. According to a research by Takeuchi et al. (2014), *Nitrospira* can oxidise methane by acquiring genes encoding the soluble or particulate methane monooxygenase bacterium in conditions where both methanol and methane are present as carbon and energy sources. In a comammox process, *Nitrospira* species convert ammonia to nitrate and then the nitrate is being taken up by plants. Methane-dependent denitrification activity has been seen in gammaproteobacterial methanotrophs such as *Methylobacter* that utilise nitrate or nitrite as a substrate (Knief, 2019). It has also been shown that the alphaproteobacterial

Methylocystis sp. strain SC2 can carry out complete denitrification of nitrate to dinitrogen using methanol as a growth substrate (Dam et al., 2013).

Additionally, environmental factors like temperature and water availability impact plant physiology and methane emissions, which in turn affect the relationship between methane metabolism and plant development. Noyce and Megonigal (2021) found that increasing the temperature of an entire ecosystem can lead to higher methane emissions from wetland plants. This is because the warming affects the characteristics of the plants and the biogeochemical processes involved. These findings have consequences for the growth of plants in warming environments, as the rise in methane emissions may coincide with changes in the availability of nutrients and the way plants function. Methane metabolism is not solely a result of microbial processes, but it can also take place through routes connected with plants. Perez-Coronel and Beman (2022) discovered that aerobic methane production in aquatic ecosystems can occur through bacterial photosynthesis, indicating that methane metabolism is interconnected with the overall metabolic networks of aquatic plants. This integration highlights the intricate nature of methane's function in plant growth, where it can serve as both a metabolic waste and a valuable resource inside the plant's biochemical framework. Overall, this comprehensive understanding of the microbial diversity and metabolic pathways involved in methane metabolism in mangrove soil could be essential for managing these ecosystems and their contribution to global methane dynamics and climate change.

5.1.3 How Nitrogen Metabolism Occurs in Mangrove Soil in Sungai Lukut?

Nitrogen fixation, which is a physiologically and phylogenetically varied microbial function, is regarded to be the primary source of combined nitrogen input in mangrove forest ecosystems (Zhang et al., 2008). Nitrogen is a essential macronutrient involved in synthesizing amino acids, proteins, nucleic acids, and chlorophyll, all of which are fundamental to plant growth (Baslam et al., 2020). The gene *nifH*, which encodes the dinitrogenase reductase enzyme, is one of the genes involved in the fixation reaction (Jayakumar and Ward, 2020). Based on mWGS's result, the *nifH* gene is abundant in soil 1, 2 and 3 which shows that the mangrove soil in Sungai Lukut are able to hydrolyse ATP giving energy to the nitrogen metabolism. In this study, nitrogen-fixing bacteria involved in nitrogen metabolism pathway are belonged to *Gammaproteobacteria*, indicating that the nitrogen-fixing group in this phlyum is predominant in the mangrove ecosystem. A number of nitrogen-fixing bacteria, identified as members of the genera *Azospirillum, Azotobacter, Rhizobium, Nitrospira, Nitrospina, Nitrococcus, Nitrobacter, Nitrosomonas Clostridium, Klebsiella,* and *Pseudomonas,* have been isolated from the rhizosphere of mangrove ecosystems (Liu et al., 2012; Walstad, 2017; Hur and Park, 2019), while nitrogen-fixing bacterial isolates described in this study were found to be members of the genera *Eubyza, Nitriliruptor, Nitrospina, Nitrospira, Nitrobacter, Nitratireductors, Nitrosomonas, Nitrococcus, Nisea, Thioalbus, Thioalkalivibrio, Thriothrix*, and others.

Nitrification is another essential process in the nitrogen cycle, where ammonia is first oxidized to nitrite and then to nitrate. The family *Nitrosopumilaceae* is crucial to world nitrogen cycles due to its function in the conversion of ammonia to nitrite

(Könneke et al., 2005). Bacteria belonging to the genera *Nitrospira* and *Nitrospina* are capable of oxidizing nitrite to nitrate. Some *Nitrospira* are capable of the commamox process, in which nitrite is used to completely oxidize ammonia to nitrate (Van Kessel et al., 2015). Moreover, *Nitrospira* bacteria that are capable of aerobic nitrite oxidation can also use hydrogen or formate as electron donors to perform the reversal process of nitrate reduction (Daims and Wagner, 2018). *Nitrosomonas* and *Nitrobacter* are two types of autotrophic bacteria; the former transforms NH_4 to NO_3, while the latter converts NO_2 to NO_3^-. Nitrifying bacteria in biological filters derive all of their metabolic energy from converting ammonium to nitrates and also provides metabolic energy for biological filters and ultimately supports plant growth (Walstad, 2017). The process of nitrogen metabolism is closely connected to carbon metabolism, especially in C3 plants. In these plants, it is crucial to convert nitrogen into organic compounds in order to produce the biomolecules necessary for photosynthesis and energy transmission (Baslam et al., 2020). This correlation demonstrates how nitrogen is critical for preventing stress-inducing nutritional imbalances and sustaining robust growth (Xu et al., 2020).

The nitrogenase enzyme (also known as dinitrogenase) are capable to reduce to ammonia from almost all of the known nitrogen-fixing prokaryotic systems. In free-living nitrogen-fixing as well as symbiotic bacteria, the nitrogenase and other enzymes participating in nitrogen fixation to produce ammonia are encoded by bacterial *nif* genes and *fix* genes. In addition to the enzyme nitrogenase, *nif* genes also code for several regulatory proteins associated with fixation of nitrogen. For example, *nifD* and *nifK* genes code for the molybdenum-iron (MoFe) protein subunits; *nifH* and *nifF* genes encode the

Fe protein and ferredoxin, while the remaining nif genes take part in the activation and processing of the enzyme complex. Plants and many bacteria can absorb and promptly reduce nitrate and nitrite to ammonia by nitrate reductase and nitrite reductase respectively in the nitrogen cycle (Kochhar and Gujral, 2020). Nitrate-reducing bacteria, as well as various heterotrophic nitrate and nitrite reducers, could complete the nitrogen cycle, generating ammonia and nitrogen gas. Nitrate, in turn, is reduced to gaseous nitrogen, and possibly to ammonia (Begmatov et al., 2021). This indicates the coupling of nitrate reduction to nitrite with reverse methanogenesis, further highlighting the intricate interactions between nitrogen metabolism and other biogeochemical processes in mangrove soil (Knief, 2019). In ecosystems, plants frequently establish symbiotic relationships with microorganisms, which can augment the availability and absorption of nitrogen. This, in turn, promotes the rapid development and productivity of the plants (De Castro et al., 2021). In conclusion, nitrogen metabolism in mangrove soil in Sungai Lukut is a complex process involving various nitrogen-fixing bacteria and genes.

5.1.4 How Sulphur Metabolism Occurs in Mangrove Soil in Sungai Lukut?

Plants need sulfur as one of the key minerals for healthy growth and development. Plants rely primarily on the soil's trace levels of the anionic form of sulfur (SO_4^{2-}) for their sulfur needs. Under sulfur deprived situations, plants can also utilise the sulfur transporter of a symbiotically associated organism, such as bacteria or fungi, to absorb sulfur from the soil (Narayan et al., 2022). In general, the amount of sulfur in soil is proportional to the amount of organic matter present, and microbial action is responsible

for the majority of the chemical transformation of sulfur forms (Kertesz and Mirleau, 2004). Sulphur metabolism is intricately connected to nitrogen metabolism, as both elements have an essential role for the production of key component of amino acids and proteins. According to the KEGG sulfur metabolism pathway, plants use photosynthesis to transform inorganic sulphur into organic forms that are then integrated into essential proteins. As a byproduct of the oxidation of C to CO_2, sulfur is produced as sulfate, which is thought to be the driving force behind biological mineralization. Most of the sulfate that is ingested is converted into organic chemicals necessary for growth of the plant's structural growth (Hawkesford and De Kok, 2007). Moreover, the significance of sulphur in stress tolerance is emphasised, especially for plants that face environmental problems such salinity, heavy metals, and pollution (Shah et al., 2022). Plants are able to enhance their N absorption and produce necessary proteins and enzymes for growth and development by efficiently metabolising sulphur (Narayan et al., 2023).

For this study, there are a lot of bacteria species that are involved in the sulfur metabolism. The major classes found in mangrove soil in Sungai Lukut are *Betaproteobacteria*, *Gammaproteobacteria*, and *Deltaproteobacteria*. *Desulfocucumis, Desulfosporosinus, Desulfotomaculum, Desulfatitalea, Desulfospira, Desulfococcus, Desulfobulbus, Desulfofustis, Desulforhopalus, Desulfotalea, Desulfuromonas,* and *Desulfacinum* are the genus found in this study. From the genera that emerged from the mangroves in India, *Desulfovibrio, Desulfotomaculum, Desulfosarcina*, and *Desulfococcus* species had been isolated (Balk et al., 2015). The bacteria belonging to the family *Desulfobulbaceae* have a wide variety of metabolic capabilities, including the

reduction of dissimilatory iron, the oxidation of elemental sulfur, and the reduction of sulfate and sulfite in the process of total oxidation of organic matter (Holmes et al., 2004; Sorokin et al., 2012). The sulfide (H_2S) is oxidized with oxygen or nitrate (Jørgensen et al., 2019). Therefore, the composition of microbial communities suggests that it is possible for a complete sulfur cycle to take place, which would involve the conversion of sulfate to sulfide and the nitrate-dependent oxidation of reduced sulfur compounds into sulfate (Begmatov et al., 2021). Moreover, *Desulfopira*, which pertains to the *Desulfobulbaceae* was discovered (Balk et al., 2015). *Desulfofustis glycolicus*, which associated with sulfate-reducing bacterial groups catalyze sulfur disproportionation (Yousuf et al., 2014). In addition, *Desulfofustis glycolicus* is able to catalyse the disproportionation of sulphur. Some organic molecules were probably metabolised and assimilated directly by these sulphate reducers (Aoyagi et al., 2021). Sulfur oxidizing chemolithotrophs that depend on denitrification, such as *Sulfurimonas sp.* HDS01, and sulfate-reducing heterotrophs, such as *Desulfobulbus spp.* and *Desulfofustis glycolicus*, were closely linked to one another through direct carbon transfer (Aoyagi et al., 2021). The microbial communities in mangrove soil suggest the possibility of a complete sulfur cycle taking place, involving the conversion of sulfate to sulfide and the nitrate-dependent oxidation of reduced sulfur compounds into sulfate (Begmatov et al., 2021). This intricate network of sulfur-metabolizing bacteria and genes in mangrove soil contributes to the biogeochemical cycling of sulfur, impacting nutrient availability and overall ecosystem functioning.

Result from this study showed that the genes of *cysJ, cysI, sir, dsrA*, and *dsrB* are abundantly formed during dissimilatory sulphate reduction and oxidation where it begins by converting sulphide to sulphite. An essential part of the enzymatic machinery for sulphate respiration in sulfate-reducing microorganisms (SRM) is a *DsrAB*-type dissimilatory sulfite reductase, which can also serve as a phylogenetic and functional identifier (Venceslau et al., 2014). Microorganisms, such as sulfite-reducing microorganisms and sulfur-disproportionating bacteria (Simon and Kroneck, 2013) contain the genes *dsrAB*. Sulphate reduction is an assimilatory process, and adenosine phosphosulfate reductase is a key regulator of this process (Narayan et al., 2022). Specifically, *cysB* controls the expression of *cysD, cysI, cysJ, cysK, cysNC, metY, tauA, tauB*, and *tauD*, all of which are involved in sulphur metabolism. While sulphide acts as an inhibitor, the *cysB* protein promotes transcription (De Kok et al., 2012). In conclusion, sulfur metabolism in mangrove soil in Sungai Lukut is a complex and dynamic process involving a diverse array of sulfur-metabolizing bacteria. These bacteria play critical roles in sulfur oxidation and reduction processes, influencing nutrient availability for plant growth and participating in the sulfur cycle. This mutually beneficial relationship can promote plant health and growth.

5.1.5 How Atrazine Compound Degrades in Mangrove Soil in Sungai Lukut?

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is a herbicide used to prevent the growth of weeds in sorghum, maize and sugarcane fields (Zhang et al., 2019). Fortunately, microorganisms offer a low-cost and efficient technique for the

degradation and removal of atrazine in the environment (Khatoon and Rai, 2020). In mangrove soil of Sungai Lukut, various bacteria play a crucial role in the degradation pathway of atrazine. Bacteria that are involved in atrazine degradation pathway in KEGG reported in this study are *Mycobacterium, Gordonia, Rhodococcus, Tetrasphaera, Clavibacter, Friedmaniella, Nocardiodes, Pseudonocardiodes, Streptomyces, Solirubrobacter, Rhodoplanes* and *Pseudolabrys*. While various microorganisms have been isolated and used to breakdown atrazine, the majority of them are from the genera *Pseudomonas, Acinetobacter, Agrobacterium, Rhodococcus, Arthrobacter, Bacillus, Norcardioide, Variovorax,* and *Citricoccus* (El Sebaï et al., 2011; Wang et al 2014; Zhao et al., 2017; Yang et al., 2018).

Four genes are associated in the atrazine degradation in Sungai Lukut which are *atzD, atzE, atzF*, and *URE*. These genes are involved in the conversion process from atrazine to biuret and urea. The *atzDEF* operon in *Pseudomonas sp.* ADP codes for the enzymes *atzD*, *atzE*, and *atzF*, which catalyse the conversion of cyanuric acid to carbon dioxide and ammonia, which can then be used by the cell as a nitrogen source (Sene et al., 2010). Atrazine can be converted into biuret by the bacteria *Chelatobacter sp.*, *Alcaligenes sp.*, and *Ralstonia sp.* (Rousseaux et al., 2002; Cheng et al. 2005). These bacteria contain the enzymes *atzA, atzB,* and *atzC*. According to Arbeli and Fuentes (2010), bacteria known to partially metabolise atrazine include *Nocardia sp.* This bacterium transforms atrazine into cyanuric acid with the aid of *atzB, atzC* and triazine hydrolase (*trzN*). However, research by Zhao et al. (2018) shows that *Arthrobacter sp.* ZXY-2 has a high capability of degrading atrazine. Complete utilisation of atrazine has

been recorded for the bacteria *Ancylobacter sp.* T10AII and *Agrobacterium sp.* (Arbeli and Fuentes, 2010; Devers et al., 2007). In order to produce cyanuric acid, a nitrogen source for many bacteria, the atrazine encoded by the genes *atzABC* is metabolised by *Pseudomonas* strain ADP and *Arthrobacter sp*. (Gao et al., 2018). FH-1, which has been shown to be *Klebsiella variicola*, is able to grow on atrazine, even though it is the only source of nitrogen. PCR and sequencing were used to find three genes (*atzC*, *trzN*, and *trzD*) in strain FH-1 that coded for enzymes that degraded atrazine. *K. pneumoniae, K. granulomatis, K. aerogenes, K. planticola, K. oxytoca*, and *K. michiganensis* were among the closely related species found by Zhang et al. (2019).

Atrazine-degrading bacteria found in mangrove habitats play a crucial role in preserving environmental well-being and mitigating the risk of pollution. Plants that have the ability to efficiently break down atrazine can avoid the growth-inhibiting effects caused by atrazine's disruption of photosynthesis and other metabolic pathways. A study conducted by Pérez et al. (2022) investigated the process of atrazine absorption, movement within the plant, accumulation, and breakdown in cattail (*Typha latifolia*). The findings of the study demonstrated that the length of time the plant is exposed to atrazine is a critical factor in determining the magnitude of its effects on plant growth. Liang et al. (2022) revealed the precise enzymes and mechanisms responsible for the breakdown of atrazine. This study showed that plants with strong degradation pathways are more effective at handling the presence of this herbicide. In conclusion, atrazine degradation in mangrove soil in Sungai Lukut involves a diverse group of bacteria that employ specific enzymes encoded by various genes. These bacteria play a crucial role in breaking down

atrazine and converting it into less harmful compounds. The presence of atrazine-degrading bacteria in mangrove ecosystems is beneficial for maintaining environmental health and preventing potential pollution.

5.1.6 How Dioxin Compound Degrades in Mangrove Soil in Sungai Lukut?

Dioxin is the most dangerous environment toxin, and it is produced by a wide range of human activities. Mineralization of organic compounds can provide carbon and energy for microorganisms that rely only on them (Saibu et al., 2020). Aerobic breakdown of these substances by bacteria occurs via the lateral and angular deoxygenation pathways, the two primary catabolic mechanisms. Special angular dioxygenases, which target the ring next to the ether oxygen, typically kick off the breakdown process (Field and Sierra-Alvarez, 2008). Extradiol (meta-cleavage) dioxygenase 2,2′,3-trihydroxybiphenyl (2,2′,3-THB)-1,2-dioxygenase is the second enzyme in the dioxin catabolic pathway. 2-hydroxy-6-oxo-6-(2′-hydroxyphenyl)-hexa-2,4-dienoic acid (HOHPDA) is produced when 2,2′,3-THB is degraded by this enzyme. Salicylate is produced when HOHPDA undergoes hydrolytic breakage of its C-C bonds, an event mediated by HOHPDA hydrolase and the final step in dibenzofurans (DF) upper metabolic route in order to quickly metabolize DF into HOBB (major metabolite), 1-hydroxydibenzofuran, and 4-hydroxydibenzofuran (Li et al., 2009). Dibenzo-p-dioxins (DD), DF, and substituted analogues were shown to be used by *Rhodococcus sp.* strain p52 aerobically as sole sources of carbon and energy through angular deoxygenation (Saibu et al., 2020).

There are many types of bacteria that may break down polychlorinated biphenyls (PCBs), DFs, and DDs (Field and Sierra-Alvarez, 2008). Result from this study highlighted that abundance bacteria that are involved in dioxin degradation pathway from KEGG database are from the genus *Mycobacterium, Mycolicibacterium, Nocardia, Eubyza, Rhodococcus, Phycicoccus, Blastococcus, Geodermatophilus, Instraporangium, Tetrasphaera, Agromyces, Athrobacter, Nocardioides, Psedunocardia, Streptomyces, Conexibacte*r, *Actinoplanes, Eubyza, Afipia, Bradyrhizobium, Devosia, Rhodoplanes, Methyloceanibacter Pseudorhodoplanes, Nicella, Novosphigobium* and *Spingomonas.* Proteobacteria (especially *Betaproteobacteria* and *Gammaproteobacteria*) and *Actinobacteria* account for the vast majority of the dioxin-degrading bacteria that have been isolated to date (Saibu et al., 2020). *Pseudomonas, Citrobacter, Cronobacter, Rhodococcus, Terrabacter*, and *Pantoea* were the most common bacterial genera in the most polychlorinated dibenzodioxins (PCDD) contaminated soils (Larentis et al., 2011). Additionally, genetic profiling of dioxin-degrading bacteria by Mahfouz et al. (2022) showed that these bacteria are members of the genus *Bacillus*. *Nocardiodes* degrade aromatic compounds and utilize a broad spectrum of carbon and nitrogen sources, including rare organic compounds and lethal pollutants from the environment (Field and Sierra-Alvarez, 2008). Compounds containing PCDD/F are susceptible to biodegradation in the environment as a component of the natural chlorine cycle. It has been shown that *Klebsiella sp.* strain HL1 and *Klebsiella sp*. strain HL7, both of which were isolated from dioxin-contaminated soil, had the ability to degrade DF (Fukuda et al., 2002). *Terrabacter sp.* strain DBF63 has been identified as a bacterium capable of degrading dibenzofuran (DF) (Rosenberg et al., 2014). Some chlorinated dibenzofurans (CDFs) and dibenzo-p-

dioxin (DD) can be broken down by the strain since it uses DF as its sole carbon and energy source.

This study revealed that there are several genes involved in the atrazine degradation pathway, including *dbfA1, dbfB, praC, xylH, dmpH, xylI, nahK, mhpD, mhpE, mhpF, bphAa, bphA1, bphA*, and *bphC*. Field and Sierra-Alvarez (2008) found that when the *Terrabacter sp.* strain DBF63 is grown on DF, both the *dfdA* and *dbfA* genes are expressed. Futhermore, *Nocardioides sp*. DF412 was found to contain a *dfdA1A2A3A4* gene cluster (Sukda et al. 2009) that codes for a ring hydroxylating dioxygenase. Numerous heterocyclic aromatics, including biphenyl, DF, and DD, have been shown to be stereospecifically oxygenated by the *bphA* genes that encode the biphenyl dioxygenase. While, *Burkholderia xenovorans* strain LB400 and *Comamonas testosteroni* strain B-356 employ both DF and DD, and their biphenyl dioxygenase (BPDO) mechanisms have been characterized (L'Abbee et al., 2005). In addition, the *bphA* proteins are well-known for catalyzing the dihydroxylation of biphenyl and several PCB congeners. Iwasaki et al. (2007) demonstrated that the *bphA* genes of *Rhodococccus sp.* RHA1 produced a biphenyl dioxygenase that was able to change DF in an effective way by both angular and lateral dioxygenation, but DD could only be converted via angular dioxygenation. The relationship between the breakdown of dioxin and the growth of plants is additionally impacted by *bphA* gne, which can alter the populations of microorganisms and their metabolic functions. Musilova et al. (2016) observed that the existence of *bphA* gene which might have either inhibitory or stimulatory effects on the decomposition of dioxins functions in polluted environments. Additionally, Seeger et al.

(2001) demonstrated that the *bphA*-encoded biphenyl dioxygenase of *Burkholderia xenovorans* strain LB400 catalyzes the angular dioxygenation of DD.

The microbes are assisting in the process of purifying the environment and assuring the availability of crucial macronutrients such as nitrogen and phosphorus, which are frequently scarce in contaminated streams. The decomposition of dioxins and the consequent emission of byproducts into the water might modify the accessibility of nutrients, which is vital for the growth of plants. Conclusively, mangrove soil in Sungai Lukut hosts a diverse group of bacteria that contribute to the degradation of dioxin compounds. The presence of dioxin-degrading bacteria in the mangrove ecosystem is crucial for detoxifying the environment and minimizing the impact of these hazardous pollutants.

5.1.7 Impact of Metabolic Processes on Plant Development

In nutrient cycling, the metabolic process refers to the series of chemical reactions that microorganisms and plants go through to transform nutrients into forms that can be easily utilized. Activities involving enzymes and microbes that are involved in the cycle of nutrients are able to maintain the plants health and the well-being of ecosystems. For examples, the carbon fixation process facilitated by enzymes including malate dehydrogenase (MDH) and fumarase, has a function in transforming carbon dioxide into organic molecules which can promote plant growth. Furthermore, the carbon skeletons required for nitrogen assimilation is helped by nitrogenase enzymes, which are encoded

by the *nifDKH* and *anfG* genes. These enzymes transform atmospheric nitrogen into ammonia. Liu et al. (2020) found that the availability of nitrogen has a substantial impact on the process of photosynthesis and the fixation of carbon in wheat. Their research revealed that when there is a lack of nitrogen, the rates of photosynthesis decrease, which in turn hinders the process of carbon fixation. Similarly, Zayed et al. (2023) found that how nitrogen metabolism is integral to the overall nutrient cycling in plants, where carbon skeletons are required to synthesize amino acids and other nitrogenous compounds. In addition, methane metabolism plays a role in both carbon fixation and nitrogen cycle. In this study, methanotrophic bacteria employ enzymes encoded by genes such as *mmoX*, *mmoY*, and *mmoZ* to convert methane into methanol. The methanol is further transformed into substances that serve as substrates for carbon fixation pathways. The process not only aids in the reduction of greenhouse gas emissions but also facilitates the functioning of the carbon and nitrogen cycles, which are required for the growth of plants (Tian et al., 2021).

Sulphur metabolism are also involves in facilitating nitrogen cycling and improving plant resistance to environmental pressures. Sulfur-oxidizing bacteria, such as *Desulfatibacillum* and *Desulfobulbus*, oxidise sulphide to form sulphate. Plants then use this sulphate to synthesise sulfur-containing amino acids, such as cysteine and methionine. These amino acids are essential for the plant's stress response and for protein synthesis. The presence of genes such as *cysJ*, *cysI*, and *dsrA* is required for the synthesis of biomolecules. This finding aligns with the research conducted by Akbudak & Filiz (2019), which stated the significance of sulphur in higher plants, specifically in relation to

environmental stresses including salt and heavy metals. Their research revealed ATP sulfurylase (ATPS) genes as pivotal components in sulphur assimilation, a process for upholding cellular activities during periods of stress. Therefore, the ATPS genes involved in sulphur metabolism that have been discovered in the Sungai Lukut soils are contributing to the maintenance of plant well-being in difficult environmental circumstances.

Based on KEGG metabolism pathway, there is a role of microbial communities in detoxifying pollutants such as atrazine and dioxins. Atrazine degradation is facilitated by a sequence of genes, namely *atzA*, *atzB*, and *atzC*, which decompose this herbicide into less toxic substances, so diminishing the toxic burden in the soil will consequently fostering a healthier environment for plants. Similarly, genes such as *dbfA1* and *dbfB* have the ability to detoxify extremely persistent environmental contaminants. These detoxification activities can hinder the accumulation of noxious chemicals that could otherwise impede plant growth (Zhang et al., 2021). In addition, Nguyen et al. (2021) studied that the biodegradation of dioxins using *Burkholderia cenocepacia* and identified genes that are responsible for this process. Hence, the role of microorganisms and their metabolic activities in promoting plant development and resistance, as well as reducing environmental contaminants, leads to a more sustainable ecosystem.

The results from this study indicate that the metabolic process from the metagenomic whole genome sequencing (mWGS) provides a comprehensive comprehension of the microbial communities and their functions within the soil as it

enables the development of biofertilizer formulations that are more effective and customised. For instance, Li et al. (2020) illustrated that the metagenomic analysis uncovered that identification of critical microbes that are potential to be a biofertilizer which can facilitate nutrient cycling and plant health. Similarly, Li et al. (2023) shown that the ability to understand nitrogen-transforming microorganisms in soils by mWGS and has a direct impact on plant development. By utilising the practical value of metagenomic characterisation, it will improve the growth-promoting microbes potential derived from agricultural residues (Mora et al., 2022). Thus, mWGS data are not only for comprehending soil microbes but also for the advancement and enhancement of biofertilizers.

5.2 N, P and K Content in Formulated Biofertilizer Influences The Growth of Duckweed and Its Protein Content

5.2.1 The Element of N, P and K Content in Formulated Biofertilizer

Biofertilizers has been recommended as a more environmentally friendly substitute for conventional chemical fertilizers and pesticides. It has been established that rhizosphere soil has a rich source of PGPB (Pii et al., 2015). In this study, bacteria were isolated from the mangrove soil. Nine bacterial strains obtained from 16s rRNA gene sequencing were grouped into three biofertilizer sets (Set A containing *Acinetobacter radioresistens, Klebsiella quasipneumonia* and *Bacillus cereus,* Set B contains *Brachybacterium paraconglomeratum, Bacillus cereus* and *Bacillus tropicus* and Set C

containing *Enterobacter cloacae*, *Paenibacillus pasadenensis* and *Bacillus thuringiensis*) with each having the ability to fix nitrogen, solubilize potassium and phosphorus, grown on the duckweed.

Based on the result obtained from this study, Set C appears to be much better than Set A and Set B as a biofertilizer set as Set C showing an increase in the content of nitrogen, potassium and phosphorus. The main advantage of Set C is its ability to enhance the phosphorus content in the soil. Several studies (Kumar et al., 2014; de Souza et al., 2015; Ngalimat et al., 2021) have reported that *Enterobacter cloacae*, one of the bacteria in Set C, is a potent inorganic phosphorus solubilizer and can significantly increase P-acquisition in plants. *Paenibacillus pasadenensis*, another component of Set C, has also been shown to be involved in the solubilization of soil phosphorus, and the production of phytohormones and antimicrobial metabolites (Govindasamy et al., 2010). Additionally, *Paenibacillus pasadenensis*, another bacterium in Set C, is known to be involved in the fixation of atmospheric nitrogen and the uptake of micronutrients, further benefiting plant growth (Grady et al., 2016). Moreover, *Enterobacter cloacae*, present in Set C, exhibits a variety of growth-promoting actions, including phosphate and potassium solubilization, as well as nitrogen fixation (Deepa et al., 2010; Ramesh et al., 2014; Chin et al., 2017). These actions contribute to improve plant health and soil fertility (Ghiglione et al., 2021). As previously mentioned, *Bacillus sp.* was also considered as an effective nitrogen-fixing bacteria (Janarthine et al., 2011). Multiple PGP favourable characteristics were found in the genus *Bacillus* including phosphate solubilization and participation in the nitrogen cycle (Stegelmeier et al., 2022). In conclusion, Set C is much better than Set A and Set B

as a biofertilizer especially for the growth of the duckweed due to its excellent ability to enhance phosphorus content in the soil, multiple growth-promoting actions, and nitrogen enhancement.

5.2.2 The Effect of Biofertilizer in Improving The Duckweed Growth and Its Protein Content

Microbes naturally found on plants play a crucial role in promoting plant growth even in challenging conditions. Extensive research on biofertilizers has demonstrated their potential to supply vital nutrients to crops, enriching crop yields without harming the environment (Kour et al., 2020). However, not all microbes are capable of interacting with plants, making it essential to analyze the interactions of plant growth-promoting bacteria (PGPB) with their natural plant hosts (Zamioudis and Pieterse, 2021). Duckweed are chosen as plant models as it known as a fast-growing aquatic plant, undergoes clonal duplication during its vegetative growth cycle, with a high number of fronds indicating healthy growth and reproduction (Tang et al., 2015). N-fixation, P solubilization, and K solubilization have been identified as the mechanisms responsible for the symbiotic connections between biofertilizers and duckweed. Studies have shown that biofertilizers from different sets can significantly enhance duckweed growth (Yoneda et al., 2021). Unlike in soil, plant-associated microorganisms in water must adhere to and colonize plant bodies to avoid being washed away by water currents. Aquatic PGPB are hypothesized to possess useful properties such as rapid adhesion and stable colonization. Based on the result obtained from this study, biofertilizer from Set A, Set B and Set C can

enhance the growth of the duckweed with Set C displayed as the best biofertilizer corresponding to the highest number of duckweed front after 15 days. The biofertilizer clearly increased the number of duckweed fronds, which is consistent with the findings of Yoneda et al. (2021). Specifically, PGPB strains from the phyla *Betaproteobacteria, Gammaproteobacteria,* and *Alphaproteobacteria* have been observed to increase the number of duckweed fronds by more than two times (Makino et al., 2022). Additionally, certain PGPB strains, such as *Pseudomonas sp.* Ps6 and *Ensifer sp*. strain SP4, have demonstrated the ability to accelerate duckweed growth (Toyama et al., 2022).

The potential for using this PGPB as a bioinoculant was demonstrated by exposing duckweed to all bacterial strains obtained in this study. The PGP effects of all tested bacterial strains on duckweeds were comparable to those of the well-studied representative PGPB, *Acinetobacter calcoaceticus* strain (Toyama et al., 2017; Makino et al., 2022). The ability of *Acinetobacter calcoaceticus* P23 to grow in both artificial media and environmental conditions makes it a potential bioinoculant for enhancing duckweed growth (Yamaga et al., 2010; Suzuki et al., 2014; Toyama et al., 2017). Several studies have shown that co-cultivation of duckweed with specific PGPB strains can lead to significant growth benefits. For instance, the *rhizobacterium* MH3 has been found to boost duckweed development, resulting in a 30% increase in frond number and a 50% increase in dry weight (Tang et al., 2015). Moreover, certain *Bacillus* strains present in different biofertilizer sets have successfully functioned as plant growth-promoting rhizobacteria (PGPR) to stimulate rapid duckweed growth (Idris et al., 2007). The hypothesis of synergistic effects arising from co-inoculation of these strains further

supports the establishment and maintenance of a mutually beneficial plant-microbe relationship. Thus, the biofertilizer set can both establish and maintain a mutually beneficial plant-microbe relationship. It's worth noting that the biofertilizer bacteria strain has the potential to promote growth and rescue plants from growth inhibition in a synergistic manner.

In this study, biofertilizer Set A, B and C showed an increase in duckweed protein corresponding with the increase in N, P and K concentration. The results obtained from Li et al. (2016) are comparable to the results obtained from this study as the protein content of the duckweed is increased as the concentrations of N and P are increased. The correlation between suggesting that higher nutrient concentrations were favourable for duckweed protein. Makino et al. (2022) reported that the introduction of PGPB to duckweed resulted in a significant rise in protein content, indicating the bacteria's ability to enhance the nutritional quality of duckweed. Additionally, Ishizawa et al. (2020) showed that the inoculation of duckweed with *Acinetobacter calcoaceticus* P23 resulted in an increase in the amount of protein and biomass produced by the plant. The ability of the bacteria to fix atmospheric nitrogen, which allows the plant to easily absorb it into its proteins, is mostly responsible for this improvement. *Spirodela polyhiza* showed the highest protein proportional to the highest growth of the duckweed growth (Li et al., 2016). According to the Femeena et al. (2023), the bacteria species can affect the duckweed growth and protein accumulation. This study showed that the protein content in Set A is higher compared to Set B and C. The different bacteria species in three biofertlizer set proved that protein content is affected by different kind of bacteria species.

The protein content in *Lemna minor* is increased after being cultured with *Azotobacter Vinelandii* for 10 days compared to the control (Shuvro et al., 2022).

Duckweed inoculated with PGPB also increases its resistance to environmental stresses, which can reduce protein synthesis in plants. Kamal (2023) investigated the possibility of establishing an artificial symbiosis between the nitrogen-fixing bacterium *Azotobacter vinelandii* and the plant *Lemna minor*. They demonstrated that this symbiotic relationship not only facilitated higher growth but also considerably boosted the amount of protein that was present in duckweed. The protein production level is decreased when the duckweed is grown under stress condition (Shuvro et al., 2022). Petersen et al. (2022) observed protein content in the duckweed were considerably high despite containing the low nutrient concentration. Ultimately, Set A show the highest amount of protein compared to control, Set B and C. To summarise, bacteria that promote plant growth have a crucial impact on increasing the element protein content of duckweed. By delving into these molecular interactions, we can find out how to make duckweed's nutritional value increase, which will open up new possibilities for its use in agriculture.