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Comparative Analysis of Bacterial Diversity in Water Bodies in UTHM Pagoh Campus

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Abstract

The study identifies the bacterial families in the water and compares the diversity between the residential college pond and the UTHM Wetland Conservation Research Station. As we can see, there is no prior information on bacterial diversity in Pagoh has been documented. So, the study aims to collect data on bacterial diversity in water bodies around the UTHM Pagoh Campus. Water quality measurements assessed levels based on the National Water Quality Standards and Water Quality Index. The study identified bacterial families in the water bodies, comparing the diversity at both sites. Methods included water sample collection, water quality measurement, bacterial culture and isolation, morphological identification, gram staining, and bacterial identification. The water quality measurements show that the UTHM Wetland Conservation Research Station has lower measurements of pH, dissolved oxygen, and temperature while having higher measurements of conductivity and biochemical oxygen demand than the pond of the residential college which conclude the UTHM Wetland Conservation Research Station falls between Class IV and V, while the residential college pond falls between Class II and III. There are 3 samples which are Gram-positive cell walls and 3 samples Gram-negative cell walls. At the UTHM Wetland Conservation Research Station, there are 4 bacterial families; Neisseriaceae, Moraxellaceae, Micrococcaceae, and Planococcaceae. In contrast, the residential college pond revealed 3 bacterial families: Corynebacteriaceae, Pseudomonadaceae, and Staphylococcaceae. The differences in bacterial diversity are attributed to various factors, including species adaptations, site nutrient levels, organisms inhabiting the sites, and environmental conditions. For further study, the study can include DNA Quantification (spectrophotometer), preparation of PCR Cocktail, gel electrophoresis, Bioedit of sample sequences to get the sequence, NCBI blast for sample, and phylogenetic tree.

1. Introduction

Approximately 71% of the Earth's surface is covered by oceans, which contain about 96.5% of all the water on the planet. Water is also found in the Earth's soil, aquifers, rivers, lakes, ice caps, glaciers, and the atmosphere as water vapor [1]. Ponds can be found in woodlands, meadows, and moors, as well as in the countryside, on farmland, floodplains, and heathlands. They are also commonly seen in villages, towns, and gardens. Wetlands are transitional areas between terrestrial and aquatic systems, where shallow water covers the ground, or the water table is frequently at or near the surface [2]. While terrestrial ecosystems support a variety of microorganisms, aquatic ecosystems also harbor diverse microorganisms that play crucial roles in stabilizing the ecosystem's chemical processes [3].

Water quality will change over time as a result of weather patterns and natural catastrophes that occur close to the UTHM Pagoh Campus, which constantly alter the flow of water toward the water bodies. Since the overflow water will carry pollutants into the water bodies, this can also lead to water pollution. The present water quality will alter as a result of the pollution. Based on the research locations, which include the UTHM Pagoh Campus and residential college, no information is presently available regarding the diversity of microorganisms in the water bodies, which can strengthen the link between water quality and microbial diversity to clarify the study's significance. Although many too many microorganisms might live in water, it is unknown which species are found in the water at the UTHM Pagoh Campus [4]. As water is around human lives and daily uses, many unknown bacteria can be consumed by humans if it is taken directly without any strict procedure. It may lead to various diseases and the evolution of new risks to human health. For public health concerns in densely populated urban areas, it may be particularly crucial to comprehend these ideas of bacterial variety and exchange. Both treated and untreated sewage commonly pollute the aquatic environment in densely populated metropolitan areas. The study supports the use of the framework for water body management as bacterial diversity plays a crucial role in maintaining the health and stability of aquatic ecosystems, along with human activities, land-use changes, and infrastructure development [5].

There are three objectives of the study which are to measure the water quality of water bodies within UTHM Pagoh Campus using multiparameter Hanna equipment, to identify the bacterial families present in the water bodies around the residential college and UTHM Pagoh Campus, and to compare the bacterial diversity between the two sites and explore its relationship with the water quality at both locations.

2. Material and Methods

The study was conducted at two distinct locations which are UTHM Wetland Conservation Research Station and the pond from the residential college The study sites were chosen based on their different geographical features, different water conditions, and environmental conditions. To prevent bias brought on by litter in the water, samples were taken at both locations away from the edge as the litter may have bacteria that do not originate from the site study. The location, date, and physical characteristics were written on the label of each bottle. To prevent the development of microorganisms, the samples were kept in a chiller at 4°C.

(a) (b)

Fig. 1 (a) Maps of Pagoh Residential College [6] (b) Maps of the UTHM Wetland Conservation Research Station land area [7]

2.1 Measurement of Water Quality

Points of study sites are chosen by taking the first point that is deep until feet level (wetland), or near the water flows (pond) and other points are 10 steps from the first point. The water quality at both locations was measured as the water sample was being collected using the multiparameter Hanna. After immersing the multiparameter in the water for a few seconds, the multiparameter Hanna was taken out. The following data were recorded: pH,

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dissolved oxygen (DO), conductivity, Biochemical Oxygen Demand (BOD) / Total Dissolved Solids (TDS), and temperature [8].

2.2 Bacterial Culture and Isolation

Nutrient agar plates were prepared to do the bacterial culture following the established protocol [9]. Repeated dilution was used to create water samples until 10^{-4} ml dilutions were achieved. The zigzag approach was used to distribute the dilution samples across the agar plates. The plates of bacteria were cultured (inverted) at room temperature (23 °C) for a day. The selected bacteria were transferred to labelled agar plates for isolation. The wire loop was flamed and used to remove a small amount of culture. The bacteria were dispersed throughout a section of the agar plates after being combined with the water drop. A day was spent incubating (inverting) the bacterial plates at room temperature. All of the bacterial plates were filtered through following a day of incubation.

2.3 Morphological Analysis of Bacteria Culture

Bacterial identification was conducted through eye observation based on colony morphology, shape, margin, optical properties, pigment, elevation, and texture.

2.4 Gram Staining

Bacteria can be divided into 2 main groups based on the reaction to the Gram stain process. End of the process there will be two colour forms which are purple (Gram-positive cell wall) and pink (Gram-negative cell wall) [10]. To achieve this, a sterile cotton swab was inserted into the bacteria plate. The slide was labelled, and the smears were allowed to dry completely. The smears became cloudy as they dried. The slide was heat-fixed by passing it through a flame 2-3 times to ensure the bacteria were fixed to the slide. The next step was the primary staining using crystal violet. The slide, containing the heat-fixed smears, was placed on a test tube rack in the sink with the smear facing up. The smear was covered with crystal violet and left for 10 seconds. This was followed by Gram's iodine staining. Both sides of the microscope slide were washed with distilled water, and the excess water was tipped off. The slide was then immediately covered with Gram's iodine, which was allowed to remain on the slide for 10 seconds. The decolorizing step followed. Both sides of the microscope slide were washed with distilled water, and the slide was rinsed with ethanol for 5 seconds. Both sides were immediately rinsed with water to remove the acetone and halt the decolorizing process. Care was taken to ensure the acetone did not remain in contact with the specimen for longer than 3 seconds. The final step was counterstaining with safranin. The slide was placed on the test tube rack in the sink with the smear side up and covered with safranin, which was left on the slide for 10 seconds. Both sides of the microscope slide were washed with distilled water and gently blotted with lens paper [11]. Observation of the slide was then conducted.

2.5 Bacterial Identification

Based on the list of results from the experiment, the bacteria were identified. The identification criteria were used to refer to websites, books, and journals such as published research articles to find the expected bacteria. The identified bacteria were then recorded up to the family level only.

3. Result and Discussion

3.1 Water Quality Measurement Using Multiparameter Hanna

Table 1 Water sample collection

Parameter	UTHM Wetland Conservation Research Station			Class Quality	Pond of the Residential College			Class Quality
	Point A	Point B	Point C		Point D	Point E	Point F	
pH	6.25	6.28	6.16	I-IV	6.50	6.46	6.52	I-IV
Dissolved Oxygen (mg/l)	1.64	1.42	1.29	IV	1.68	1.8	1.57	IV
Conductivity (µs/cm)	184	99	68	IIB-III	6	8	8	IIB-III
Biochemical Oxygen Demand (ppm)	92	50	34	V	3	4	4	IIA-IIB

Temperature	27.25	27.79	28.09	IIA and III	29.78	29.96	29.57	IIA and III

Table 1 shows that the water at the pond of the residential college has a closer pH towards neutral. The diversity of water composition can cause the water to become closer to neutral as it has more processes such as photosynthesis, respiration, and decomposition which affect carbon dioxide levels [11]. Water at the pond of the residential college has a higher percentage of dissolved oxygen (DO). Many biological, chemical, and physical factors affect the DO concentration in the pools such as dead organisms in the water, materials flow from sewage, and moss growth. Dissolved oxygen concentration changes very quickly in ponds due to various factors like low diffusion, low solubility, and rapid utilization. High DO levels may support aerobic bacteria, which play a role in nitrogen cycling while low DO levels may promote anaerobic bacteria [12].

Water at the pond of the residential college has lower conductivity than the UTHM Wetland Conservation Research Station. One of the most important factors in assessing the quality of water is conductivity [12]. Temperature and salinity both cause conductivity to increase, which might be harmful to the quality of the water. This is due to the fact that water with higher conductivity has more pollutants (dissolved materials, chemicals, and minerals) High metal concentrations can be toxic to most bacteria, reducing microbial diversity. The wetland's increased conductivity may be a sign of contaminants that are lowering the water's quality. Sewage leaks close to the wetland can be the cause. The Biochemical Oxygen Demand (BOD) of the residential college's pond water is lower than that of the UTHM Wetland Conservation Research Station. The BOD may also be interpreted as a measurement of the quantity of organic materials that can serve as a substrate to support the growth of microorganisms. High BOD indicates the growth of heterotrophic bacteria, while decreasing oxygen-sensitive species. Sewage with a high BOD content might cause water to have less oxygen, which can kill some species [13]. This means that water at the wetland has lower quality as it has higher BOD and lower DO in water quality assessment. Both sample sites have slightly different temperatures as both sites have the same environmental conditions and are near each other.

Based on the National Water Quality Standards and Water Quality Index, we can see that UTHM Wetland Conservation Research Station has a higher class since it has a higher measurement on BOD measurement. UTHM Wetland Conservation Research Station can be put between Class IV and V while the pond of the residential college can be put between Class II and III as follows the Water Quality Index which is based on the uses of the water area. It only covers three points of the study site, and the points of wetland are near the sewage and oil palm plantations, the fertilizer or leakage from the sewage can cause the pollution to occur. As the pollution occurs, the BOD measurement will be high as the water will lack oxygen and the organisms around the study site points will need a higher level of oxygen [13]. The measurement can be different as the water samples are at deeper or other places. From the pond at the residential college, the measurements show that even though they have lower classes, conventional treatment is required as the pH is on a high scale and the dissolved oxygen is on a lower scale. Unlike UTHM Wetland, the size of the pond is smaller and the different site points may occur with similar measurements. As for UTHM Wetland, the size is bigger and the different site points may have different measurements as at some points, the water levels are deeper and far from the oil palm plantations. The expansion of urban infrastructure near UTHM Pagoh Campus may influence local aquatic ecosystems by introducing pollutants and altering water parameters. Monitoring water quality parameters can help detect early signs of ecosystem degradation, guiding sustainable water management and conservation efforts.

3.2 Gram Staining

Table 2 Gram Staining Result

Parameter	UTHM Wetland Conservation Research Station			Pond of the Residential College			
	Point A	Point B	Point C	Point D (1)	Point D (2)	Point E	Point F
Gram Staining Result	-	Gram-negative cell wall	Gram-positive cell wall	Gram-positive cell wall	Gram-negative cell wall	Gram-negative cell wall	Gram-positive cell wall

Some study states that bacteria are greatly impacted by the existence of ponds and wetlands, which changes their survival, variety, and abundance [14]. The design and environmental conditions of constructed ponds and wetlands, which are used for water treatment, can have a variety of effects on bacterial populations [15]. Depending on variables including nutrient availability, sediment properties, and outside contaminants, these

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systems can either increase or decrease bacterial populations. Since they better retain the small particles that bacteria attach to, constructed wetlands are more successful than ponds at lowering bacterial loads and preventing their persistence. Because they cling to tiny particles and shield themselves from predators, bacteria like thermotolerant coliforms have higher survival rates in ponds [16]. The findings imply that the treatment procedures in these wetlands change the general structure of the bacterial community by affecting the number and composition of both Gram-positive and Gram-negative bacteria [17].

3.3 Morphological Identification by Using Eye Observation

Table 3 Morphology of Samples

Samples	Colour	Shape	Elevation	Form	Margin
Point B	Whitish	Coccus	Raised	Irregular	Undulate
Point C	Yellowish	Coccus	Flat	Irregular	Undulate
Point D (1)	Yellowish	Rod	Flat	Circular	Entire
Point D (2)	Greenish	Rod	Flat	Circular	Entire
Point E	Greenish	Rod	Flat	Circular	Entire
Point F	Whitish	Coccus	Raised	Circular	Entire

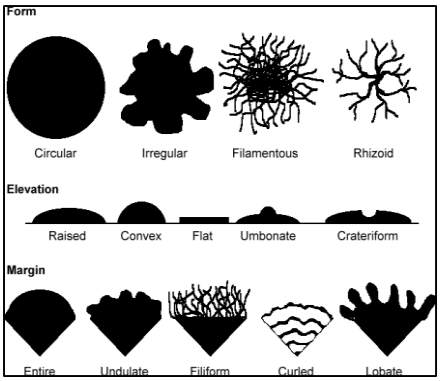

Fig. 2 Examples of form, elevation, and margin of bacteria on the plates

Bacterial identification was conducted through eye observation based on colony morphology. The results have been recorded based on colour, shape, elevation, form, and margin. Based on the data above, 2 samples were found in the water of UTHM Wetland Conservation Research Station while 4 samples were at the residential college pond. The sample from the pond has unique samples as it has a greenish colour has been found. A study state that it forms from a bacterium that can produce pyocyanin, which can give the agar a greenish-blue colour. Different strains generate different amounts of pigment, and when the bacterium is cultivated on a rich media, it can occasionally emit a pleasant grape-like odor due to 2-aminoacetophenone [18].

The shapes of samples are commonly coccus and rod-shaped. Due to their capacity to adhere to surfaces, their functions in the breakdown of organic waste, and their ability to adapt to changing environmental factors like temperature and nutrient availability, which affect their growth, coccus and rod-shaped bacteria are prevalent in water [19]. Their forms improve survival; spherical cells may generate more offspring per gram of resources, optimizing nutrition accumulation in aquatic conditions, whereas rod-shaped bacteria migrate effectively toward food sources [19]. Additionally, these forms improve stability against osmotic pressure, which is essential for aquatic life [20].

3.4 Bacteria Identification by the Article Papers

Table 4 List of identified families of bacteria founded

Habitat	Sample	Bacterial Family	Notable genera	References
UTHM Wetland Conservation Research Station	Point B	Neisseriaceae	*Neisseria*	[21]
		Moraxellaceae	*Moraxella*	[22]
	Point C	Micrococcaceae	*Micrococcus*	[23] & [24]
		Planococcaceae	*Planococcus*	[25]

Pond of the Residential College	Point D (1)	Corynebacteriaceae	*Corynebacterium*	[26]
	Point D (2)	Pseudomonadaceae	*Pseudomonas*	[18]
	Point E			[27] & [28]
	Point F	Staphylococcaceae	*Staphylococcus*	[29]

The Neiseriaceae family can survive in wetland water habitats because of several unique characteristics. These modifications are essential for surviving in wetlands anaerobic environments and variable water levels. Radial oxygen transport which supports aerobic respiration in anaerobic soils, Neiseriaceae species have evolved systems to move oxygen from their aerial portions to their roots [30]. Maintaining aerobic conditions in the root zone, which supports plant growth and health, depends on better gas exchange made possible by greater root porosity [31]. The majority of Neisseriaceae species are found in human and animal hosts, and they prefer mucosal surfaces over watery habitats [32]. Many Neisseriaceae species can switch between aerobic and anaerobic respiration, allowing them to survive in wetlands where oxygen levels fluctuate due to seasonal flooding or microbial activity. Certain Neisseriaceae species interact with wetland plants by colonizing their rhizospheres (root zones). The wetland may have a higher of animal hosts than the college residential pond. Nutrient-rich habitats, which are less prevalent in ordinary pond settings, are frequently associated with their biological niche. Ponds that are oligotrophic (poor in nutrients) may not provide the particular growth conditions needed by many aerobic Neisseriaceae species [33]. By eluding nutritional immunity, the pathogenic Neisseria species including *Neisseria meningitidis* and *Neisseria gonorrhoeae* can cause massive morbidity and mortality in humans, resulting in severe infections like meningitis and gonorrhea that can cause major health complications.

Members of the Moraxellaceae family are able to survive in wetland water habitats due to a number of unique characteristics. These adaptations, which together improve their survival and ecological success in such environments, include physical traits, metabolic flexibility, and stress tolerance systems. According to their cell structure, Moraxella species are usually rods or cocci that are Gram-negative. They frequently display pleomorphism, which enables them to adjust to different environmental circumstances. Despite lacking flagella, several species have surface-bound "twitching motility," which makes it easier to move across damp surfaces [34]. According to oxygen consumption, certain species can thrive in anaerobic environments, enabling them to make use of a variety of niches found in wetland ecosystems, despite their primary aerobic growth [35]. Moraxella strains have notable stress-resistant responses to environmental stressors such as heat, osmotic pressure, and oxidative stress, especially when carbon-starved, according to survival mechanisms. This flexibility is essential for surviving in the changing environment of wetlands [36]. These bacteria are chemoorganotrophic, relying on organic matter for sustenance. Ponds with low organic content may not support their growth [35]. A member of the Moraxellaceae family, *Moraxella catarrhalis* is a newly discovered respiratory pathogen that has a substantial clinical impact on human health. It causes recurrent respiratory infections in individuals with chronic obstructive pulmonary disease and acute otitis media in children.

The family Micrococcaceae has several unique characteristics that allow them to survive in marsh water settings, especially under harsh circumstances. Rapid microdiversification enables members of the Micrococcaceae family to adjust to changing environmental circumstances in wetlands [37]. The ability of Micrococcaceae to inhabit certain niches within wetlands enables them to efficiently utilize local resources [37]. These bacteria generate secondary metabolites that improve nutrient cycling and pollutant degradation by facilitating interactions with plants and other microorganisms, according to their production of microbial volatiles [38]. Their ecological success in a variety of wetland habitats is facilitated by their capacity to adapt to seasonal and spatial changes [38]. Since they prefer habitats with greater levels of organic matter and nutrient availability—conditions that are uncommon in many freshwater ecosystems—they are less frequently found in aquatic systems, such as ponds [39]. The tiny genomes of Micrococcus species may also limit their capacity to adapt and survive in a variety of ecological niches, which might explain their restricted presence in aquatic habitats [40]. Members of the Micrococcaceae family, including Micrococcus species, can cause illnesses like peritonitis in immunocompromised people by acting as opportunistic pathogens. Despite their modest virulence, these infections have the potential to result in refractory peritonitis and peritoneal dialysis failure.

Due to several unique adaptations, the Planococcus family can thrive in wetland water environments, which are characterized by fluctuating water levels and varying redox conditions. These adaptations include interactions with other microbial populations, metabolic flexibility, and specific genetic traits [41]. The presence of distinct gene clusters in its genome suggests specialized adaptations for stress responses, which are crucial for survival in dynamic wetland habitats. Planococcus species can utilize various electron acceptors, such as nitrate and sulfate, which are commonly found in wetland soils with shifting redox conditions [42]. Additionally, Planococcus interacts with wetland plants to enhance gas exchange and nutrient cycling, both of which are vital for survival in wet environments [42]. Disease-causing pathogenic bacteria, such as those in the Planococcus family, may infiltrate host cells, stick to tissues, and avoid the immune system.

It is known that Corynebacteriaceae may be found in both freshwater and marine habitats [43]. By offering more nutrients and habitat, microalgae in ponds can increase the variety of bacteria, including Corynebacteriaceae [44]. Ponds are perfect homes for Corynebacteriaceae because they frequently include nutrients and organic debris that promote bacterial development. The prevalence of these bacteria in aquatic environments can be further enhanced by the interaction between algae and bacteria, which can increase microbial diversity [44]. The wetland's supply of nitrogen and phosphorus, which are essential for bacterial development, may be limiting the number of Corynebacteriaceae [45]. Proteobacteria and Bacteroidetes are the most common phylum in wetlands, frequently outcompeting Corynebacteriaceae for resources [46]. Particularly in immunocompromised people, Corynebacterium species can cause respiratory conditions such bronchitis, rhinosinusitis, pharyngitis, and tracheitis.

Due to their widespread presence in soils, freshwater, and marine settings, Pseudomonas species are able to colonise a variety of aquatic habitats. Their ability to endure harsh environments, including nutrient-poor waters, is essential to their tenacity in ponds [47]. In aquaculture ponds, *Pseudomonas aeruginosa* efficiently breaks down contaminants such heavy metals, playing a vital role in bioremediation [48]. Because of increased runoff and nutrient intake during rainy seasons, *Pseudomonas aeruginosa* population density in aquatic habitats can fluctuate with seasonal fluctuations, peaking during these times [49]. Higher temperatures (around 37ºC) are ideal for Pseudomonas species, especially *Pseudomonas aeruginosa*. Lower temperatures may prevent the formation of natural wetlands, resulting in counts below 1 CFU/100 mL [50]. Diverse microbial communities that compete for resources are abundant in wetlands. Pseudomonas may not be as effective as other bacteria in using the resources at their disposal due to this competition, which can restrict their development [51]. Despite these difficulties, Pseudomonas may still be found in artificial wetlands, where the environment may be better regulated, facilitating its development and its use in wastewater treatment [52]. Concerns regarding the ecological effect and control of Pseudomonas strains in wetlands are raised by the variation in antibiotic resistance among these organisms in various habitats [51]. Human illnesses can be brought on by the Pseudomonas family, notably by species like *Pseudomonas aeruginosa*, which can cause infections that are frequently severe, especially in those with weakened immune systems. *Pseudomonas fluorescence* and *Pseudomonas putida* are two more harmful species that cause a number of health problems.

The presence of organic elements and pathogens in fish pond wastewater frequently promotes the growth of Staphylococcus species [53]. Because of their adaptability, staphylococci may live in a variety of aquatic habitats, such as lakes and rivers, where they can be separated from surface waters. Staphylococcus bacteria can thrive in environments that are eutrophicated by fertilizer runoff [54]. Diverse microbial communities may be found in wetlands, with prominent species including Firmicutes and Proteobacteria outcompeting Staphylococcaceae for resources. Microbial diversity is greatly impacted by variations in water quality. For example, diverse bacterial communities can be supported by high-quality water sources, which may restrict the abundance of Staphylococcaceae. Certain microhabitats, such as those linked to animal or human activity, which are frequently not duplicated in natural wetland settings, may be preferred by Staphylococcaceae but are less common in wetlands [51]. From minor skin infections to serious illnesses including sepsis, toxic shock syndrome, pneumonia, and infective endocarditis, Staphylococcus aureus may cause a wide range of illnesses. Immune evasion and disease severity are greatly influenced by its virulence components, which include cytolysins and superantigens.

4. Conclusion

The water quality of UTHM Pagoh Campus measurements shows that the UTHM Wetland Conservation Research Station is put between Class IV and V while the pond of the residential college can be put between Class II and III. Bacteria diversity also can be identified by finding the expected family classes of bacteria that be found from the study based on bacterial culture, bacterial isolation, morphological identification, and Gram staining. There are 3 samples which are Gram-positive cell walls and 3 samples Gram-negative cell walls. Neisseriaceae, Moraxellaceae, Micrococcaceae, and Planococcaceae were identified in the UTHM Wetland Conservation Research Station while Corynebacteriaceae, Pseudomonadaceae, and Staphylococcaceae identified in the pond of the residential college. The geographic, nutrient, and environmental conditions of the site can affect the diversity of bacteria that live in the water since each species of bacteria has its own conditions to live. The modifications of bacterial cells are essential for surviving in wetlands' anaerobic environments and variable water levels. Some bacteria can endure harsh environments, including nutrient-poor waters, which is essential to their tenacity in ponds.

For further study, many other methods can help in the identification of bacteria diversity. For example, single-colony PCR and bacterial DNA extraction using boiling method. The study will include DNA Quantification (spectrophotometer), preparation of PCR Cocktail, gel electrophoresis, Bioedit of sample sequences to get the sequence, NCBI blast for sample, and phylogenetic tree. Future studies will be done to get the sample's full identification with the morphological identification that has been done. Anthropogenic activities alter bacterial diversity in aquatic ecosystems, influencing ecosystem stability, water quality, and microbial functionality. Understanding these impacts is crucial for developing strategies to mitigate environmental degradation, promote

sustainable urban planning, and enhance conservation efforts in urbanized environments like UTHM Pagoh Campus.

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Conflict of Interest

Authors declare that there is no conflict of interests regarding the publication of the paper.

Author Contribution

The authors confirm their contributions to the paper as follows: ***study conception and design:*** *Muhammad Amri Ibrahim, Furzani Pa'ee;* ***data collection:*** *Muhammad Amri Ibrahim;* ***analysis and interpretation of results:*** *Muhammad Amri Ibrahim, Furzani Pa'ee;* ***draft manuscript preparation:*** *Muhammad Amri Ibrahim, Furzani Pa'ee. All authors reviewed the results and approved the final version of the manuscript.*

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