water

MDPI

Article

Bacterial Community Composition and Function in a Tropical Municipal Wastewater Treatment Plant

Yaqiong Gu, Beiying Li, Xiang Zhong, Conghe Liu and Bin Ma *

Key Laboratory of Agro-Forestry Environmental Processes and Ecological Regulation of Hainan Province, School of Ecology and Environment, Hainan University, Haikou 570228, China; 15708916637@163.com (Y.G.); libeiying09@163.com (B.L.); zx01120008@163.com (X.Z.); lch1040325098@163.com (C.L.)
* Correspondence: mady7802@163.com; Tel.: +86-898-66292091

Abstract: Bacterial diversity and community composition are of great importance in wastewater treatment; however, little is known about the diversity and community structure of bacteria in tropical municipal wastewater treatment plants (WWTPs). Therefore, in this study, activated sludge samples were collected from the return sludge, anaerobic sludge, anoxic sludge, and aerobic sludge of an A^2O WWTP in Haikou, China. Illumina MiSeq high-throughput sequencing was used to examine the 16S ribosomal RNA (rRNA) of bacteria in the samples. The microbial community diversity in this tropical WWTP was higher than in temperate, subtropical, and plateau WWTPs. *Proteobacteria*, *Bacteroidota*, *Patescibacteria*, and *Chloroflexi* were the dominant phyla. Nitrification bacteria *Nitrosomonas*, and *Nitrospira* were also detected. *Tetrasphaera*, instead of *Candidatus Accumulibacter*, were the dominant polyphosphate accumulating organisms (PAOs), while, glycogen accumulating organisms (GAOs), such as *Candidatus Competibacter* and *Defluviicoccus* were also detected. The bacterial community functions predicted by PICRUSt2 were related to metabolism, genetic information processing, and environmental information processing. This study provides a reference for the optimization of tropical municipal WWTPs.

Keywords: tropical municipal wastewater treatment plant; bacterial community; functional genes

check for updates

Citation: Gu, Y.; Li, B.; Zhong, X.; Liu, C.; Ma, B. Bacterial Community Composition and Function in a Tropical Municipal Wastewater Treatment Plant. *Water* **2022**, *14*, 1537. https://doi.org/10.3390/w14101537

Academic Editors: Christos S. Akratos, Alexandre T. Paulino and Jesus Gonzalez-Lopez

Received: 4 March 2022
Accepted: 8 May 2022
Published: 11 May 2022

1. Introduction

Wastewater treatment plants (WWTPs) are widely used globally to remove pollutants from wastewater, and provide a significant contribution to the environment and human health [1,2]. Most municipal WWTPs depend on the activated sludge, which is based on microbial metabolic actions to remove organic pollutants, nitrogen, phosphorus, and other pollutants from municipal wastewater [3,4].

Nitrifying and denitrifying bacteria are needed for efficient nitrogen removal: they transform ammonia into nitrate and then convert nitrate into nitrogen gas. Nitrifying bacteria include ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB). AOB are represented by five recognized genera in two phylogenetically distinct groups, in the subclasses of *Betaproteobacteria* and *Gammaproteobacteria*. Four genera of AOB, including *Nitrosospira*, *Nitrosomonas*, *Nitrosovibrio*, and *Nitrosolobus*, are grouped in the *Betaproteobacteria*, while one genera of *Nitrosococcus* is within the *Gammaproteobacteria* [5]. *Nitrosomonas* and *Nitrosospira* are the most extensively studied AOB. Gram-negative NOB, are more phylogenetically distinct and widespread than AOB, including *Nitrospira*, *Nitrococcus*, *Nitrobacter*, and *Nitrospina* [5]. There are numerous denitrifying bacteria that are able to realize denitrification and the removal of organic materials during anoxic or aerobic processes, such as *Bacillus*, *Pseudomonas*, *Thauera*, *Acinetobacter*, *Hyphomicrobium*, *Dokdonella*, *Azoarcus*, *Rhizobium*, *Denitratisoma*, *Zoogloea*, and *Sulfuritalea* [6].

Compared with chemical phosphorus removal, the enhanced biological phosphorus removal (EBPR) process is an effective, economical, and environmentally friendly phosphorus (P) removal technology. During the EBPR process, polyphosphate (polyP) accumulating organisms (PAOs) anaerobically utilize the energy and reduce the power gained from the hydrolysis of glycolysis, intracellular polyPs and the tricarboxylic acid (TCA) cycle to absorb volatile fatty acids (VFAs) and store them as polyhydroxyalkanoates (PHAs). In the successive anoxic/aerobic phase, PAOs use the reserved PHAs as carbon sources and energy for polyP generation, depending on an excess of uptake inorganic orthophosphate released in the anaerobic stage, for glycogen synthesis and biomass growth [7]. *Candidatus Accumulibacter* is a crucial PAO in laboratory- and full-scale EBPR systems [8–10], and main carbon sources are attributed to easily degradable small molecule compounds, such as VFAs. Another vital PAO is *Tetrasphaera*, belonging to *Actinobacteriota*. *Tetrasphaera* are more abundant than *Candidatus Accumulibacter* in WWTPs which have lower wastewater temperatures, such as Portugal (8–25 °C) [11] and Denmark (9–18 °C) [3].

The microbial diversity and activity of activated sludge help the stability of WWTPs. Microbial communities are correlated with the successful operation of WWTPs. Nevertheless, the link between biological knowledge and treatment technologies is always deemed as “black boxes” [12]; therefore, many problems are frequently encountered during the operation of WWTPs, such as nitrification failure [12], activated sludge bulking, and foaming [13].

A thorough understanding of the microbial community in full-scale WWTPs is crucial to provide intelligent management for operating and optimizing the processing effect to meet the stricter discharge standards [14]. One hundred ten activated sludge samples from 21 cities in the north and south of China were investigated using high-throughput 16S ribosomal DNA (rDNA) sequencing to explore the regional effects on microbial community structure [15]. The Global Water Microbiome Consortium (GWMC) recently carried out systematic investigations on microbial communities and identified core microbial taxa of global WWTPs [2]. Reviewing the data from these studies showed that most activated sludge samples were from mid-latitude WWTPs. Low-latitude tropical WWTPs are poorly studied, and the microorganisms they contain are mostly unknown.

Therefore, to understand the microbial community structures and related metabolic potential of activated sludge in tropical municipal WWTPs, four sludge samples were obtained from a tropical WWTP in Haikou China. Illumina MiSeq high-throughput sequencing was used to evaluate the microbial community diversity and structure. The community structure associated with nitrogen and phosphorus removal was analyzed. The corresponding metabolic activities of microorganisms were studied by identifying the functional pathways and genes.

2. Materials and Methods

2.1. WWTP and Sample Collection

In the study WWTP, located in Haikou, the biggest city on the tropical island of China, a conventional A^2O process is employed for effectively removing nitrogen and phosphorus (Table 1). The temperature of the mixed liquor in the studied wastewater treatment plant is 23.2–32.3 °C throughout the year. These are higher than that in the temperate zones (Beijing WWTP, 12.8–26.6 °C). The temperature of the samples collected in this study were 22.6–23.2 °C.

Table 1. The influent and effluent organic matter, nitrogen, and phosphorus concentrations of the studied WWTP from January 2020 to June 2021.

	BOD_5 (mg/L)	**COD (mg/L)**	**NH_4^+-N (mg/L)**	**TN (mg/L)**	**TP (mg/L)**
Influent	36–73	79–232	18.2–37.7	25.0–43.1	1.56–2.06
Effluent	4.5–9.2	6–25	0.18–1.76	7.37–9.67	0.06–0.36

The site survey and sample collection were conducted in January 2021. Suspended sludge was collected from the anaerobic sludge (HN_YY), anoxic sludge (HN_QY), aerobic sludge (HN_HY), and return sludge (HN_HL) (each sludge sample was collected in triplicate and combined into a single sample). The samples were put in 500-mL sterile polyethylene bottles and kept on ice for bringing back to the laboratory. The dewatered sludge was stored at −80 °C until DNA extraction, 16S rRNA gene PCR amplification, and Illumina MiSeq sequencing.

2.2. DNA Extraction and PCR Amplification

DNA extraction was carried out using the FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA). A NanoDrop 2000 UV–vis spectrophotometer (Thermo Scientific, Wilmington, USA) was used for quantitative and qualitative DNA analysis, and DNA quality was checked by 1% agarose gel electrophoresis. The V3–V4 region of the bacterial 16S gene was amplified using the sequencing primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). Polymerase chain reactions (PCR) were performed in a 20-μL system with 4 μL of 5 × FastPfu buffer, 2 μL of dNTPs (2.5 mM), 0.8 μL forward primer(5 μM), 0.8 μL of reverse primer (5 μM), 0.4 μL of FastPfu polymerase, 2 μL of BSA, and 10 ng of template DNA. The PCR program comprised an initial denaturation at 95 °C for 3 min, after 27 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, with a final extension at 72 °C for 10 min [16].

2.3. Analytical Methods and Statistical Analysis

The Illumina platform was used for high-throughput sequencing. Samples were sent to Majorbio Biopharm Biotechnology Co., Ltd. (Shanghai, China) for DNA sequencing. Operational taxonomic units (OTUs) with 97% similarity were clustered, the data were analyzed via the online Majorbio Bioinformatic Cloud Platform (https://cloud.majorbio.com/) accessed on 3 April 2021, and the alpha diversity index of activated sludge (AS) samples were calculated via Mothur (version 1.30.2, https://www.mothur.org/wiki/Download_mothur, Patrick D. Schloss, USA, accessed on 3 April 2022); curves were generated using the R tool (version 3.3.1). This taxonomic analysis can determine the community structure composition at different classification levels. The potential functions and metabolic pathways of bacteria were predicted by PICRUSt2 software (version 2.2.0, https://github.com/picrust/picrust2/, Gavin M. Douglas, Canada accessed on 3 April 2021) and compared with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, and the closed OTU table from QIIME (version 1.9.1, http://qiime.org/install/index.html accessed on 3 April 2021) was used to obtain different functional prediction information. The raw data from this study has been added to the National Center for Biotechnology Information (NCBI) database under the accession numbers SRR17717417, SRR17717418, SRR17717419, and SRR17717420.

3. Results and Discussion

3.1. Bacterial Community Diversity

The microbial diversity of AS is propitious to the efficiency and stability of WWTP performances; therefore, the microbial community diversity of the WWTP were analyzed. After removing low-quality sequences and chimeras, at least 49,601 valid sequences were obtained from each sample by high-throughput sequencing (Table 2), with an average length of 435 bp. Rarefaction curves demonstrated that all the samples were sequenced almost to saturation (Figure 1). These results were also confirmed by Good’s coverage values, all of which were higher than 99% (Table 2). Alpha diversity is an indicator that reflects the number of species and the relative abundance of species within the community, mainly indicating the species composition and the distribution characteristics of the number of individuals in the community. The higher the Chao or ACE index, the greater the community richness. The community richness in the aerobic area was the highest by Chao or ACE index (1461 or 1481), while that in the anaerobic area was the lowest (1405 or 1415). The Heip index values were from 0.1667–0.1758, which characterizes the evenness of microbes. The Shannon and Simpson indexes, which imply the species diversity, accounted for 5.297–5.344 and 0.01072–0.01131, respectively. The lower the Simpson index and the higher the Shannon index, the greater the diversity. These two indexes both indicate that the diversity of the anaerobic zone is highest. Additionally, the total number of OTUs in the anaerobic, anoxic, aerobic, and return units were 1182, 1232, 1193, and 1256 respectively, which was slightly lower than in other municipal WWTPs (1361–2599) [17,18]. Compared with the Chao and Shannon indexes of 870–1207 and 4.57–5.17 in a temperate [19], subtropical [20] and plateau [21] WWTPs, the Haikou sewage treatment plant has a higher diversity because some temperature-sensitive microorganisms cannot survive in cold environments. Samples from industrial petrochemical-activated sludge also showed lower diversity than samples from the Haikou domestic activated sludge, which might be due to the toxic material from industrial petrochemical wastewater [4].

Table 2. Sequence, OTUs, coverage, and Alpha diversity of microbial communities in the anaerobic sludge (HN_YY), anoxic sludge (HN_QY), aerobic sludge (HN_HY), and return sludge (HN_HL).

Sample\Estimators	Sequence	OTU	Shannon	Simpson	Ace	Chao	Heip	Coverage
HN_YY	49601	1182	5.340	0.01072	1415	1406	0.1758	0.9919
HN_QY	52793	1232	5.323	0.01124	1442	1434	0.1657	0.9936
HN_HY	51282	1193	5.297	0.01131	1481	1461	0.1667	0.9904
HN_HL	52960	1256	5.344	0.01076	1448	1430	0.1660	0.9941

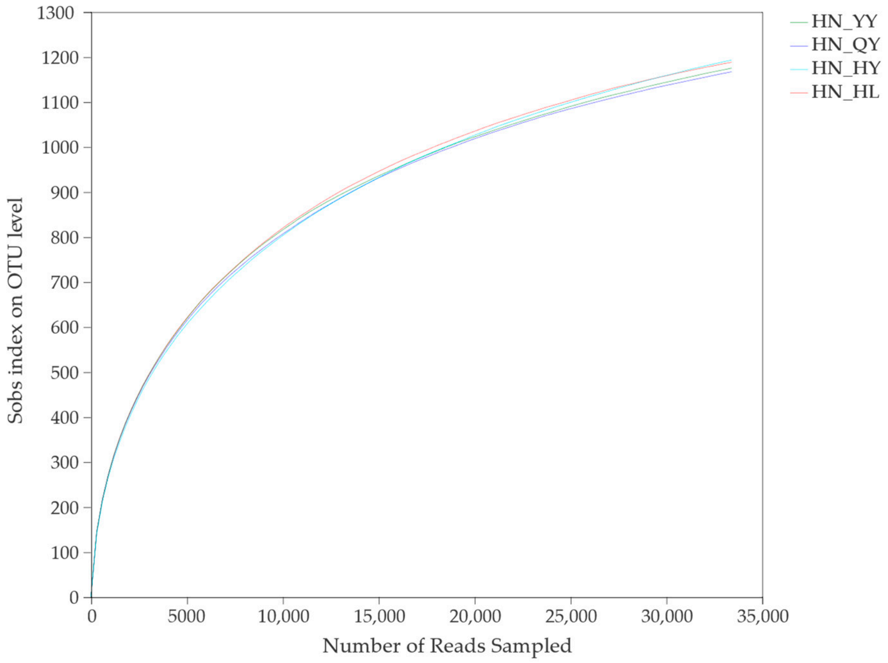

Figure 1. Rarefaction curves of OTUs clustered at 97% sequence identity across in the anaerobic sludge (HN_YY), anoxic sludge (HN_QY), aerobic sludge (HN_HY), and return sludge (HN_HL).

3.2. Bacterial Community Structure

At the phylum level, the four sludge samples obtained from different units of Haikou WWTP shared 36 phylum, accounting for 94.7% of the total, while there was one unique phylum, demonstrating that the phylum in AS from different units is generally similar. Due to variation in the environmental situation, each operation unit will form a different microbial community structure. However, in consequence of the return system, the microorganism cycles in each operation unit, and decay and growth kinetics are too slow to observe changes in hydraulic retention time (a few hours), so microorganisms of different units have a lot in common.

The predominant phylum among the four sludge samples was *Proteobacteria*, with relative abundances of 24.26–28.31% (Figure 2), which are of great significance to the global carbon, nitrogen, phosphorus, and sulfur cycles [22]. This is consistent with previous studies, indicating that *Proteobacteria* are widely spread in full-scale WWTPs [2,15,17]. *Proteobacteria* have five major classes, including *Alphaproteobacteria*, *Betaproteobacteria*, *Deltaproteobacteria*, *Gammaproteobacteria*, and *Epsilonproteobacteria*, which were generally detected in the other WWTPs [18]. In this study, *Gammaproteobacteria* accounted for the most significant proportion of 15.62–18.45% in the four samples, followed by *Alphaproteobacteria* (8.64–11.44%). These results showed good agreement with the findings of Xue et al. [23], who found that *Gammaproteobacteria* were the crucial subclass of *Proteobacteria* in WWTPs in North America, based on their relative abundance. In contrast, Zhang et al. [24] found that *Betaproteobacteria* were the most abundant in AS from 14 WWTPs. *Bacteroidota* (17.4–19.93%), *Patescibacteria* (15.97–18.11%), *Chloroflexi* (13.54–16.74%), *Actinobacteriota* (8.47–11.07%), *Acidobacteriota* (3.17–5.90%), *Firmicutes* (2.9–4.50%), and *Myxococcota* (1.01–1.28%) were the dominant phyla (Figure 2). Other phyla occupied less than 1% of the relative abundance of bacteria. *Bacteroidetes* have a strong degradation capacity to metabolically break down proteins, lipids, and other macromolecules, particularly generating VFAs [1,6]. *Patescibacteria* usually occur in both groundwater and surface water and are favorable to denitrification. Furthermore, their distribution does not directly rely on a specific host, but is attracted by conditions that meet a fermentative lifestyle [25]. However, *Patescibacteria*, is not commonly reported as a dominant phylum in WWTPs, while it had a much higher abundance in this study. Members of *Chloroflexi* remain as flocculent skeletons of sludge colloidal flocs and are beneficial for forming granular sludge and the decomposition of hard-degraded organic sources [17]. *Actinobacteria* play a key role in EBPR systems [26]. *Firmicutes* often inhabit AS and contain genera that degrade pollutants, demonstrating their significance in wastewater treatment [19]. *Myxococcota* has been indicated to have a pivotal role in dissimilarity sulfate reduction, nitrate reduction, and fermentation [27].

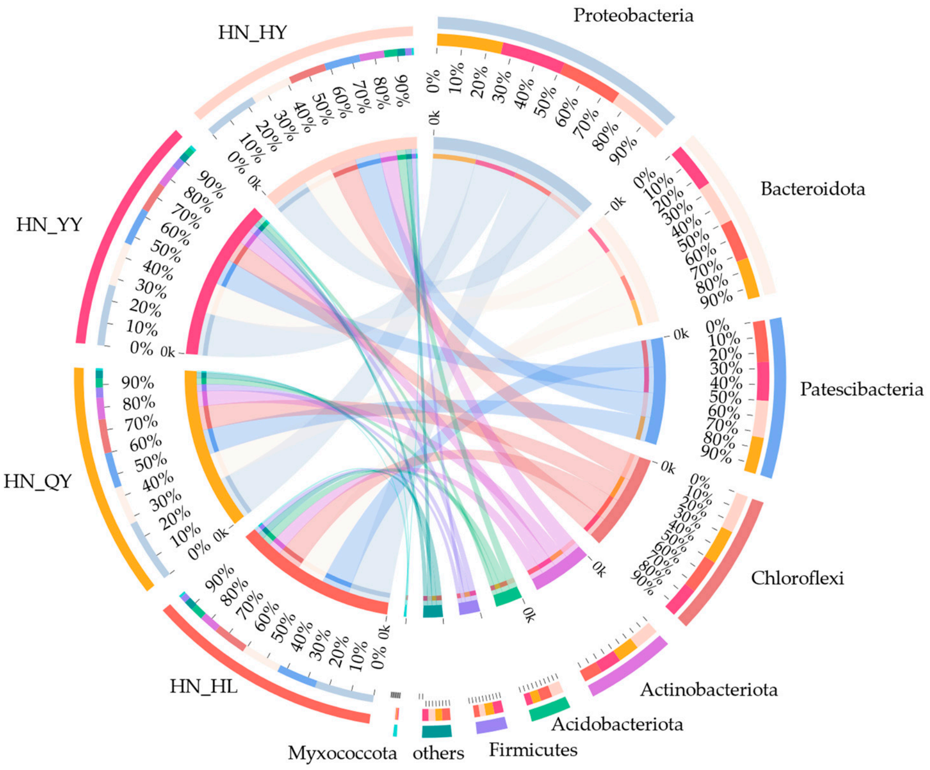

Figure 2. Four activated sludge samples and species relationship at the phylum level, including the anaerobic sludge (HN_YY), anoxic sludge (HN_QY), aerobic sludge (HN_HY), and return sludge (HN_HL).

To a certain extent, the activities and abundance of functional microorganisms involved in AS are the key elements promoting nutrient removal in wastewater. The 16S rRNA analysis provides information on functional genera with important sewage processes, such as nitrification, denitrification, EBPR, and hydrolysis.

The removal of ammonium and other nitrogen species from wastewater is necessary. In this study, *Nitrosomonas* (0.14–0.2%) was the only AOB and *Nitrospira* (0.22–0.47%) was the only NOB found in the four sludge samples (Figure 3), highlighting that the diversity of nitrifying bacteria was low.

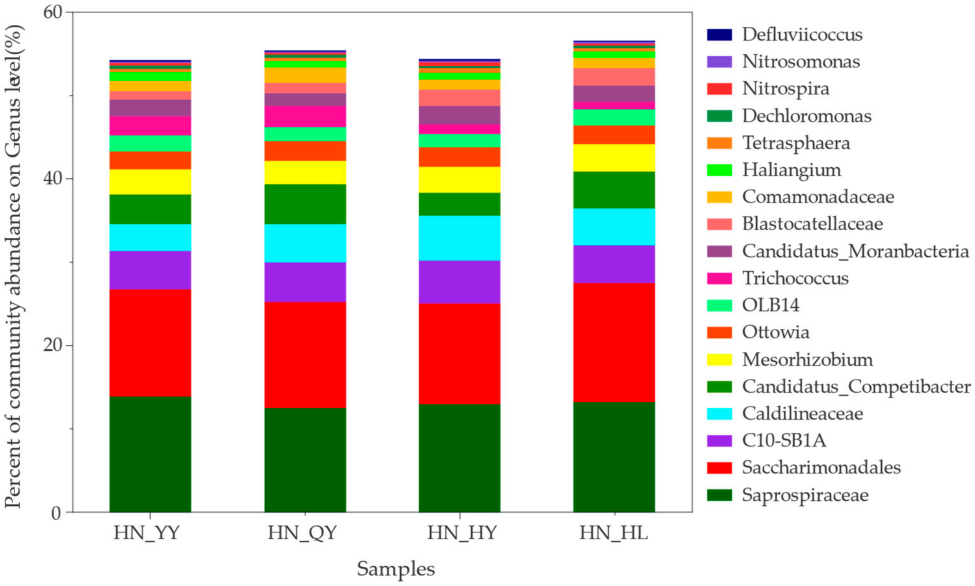

Figure 3. Relative abundance of the predominant organic matter, nitrogen, and phosphorus removal organnisms (genus level) in the anaerobic sludge (HN_YY), anoxic sludge (HN_QY), aerobic sludge (HN_HY), and return sludge (HN_HL).

Nitrosomonas is a dominant AOB genus in many AS and seems to be favored at higher temperatures compared with *Nitrosospira* [28]. In addition to oxidizing ammonium, *Nitrosomonas* is also considered to potentially degrade triclosan [29], which is an emerging pollutant in personal care products widely used as a broad-spectrum antimicrobial agent [30]. Unfortunately, triclosan concentration was not measured in this studied tropical WWTP. The genus *Nitrospira* (phylum *Nitrospirae*) as NOB has been recently identified as some species that could achieve complete oxidation of ammonia to nitrate, rather than two-step ammonia oxidizing by AOB and NOB [31]. However, the dominance of NOB *Nitrospira* in WWTPs remains controversial. Yao and Peng suggested that *Nitrospira* (4.02%) was the dominant NOB in 10 full-scale biological nutrient removal warm temperate WWTPs [32]. Ju et al. analyzed an A/O subtropical WWTP and found that *Nitrospira* was the most abundant genera (6.1 ± 4.2%) [33]; however, Saunders et al. investigated bacterial community compositions of 13 temperate WWTPs and reported that *Nitrotoga* was the primary nitrite-oxidizer rather than *Nitrospira* [3]. Alawi et al. verified that the three genera of a novel cold-adapted nitrite oxidizer, *Nitrobacter*, and *Nitrospira* inhabited AS, and differences in dominance among these genera might be due to temperature variations [34].

Given the relatively low abundance of *Nitrosomonas* in all four units, the question that needs to be discussed is which AOB oxidizes ammonia. Given the low ammonia effluent of the investigated WWTP, ammonia oxidation obviously occurred. One possible explanation is that complete ammonia oxidizers (comammox) are involved in at least the partial oxidation of ammonia. No direct evidence for comammox was found as they could not be distinguished from other *Nitrospira* populations by 16S rRNA amplicon sequencing [35]. However, there is some indirect evidence to support their presence. Firstly, the most common comammox in the environment are members of *Nitrospira* phylogenetic grouping [31]. Secondly, it is universally accepted that comammox survive selectively over other nitrifiers under an oligotrophic lifestyle, such as low ammonium concentrations [36].

Another possible explanation is that unknown ammonia oxidizing bacteria contribute to ammonia oxidation [15].

NO_3^--N removal is governed by heterotrophic denitrifiers, which are responsible for reducing nitrate and nitrite to nitrogen gas under anoxic conditions [18]. As shown in Figure 3, *Caldilineaceae* (3.24–5.4%), *Ottowia* (2.12–2.37%), *OLB4* (1.57–1.95%), and *Haliangium* (0.77–1.04%), accounted for the majority of denitrifying bacteria genera [14,37,38]. *Candidatus Competibacter* (2.74–4.79%) and *Defluviicoccus* (0.14–0.16%) were the dominant glycogen accumulating organisms (GAOs), which are thought to compete limited carbon sources with PAOs but do not contribute to phosphorus removal, and act as two other typical denitrifying bacteria for endogenous nitrate removal [39]. *Mesorhizobium*, in the class of *Alphaproteobacteria*, which is regarded as not only a nitrogen-fixing bacterium but also as a denitrifier both in aerobic and anaerobic conditions [40,41], accounted for 2.81–3.29% in the four samples. The abundances of putative denitrifying bacteria were higher than nitrifiers in the four AS samples, while the nitrification performance in the Haikou WWTP was excellent. It also found that expression of nitrification-related genes was much lower than that of the denitrification-related genes in an A/O AS system [42]. This may be because most denitrifying bacteria are heterotrophic, leading to faster growth than autotrophic nitrifying bacteria. Different from tropical wastewater treatment plants, *Hydrogenophilaceae* and *Thauera* are common denitrifying bacteria in subtropical wastewater treatment plants [14,15,18], and even *Hydrogenophilaceae* dominates in the A^2/O process [43]. This may be due to different types of carbon sources. We should further study the effects of different types of wastewater in the tropical WWTPs, focusing on denitrifying bacteria.

PAOs are essential, and their activity is highly involved in phosphorus removal efficiency. No *Candidatus Accumulibacter* were detected in the four samples, which is the same as the results of Wei [44]. However, *Tetrasphaera* was detected at relative gene abundances of 0.37–0.58% in our study. Previous studies have implied that the abundance of *Tetrasphaera* is higher in temperate climates (3.6–28%) [11,26] than in tropical climates (0.23–1.8%) [9] in full-scale WWTPs. It is suggested that *Tetrasphaera* could make a comparative or even greater contribution to phosphorus removal in Denmark full-scale WWTPs compared with *Candidatus Accumulibacter* [10]. *Tetrasphaera* are more versatile in their substrate utilization capabilities than *Candidatus Accumulibacter*, as they can uptake both amino acids and glucose and have the ability to ferment complex organics and accumulate fermentation by-products [7,26]. Some *Tetrasphaera* have been shown to be VFA users; however, it is always considered that *Tetrasphaera* cannot synthesize and store intracellular PHAs [26]. Interestingly, not only could *Tetrasphaera* be beneficial to direct phosphorus removal, but also by supplying VFAs to *Accumulibacter* from their fermentation [7]. The type of carbon source and temperature may be factors for distinguishing the two groups of PAOs. DNA extraction bias might partly lead to underestimation of the abundances of *Tetrasphaera* because of extracting DNA from Gram-positive bacteria (e.g., *Tetrasphaera*) [45]. Petriglieri et al. confirmed that newly discovered PAO organisms (*Dechloromonas*) are actively correlated to phosphorus removal in WWTPs and should be considered as crucial as *Candidatus Accumulibacter* and *Tetrasphaera* [46]. *Dechloromonas*-related putative PAOs (0.24–0.4%) have also been found to use oxygen, nitrite, and nitrate as various electrons acceptors [8]. Only Ge et al. identified a novel PAO organism, in the *Comamonadaceae* family, which was dominant and drove phosphorus removal in treating abattoir wastewater with short solid retention time (SRT; <4 days), and this genus was also found in our samples (1.21–1.82%) [47].

The efficient removal of phosphorus is not only related to the abundance but also the activity. Fernando et al. surmised that *Candidatus Accumulibacter* and *Tetrasphaera* are only responsible for 24–70% of total phosphorus removal in Danish WWTPs, suggesting that complete phosphorus removal is also accompanied by chemical/biological precipitation of phosphorus or other unidentified PAOs [10]. Further study to identify these novel PAOs and determine not only their physiological and biochemical functions, but also the competitive ability of phosphorus removal, energy, and mass balance via combining multiple methods in situ, and the relation of other bacteria, will likely improve our ability to manage EBPR.

Hydrolysis, which is carried out by a few specific species, mainly filamentous bacteria, is essential to biological nutrient removal (BNR) in WWTPs. Hydrolysis provides carbon substrates, such as short-chain fatty acids, which are limited substrates for polyphosphate-accumulating organisms and denitrifying bacteria, while also being a habitat for other functional bacteria to grow. The dominant hydrolysis bacteria in the four samples were *Saccharimonadales* (12.06–14.29%) and *Saprospiraceae* (12.53–13.89%), which degrade complex organic compounds, such as protein to generate amino acids as energy and carbon sources [1,48]. The presence of *C10-SB1A* (4.53–5.17%) indicates the hydrolysis ability of the macromolecular organic compounds in municipal wastewater [49]. Other species were *Trichococcus* (1.16–2.56%), which can decompose carbohydrates into lactate, formate, acetate, ethanol, and carbon dioxide [50] and *Candidatus Moranbacteria* (1.52–2.23%) and *Blastocatellaceae* (1–1.97%), which can degrade complex substrates, such as peptone, casamino acids, and ethyl tert-butyl ether [51].

3.3. Functional Predictive Analysis

Apart from the functional bacteria, the associated metabolic activities of microorganisms in WWTPs is essential to nutrient removal performance. Therefore, the functional pathways and enzymes were determined based on the KEGG database. Metabolism (77.72–78.18%), genetic information processing (7.23–7.20%), environmental information processing (5.09–5.27%), cellular process (4.26–4.38%), and human diseases (3.44–3.66%) pathways had the highest gene hits (Figure 4). The relative abundance of gene hits related to metabolism account for greatest, suggesting that the microbial community plays a pivotal role in removing various pollutants. However, the abundance of other pathway genes remains at a similar level between the four samples.

As shown in Figure 4, frequency of ammonia monooxygenase (*amo*) ranged from 1895.02–2645.5 in the four samples. *Amo* is responsible for the first step in the reaction of ammonia oxidation to nitrite. Frequency was exhibited for hydroxylamine dehydrogenase (*hao*), from 121.5–175.5 in the four samples. *Hao* transforms hydroxylamine into nitrite during the nitrification process. The functional enzymes of denitrification expressed by the total genes were sufficient than those of nitrification in all the samples, which could have resulted in higher denitrification activities. Surprisingly, nitrate reductase (*nar* and *nap*), which is associated with the conversion of nitrate to nitrite, had greater frequency (8687.28–14204.1) than *amo* and *hao*. In addition, nitrite reductase (*nir*; 1819.74–2660.99), nitric oxide reductase (*nor*; 5049.65–8873.16), and nitrous oxide reductase (*nos*; 3936.08–5425.66), which are involved in nitrate and nitrite reduction to nitrogen gas [52], were similarly expressed. This provides a solid foundation for total nitrogen removal from wastewater.

The phosphorus removal was depicted in the hits of adenylate kinase (*adk*) and exopolyphosphatase (*ppx*), which are generally related to anaerobic phosphorus release, while polyphosphate kinase (*ppk*) is considered to be catalysis of aerobic phosphorus uptake [8]. However, the proportions of *adk* (29,753.23–38,808.08), *ppx* (30,603.69–39,114.94), and *ppk* (25,706.61–33,883.99) showed little variation among samples. The high frequencies of *adk* and *ppk* results in stable and efficient phosphorus removal.

It should also be noted that the function of microorganisms is not directly reflected by the 16S rRNA data. The PICRUSt2 method was used for the prediction of functional enzymes. Although the ability to reveal functional profiles of microbial communities accurately and efficiently with this method has been shown by numerous studies, it also has limitations. The functional prediction may be partly due to the limited number of generic genes using 16S rRNA gene sequencing. Therefore, it is necessary to use metagenomics, metabolomics, transcriptomics, proteomics, and other omics approaches to further study microorganisms comprehensively and objectively.

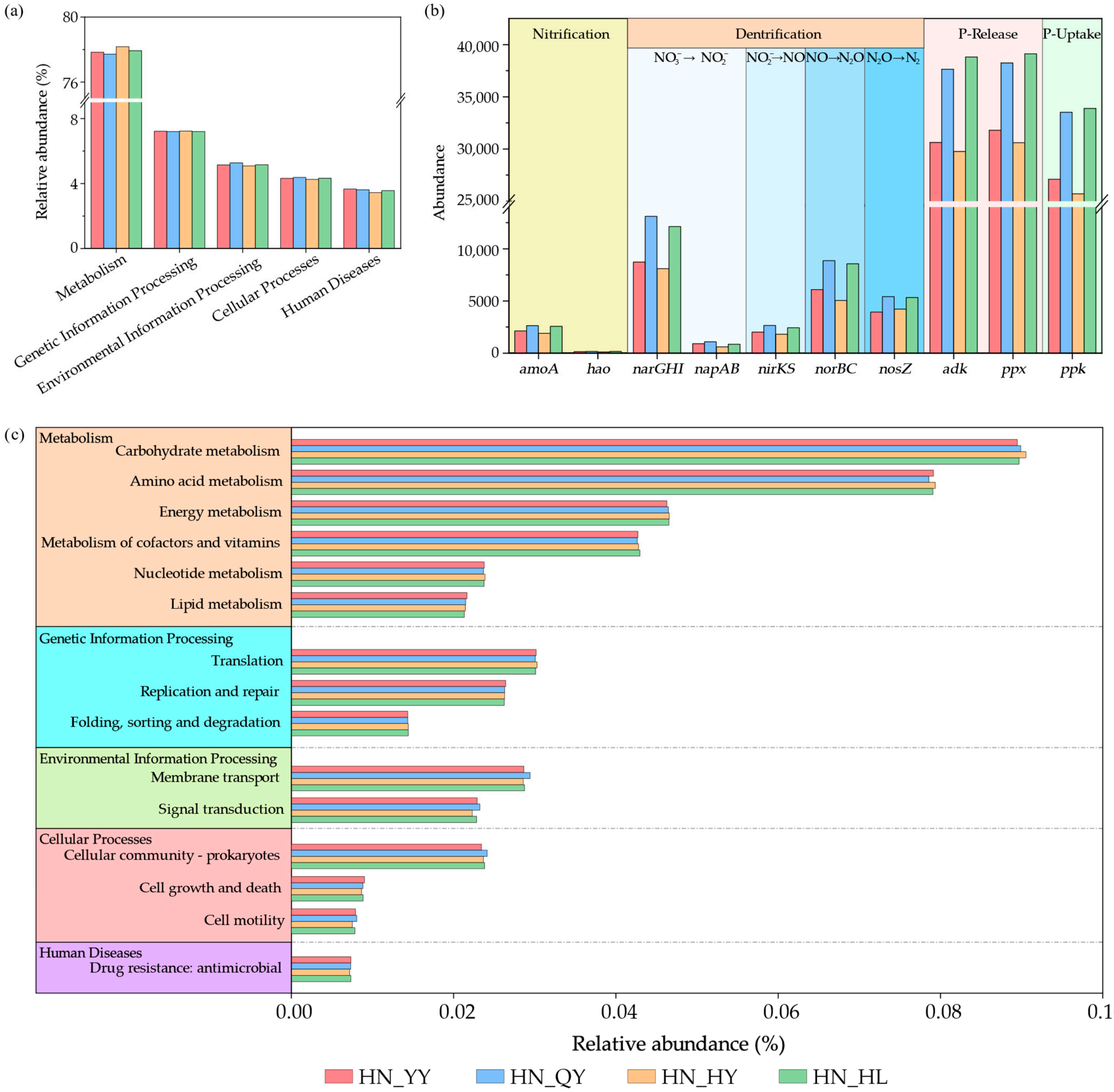

Figure 4. Bacterial function pathways according to KEGG in the anaerobic sludge (HN_YY), anoxic sludge (HN_QY), aerobic sludge (HN_HY), and return sludge (HN_HL) of Haikou WWTPs. (**a**) Function pathways at level 1; (**b**) frequencies of the key genes including nitrogen and phosphorus removal according to the KO numbers assigned to genes; and (**c**) function pathways at level 2.

3.4. The Elimination of Primary Sedimentation Tank May Enhance Biological Phosphorus Removal in Tropical WWTPs

Tetrasphaera, instead of *Candidatus Accumulibacter*, were the dominant PAOs in the studied tropical WWTP. *Tetrasphaera* can take up phosphorus using energy generated by fermentation of complex organic matter, such as carbohydrates and amino acids (including glucose, aspartate, glutamate). *Candidatus Accumulibacter* used the readily biodegradable COD (rbCOD) in wastewater as organic carbon source for phosphorus removal. Except for rbCOD, municipal wastewater also contains part of the slowly biodegradable, particulate COD (pCOD), which is usually removed in the primary sedimentation tank of WWTPs. Toja Ortega et al. found that *Tetrasphaera* increased in sludge flocs with an increased influent pCOD concentration [53]. This was attributed to the fact that sludge flocs can retain more pCOD and have a higher hydrolysis ability, making monomers, such as amino acids and monosaccharides, available to *Tetrasphaera*. In this studied tropical WWTP, the primary sedimentation tank is not employed, so that pCOD directly enters the anaerobic tank. This may benefit phosphorus removal through enriching *Tetrasphaera* in sludge flocs. Therefore, the elimination of the primary sedimentation tank may enhance biological phosphorus removal in tropical WWTPs.

4. Conclusions

The diversity of microbial communities in this tropical WWTP was higher than the plateau WWTP. The dominant phyla were *Proteobacteria* (24.2–28.31%), *Bacteroidota* (17.45–19.93%), *Patescibacteria* (15.97–18.11%), and *Chloroflexi* (13.54–16.74%). *Nitrosomonas* (0.14–0.18%) and *Nitrospira* (0.22–0.47%) were detected for nitrification in this WWTP. *Tetrasphaera*, instead of *Candidatus Accumulibacter*, was the dominant PAO, while GAOs, such as *Candidatus Competibacter* (2.74–4.79%) and *Defluviicoccus* (0.14–0.16%), were also detected. The functions of the bacterial communities predicted by PICRUSt2 were related to the metabolic pathways of metabolism (77.72–78.18%), genetic information processing (7.23–7.20%), and environmental information processing (5.09–5.27%). The elimination of the primary sedimentation tank may enhance biological phosphorus removal in tropical WWTPs, since the enriched PAO, *Tetrasphaera*, can use particulate organic matter in wastewater for phosphorus removal.

Author Contributions: Conceptualization, Y.G. and B.M.; investigation, Y.G., X.Z. and C.L.; supervision, B.M.; visualization B.L.; writing—original draft, Y.G.; writing—review and editing, Y.G. and B.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research project was financially supported by Hainan Provincial Natural Science Foundation of China for High-level Talents (2019RC017), the Key Research and Development Plan of Hainan Province (ZDYF2021SHFZ061), Haikou major science and technology projects (2021-003) and the C-level Excellent Talent Project of Hainan University (KYQD(ZR)1867).

Institutional Review Board Statement: Not applicable for studies not involving humans or animals.

Informed Consent Statement: Not applicable for studies not involving humans.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: We thank Xiuhuang Li, Yin Liu, Huiting Jia, Yanbing Liang and Rongping Li for the help in this study.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

References

1. Nielsen, P.H.; Saunders, A.M.; Hansen, A.A.; Larsen, P.; Nielsen, J.L. Microbial communities involved in enhanced biological phosphorus removal from wastewater—A model system in environmental biotechnology. *Curr. Opin. Biotechnol.* **2012**, *23*, 452–459. [CrossRef] [PubMed]
2. Wu, L.; Ning, D.; Zhang, B.; Li, Y.; Zhang, P.; Shan, X.; Zhang, Q.; Brown, M.; Li, Z.; Van Nostrand, J.D.; et al. Global diversity and biogeography of bacterial communities in wastewater treatment plants. *Nat. Microbiol.* **2019**, *4*, 1183–1195. [CrossRef] [PubMed]
3. Saunders, A.M.; Albertsen, M.; Vollertsen, J.; Nielsen, P.H. The activated sludge ecosystem contains a core community of abundant organisms. *ISME J.* **2016**, *10*, 11–20. [CrossRef] [PubMed]
4. Wang, Q.; Liang, J.; Zhang, S.; Yoza, B.A.; Li, Q.X.; Zhan, Y.; Ye, H.; Zhao, P.; Chen, C. Characteristics of bacterial populations in an industrial scale petrochemical wastewater treatment plant: Composition, function and their association with environmental factors. *Environ. Res.* **2020**, *189*, 109939. [CrossRef]
5. Ge, S.; Wang, S.; Yang, X.; Qiu, S.; Li, B.; Peng, Y. Detection of nitrifiers and evaluation of partial nitrification for wastewater treatment: A review. *Chemosphere* **2015**, *140*, 85–98. [CrossRef]
6. Zhao, W.; Peng, Y.; Wang, M.; Huang, Y.; Li, X. Nutrient removal and microbial community structure variation in the two-sludge system treating low carbon/nitrogen domestic wastewater. *Bioresour. Technol.* **2019**, *294*, 122161. [CrossRef]

7. Marques, R.; Santos, J.; Hien, N.; Carvalho, G.; Noronha, J.P.; Nielsen, P.H.; Reis, M.A.M.; Oehmen, A. Metabolism and ecological niche of Tetrasphaera and Ca. Accumulibacter in enhanced biological phosphorus removal. *Water Res.* **2017**, *122*, 159–171. [CrossRef]
8. Roy, S.; Guanglei, Q.; Zuniga-Montanez, R.; Williams, R.B.H.; Wuertz, S. Recent advances in understanding the ecophysiology of enhanced biological phosphorus removal. *Curr. Opin. Biotechnol.* **2021**, *67*, 166–174. [CrossRef]
9. Qiu, G.; Zuniga-Montanez, R.; Law, Y.; Thi, S.S.; Thi Quynh Ngoc, N.; Eganathan, K.; Liu, X.; Nielsen, P.H.; Williams, R.B.H.; Wuertz, S. Polyphosphate-accumulating organisms in full-scale tropical wastewater treatment plants use diverse carbon sources. *Water Res.* **2019**, *149*, 496–510. [CrossRef]
10. Fernando, E.Y.; McLlroy, S.J.; Nierychlo, M.; Herbst, F.-A.; Petriglieri, F.; Schmid, M.C.; Wagner, M.; Nielsen, J.L.; Nielsen, P.H. Resolving the individual contribution of key microbial populations to enhanced biological phosphorus removal with Raman-FISH. *ISME J.* **2019**, *13*, 1933–1946. [CrossRef]
11. Lanham, A.B.; Oehmen, A.; Saunders, A.M.; Carvalho, G.; Nielsen, P.H.; Reis, M.A.M. Metabolic versatility in full-scale wastewater treatment plants performing enhanced biological phosphorus removal. *Water Res.* **2013**, *47*, 7032–7041. [CrossRef] [PubMed]
12. Xia, Y.; Wen, X.; Zhang, B.; Yang, Y. Diversity and assembly patterns of activated sludge microbial communities: A review. *Biotechnol. Adv.* **2018**, *36*, 1038–1047. [CrossRef] [PubMed]
13. Wang, P.; Yu, Z.; Qi, R.; Zhang, H. Detailed comparison of bacterial communities during seasonal sludge bulking in a municipal wastewater treatment plant. *Water Res.* **2016**, *105*, 157–166. [CrossRef] [PubMed]
14. Cao, J.; Zhang, T.; Wu, Y.; Sun, Y.; Zhang, Y.; Huang, B.; Fu, B.; Yang, E.; Zhang, Q.; Luo, J. Correlations of nitrogen removal and core functional genera in full-scale wastewater treatment plants: Influences of different treatment processes and influent characteristics. *Bioresour. Technol.* **2020**, *297*, 122455. [CrossRef] [PubMed]
15. Zhang, J.; Liu, G.-H.; Wei, Q.; Liu, S.; Shao, Y.; Zhang, J.; Qi, L.; Wang, H. Regional discrepancy of microbial community structure in activated sludge system from Chinese WWTPs based on high-throughput 16S rDNA sequencing. *Sci. Total Environ.* **2021**, *818*, 151751. [CrossRef]
16. Li, W.; Peng, Y.; Gao, R.; Zhang, Q.; Li, X.; Kao, C.; Li, J. Effect of low salinity on nitrogen removal from municipal wastewater via a double-anammox process coupled with nitritation and denitratation: Performance and microbial structure. *Bioresour. Technol.* **2022**, *346*, 126633. [CrossRef]
17. Zhang, L.; Shen, Z.; Fang, W.; Gao, G. Composition of bacterial communities in municipal wastewater treatment plant. *Sci. Total Environ.* **2019**, *689*, 1181–1191. [CrossRef]
18. Xiang, J.; Wang, C.; Lv, W.; Liu, Y.; Sun, J.; Gong, T. Differences of bacterial communities in two full-scale A(2)/O municipal wastewater treatment plants and their effects on effluent total nitrogen removal. *Environ. Technol. Innov.* **2021**, *21*, 101317. [CrossRef]
19. Luo, Y.; Yao, J.; Wang, X.; Zheng, M.; Guo, D.; Chen, Y. Efficient municipal wastewater treatment by oxidation ditch process at low temperature: Bacterial community structure in activated sludge. *Sci. Total Environ.* **2020**, *703*, 135031. [CrossRef]
20. Zhang, B.; Xu, X.; Zhu, L. Structure and function of the microbial consortia of activated sludge in typical municipal wastewater treatment plants in winter. *Sci. Rep.* **2017**, *7*, 17930. [CrossRef]
21. Fang, D.; Zhao, G.; Xu, X.; Zhang, Q.; Shen, Q.; Fang, Z.; Huang, L.; Ji, F. Microbial community structures and functions of wastewater treatment systems in plateau and cold regions. *Bioresour. Technol.* **2018**, *249*, 684–693. [CrossRef] [PubMed]
22. Al Ali, A.A.; Naddeo, V.; Hasan, S.W.; Yousef, A.F. Correlation between bacterial community structure and performance efficiency of a full-scale wastewater treatment plant. *J. Water Process Eng.* **2020**, *37*, 101472. [CrossRef]
23. Xue, J.; Schmitz, B.W.; Caton, K.; Zhang, B.; Zabaleta, J.; Garai, J.; Taylor, C.M.; Romanchishina, T.; Gerba, C.P.; Pepper, I.L.; et al. Assessing the spatial and temporal variability of bacterial communities in two Bardenpho wastewater treatment systems via Illumina MiSeq sequencing. *Sci. Total Environ.* **2019**, *657*, 1543–1552. [CrossRef]
24. Zhang, T.; Shao, M.-F.; Ye, L. 454 Pyrosequencing reveals bacterial diversity of activated sludge from 14 sewage treatment plants. *ISME J.* **2012**, *6*, 1137–1147. [CrossRef] [PubMed]
25. Frey, B.; Rime, T.; Phillips, M.; Stierli, B.; Hajdas, I.; Widmer, F.; Hartmann, M. Microbial diversity in European alpine permafrost and active layers. *Fems Microbiol. Ecol.* **2016**, *92*, fiw018. [CrossRef] [PubMed]
26. Liu, R.; Hao, X.; Chen, Q.; Li, J. Research advances of Tetrasphaera in enhanced biological phosphorus removal: A review. *Water Res.* **2019**, *166*, 115003. [CrossRef]
27. Murphy, C.L.; Yang, R.; Decker, T.; Cavalliere, C.; Andreev, V.; Bircher, N.; Cornell, J.; Dohmen, R.; Pratt, C.J.; Grinnell, A.; et al. Genomes of Novel Myxococcota Reveal Severely Curtailed Machineries for Predation and Cellular Differentiation. *Appl. Environ. Microbiol.* **2021**, *87*, e01706-21. [CrossRef]
28. Karkman, A.; Mattila, K.; Tamminen, M.; Virta, M. Cold Temperature Decreases Bacterial Species Richness in Nitrogen-Removing Bioreactors Treating Inorganic Mine Waters. *Biotechnol. Bioeng.* **2011**, *108*, 2876–2883. [CrossRef]
29. Zhao, Y.; Gao, J.; Zhang, W.; Wang, Z.; Cui, Y.; Dai, H.; Li, D.; Zhang, Y. Robustness of the partial nitrification-anammox system exposing to triclosan wastewater: Stress relieved by extracellular polymeric substances and resistance genes. *Environ. Res.* **2022**, *206*, 112606. [CrossRef]

30. Emmanouil, C.; Bekyrou, M.; Psomopoulos, C.; Kungolos, A. An Insight into Ingredients of Toxicological Interest in Personal Care Products and A Small-Scale Sampling Survey of the Greek Market: Delineating a Potential Contamination Source for Water Resources. *Water* **2019**, *11*, 2501. [CrossRef]
31. Daims, H.; Lebedeva, E.V.; Pjevac, P.; Han, P.; Herbold, C.; Albertsen, M.; Jehmlich, N.; Palatinszky, M.; Vierheilig, J.; Bulaev, A.; et al. Complete nitrification by Nitrospira bacteria. *Nature* **2015**, *528*, 504–509. [CrossRef] [PubMed]
32. Yao, Q.; Peng, D.-C. Nitrite oxidizing bacteria (NOB) dominating in nitrifying community in full-scale biological nutrient removal wastewater treatment plants. *Amb Express* **2017**, *7*, 25. [CrossRef] [PubMed]
33. Ju, F.; Guo, F.; Ye, L.; Xia, Y.; Zhang, T. Metagenomic analysis on seasonal microbial variations of activated sludge from a full-scale wastewater treatment plant over 4 years. *Environ. Microbiol. Rep.* **2014**, *6*, 80–89. [CrossRef] [PubMed]
34. Alawi, M.; Off, S.; Kaya, M.; Spieck, E. Temperature influences the population structure of nitrite-oxidizing bacteria in activated sludge. *Environ. Microbiol. Rep.* **2009**, *1*, 184–190. [CrossRef] [PubMed]
35. Zhao, Z.; Huang, G.; He, S.; Zhou, N.; Wang, M.; Dang, C.; Wang, J.; Zheng, M. Abundance and community composition of comammox bacteria in different ecosystems by a universal primer set. *Sci. Total Environ.* **2019**, *691*, 146–155. [CrossRef] [PubMed]
36. Kits, K.D.; Sedlacek, C.J.; Lebedeva, E.V.; Han, P.; Bulaev, A.; Pjevac, P.; Daebeler, A.; Romano, S.; Albertsen, M.; Stein, L.Y.; et al. Kinetic analysis of a complete nitrifier reveals an oligotrophic lifestyle. *Nature* **2017**, *549*, 269–272. [CrossRef]
37. Liu, T.; He, X.; Jia, G.; Xu, J.; Quan, X.; You, S. Simultaneous nitrification and denitrification process using novel surface-modified suspended carriers for the treatment of real domestic wastewater. *Chemosphere* **2020**, *247*, 125831. [CrossRef]
38. Sheng, H.; Weng, R.; Zhu, J.; He, Y.; Cao, C.; Huang, M. Calcium nitrate as a bio-stimulant for anaerobic ammonium oxidation process. *Sci. Total Environ.* **2021**, *760*, 143331. [CrossRef]
39. Chu, G.; Yu, D.; Wang, X.; Wang, Q.; He, T.; Zhao, J. Comparison of nitrite accumulation performance and microbial community structure in endogenous partial denitrification process with acetate and glucose served as carbon source. *Bioresour. Technol.* **2021**, *320*, 124405. [CrossRef]
40. Zhang, F.; Li, P.; Chen, M.; Wu, J.; Zhu, N.; Wu, P.; Chiang, P.; Hu, Z. Effect of operational modes on nitrogen removal and nitrous oxide emission in the process of simultaneous nitrification and denitrification. *Chem. Eng. J.* **2015**, *280*, 549–557. [CrossRef]
41. Okada, N.; Nomura, N.; Nakajima-Kambe, T.; Uchiyama, H. Characterization of the Aerobic Denitrification in *Mesorhizobium* sp. Strain NH-14 in Comparison with that in Related Rhizobia. *Microbes Environ.* **2005**, *20*, 208–215. [CrossRef]
42. Cao, J.; Zhu, Q.; Zhang, T.; Wu, Y.; Zhang, Q.; Fu, B.; Fang, F.; Feng, Q.; Luo, J. Distribution patterns of microbial community and functional characteristics in full-scale wastewater treatment plants: Focusing on the influent types. *Chemosphere* **2021**, *281*, 130899. [CrossRef] [PubMed]
43. Chen, Y.; Zhao, Z.; Peng, Y.; Li, J.; Xiao, L.; Yang, L. Performance of a full-scale modified anaerobic/anoxic/oxic process: High-throughput sequence analysis of its microbial structures and their community functions. *Bioresour. Technol.* **2016**, *220*, 225–232. [CrossRef] [PubMed]
44. Wei, S.P.; Stensel, H.D.; Bao Nguyen, Q.; Stahl, D.A.; Huang, X.; Lee, P.-H.; Winkler, M.-K.H. Flocs in disguise? High granule abundance found in continuous-flow activated sludge treatment plants. *Water Res.* **2020**, *179*, 115865. [CrossRef] [PubMed]
45. Albertsen, M.; Karst, S.M.; Ziegler, A.S.; Kirkegaard, R.H.; Nielsen, P.H. Back to Basics—The Influence of DNA Extraction and Primer Choice on Phylogenetic Analysis of Activated Sludge Communities. *PLoS ONE* **2015**, *10*, e0132783. [CrossRef]
46. Petriglieri, F.; Singleton, C.; Peces, M.; Petersen, J.F.; Nierychlo, M.; Nielsen, P.H. "Candidatus Dechloromonas phosphoritropha" and "Ca. D. phosphorivorans", novel polyphosphate accumulating organisms abundant in wastewater treatment systems. *ISME J.* **2021**, *15*, 3605–3614. [CrossRef]
47. Ge, H.; Batstone, D.J.; Keller, J. Biological phosphorus removal from abattoir wastewater at very short sludge ages mediated by novel PAO clade Comamonadaceae. *Water Res.* **2015**, *69*, 173–182. [CrossRef]
48. Li, S.; Li, J.; Yang, S.; Zhang, Q.; Li, X.; Zhang, L.; Peng, Y. Rapid achieving partial nitrification in domestic wastewater: Controlling aeration time to selectively enrich ammonium oxidizing bacteria (AOB) after simultaneously eliminating AOB and nitrite oxidizing bacteria (NOB). *Bioresour. Technol.* **2021**, *328*, 124810. [CrossRef]
49. Liu, W.; Shen, C.; Liu, C.; Zhang, S.; Hao, S.; Peng, Y.; Li, J. Achieving stable mainstream nitrogen and phosphorus removal assisted by hydroxylamine addition in a continuous partial nitritation/anammox process from real sewage. *Sci. Total Environ.* **2021**, *794*, 148478. [CrossRef]
50. Yan, P.; Zhao, Y.; Zhang, H.; Chen, S.; Zhu, W.; Yuan, X.; Cui, Z. A comparison and evaluation of the effects of biochar on the anaerobic digestion of excess and anaerobic sludge. *Sci. Total Environ.* **2020**, *736*, 139159. [CrossRef]
51. van der Waals, M.J.; Plugge, C.; Meima-Franke, M.; de Waard, P.; Bodelier, P.L.E.; Smidt, H.; Gerritse, J. Ethyl tert-butyl ether (EtBE) degradation by an algal-bacterial culture obtained from contaminated groundwater. *Water Res.* **2019**, *148*, 314–323. [CrossRef] [PubMed]
52. Lu, H.; Chandran, K.; Stensel, D. Microbial ecology of denitrification in biological wastewater treatment. *Water Res.* **2014**, *64*, 237–254. [CrossRef] [PubMed]
53. Toja Ortega, S.; Pronk, M.; de Kreuk, M.K. Effect of an Increased Particulate COD Load on the Aerobic Granular Sludge Process: A Full Scale Study. *Processes* **2021**, *9*, 1472. [CrossRef]