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Defining the nitrogen fixation community size, structure and relevancy in Nile tilapia aquaculture pond waters

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Abstract
Nitrogen fixation microbial communities within 3 different intensity Nile tilapia (*Oreochromis niloticus*) pond water columns were identified and correlated to environmental factors, influencing their summer pond productions in Yi Xing China. The microbial communities encoding the *nifH* - gene and known to promote plant growth, reduction of pond nitrate leaching and pollution were taxonomically identified to their most probable genera, revealing *Azospirillum, Azoarcus, Anaeromyxobacter, Azotobacter, Geobacter, Trichormus,* and *unclassified_Opitutaceae,* as the most dominant. Of the studied environmental factors, ammonium concentrations with the greatest influence on the communities recorded the highest significant differences of 3.18, 1.98 and 3.41 mg/L for the 3 studied ponds. The monthly mean temperatures, dissolved oxygen and pH values were documented and observed to be above pond culture optimal, while the chemical oxygen demand and total organic carbon nutrient content revealed an increment in energy sources within the pond systems. Quantitative analysis of the microbes encoding *nifH* gene abundances were established as 3.65 x 10^6, 2.66 x 10^6 and 3.11 x 10^6 copies/g wet weights in ponds 1, 2 and 3 respectively. Linear discriminant analysis effect size revealed *Chromatiaceae* and *Thiocapsa* as the closest relatives in the three ponds, while the Redundancy analysis tests indicated ammonia nitrogen, total phosphorous and dissolved Oxygen were the most important factors shaping the bacterial communities.

Keywords: Nitrogen fixation bacteria, *nifH* - encoding gene, Bacteria community composition, Cyanobacteria, functional gene analysis, *LEfSe*, Total organic carbon

1. Introduction
Boosting food security and improving health options with less or cholesterol free products are considered aquaculture options for feeding the escalating world population, that faces sustainable productivity challenges [1]. Fish production is boosted through intensive farming, increment in artificial feeding, pond fertilization amongst other techniques used. However, nitrogen deposits from uneaten feeds, animal wastes or the fertilizers to propagate pond production are increasingly becoming a problem through nitrate leaching and pollution [2]. Surface runoff and expired water released after production increases water quality degradation and eutrophication of natural water bodies [3].

In the quest to reduce or eliminate the nitrogenous wastes within the ponds by propagating vegetables alongside fish production for the quick uptake of the broken-down amino acids, we accordingly embarked on biological mechanisms in this study. However, the only natural biological source of fixing nitrogen in the biosphere is by nitrogen-fixing microbes [4]. Although some nitrogen fixing bacteria have been cultivated in the laboratory, a great diverse taxa have not yet been identified [5]. This marks the most crucial point of understanding microbial community structures, composition and the main functional genes, laying in the prokaryotic conversion process [6]. The Prokaryotic conversion, a proposed step towards the promotion of generating microbes to clean nitrogenous wastes in aquaculture ponds would require substantial knowledge concerning the overall community structure, population dynamics and metabolism of different organic carbon sources sustaining the environmental microbes that remains vividly documented. We therefore explored other limiting steps containing the nitrogenase enzyme whose multiple subunits are encoded by the genes such as *nifH*, *nifD*, and *nifK* [4]. This study focuses on microbes influenced by the dinitrogenase reductase transfer mechanisms of reducing equivalents to the core enzyme complex since, this creates reducing equivalents used to convert N_2 into NH_3 equating to the partial nitrogen fixation process [4].

The diversity of functional genes play important roles in the function of the N cycle [7]. Which could probably be constrained by the physical and chemical complexity of the environment. To characterize the diversity of the functional genes encoding to *nifH,* it's necessary to evaluate the microbial community diversity in relation to environmental factors and ecosystem complexity. Using DNA microarray technologies of functional gene detection [8], genotyping of bacteria based on genomic DNA-DNA similarities [9], Illumina through put sequencing and population genetics, we improve on the techniques of characterization and functions in the environment. Primer analyses of the microbial gene sequences serve as functional biomarkers and facilitate annotated databases observed in phylogenetic analyses evaluated by polymerase chain reactions (PCR) [10, 11].

From our related unpublished work, we considered the *nifH* gene, a widely studied ecological and evolutionary bio-marker [12]. The objective of this study was to identify bacterial communities involved in nitrogen fixation within 3 different intensity Nile tilapia (Oreochromis niloticus) pond water columns and correlate them to environmental factors which influence summer productions. As it is well known that environmental factors influence the abundant distribution and diversity in spatial and temporal conditions of nitrogen-fixation microbes across habitats, this study would also attempt to discuss and relate the findings pertaining to the environmental ecology of microbial communities within the summer tilapia grow-out ponds. These studies further attempt to have a better understanding of the communities deemed relevant in eliminating nitrogenous wastes within the tilapia production with the aim of improving water quality by avoiding nitrate leaching and pollution in the ponds and related production systems of hydroponic and aquaponics production.

2. Materials and methods

2.1. Study area

The study was conducted in Yi Xing (N31° 27' 48.2" E 119° 51' 1.7"), on a thirteen year old Research Facility belonging to Freshwater Fisheries Research Center (FFRC), affiliated to Nanjing Agriculture University under Chinese Academy of Fisheries Sciences (CAFs), Jiangsu Province, P R. China. The study focused on nitrogen fixation microbes within Nile tilapiine (*Oreochromis spps*) culture ponds practicing intensive mono and poly culture systems. An in-depth purely observational study of the effects of environmental and physiochemical parameters on the microbial community was conducted to obtain solutions for aquaculture farms using target bacterial agents. The fish within the ponds were bred, hatched and nursed at the facility nursery until the juvenile stage before being transferred to the study ponds. During the study, only under excessive or intensive heat temperature was a volume of 1 to 3% of water added to each pond to refill any water lost by evaporation. This described field study required neither specific written permission nor approval permits from the Environmental ethics committee of the FFRC of the Chinese Academy of Fisheries Sciences (CAFs), however the committee reviewed and approved the study.

2.2. Sampling procedure

Water samples from three tilapia grow-out ponds were collected between May and October, 2014. A total of 117 samples were collected for physiochemical analyses, while 36 samples were used in the bacterial studies.

Samples were collected at three adjacent spots, i.e. near inlet, close to outlet and close to the pond center using 1 liter van Dorn water sampler, mixed and concentrated in 3 liter cans, while 200mls of water from the adjacent spots were collected and stored in sterilized bottles for physiochemical (former) and bacterial (latter) analyses respectively. The samples were transported to the laboratory within 4 h of collection for further analysis. For nutrient determination, the water was collected on weekly intervals for 13 weeks while bacterial samples were collected on a monthly basis (4 times). Bacterial communities per pond were obtained by filtration using 0.22µm filter membranes in triplicates of 200mls concentrated water samples and stored at -80°C, until further laboratory analysis. Samples were labeled depending on: the month of collection (*M*-May, *J*-July, *S*-September, or *O*-October); type of sample (*W*-water); pond number (1, 2 or 3) and sample number (1, 2 or 3). i.e. *MW11*, represented May water in pond 1 for sample 1 and *JW21* represented July water in pond 2 for sample 1. Similarly September (*S*) and October (*O*) had matching label designs.

2.3. Physiochemical analyses

Dissolved oxygen (DO, mg/L), water transparency, Oxidation-Reduction Potential (ORP), pH and temperature (°C) of pond water were measured using a 3-Star DO meter (Thermo, Beverly, MA), Secchi disc, ORP and S20 Seven Easy digital pH meter (Mettler Toledo, Switzerland) with an inbuilt mercury thermometer respectively. Ammonia nitrogen (NH_4^+-N) concentrations were measured by Nessler colorimetric method, Nitrate nitrogen (NO_3^--N), Nitrite nitrogen (NO_2^--N), Sulphate ions were determined using the 3000 ion chromatography Dionex machine. TOC was determined using the TOC analyzer machine, COD was determined using the oxidation method, while Total Nitrogen (TN) and Total Phosphorous (TP) were measured using the UV Spectrophotometry method [13].

2.4 DNA extraction

The genomic DNA in the water was extracted using the Power water™ DNA Isolation Kit (Mo Bio Laboratories, Inc., Solana Beach, CA, USA) according to the manufacturer's protocols. Extraction yield and the quality of the DNA were verified by agarose gel electrophoresis and Spectrophotometry. The extracted DNA was further purified using the DNA Pure-spin Kit (Vigorousbio, Beijing, China) to minimize PCR inhibition. [14]. the quantified extracts were stored at -20°C until further analysis. Prior to analysis, the extracted DNA samples were diluted 10-fold to minimize the possible influence of the PCR inhibitors.

2.5 PCR and Pyro-sequencing

A universal PCR primer *nifh-1F,* 5'-AAAGGCGGAATCGGCAAGTC-3', and its reverse *nifh-2R,* 5'-TTGTTCGCGGCGTACATG-3', were used in quantitative real-time PCR to quantify the gene copy numbers in the environment, for expression studies [15]. Real-time PCR targeting the nitrification function genes *nifH* was conducted using an ABI 9700 instrument with Sequence Detection Software v 2.0.5 (Applied Biosystems). The reaction mixture of 20 µl contained 0.25 µM of each primer, 10 µl of Power SYBR Green PCR master Mix (Applied Biosystems, Foster City, CA, USA), and 1 µl of the purified DNA template. Bovine serum albumin (BSA, 500ng/reaction) and dimethylsulfoxide (DMSO, 12.5µmol/reaction) were used to enhance the PCR efficiency. The modified primer pair *nifh-1F* and *nifh-2R* [16]. *as in* [15]. was used for amplification. Agarose

gel electrophoresis was employed after denaturation reactions of 7 min at 95 ºC, 40 cycles of 15s at 95 ºC, 30s at 60 ºC annealing temperatures and 30s at 72 ºC extension. qPCR reactions were run using 1ng of environmental DNA or 3 ng cDNA, a mixture of 10^4 copies of all standard clones or the same mixture without the target standard clone as template to confirm primer specificities. The standard curves were constructed with plasmids containing an insert of the respective genes with PCR efficiency determined using the curve standard formula $E = 100*(10^{(-1/slope)} - 1)$.

All related procedures were performed under the high-throughput multiplex barcode pyro-sequencing following Genome Sequencer FLX System manufacturer's instructions (Roche, Nutley, New Jersey, USA). In brief, the region of 454 bps in the *nifH* gene was selected to construct the community library through tag pyrosequencing and determined by employing the Roche GS-FX 454 pyrosequencer and after filtration high-quality sequences were assigned to samples according to related barcodes. Sequences were aligned in accordance with Usearch alignment [17] and clustered into operational taxonomic units (OTUs) using GAST [18] with a version of the green genes 13_5 database [19] trimmed to the V_6 region. The OTUs were analyzed with QIIME v 1.7 [20].

2.6 Data and Statistical analysis

One way ANOVA and Duncan's multiple range *tests* HSD were used to determine the average means ± SD and the significant differences among the microbial communities and the water physiochemical parameters. OTUs that reached 94 - 95% similarity level were used for determining indices of gene diversity (Shannon-Wiener [H], Simpson diversity [D]) and Good's coverage analysis by using the software package MOTHUR 1.15.0 [21]. Rarefaction analysis and two non-parametric richness estimators, the abundance-based coverage estimator (S_{ACE}) and the bias-corrected Chao-1 (S_{Chao1}), were calculated using MOTHUR [21].

Community classification of the *nifH*-carrying microbial assemblages was performed with Fast Uni-Frac environmental clustering [22]. Correlations between the *nifH*-harboring microbial assemblages and environmental factors were determined according to previously described procedures [23]. Weighted *nifH* OTU and class data were used to identify the most significant environmental factors that had the strongest influence on the community structure and spatial distribution of the *nifH*-harboring microbial assemblages in Yi Xing. Pearson correlation analyses (significance level 0.05) of the abundance of the water *nifH* genes with environmental factors were performed with the statistics software SPSS 16.0V as detailed previously [24, 25]. The *nifH* gene sequences derived from Pyro-sequencing were deposited in the NCBI Sequence Read Archive which can be obtained through Nucleic acid sequence accession numbers at (http://www.ncbi.nlm.nih.gov/Traces/sra)

3. Results and Discussion

3.1 Results

3.1.1 The nutrient composition and physiochemical conditions:

The average means ± SD for the nutrient components i.e. TN, TP, NH_4^+- N, NO_2^-- N, NO_3^-- N, COD and TOC in the Water are shown in (Table 1). Results on nutrient components showed that TP and COD were significantly high ($p < 0.05$) in P2, P1 and P3 in that respective order. While NH_4^+- ions were observed with significant differences in P3, P1 and P2. TN and NO_3^--N concentrations had no significant difference in all the three Ponds. Taking into account of all analyzed environmental parameters, the NH_4^+- N ion concentrations registered the highest significant differences across all ponds. From the studied ponds, we registered temperature (T ºC), pH, DO and ORP on a weekly basis as reported in (Table 1), and on average the observed parameters had no significant differences, from baseline survey to harvest data. During the sample collections, it was observed that DO values were always high within the month of May *MW* recording the highest mean DO concentration at 8.99 mg/L, followed by 6.31, 7.39 and 8.77 mg/L in *JW, SW* and *OW* samples respectively. These concentrations are within the recommended aquaculture production settings. Temperatures during the production period of May to October ranged from 27.3 ºC to 34.3 ºC, while the (SD) water transparency decreased from the baseline depth in May (*MW*) at 0.41m to 0.29m in October (*OW*) at the time of harvest. The average pH values obtained during the sampling period were optimal.

Table 1 Footnote: Each point represents a mean value and Standard error of 3 replicates ($P<0.05$) for TN, TP, NH_4^+-N, NO_2^--N COD Temperature, DO, ORP, pH and Water transparency (SD)for water samples from P1, P2 and P3 [a, b] indicate significantly different values from the least observed Pond values according to the Duncan multiple regression analysis test. [†] represents no significant difference from baseline observed values

Table 1: Physiochemical Characteristic and Nutrient composition results for water samples in Yi Xing Ponds 1, 2 & 3

	Culture system	***Intensive***	
	Ponds		
Parameters	**Pond 1**	**Pond 2**	**Pond 3**
TN [†](mg/L)	5.18 ± 0.39	4.53 ± 0.38	4.90 ± 0.45
NH_4^+ - N (mg/L)	$3.18^{b,c}$ ± 0.47	$1.98^{a,b}$ ± 0.40	3.41^{c} ± 0.47
NO_2^- - N (mg/L)	0.40^{b} ± 0.07	0.20^{a} ± 0.04	0.38^{b} ± 0.03
NO_3^- - N [†](mg/L)	0.24 ± 0.12	0.12 ± 0.04	0.20 ± 0.14
TP (mg/L)	$0.91^{a,b}$ ± 0.12	$1.06^{a,b}$ ± 0.15	0.71^{a} ± 0.09
COD (mg/L)	$14.07^{a,b}$ ± 0.79	$14.28^{a,b}$ ± 0.77	12.16^{a} ± 0.82
TOC (mg/L)	23.04^{b} ± 1.00	$21.75^{a,b}$ ± 1.27	18.89^{a} ± 1.19
pH	7.78 ± 0.31	7.71 ± 0.18	7.49 ± 0.18
Temp (ºC)	29.31 ± 0.96	29.19 ± 0.90	30.38 ± 0.95
DO (mg/Kg)	7.86 ± 0.83	8.32 ± 0.53	6.87 ± 0.64
ORP (	177.83 ± 9.17	178.50 ± 11.96	165.08 ± 6.45
SD (m)	0.34 ± 0.03	0.29 ± 0.01	0.34 ± 0.02

3.1.2 Detection of *nifH* - encoding nitrogen fixation bacteria

Amplifications and Standard curves generated as references were determined by extrapolating and calculating the concentrations of environmental DNA in the samples and the efficiency of the PCR amplification of the *nifH* genes was observed to be over 80% (Supplementary Data SD1). Results showed that the Mean *nifH* gene abundance in P1, P2 and P3 were 3.65 x 10^6; 2.66 x 10^6 and 3.11 x 10^6 copies/g wet weights (ww) respectively. Comparing with sediment data, results from our unpublished work obtained from the same ponds, the results showed significant differences i.e. P1 with a stocking density of 1,500 fish per 667 sq. M displayed the highest abundance of *nifH* genes followed by P3 and P2 abundances.

3.1.3 Microbial diversity and bacterial communities

The bacterial community diversity assessed through operational taxonomic units (OTUs) generated was revealed

in the microbial communities richness and diversity per month (Table 2). Filtering a total of 768,373 high-quality sequence reads with an average length of 431 bp 1730 OTUs at 0.97 sequence similarities were used in our analysis. In contrast to results from our unpublished work on the determination of *nifH* encoding bacteria in sediment, the lowest number of OTUs was observed in P2 and these were significantly different from those in P1 and P3 both in numbers and identification of the micro biota amongst the ponds. The highest divergent rarefied OTUs were observed in P3 revealing 49 OTUs followed by P1 and P2 samples with 37 and 35 OTUs respectively. The non-parametric richness indices of Ace and Chao, evaluated at 3% dissimilarity, revealed similar comparative trends in the prediction of the number of OTUs for each similar pond sample. The most observed monthly OTUs of all samples were in *MW* denoting the highest richness (Ace = 6134, Chao = 6258), whereas the *OW* had the lowest monthly total richness (Ace = 4496, Chao = 4580). The highest bacterial diversity measured (Shannon = 5.897) was revealed in P3 baseline records. On the contrary, the lowest diversity was also observed within P3 in the *July* sample (Shannon = 3.447). The Coverage index range was above 0.992 whereas the Simpson index varied from 0.0058, in the baseline record of P3, to 0.1391, in *September P1* samples. (Also see supplementary data SD1)

Table 2 Footnote: Total values for samples n=3, ($P < 0.05$) MW denotes month for May Water sample, JW=July Water, SW =September Water and OW =October Water. Experimental Ponds are numbered 1, 2, and 3. Values in brackets are 95% confidence intervals as calculated by MOTHUR.

Table 2: Summary of Total Richness and Diversity of bacterial community in P1, P2 and P3 water samples of Yi Xing

	Reads	Reads-(Mean)	OTUs	Ace	Chao	Shannon†	Simpson†	Coverage†
P1	263581	21965	$593.58^{a,b} \pm 51.0$	$629.58^{a,b} \pm 52.8$	$644.08^{a,b} \pm 55.$	4.33 ± 0.3	0.087 ± 0.02	0.997 ± 0.0
P2	205482	17123	$490.58^{a} \pm 30.2$	$535.00^{a} \pm 34.1$	$543.58^{a} \pm 35$	4.10 ± 0.1	0.082 ± 0.01	0.995 ± 0.0
P3	250455	20871	$647.33^{b} \pm 37.6$	$689.25^{b} \pm 44.2$	$700.17^{b} \pm 45$	4.72 ± 0.3	0.050 ± 0.01	0.996 ± 0.0

3.1.4 Taxonomic Classification

This study revealed microbial representation of 2 domains, 8 phyla, 12 classes, and 49 most probable genera distributed across the three ponds (Supplementary data SD 2). At a 97% similarity microbial identification in 594 filtered OTUs from 263,581 sequences P1, revealed its communities belonged to 6 phyla, 10 classes, and 36 most probable genera. Similarly the P2 classified communities were closely related with P1 communities but revealed existence of phylum Firmicutes microbes that were missed in P1. P2 was also found to exhibit fewer genera (33) while P3 exhibited the most, i.e. 8 phyla distributed in 12 classes revealed in 46 most probable genera. The phylogenetic classification within the three ponds revealed that, phylum *Proteobacteria* was significantly dominant (88.78%), in all the ponds. Phyla *Verrucomicrobia, Cyanobacteria* and *Euryarchaeota* were observed at 4.79, 3.28 and 1.64% with the remaining phyla observed under 1% in the various communities as summarized in (Fig 1 & Supplementary data SD 2)

Fig 1 Footnote: Bacterial community distribution in pond 1, 2 & 3 sediment samples. The abundances are presented in terms of taxon numbers affiliated to that phylum divided by the total effective bacteria taxa.

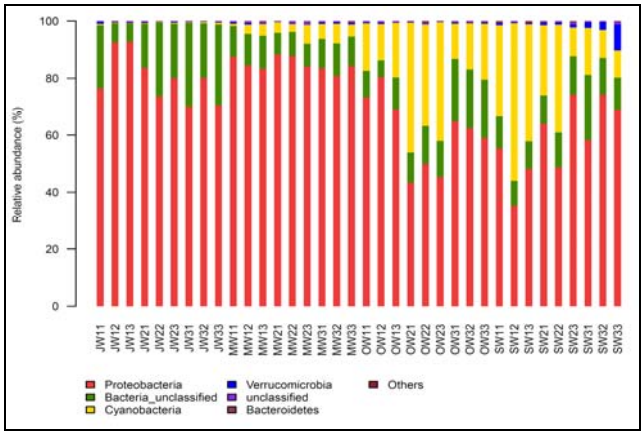

Fig 1: Phylum Distribution Bar graphs for sampled microbial communities in Ponds 1, 2 and 3 of Yi Xing city, China

From the identified microbes, only P2 and P3 had OTUs revealed with the most probable identity of microbes in phylum *Firmicutes* exhibited, even though in P2 they were only revealed within the baseline samples. Phylum *Chlorobi* was revealed in P1 and P3 and only observed in the July samples. As presented in (Fig SF1), P2 exhibited Proteobacteria, Cyanobacteria, Bacteriodetes Firmicutes and Verrucomicrobia as the only phyla distributed throughout the growing period. P1 and P3 were observed to exhibit more related microbes as they were the only ones to reveal *Environmental samples* in July.

At class level, P3 was observed to exhibit all classes (12), while P1 and P2 had 10 classes in each differing in Environmental no_rank and *Clostridia* classes respectively. Results from temporal data revealed that Environmental no_rank class flourished at 80% *in May (MW),* P1 and P3 but completely missed in P2 while *Beta Proteobacteria*, *Chlorobi* and *Bacteriodia* were exhibited over 80% in July (*JW).* The hierarchy of the five dominant classes was observed as *Delta, Beta, Alpha and Gamma* (Proteobacteria), and *Opitutae* (Verrucomicrobium) at 65.8, 9.71, 6.57, 5.88 and 4.79% respectively.

Interestingly across the ponds, classes Chlorobi, Epsilonproteobacteria, Clostridia and the Environmental samples were exhibited at 100% in a particular community in a one specified months i.e. P1 exhibited classes Epsilonproteobacteria and Chlorobi at 100% in May and July respectively while P2 revealed *Environmental samples*, *Clostridia* and *Epsilon Proteobacteria* flourishing at 100% in October for the former and May for the latter two. For P3 *Environmental samples* flourished at 100% in July, while *Chlorobi* and *Epsilon proteobacteria* communities flourished at 95% in May (Fig 2).

Fig 2 Footnote: Fig 2 Footnote: Bacterial community distribution in pond 1, 2 & 3 water samples. The abundances are presented in terms of taxon numbers affiliated to that class divided by the total effective bacteria taxa.

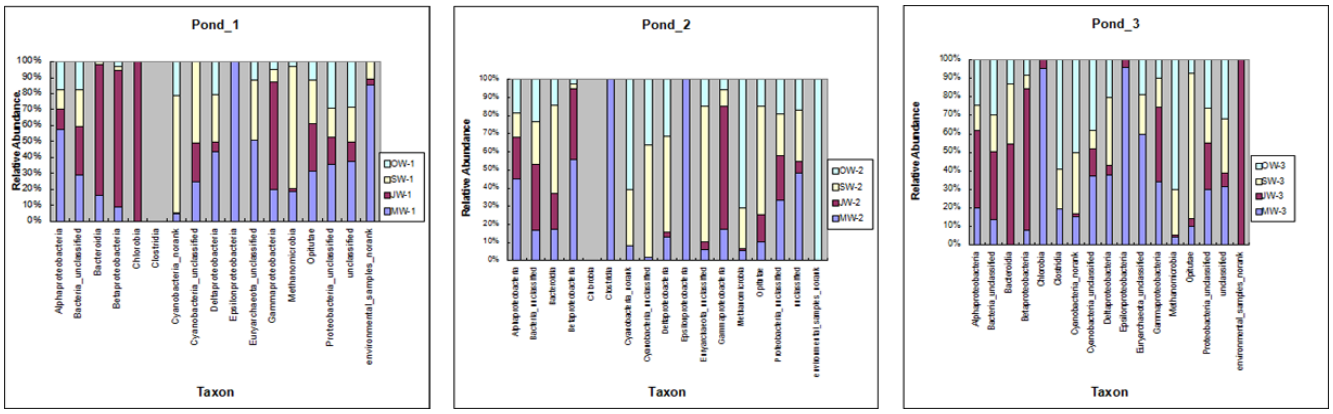

Fig 2: Class Distribution Bar graphs for microbial communities in Ponds 1, 2 and 3 of Yi Xing city, China.

At genus level, this study revealed that Yi Xing ponds had approximately 49 nitrogen fixation bacterial genera displayed with the major genus being observed as *Geobacter*. Also in abundance were 30 other genera including *Azoarcus, Azohydromonas, Azospira, Azotobacter, Bradyrhizobium, Candidatus_Accumulibacter, Dechloromonas, Desulfobulbus, Desulfovibrio, Desulfomicrobium, Lyngbya, Methylomonas, Rhodomicrobium, Rhodopseudomonas, Sinorhizobium* and *unclassified_Opitutaceae* among other that dominated through succession in the different ponds (Fig 3 & Supplementary data SD3). Findings from the study also revealed the significantly different bacterial communities within the different pond that included: *Leptothrix, Polaromonas, Pseudomonas* and *Sulfuricurvum* in P1, *Klebsiella and Clostridium* in P2. P3 that practiced polyculture was observed to exhibit 13 genera that differed from the other pond communities that included: *Allochromatium, Azospirillum, Azovibrio, Chloroherpeton, Dickeya, Methylocystis, Rhodospirillum, Rubrivivax, Sideroxydans, Skermanella, Stenotrophomonas, Tolumonas* and *Acetobacterium* (Supplementary data SD3)

Fig 3 Footnote: Bacterial community at genus distribution in pond 1, 2 & 3 water samples. The abundances are presented percentages of taxon numbers affiliated to that genus within the total effective bacteria taxa.

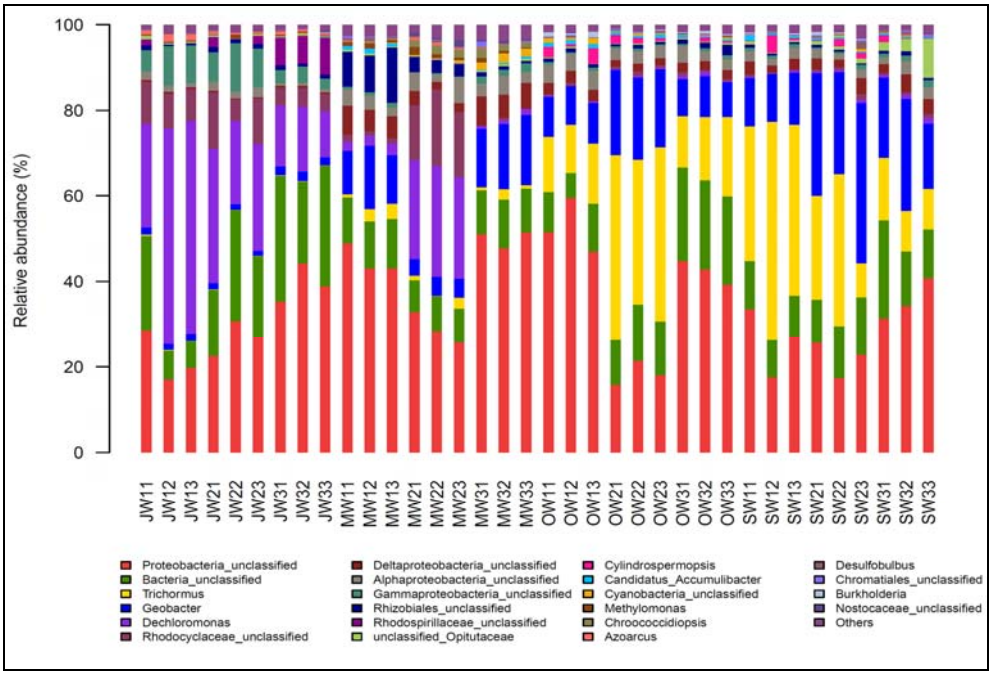

Fig 3: Relative Abundance of genus distribution of Bacteria within the sampled sites of Ponds 1, 2 and 3 in Yi Xing

The richness among the three groups is represented in a Venn diagram (Fig.4), displaying the overlaps between OTUs shared among the communities in the three ponds. Out of the total sum of 719,518 sequence reads, from the three ponds, this study revealed 730 rarefied OTUs that were used to identify the microbes in all the three communities. In the entire bacterial community 1,263 OTUs (33.42%) were shared by the three ponds and were revealed in phyla Proteobacteria, Cyanobacteria, Bacteroidetes, Verrucomicrobia and Euryarchaeota. A total of 997 OTUs (26.38%) were shared by at least two ponds and the study analyses of the shared OTUs between two ponds i.e. P1 and P2 shared 287 *OTUs* revealed uniquely in genus *Anaeromyxobacter and Environmental samples* among others. P2 and P3 shared 290 OTUs revealed uniquely in *unclassified_Opitutaceae* a genus in Phylum Verrucomicrobia and *Klebsiella* while P1 and P3 shared 420 OTUs that we identified to probably come from microbial communities of genus *Leptothrix* and *Sulfuricurvum*. For independent representation P1, P2 and P3 had 490, 314 and 715 un-shared OTUs (Fig 4), with P2 and P3 revealing the presence of genus *Clostridium* and *Acetobacterium* respectively from Phylum Firmicutes.

Fig 4 Legend: Bacterial communities of ponds 1, 2 & 3 waters based on the sequential identification of (97% similarity) shared OTUs.

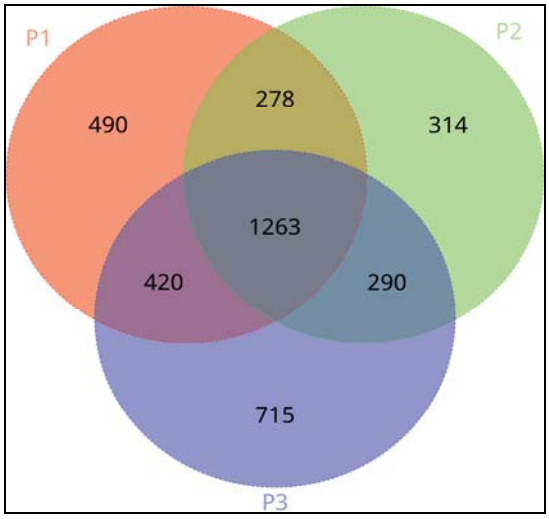

Fig 4: Venn diagram displaying overlaps between genus groups of microbial communities within Tilapia ponds at different Stocking Densities

Using the Hierarchical cluster analysis at phylum level, three bacterial community clusters (left side) were revealed. The heat map representing all ponds, showed the intensity of the relative abundance of each phyla as indicated by a gradient of colors i.e. green (low abundance) to red (high abundance) (Fig.5). Complete linkage clustering of samples based on the phyla composition and abundance within the ponds was observatory across all the ponds. Three clusters were recorded with the 1st one displaying September and October microbiota in P1 and P2 as having a high abundance of phyla Bacteroides, Verrucomicrobia and Euryarchaeota classified to there probably genera of *Paludibacter*, *unclassified_Opitutaceae* and *Methanosaeta* respectively. The 2nd Cluster did also exhibit microbes of *September* and *October* although these distinctive microbial communities from P3 revealed in abundant *Cyanobacteria* were classified most probably as *Chroococcidiopsis, Cylindrospermopsis, Lyngbya* or *Trichormus genera*. The last cluster exhibited bacterial composition across all ponds in abundances of *Proteobacteria, Chlorobi and Environmental samples* with communities identified as *Methylomonas*, *Chloroherpeton, no_rank (environmental)* and *Acetobacterium (Firmicutes)* among other genera. Similarly, using the complete linkage clustering, the microbial genera in the water samples with significant representation across all three ponds were defined in (Fig SF2). As shown on top of the chart 5 microbial clusters were revealed with Cluster 1 at the far left exhibiting microbial communities of P3 in *July* well represented by *Dechloromonas, Rhodopseudomonas, Azoarcus* and *Methylomonas* genera among others. Cluster 2 displayed communities of P2 observed in *May* and *July* represented by *Dechloromonas, Rhodospirillum, Methylomonas and Sulfuricurvum* as the significantly abundant genera. Cluster 3, was observed to harbor *September* and *October* P2 communities and *P1 September microbes* dominated by *Trichormus, Desulfovibrio* and *Geobacter* genera that were closely related to the communities of cluster 4. The 5th cluster revealed presence of dominant microbial communities from *Burkholderia, Thiocapsa, Tolumonas, Geobacter and unclassified_Opitutaceae* among others

Figs 5 Legend: Abundances are determined with color differentiation, lighter green displays low abundance and red the highest abundance

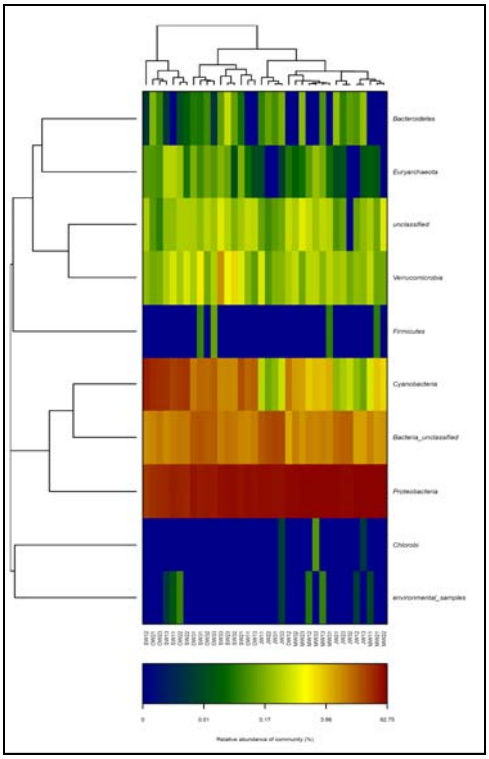

Fig 5: *Heat chart showing hierarchical cluster at the phylum level of microbiota communities within Tilapia grow out ponds in Yi Xing.*

Analyses of key factors regulating the bacterial community were presented using the linear discriminant analysis (LDA) effect size (*LEfSe*) method (Fig. 6a), OTU features and effects of the ponds to the small scale compartments within the microbial communities in their spatial and temporal community sizes were determined. These samples revealed microbial presence in only two pond densities i.e. P3 with highest abundance (green) and P2 with least abundances (red) as compared to the sediment communities, P3 revealed two taxa, *Chromatiaceae* and *Thiocapsa* at LDA scores of 2.65 and 2.5 respectively clustered together signifying their closer evolutionary relationship while P2 revealed four taxa including *Syntrophobacterales, Syntrophaceae, Desulfobacca* and *Environmental samples* with negative LDA scores of -3.25, -3.30, -3.50 and -4.00 respectively. Considering the temporal revelation of microbial communities (Fig. 6b), the baseline survey (May samples) disclosed the dominant phyla Proteobacteria revealing the most probable genera as *Desulfovibrio, Desulfomicrobium, and Methylomonas.* July revealed *Rhodospirillum, Xanthobacter, Bradyrhizobium* and *Paludibacter* as the dominant genera, while September exhibited *Geobacter, unclassified_Opitutaceae,* and *Methanosaeta* genera among others. Lastly, October revealed the existence of genus *Bradyrhizobium,* these revelations signified the presence of the nitrogen fixation microbes in Yi Xing ponds

Fig 6a & 6b Legend Titles: LEfSe diagrams showing microbial differences observed within the ponds of different stocking density communities, phylogenetic distribution and Ribosomal data bearing observed microbial taxa. Green color represents the highest pond density, blue represents the medium density and red represents the least density

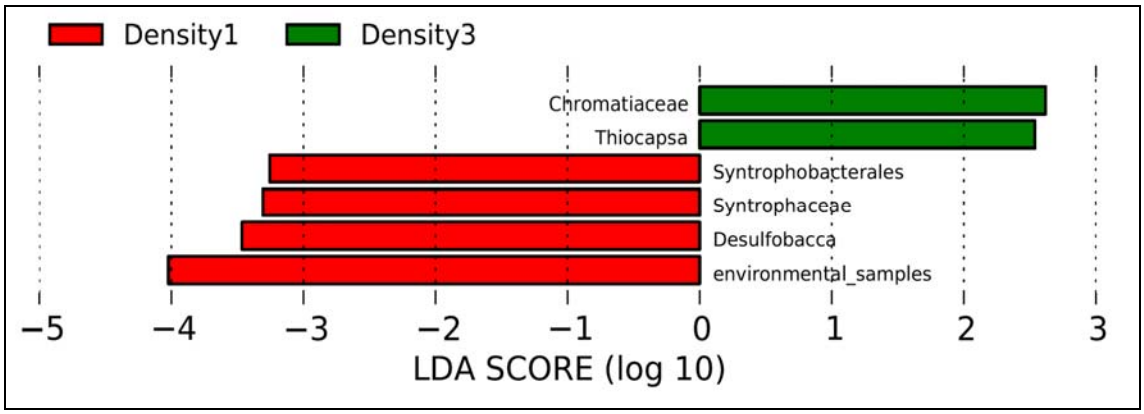

Fig 6a: Bacterial clades, showing statistically significant and biological consistent microbial differences in P1 and P3Fish densities

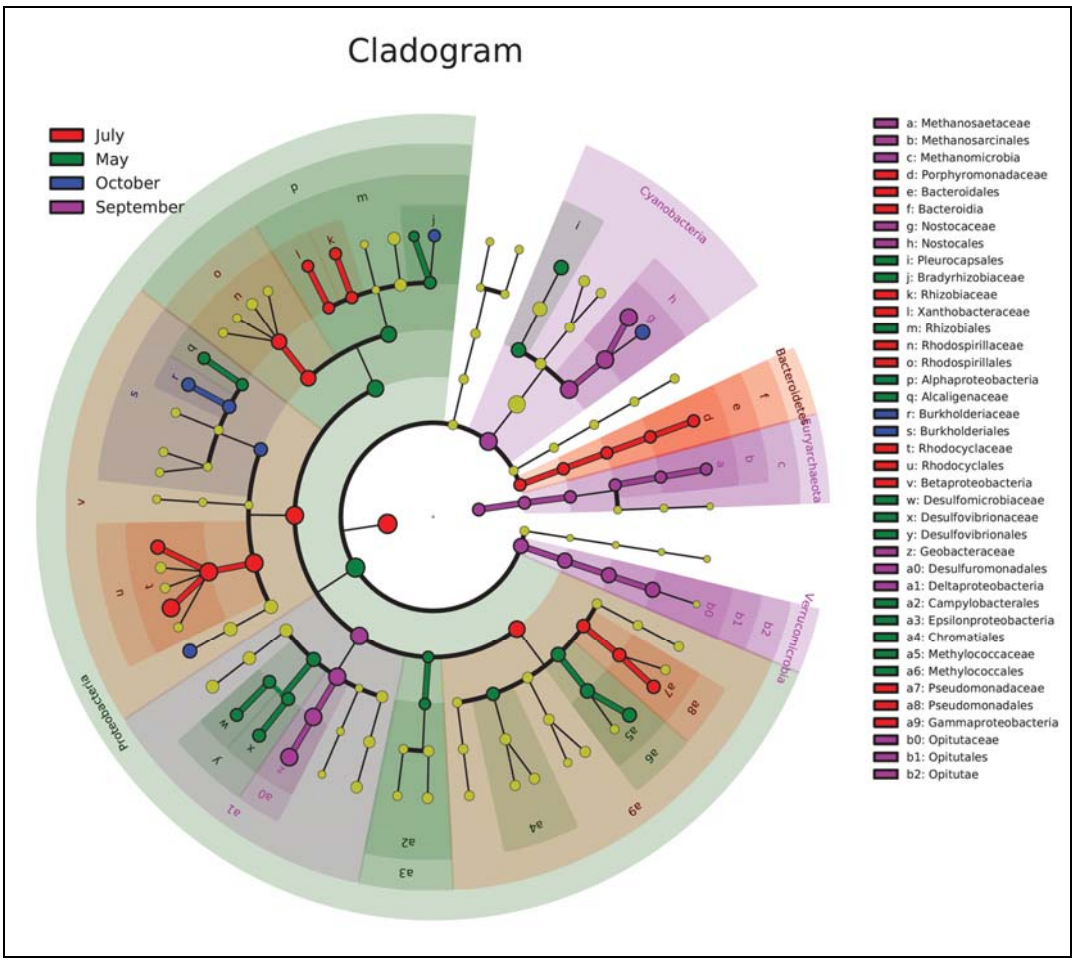

Fig 6b: Cladogram chart showing microbial representation within ponds 1, 2 and 3stocking Densities

Rank distribution abundance curves (Fig SF3) were used to estimate the species richness and determine similarity levels of microbiota among the three stocking densities. The results revealed that the highest and lowest species richness in pond microbiota were exhibited in P1 and P2 respectively especially in the baseline samples. The curves determined the majority OTUs significantly present within each sample and their shape, (sigmoid), revealed that the total richness of the microbial community had been sampled completely limiting any option of having unattended to OTUs in the clone library.

Rare-fraction measured results analyzed by Shannon Weiner Rarefaction index revealed the majority OTU pairs present had significantly low abundances in all the ponds. The generated graph, suggested the number of similar OTU pairing diversities had leveled off revealing no further rarefaction measures even if the number of reads were to be increased (Fig 7). P2 and P3 July samples in *JW22, JW33* and *JW31* together with *SW12*, exhibited the lowest diversity pairing at the rarefaction measure: r_Shannon ratio of 3.3. During the baseline survey at 0.97% similarity, P3 samples revealed the highest pairing diversity ratios with *MW32* showing a 5.75 pairing linkage factor obtained under 16,000 sequential reads. The highest sequential readings in the entire study were observed in P3 (*OW33)* towards the harvest (October), revealing 4.51 pair linkage factor in 27,989 reads.

Fig 7 Legend: Rarefaction analysis of the nifH – gene clone library using the specified nifH1 & nifH2 primers on water samples of Ponds 1, 2 & 3.

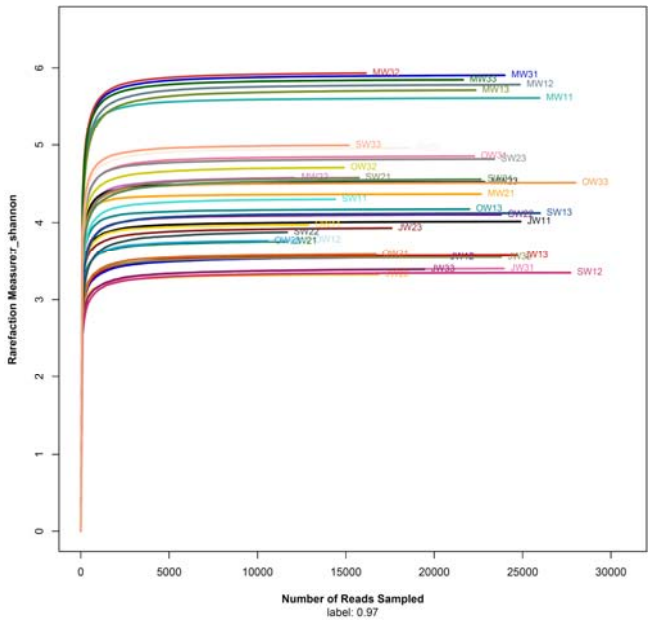

Fig 7: Showing Rare-fraction measures within the ponds analyzed with the r_Shannon index

The Environmental factors influencing the bacterial community were analyzed by Redundancy Analysis test (RDA) and the correlations between the microbial genera, fish stocking densities, environmental and nutrient variables presented in (Fig.8). The study results revealed that NH_4^+-N, TP, TOC, DO and Secchi depth (SD) in that order were the most important factors defining the bacterial communities. NH_4^+-N content revealed the highest effective explanatory factor for variance of the *Geobacter* genera used to assess the microbial reactions in P1, P2 and P3 for the minimum inhibitory determination causing total lack of expression represented in the evaluation of ordinates on the RDA1 axis. RDA2 was determined by more extractable variables including DO, SD, TP, TOC, SO_4^{2-}, NO_3^-, NO_2^-, TN and COD. The analysis further showed that NH_4^+-N, DO, and SD in that order correlated significantly with RDA 1, while TP, TOC and SO_4^{2-} in a descending order of significance correlated with RDA 2. The score plots revealed that NH_4^+-N accounted for the 40.14% of genus density variance in the majority of P1 and P3 microbial communities grouped to the positive side of the graph while the other environmental variables revealed a 26.23% variance in all the ponds. P2 microbial communities were observed on the negative sides of both axes suggesting this community might have had differing environmental influences compared to P1 and P3 communities. Similar results in the temporal analysis focusing on the monthly distributions (results not shown) revealed that September was the month with the highest significant correlation between the environmental samples and microbial communities mainly in P3 and P2.

Fig 8 Footnote: RDA ordination plots for the first two principal dimensions of the relationship between the environmental parameters of the ponds and the fish stocking densities for the water harboring nifH-gene microbial assemblages analyzed using data of the nifH OTUs.

Fig 8 Legend: Correlation between environmental variables and RDA axes are represented with the arrow angle and length, the more acute the angle and the longer the arrow length the high the significance in correlation.

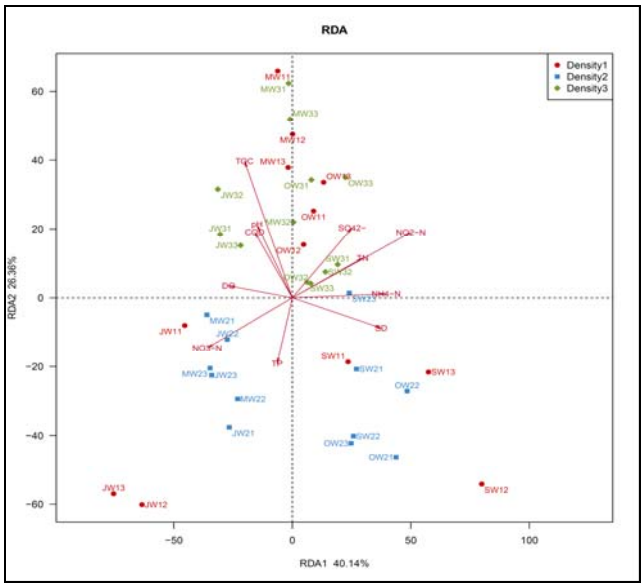

Fig 8: Redundancy Analysis plot for the microbial genus in Ponds 1, 2 and 3 of Yi Xing

3.2 Discussion

In this study identification of key taxons of nitrogen fixation bacteria encoding *nifH* - gene, to promote biological degradation in commercial farms and mediate mechanisms to short term regulations of microorganisms observing nitrogen fixation bioreactors were understood through environmental interactions.

The phylogenetic analysis, *nifH*-gene sequencing and quantification of the copies of the *nifH* gene encoding microbial abundances congruently together continued to reveal class Deltaproteobacteria exhibiting genus *Geobacter* and *Desulfobulbus* were the most dominant N_2 fixation microbes in the water columns. All the three ponds in this study revealed the identified taxa to be closely related and they included α, β, δ, ε and γ classes of phylum Proteobacteria represented, alongside microbial communities identified in classes of Bacteriodia, Methanomicrobia, Opitutae, Chlorobi and Clostridia.

Environmental microbes encoding *nifH* gene in this study in ponds P1, P2 and P3 revealed there is limited knowledge of diazotrophic microbial communities [5]. Most of the identified communities were under no_rank genus, attributing the fact that all detected *nifH* encoding sequences may not have originated from the active diazotrophic microbes [24]. This implies not all *nifH* encoding sequences originally defined at phylum, class or genus levels of environmental samples were characterized and some may not be involved in N_2 fixation [12, 26].

Although several studies have suggested that environments may select among the dispersed microbes, such as habitat specificity of different types of bacteria [27], similar environmental settings select for similar bacterial communities [28], our belief is that the composition of the free living microbial communities can be defined through the interacting organisms surrounding them through succession.

The results revealed that pH, DO, SD, TOC, NH_4^+-N and TP were the predominant environmental factors observed to control nitrogen fixation community functions in our study (Table 2), and this was based on the fact that nitrogen fixing activities of free living non photosynthetic aerobic bacteria are strongly dependent on favorable moisture conditions, oxygen concentrations and supply of the organic carbon

substrates. [29]. Furthermore, pH and dissolved oxygen did not only influence the overall nitrogen removal but also resulted in long term succession of community structure and diversity [30, 31].

Since environmental conditions play key roles in shaping the abundance and spatial distribution of nitrogen fixation bacteria, it was observed that only P3 revealed all phyla present in the study ponds. P1 & P2 had no representations of phyla *Firmicutes* and *Chlorobi* respectively, as shown in (Figs 1 & SF1) and it is suggested that these microbes exhibition in P3 alone could be associated with the model of farming (Poly-culture) rather than environmental conditions. However, in order to have a better understanding of the mechanisms involved, further studies are required.

Although limited, the *nifH* database was used to account for the specificity of the obtained primers, used in combination to evaluate the diversity of *nifH* genes in the different environments [32, 33]. And environmental surveys of *nifH* diversity [34]. This study design computed abundances of *nifH-1F* and *nifH-2R*-encoding nitrogen fixation bacteria in the experimental ponds estimating the quantity of the *nifH* functional genes that compared to Wang's works [30]. Thus, our observations indicate the presence of *nifH*-encoding nitrifying and nitrogen fixation bacteria in the pond water samples.

As suggested in many studies, PCR community based characterization, have potential biases including differential DNA extraction efficiencies [35], however in the study design, we adopted the Illumina throughput characterization technique to increase the feasibility of numbers of sequences in a single study allowing deeper coverage and provision of new insights regarding microbial community interactions [36]. Contrary to other studies, this study was limited to a single gene pair (i.e., *nifH*-encoding community) in the nitrogen fixation pathway and thus we deduce that the representation may have a partial reflection of the potential nitrogen fixation communities within the ponds.

The revelation that Yi Xing pond waters displayed a unique environment with great diversity and novelty within the *OTU* sequences was clearly understood through the Venn diagram display (Fig 4). The intersection with multitudes of divergent OTUs (1263) shared amongst the ponds revealed that most of the microbes in P1, P2 and P3 were related or had similar evolutionary background with the unique microbes only observed in one or two of the adjacent ponds. Several conditions may have played major roles in these observations [30]. Various studies, state that environments select among the diverse microbes such as habitat specificity [37] and similar environmental settings [38] that play great roles in modifying the different types of bacteria and selection for similar bacterial communities.

The heated maps (Figs 5 & SF2), show sampled community clusters were based on the complete linkage method [39]. The relative abundance observations of the most abundant OTUs (3% distance) clusters in terms of sequence reads by the phylum taxon were well represented in *Proteobacteria (88.78%), Verrucomicrobia (4.79%)* and *Cyanobacteria* (3.28%) that represented the free living heterotrophic or autotrophic microbes frequently reported as plant growth promoters that transform of nitrogen from the unavailable gaseous form in the atmosphere to forms up taken by plants and other organisms [40]. Other phyla were scantly represented across the three clusters that included *Firmicutes, Chlorobi* and *Environmental samples* (Fig 8). At the genera taxon for the revealed microbes, we would consider those that are beneficial or neutral to the plants for future study for example across all ponds *Azospirillum* a facultative entophytic diazotroph capable of colonizing the surfaces and interior of roots can initiate the biological nitrogen fixation process with non-leguminous plants that in turn benefits the plant's water and mineral uptake [41]. In freshwater and marine systems Cyanobacteria are major nitrogen fixers [42]. Able to survive extreme environments because of unique adaptations to resist desiccation and fix nitrogen, but above all are used as biofertilizers in modern agriculture [43]. And we would suggest they are considered for aquaculture pond biofertilization. These observations are significant in identifying the most suitably diverse communities to focus upon in obtaining the best strain for the nitrogenous waste cleaning purposes.

The *LEfSe* results observed further explained the study results provided in three main outputs describing the effect sizes of differences observed among stocking density communities with the number of clades being regulated to 2 within the highest abundant density pond (P3) and 4 in the least density microbial communities. The phylogenetic distribution of these differences although sparsely distributed showed linkages and relationships amongst *Syntrophaceae, Syntrophbacterales* and *Chromatiaceae* communities. The driving force behind these effects formed the third output with similar OTUs performing similar functionalities to expression [44, 45]. i.e. *LEfSe* doubled in determining features of organisms, clades, genes or functionality in the *OTUs* [46]. The revealed observations in (Figs 6a & 6b) clearly indicated no clades were consistently present in all the experimental pond sites that clearly demonstrated differences among the population structures of these communities in space and time.

Rank distribution curves determining the majority of significant OTUs present in each pond, (Fig SF3), defined the bacterial communities through relation of similarities near 97% OTUs analyzed by the r Shannon index. The shape of the *nifH* curves indicated no further OTUs would be expected even if more clones were sequenced [47]. Characterization of the bacterial taxa based on DNA extractions was dependent on the quality of the DNA retrieved, the PCR amplification and significantly the primers employed. The flattened curves displayed graphically indicated the end of amplification cycle that deduced the best results (Fig 7) although at this stage, all environmental sample analyses yielded almost exclusively unknown sequences [48].

The RDA results defined the interactions amongst the physiochemical parameters, nutrient variables and the bacterial communities analyzed as shown in (Fig 8). The effects and influences of all above variables on the genera stocking densities, the spatial and temporal distribution were registered.

Of all environmental variables analyzed, dissolved oxygen (DO), Secchi depth (SD) and pH were observed to dominantly contribute to the heterogeneous community structure and spatial distribution in the sampled water with *nifH*-encoding microbial assemblages. Although the results suggested SD may have had limited control on the correlation of the *nifH*-encoding microbes. It could also be explained as having limited N_2 fixation bacteria within the samples however, other aerobic bacterial microbes may be the major constituents of the microbiota in the surface water (down to a 5-cm depth) in the ponds.

Since biological nitrogen fixation is mediated by nitrogenase enzyme a process more important to the biological activity in

the ponds, this depends on the ecological conditions in association with the specific nitrogen fixation capabilities of certain microorganisms and plant genotypes under various climatic conditions. From our study results it's revealed that TP and NO_3^--N are of great importance in determining the high bacterial densities in P2 a result that envelops Dang's cautious suggestion for the *nifH*-encoding microbial population actively consumed phosphorous and organic matter. Considering the fact that evolutionary trajectory of adaptive mechanisms protect nitrogenase from molecular and reactive O_2 species discerned in physio-ecological patterns in microbial morphology, biochemistry, physiology and community structure along gradients of anaerobic to fully aerobic environments [49]. It sets stage for future investigations on enzymatic and oxidative catalysis of the characterized microbes in situ at species levels.

Fig SF1 Footnote: Bacterial community distribution in pond 1, 2 & 3 sediment samples. The abundances are presented in terms of taxon numbers affiliated to that phylum divided by the total effective bacteria taxa.

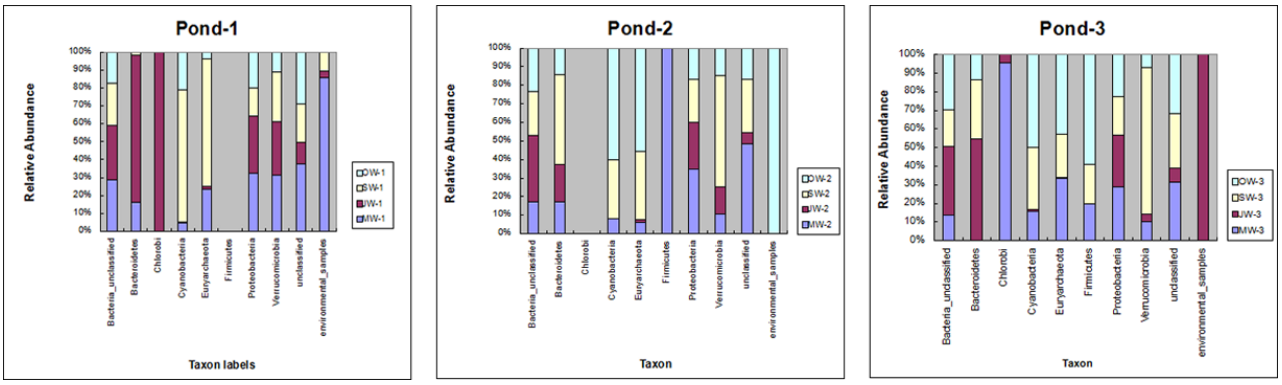

Fig SF1: Phylum Distribution Bar graphs for monthly sampled microbial communities in Ponds 1, 2 and 3 of Yi Xing city, China

Fig SF2 Legend: Abundances are determined with color differentiation, lighter green displays low abundance and red the highest abundance

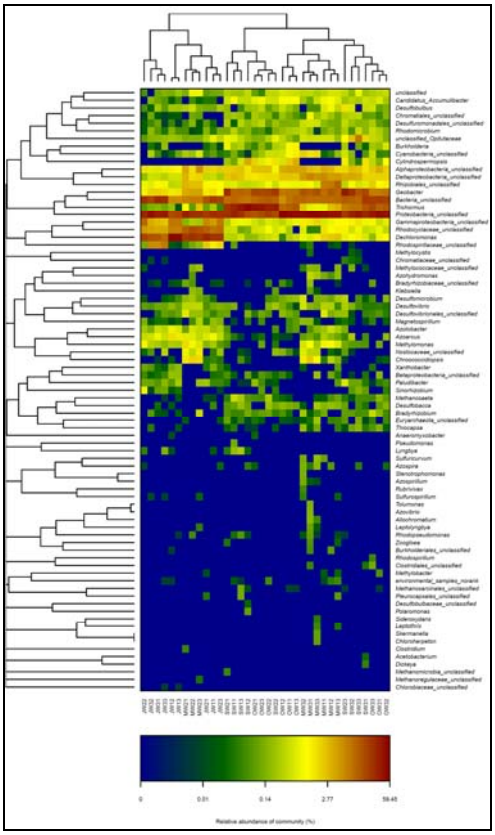

Fig SF 2: Heat chart showing hierarchical cluster at the genus level of microbiota communities within Tilapia grow out ponds in Yi Xing.

Fig SF2 Legend: Abundances are determined with color differentiation, lighter green displays low abundance and red the highest abundance

Fig SF3 Footnote: These determine the majority OTUs significantly present within all ponds Curves exponentially raise then level off as no new OTU sequential numbers tend to be read.

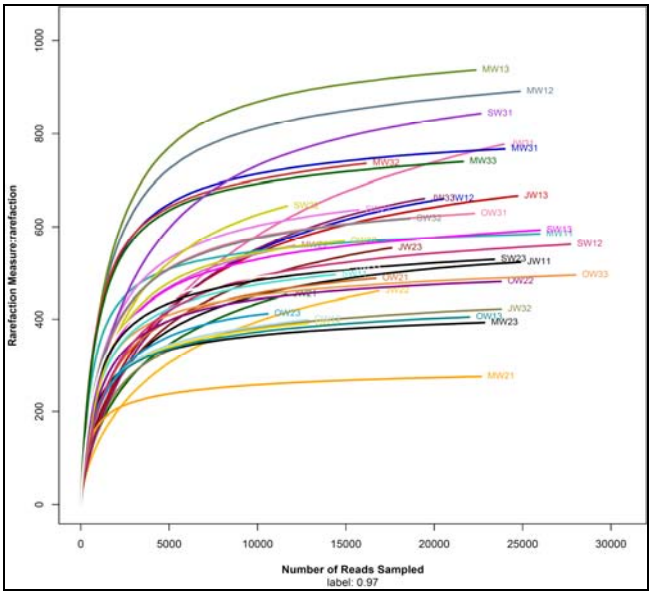

Fig SF3: Rank distribution abundance curve, showing the tails in the OTU ranks relative abundance curves.

4. Conclusion

In this study nitrogen fixation bacterial identity, characterization and diversity in the summer pond production systems of Yi Xing, China were evaluated with the illumina high throughput multiplex barcode pyrosequencing and functional gene analysis. The results elucidated that the ponds harbored a gradient of nitrogen fixation bacteria taxonomically identified and correlated with the environmental factors revealing the existence of a multitude of genera being dominated by *Geobacter* and *Desulfobulbus.*

This provides a novel insight in the presence of *nifH* – gene encoding bacteria being targeted as nitrogenous waste removers in pond culture a significant factor to consider since these microbes double as plant growth promoters in aquaponics, hydroponic and pond culture systems an intended applied research for promoting sustainable production.

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