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Characterization and functional analysis of *nifH* encoding Nitrogen fixation bacteria in Nile Tilapia pond sediment

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In this study, an investigation was carried out on bacterial communities involved in nitrogen fixation in three different intensity Nile tilapia (*Oreochromis niloticus*) culture ponds. Summer physicochemical factors defining the sediment degradation were determined. Results revealed that pond temperatures ranged from 27.3 to 34.2ºC, dissolved oxygen 6.31 to 8.99 mg/L and pH was between 7.18 and 7.98. Nutrient levels differed significantly (P < 0.05) amongst the ponds: Total phosphorous values in the ponds were 0.034, 0.038 and 0.028 % while total organic carbon values (P < 0.05) were 4.33, 4.93 and 4.16 mg/Kg for the three ponds respectively. There were no significant differences (P<0.05) in total nitrogen and nitrite nitrogen registered throughout the study. The nitrogen fixation microbial communities presumed to significantly reduce pond nitrification were taxonomically identified to their most probable genera that included; *Bradyrhizobium, Magnetospirillum, Rhodomicrobium, Rhodospirillum Sinorhizobium, Azotobacter, Methylobacter, Methylomonas, Thiocapsa, Geobacter, Anaeromyxobacter, Desulfobacca, Desulfobulbus, Desulfomicrobium, Desulfovibrio and Syntrophobacter* genera. The quantitative analysis revealed the *nifH* gene mean abundances were 3.02 x 10^7, 4.06 x 10^7 and 4.85 x 10^7 copies/g wet weights in ponds 1, 2 and 3 respectively. The Redundancy analysis indicated that total phosphorous and total organic carbon were the most important factors in shaping the bacterial communities while stocking densities of 1,800 or less fish per 667 sq. M were not regulating factors for the microbial abundances. This study would set stage for future investigations on enzymatic catalysis and oxidative roles of the identified microbe communities to species level, for wide adoption in cultures.

Key words: Bacteria community composition, nitrogen uptake, N_2-fixation bacteria, *nifH*-gene, functional analysis, total organic carbon, Proteobacteria, *rhizobia*

INTRODUCTION

As wild fish stocks continue to decline, the need to boost production through aquaculture intensification is a necessity (Johnson J 2013). Through intensification pond fertilization is required yet artificial feeds and fish wastes already produce excessive nitrogenous additives to the water. Recent studies suggest that an excessive use of

chemical fertilizers induces environmental pollution such as nitrate leaching and nitrous oxide emissions (Bagali SS 2012). There is a major growing challenge of water quality deterioration, due to high metabolite concentrations, and limited feed utilization. N_2 is becoming one of the major concerns as a pollutant in terrestrial ecosystems (Bagali SS 2012). Currently, there is a great need for efficient water utilization and environmentally friendly production systems (Avnimelech Y 2007). Water treatments, done by chemical processes are inherently disadvantaged, as there are additives that tend to stay for a longer time in the environment and some accumulate in aquatic animal organs rendering it unsafe for human consumption. This makes biological nutrient recycling processes the most efficient approach to treat waste-water for reuse or discharge in aquaculture.

Considering that the biological processes, are the most important ones with respect to aquaculture waste water treatment, nitrogen-fixing microbes were identified as the only biological source for fixing nitrogen in the biosphere (Rubio LM 2002). Understanding this process, one requires studies relating to the mechanism of catalysis that should be performed on the nitrogenase enzyme, whose multiple subunits are encoded by genes such as *nifH, nifD*, and *nifK* (Rubio LM 2002). The process of breaking down free nitrogen and inorganic nitrogenous wastes, such as NH_3^+ to amino acids for quick uptake, in aquaculture ponds and aquaponics production systems requires substantial knowledge concerning the overall community structure, population dynamics, metabolism, main functional genes and organic carbon sources of different microbes (Huijie L 2014).

To aid the identification of nitrogen fixation bacteria, the *nifH* gene, a widely studied ecological and evolutionary bio-marker is considered (Raymond J 2004). Since dinitrogenase reductase encoded by the *nifH* gene is relatively conserved in all known organisms (Gonzalez LJ 2005) its, vital to develop suitable probes to screen for the occurrence of nitrogenase in bacteria (Gaby JC 2012) Microbial identification by Illumina through put (Thomas F 2014), and developments of molecular methods as environmental bacterial diversity distribution assessments, allows identification of total bacterial population monitoring based on gene probing DNA sequences (Mergel 2001). Furthermore, this study tries to understand the microbial diversity and water quality factors related to the thriving of the significant anaerobic microbial communities within the culture systems. Different identification techniques, of these communities, were undertaken, given the fact that; nitrogen fixation bacteria were sensitive organisms that displayed extreme susceptibility to a wide variety of inhibitors (Huijie L 2014).

The objective of this study was therefore to provide information on the nitrogen fixation bacteria communities, that were identified during a summer production period, in tilapia grow-out ponds, through characterizing and defining these microbial communities to their closely related genus levels for future studies on the catalytic process of the enzyme and their wide adoption for culture to be used in promoting vegetable plant growth alongside fish production in ponds or in systems like aquaponics or hydroponics.

MATERIALS AND METHODS

Study Area

The study was conducted at a Research facility in Yi Xing, Jiangsu Province (N31° 27' 48.2" E 119° 51' 1.7") PR. China. Nitrogen fixation microbes from a summer production Nile tilapia (*Oreochromis spp*) pond sediment were focused on. Data and samples were collected from two intensive monoculture ponds, i.e. pond 1 (P1) and pond 2 (P2) with stocking densities of 1,500 and 1,200 tilapia fish per 667sq.M respectively, and a Polyculture production, pond 3 (P3) stocked with 1,800 tilapia fish per 667sq.M, 30 pieces of Big head carps and 60 pieces of Silver carps cultivated together. Each pond had an area of 1334 sq.M, a representation of 2 Chinese *Mu*. The Research facility belonged to Freshwater Fisheries Research Center (FFRC) affiliated to Nanjing Agriculture University under the Chinese Academy of Fisheries sciences (CAFs).

Sampling procedure

Sediment samples from two Mono-culture and one Poly-culture cultivation systems were collected, between May and October 2014. The study collected a total of 144 samples of which 108 samples were analyzed for physiochemical while 36 samples were used in the bacterial studies.

Sample collections were done at three adjacent spots, i.e. near inlet, close to outlet, and close to the pond center, using a mud grabber. For each sample ten or five spatulas of mud were scooped separately into sterilized plastic vials, with the 5 spatula mud vials being transferred immediately to liquid nitrogen for preservation. All the samples were placed in insulated containers and transported to the laboratory within 4 hours of collection. Samples for bacterial analysis were

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stored in a fridge at -80°C, while those for nutrient analysis were frozen dried at -80°C for 12 hours before storage at -20°C pending further laboratory analysis. The studied samples were analyzed in triplicate and labeled depending on; the month of collection i.e. (*M*-May, *J*-July, *S*-September, and *O*-October); sample type (*S*-sediment); pond numbering (1, 2 or 3) and sample numbering (1, 2 or 3) i.e. *MS11*, represented May Sediment in pond 1 for Sample 1. Similarly *JS32* represented July Sediment in pond 3 for Sample 2. While September denoted as (*S*) and October (*O*) had similar label designs.

Physiochemical analyses

Dissolved oxygen (DO, mg/L), water transparency, Oxidation-Reduction Potential (ORP), pH and temperature (°C) of pond water logged sediment 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. The sediment nutrient concentrations, i.e. ammonium nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N) and nitrite nitrogen (NO_2^--N) were measured using the Nessler colorimetric method, TOC was determined using the oxidation method, while TN and TP were measured using the Kjeldhal method and UV Spectrophotometry methods respectively (Wei 2002).

DNA extraction

DNA from 0.25g per sediment sample in triplicate (108 samples) was extracted using the Power Soil®- htp 96 Well Soil DNA Isolation kit (MO-BIO) following the manufacturer's instructions and purified using the Power Clean DNA Clean-Up kit (MO-BIO). Each sample was extracted in triplicate to avoid bias and the extracts from the same samples pooled together. The extracted DNA was stored at -20ºC until use.

Amplification and Pyrosequencing

The PCR amplification and pyrosequencing were performed according to established protocols (Eren AM 2013). The V_3-V_4 region of the *nifH* genes was amplified using the modified primer pairs, for the bacterial community with partial sequences of 5'-AAAGGCGGAATCGGCAAGTC-3' and 5'-TTGTTCGCGGCGTACATG-3' gene codes for *nifH-1F and nifH-2R* respectively (Baker 2003). A 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-FLX 454 pyrosequencer. All related procedures were performed following Genome Sequencer FLX System manufacturer's instructions (Roche, Nutley, New Jersey, USA). The *nifH*-encoding nitrogen fixation gene sequences derived from pyro-sequencing were deposited in the NCBI Sequence Read Archive under accession number obtained at (http://www.ncbi.nlm.nih.gov/Traces/sra).

PCR

The PCR was carried out with modification of (Thomas F 2014) procedure in triplicate with total reaction volume of 50µl; 10µl of 5X RT Buffer; 4µl of dNTP mix; 1µl of the template DNA; 1µl of each primer; 0.5µl of AmpliTaq DNA and the remaining reaction volume made up of 32.5µl of dH_20. The amplification program consisted of an initial degeneration stage at 97°C for 7 minutes, followed by 34 cycles, of 94°C for 20 s denaturing, 65°C for 30 s annealing, 72°C for 40 s extension and the final extension of 72°C for 10 minutes. Replicate PCR products of the same samples were assembled within PCR tubes and visualized on agarose gel 1% in TAE buffer, containing Ethidium bromide (EB). Additionally, the double stranded DNA assay (in vitro) was quality controlled by Agilent 2700 bioanalyzer (Agilent 2,700 USA). Following the SYBX green assay quantification; the amplicons from each reaction mixture were pooled in equimolar ratios based on concentrations and subjected to emulsion PCR to generate, amplicons libraries. The libraries were cleaned using MinElute kits and sequenced in a single paired end lane of illumina, while the overlapping paired end reads were kept for further analysis (Eren AM 2013). The high-quality sequences after filtering were assigned to samples according to barcodes. Sequences were aligned in accordance with Usearch (Schloss PD 2011, Quast C 2013) and clustered into operational taxonomic units (OTUs) using GAST (Huse SM 2008), with a version of the Greengenes 13_5 database (McDonald D 2012) trimmed to the V_3-V_4 region. OTUs were analyzed with QIIME v 1.7 (Caporaso JG 2010) A Venn diagram displaying unique OTUs was drawn to depict the similarities and differences among Ponds 1, 2 and 3 communities. For taxonomy-based analysis, the Usearch (Version 7.1) database project (Schloss PD 2009, Edgar RC 2010) was used as a repository for aligned rDNA sequences. Data sets were rarefied to lowest number of sequences per sample and the weighted *nifH* OTUs and genus data were used to identify the most significant environmental factors that had the strongest influence on the community structure and spatial distribution of *nifH* harboring microbial assemblages in the pond sediment. (Dang 2010).

Determining bacterial communities within the Ponds

The microbes encoding *nifH* gene within the ponds were identified using RT - qPCR. The Allele ID 7.75 software (PREMIER Biosoft) was used on alignments of nitrate reductase sequences to design a suite of primers targeting divergent phylotypes and the obtained primers were checked against the nr/nt database using the primer BLAST tool on the NCBI server (Ye J 2012). For each

target phylotype DNA standards were prepared by linearizing plasmids from one representative clone. The study reactions were performed on an Mx3005P thermo-cycler (Strategene) using the Maxima SYBR Green qPCR Master Mix containing 0.01 µM ROX (Thermo Scientific). For each primer set, qPCR conditions were optimized on serial dilutions of the respective standard clone (10-10^5 copies) to ensure satisfying specificity and efficiency above 80%. Reactions were denatured 7 min at 95ºC, followed by 40 cycles of 15 s at 95ºC, 30 s at 60ºC annealing temperatures and 30 s at 72ºC extension. Dissociation curves were obtained by heating up the reactions from 65 to 95ºC. PCR efficiency was determined using the standard curve by the formula $E = 100*(10^{(-1/slope)} - 1)$. To check the specificity of each primer set, qPCR reactions were run using 1 ng 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. A single band of the expected size was observed on a 1% (wt/vol) agarose gel for the former two cases and no amplifications were detected in the latter case. The reaction carried out in 96 wells had a total reaction volume of 25µl; with 12.5µl of SYBR Green; 2.5µl of Primer mix (F+R, 4µM); 5µl of the *c*DNA and the remaining reaction volume made up of 5µl of dH_20. Each phylotype reaction was duplicated on the same plate as the environmental samples to obtain fresh standard curves and determine the assay performance. A single lot of the cDNA was used to minimize the variability due to reverse transcription. To ensure no contribution of the background signal to gene quantification, C_T cut off thresholds were set 3.3 cycles lower than that of the no-template control if detected (Smith CJ 2006).

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 between the microbial community and sediment physiochemical parameters. OTUs reaching 94 - 95% similarity levels were used for Abundance based coverage estimations (Ace); Richness (Chao); Shannon; Simpson diversity indices and Good's coverage were analyzed using the software package MOTHUR 1.15.0 (Schloss PD 2009). Hierarchical cluster analysis was performed using the g plots package of R (http://projects.gnome.org/gedit/) in LinuxWin32. The relationships between the bacterial community composition; diversity; functional gene abundance; and environmental factors, were analyzed by Redundancy Analysis (RDA); using Canoco for Windows 4.5. All the variables were normalized via $\log_{10}$ (N + 1) transformation and Monte Carlo permutation tests were used to assess the statistical significance of the relationships.
All the above correlation analyses, were performed using statistical package SPSS 16.0V.

RESULTS AND DISCUSSION

Results

Physiochemical conditions & nutrient composition of the pond sediment

Results for the Average Means ± SD for the temperatures (T), pH, DO, and ORP during the four months sampling period are presented in Table 1. The lowest DO and pH were observed in the July sediment sample (*JS*). The average pH values, obtained during the sampling period, ranged between 7.0 and 8.0 and the DO range throughout the production period was observed to be significantly high ($P < 0.05$). All physiological parameters determined within the three ponds were observed to have no significant differences ($P < 0.05$) and the trend of the results in DO and ORP were as expected i.e. least in P3, P1 and P2 having stocking densities of 1800, 1500 and 1,200 tilapia fish head per 667 sq. M. The trend in temperature fluctuations even though not significantly different amongst the ponds was positive to our norm. On the contrary SD deviated from our expected norm, the fewer the fish numbers the smaller the depth size which could have resulted with the presence of algal bloom. The recorded temperatures across the production period from May to October ranged between 27.31ºC and 34.26ºC while the water transparency was observed to be decreasing from the initial Baseline depth in May (*MS*) at 0.41m to 0.29m in October (*OS*) during the harvest period.

The determined nutrient components in the sediment i.e. TN, TP, COD, NO_2^-- N, and NH_4^+- N showed that P2 had the highest registered concentration values of all the studied parameters (Table 1). Analyses using Duncan's regression showed that only TN, and NO_2^--N concentrations were not significantly different ($P < 0.05$) in all the Ponds. NH_4^+-N, TP and TOC concentrations were observed to be significantly different ($P < 0.05$) amongst all the ponds. P2 registered the highest organic carbon and total phosphorous concentrations that were steadily followed by P1 and P3 concentrations showing a positive trend with the expected norm. On the contrary between P1 and P3, it was observed that NH_4^+-N concentrations in P1 were far less than those in P3 diverging from the norm; however it could be suggested that the stocking densities and culturing methods would have influenced the levels of NH_4^+ - N concentration.

Detection of nifH - encoding nitrogen fixation bacteria

The efficiency of the PCR amplification of the *nifH* genes generated from the SYBR Green standard curve was observed to be 90% (Supplementary Data SD1) and the results showed that the mean *nifH* gene abundances in P1,

Table 1: Physiochemical Characteristics and nutrient composition results for Sediments in Yi Xing Ponds, (1, 2 & 3), Sampled within four Months

	Culture system	*Intensive*	
	Ponds		
Parameters	**Pond 1**	**Pond 2**	**Pond 3**
pH †	7.78 ± 0.31	7.71 ± 0.18	7.49 ± 0.18
Temp (°C) †	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
TN (%) †	0.30 ± 0.02	0.34 ± 0.01	0.31 ± 0.01
TP (%)	0.034^{b} ± 0	0.039^{c} ± 0	0.027^{a} ± 0.00
NH_4^+ - N (mg/Kg)	73.191^{a} ± 4.11	115.50^{b} ± 14.95	$94.69^{a,b}$ ± 7.26
NO_2^- - N (mg/Kg) †	1.24 ± 0.40	1.54 ± 0.39	0.92 ±0.08
TOC (mg/Kg)	$4.33^{a,b}$ ± 0.26	4.93^{b} ± 0.24	4.16^{a} ± 0.18

Each point represents a mean value and Standard error of 3 replicates ($P<0.05$) for T(°C), DO, ORP, pH, SD (Water transparency), TP, TN, NH_4^+-N, NO_2^--N & COD in sediment samples of the month May (MS), July (JS), September (SS) and October (OS). $^{a, b, c \& d}$ indicate significantly different values from the baseline survey according to the Duncan's multiple regression analysis test. † represents no significant difference from baseline observed values. Total area per pond is "2 Mu". Each "Mu" is equivalent to 667 sq. M

P2 and P3 were 3.02 x 10^7, 4.06 x 10^7 and 4.85 x 10^7 copies/g ww. P3 with the highest stocking density, i.e. 1,800 tilapia fish per 667 sq. M, yet practiced polyculture of Bighead (30) and Grass carps (60) registered an observatory result showing the highest abundance of *nifH*-encoding gene nitrifiers. P1 with the second highest stocking density had the least *nifH* gene copies / g (ww).

Microbial diversity and dominant bacterial communities

After filtering a total of 744,352 high-quality rarefied sequential reads, a total of 3,636 OTUs at 0.96 of sequence similarity with a read length of 434.44 bp, were identified in the three ponds for the analysis. The least number of OTUs in a pond were observed in P1, although this had no significant difference ($P < 0.05$) as compared to the other ponds (Table 2). The majority of the OTUs observed in all the ponds (60.8%), came from phylum *Proteobacteria*; Class *Deltaproteobacteria* and genus *Geobacter*.

The non-parametric richness indices of Ace, Chao, and Shannon evaluated at 96% similarity, showed similar comparative trends in the prediction of the number of OTUs for each related pond sample. These observed results suggested an existence of similar microbial taxa in the three ponds with the difference being recorded in the amounts counted ± SE. P2, had the most observed OTUs with the highest Richness displaying significant differences ($P < 0.05$) in Chao and Simpson parametric indices . The lowest total richness was observed in P1 specifically in the baseline sediment in the month of May as there were limited feeds given to the fish.

P1, although not significantly different with other ponds ($P < 0.05$), recorded the highest sediment bacterial diversity measurements, while P2 registered the least diversities. The coverage index was over 96% and ranged between 0.9652 and 0.9675. The Simpson index showed significant difference variations ($P < 0.05$) between 0.0418^{a} to 0.0835^{b} amongst the three ponds.

Taxonomic classification

Generally this study observed 3 domains, which included 10 phyla, 17 classes, 33 orders, 45 families and 62 genera distinctively distributed across the three ponds. Classifiable sequences at the distance of 3%, observed that P1 with a total of 151,580 filtered sequence reads and 1,156 OTUs (Table 2) registered microbes that belonged to 9 phyla, 15 classes, 26 orders, 32 families and 36 genera. P2 microbes were classified into 8 phyla, 14 classes, 23 orders, 31 families and 34 genera while P3 micro-biota belonged to 10 phyla, 16 classes, 27 orders, 34 families and 38 genera. Observing the phylogenetic classification of the sequences from the three ponds at considered taxa levels, out of the 10 phyla, Phylum *Proteobacteria* was the most dominant representing 91% in all the ponds, followed by the *Verrucomicrobia*, Cyanobacteria and Euryarchaeota an Archaea at 4, 2 and 2% while the remaining phyla,

Table 2: Summary of Total Richness and Diversity of bacterial communities from three stocking density Sediment samples of Yi Xing Ponds in Jiangsu Province China

Pond name	Reads	0.97 OTU	*Ace* †	*Chao*	*Coverage* †	*Shannon* †	*Simpson*
P1	151,580	1156	1572 ± 40.9	1545[a] ± 41.2	0.9669 ± .003	5.0908 ± 0.11	0.0528[a] ± 0.008
P2	170,583	1285	1739 ± 60.3	1708[b] ± 57.0	0.9675 ± .003	4.8950 ± 0.14	0.0835[b] ± 0.013
P3	156,347	1195	1670 ± 78.7	1601[a, b] ±50.2	0.9652 ± .004	5.0458 ± 0.09	0.0418[a] ± 0.007

Total values for samples n=12, each point represents a mean value and Standard error of 3 replicates (P<0.05) P1 denotes Pond 1, P2 = Pond 2 and P3 = Pond 3 OUT representations within sediment samples obtained in the months of May, July, September and October 2014. [a, b] indicate significantly different values from the least observed Pond values according to the Duncan multiple regression analysis test while † represents no significant difference. Values are at 95% confidence intervals as calculated by MOTHUR.

Bacteroides, Chlorobi, Firmicutes and Environmental samples were under 1%.

As presented in (Figure 1); all phyla were present in the months of May, July and September, (the early and mid stages of grow out periods), however in the harvest stage, October (*OS*), the phylum *Chlorobi* was missing in P1 and P2. Based on comparison of the spatial distribution of Phylum *Chlorobi*, amongst the ponds, it was distinctively observed to flourish more dominantly in P2 while its temporal dominance was limited to July (*JS*). The study observations also revealed that only P3 registered all phyla being represented at all stages in the sample results.

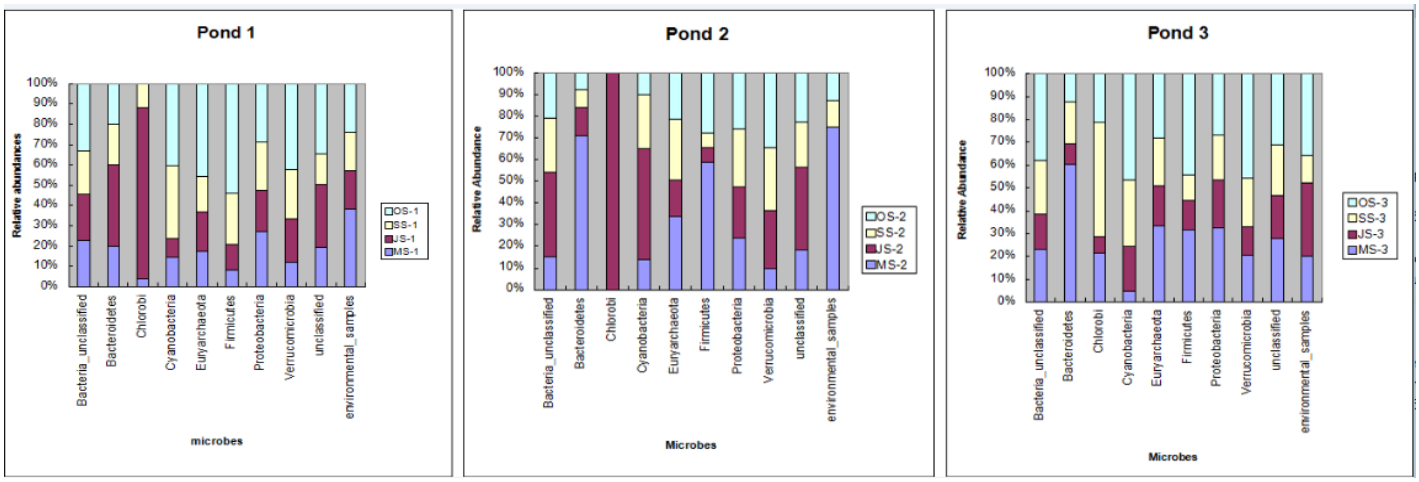
The five most dominant classes registered in all the ponds were mainly from Phylum Proteobacteria (Supplementary Figures SF1 & SF2). Observations in P1 revealed the representative abundances of *Proteobacteria_unclassified*, *Deltaproteobacteria, Bacteria_unclassified, Alphaproteobacteria* and *Gammaproteobacteria* were 45.69, 39.40, 6.81, 3.34 and 1.81% respectively. P2 was dominated by *Deltaproteobacteria, Proteobacteria_unclassified, Bacteria_unclassified, Alphaproteobacteria* and *Gammaproteobacteria* at 58.37, 37.83, 8.09, 3.35 and 1.32% observable ratios respectively. P3 registered *Deltaproteobacteria, Proteobacteria_unclassified Bacteria_unclassified, Alphaproteobacteria* and *Gammaproteobacteria* as respective dominants at 46.12, 44.40, 5.82, 2.30 and 2.18%.

The relative abundance at the genus level displaying the five major genera that dominated against each other through succession in the different ponds are represented in Figure 2; P1 and P3 had *Proteobacteria_unclassified*, *Geobacter, Bacteria_unclassified*, *Deltaproteobacteria_unclassified* and *Alphaproteobacteria_unclassified* dominating with 7 and 6% of the other unclassified genera for the former and latter ponds respectively. Meanwhile P2 had *Geobacter*, *Proteobacteria_unclassified*, *Bacteria_unclassified*, *Deltaproteobacteria_unclassified* and *Alphaproteobacteria_unclassified* registered in that respective order with 8 other unclassified genera. The abundances were tabulated based on identified taxa at class levels i.e. *Alphaproteobacteria_unclassified* comprised of *Bradyrhizobium*, *Magnetospirillum*, *Rhodomicrobium*, *Rhodospirillum & Sinorhizobium* genera. *GammaProteobacteria* consisted of *Azotobacter*, *Methylobacter*, *Methylomonas* and *Thiocapsa* genera. All bacteria under the phylum Proteobacteria but unidentified beyond class level were considered as *Proteobacteria_unclassified*. *Geobacter* although a Deltaproteobacteria was considered independent while the rest of the microbes i.e. *Anaeromyxobacter, Desulfobacca, Desulfobulbus, Desulfomicrobium, Desulfovibrio* and *Syntrophobacter* were reported under *Deltaproteobacteria_unclassified*.

To further understand the shared richness, among the three groups, a Venn diagram (Figure 3); displaying the overlaps between groups was developed to evaluate the distribution of OTUs among the ponds. Out of the total sum of 3636 identified OTUs (Table 3), 3185 OTUs with a 97% sequence similarity were observed within the three communities. From the entire bacterial community, 60.75% of the total OTUs were shared amongst the three ponds, while 22.42% were shared by at least two ponds i.e. P1 and P2; P2 and P3; then P1 and P3 shared 9.54, 9.36 and 3.52% OTUs respectively amongst the ponds. P1, P2 and P3 had 145, 186 and 205 un-shared OTUs respectively. Of the unshared OTUs it was observed that P1 differed from other ponds by exhibiting *Leptolyngbya*, *Chroococcidiopsis* and *Methylosoma* genera with the former pair being classified under Cyanobacteria and the latter one under Proteobacteria phyla (Supplementary Data SD2). The unshared microbes of P2 included; *Desulfovibrio*, *Tolumonas*, *Burkholderia* and *Cylindrospermopsis* with the former trio classified under Phylum Proteobacteria and the latter Cyanobacteria. For P3, the distinctiveness lied in registered *Azospira and Thiocapsa*, genera classified under phylum Proteobacteria, *Chroococcidiopsis* under Cyanobacteria *and Acetobacterium* under the Firmicutes phyla.

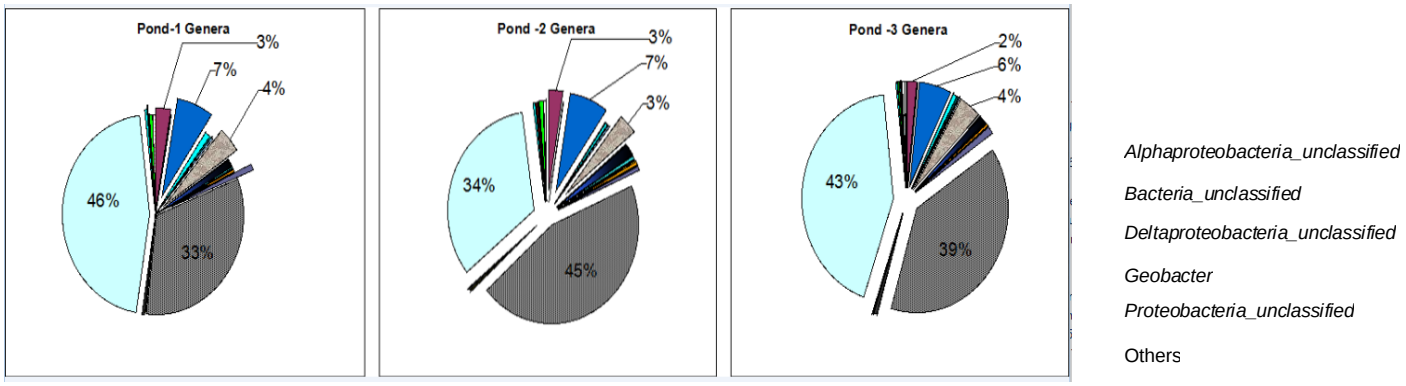
Results from the heat map, (Figure 4); revealed the intensity of the relative abundance of each genus, as represented by a gradient of colors observed from green (low abundance) to red (high abundance). The genus composition and abundance of nitrogen fixation microbes in the ponds was based on complete linkage clustering. The abundant genera clustered included *Proteobacteria_unclassified, Candidatus_Accumulibacter, Bacteria_unclassified, Deltaproteobacteria_unclassified,*

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



Bacterial communities distributed 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 with percentage representation in the four months sediment samples (May –MS; July – JS; September – SS and October- OS).

Figure 2: Relative Abundance of genera distributions of Bacteria within the community structures of Pond 1, 2 and 3 in Yi Xing



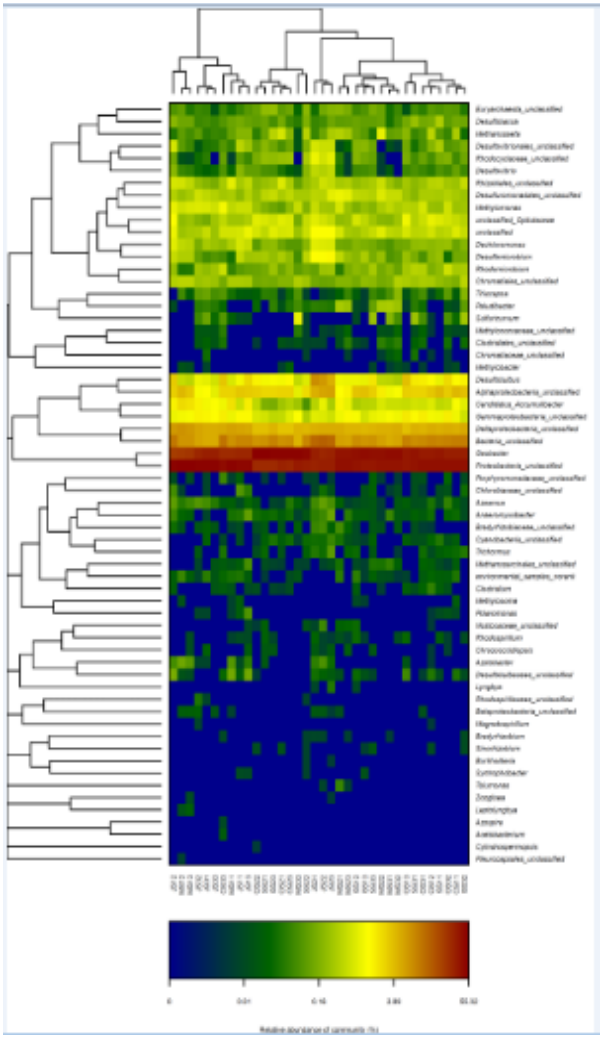
Bacterial community distribution at genus levels in pond 1, 2 & 3 sediment samples. The abundances are presented in percentages of taxon numbers affiliated to that genus within the dominant effective bacteria taxa.

Figure 3: Venn diagram displaying overlaps between genus groups of microbial communities within Tilapia ponds at different Stocking Densities

Bacterial communities of ponds P1, P2 & P3 sediment based on the sequential identification of (97% similarity) shared OTUs. P1is stocked with 1,500 fish, P2 with 1,200 fish while P3 has 1,800 fish per 667sq.M

Desulfobulbus, Gammaproteobacteria_unclassified, Candidatus_Accumulibacter, and Alphaproteobacteria_unclassified. Distinctive bacterial compositions were found in the nitrogen fixation microbiota of P3, which practiced polyculture; this was significantly associated with the Shannon index. Another observation while comparing pond bacterial communities revealed 5 clusters from the number of sequences affiliated with OTUs and displayed at the top of the heat map, i.e. cluster 1(at the extreme right) initially disclosed microbial communities flourishing in P1 and P3 being grouped together, revealing a relationship between those communities, that eventually interrelated with other clusters, that included: Cluster 2, with microbe communities in all the ponds; Cluster 3 revealed communities in P2 and P3; Cluster 4 with communities of P2 and P3: while cluster 5 had representation similar to cluster 1.

Figure 4: Heat chart showing hierarchical cluster at the genus level of micro-biota communities within Tilapia grow out ponds of Yi Xing.



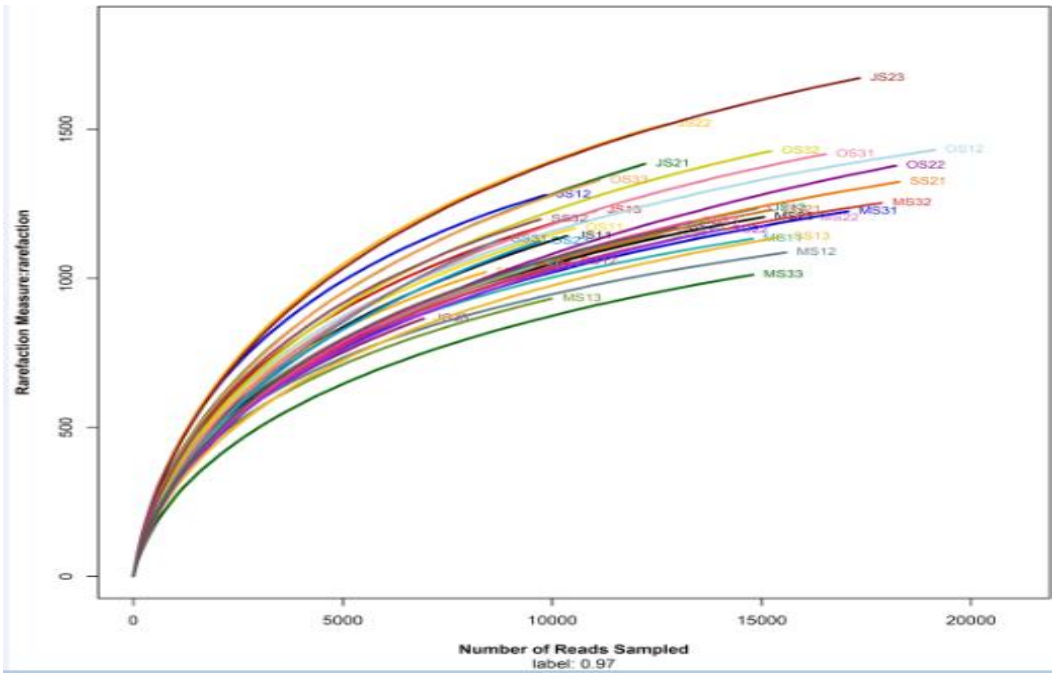
Abundances are determined with color differentiation, lighter green displays low abundance and red the highest abundance

To estimate the species richness, within the study ponds at different stocking densities Rarefaction curves were used to determine similarity levels of microbiota. Results showed that, the species richness was significantly high in P2 micro-biota with the highest rarefaction measure being 1,672 OTUs out of 17,330 sample reads in *JS* 23. The lowest measure readings were observed in pond 1 in the month of May especially in *MS* 13 with a rarefaction measure of 932 OTUs out of 9980 sample readings. However, the shape of the curve revealed that the total richness of the microbial community might have not reached completion (Figure 5).

From our observed results, Shannon Weiner Rarefaction curves are obtained after the calculus to estimate richness (Katherine RA 2013) (at a 96% similarity level) of nitrogen fixation micro-biota reflected among the three groups at genus level. Graphically presented in (Supplementary Figure SF3); on average, October and September microbe samples registered the most and least number of reads respectively. The least registered sequential number of OTUs was 4 OTUs in pond 2 in October (*OS*22) and the highest registered 6 OTUs in pond 1 in July (*JS* 12) samples respectively.

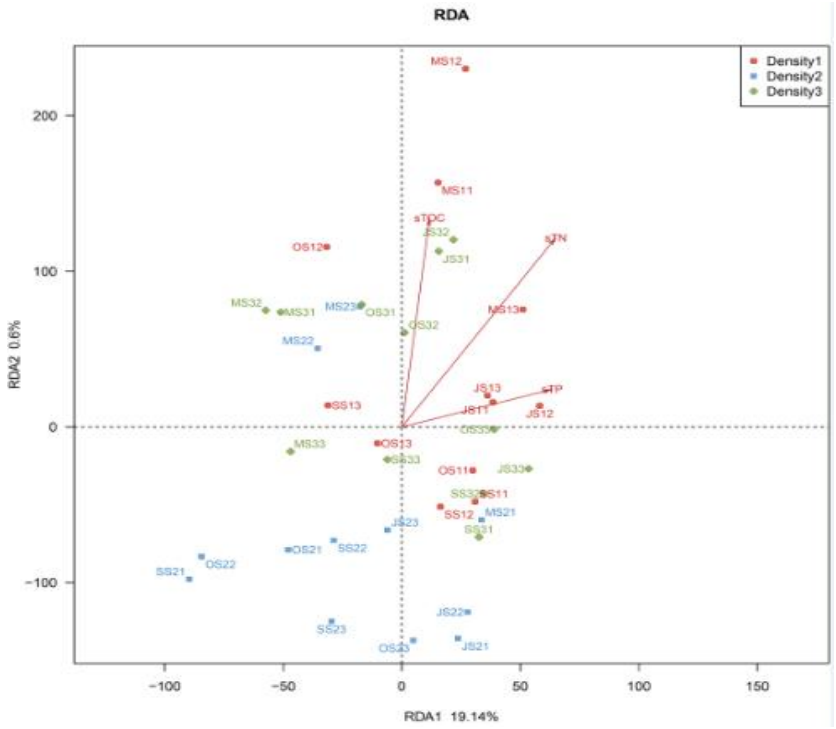
The Environmental parameters', influences on the bacterial community were analyzed by Redundancy Analysis, (RDA), in (Figure 6). As shown by the arrows the introduced environmental variables influenced the occurrence of the *nifH* microbes differently both in extent and direction. TOC, content proved to be the highest effective explanatory factor for variance of the *Geobacter* genera that we used to assess the microbial reaction in P1, P2 and P3 for the minimum inhibitory determination causing total lack of expression. Furthermore, RDA1 revealed the most important in the evaluation of ordinates was predominantly determined by the TOC content in the soil with r = 0.897 values. RDA2 was determined by more variables i.e. extractable TP, TN, pH and the RDA1 and RDA2 axes together explained the data variations. The score plots revealed that TOC accounted for 19.14% of genus density variance in the majority of P1 and P3 microbial communities grouped to the positive or upper side of the graph while the other environmental variables revealed a 0.6% variance in all ponds. The correlation between TOC and RDA 1 highly influenced the microbial communities of *July* and *October* samples of P3 that included *Desulfobulbus, Candidatus_Accumulibacter, Geobacter, a*nd *Methylomonas.* TP that best correlated with RDA 2 measures influenced the July micro-biota within P1 (*JS12* and *JS11* samples). From the microbial RDA plot (*Figure not presented*) under the temporal specificity observed results revealed that the 80% of the microbe communities in all ponds were well distributed on the positive side of the graph in July and on the negative side in October with TOC showing the best ordination as above that accounted for a density variances of 19.14%. In May and September P1 and P3 communities were grouped together on the positive side signifying closer relationships between the ponds while on the contrary P2

Figure 5: Rank distribution abundance curve, showing the tails in the OTU rank relative abundance curves.



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.

Figure 6: Redundancy Analysis plot for the microbial genus densities in Ponds 1, 2 and 3 of Yi Xing



RDA ordination plots for the first two principal dimensions of the relationship between the environmental parameters of the ponds and the nifH-gene densities of the sediment harboring nifH-gene microbial assemblages analyzed using data of the nifH OTUs. 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.

communities were on the negative side. RDA2 variances similar to those of the density above revealed that only in May were communities of P1, P2 and P3 grouped together on the right side suggesting positive environmental influences to these closely related microbes.

DISCUSSION

In this study, diverse microbes, encoding *nifH* were identified and characterized. *nifH* - *gene* sequencing, phylogenetic analysis and quantification of the copies of the *nifH*-gene encoding microbiota abundances together congruently showed that phylum Proteobacteria especially genus *Geobacter* were the most dominant nitrogen fixation microbes in the pond sediment populations. The identified taxa were closely related in all the three ponds and included: *Bradyrhizobium, Magnetospirillum, Rhodomicrobium, Rhodospirillum Sinorhizobium, Azotobacter, Methylobacter, Methylomonas, Thiocapsa, Geobacter, Anaeromyxobacter, Desulfobacca, Desulfobulbus, Desulfomicrobium, Desulfovibrio* and *Syntrophobacter* genera together with *Proteobacteria_unclassified, Alphabacteria_unclassified Deltabacteria_unclassified* and *Bacteria_unclassified among others*. P2 uniquely exhibited distinctive OTUs probably from the *Burkholderia & Tolumonas* genera and P3 displayed an OTU most probably found in genus *Azospira.* The *Azotobacter,* Rhizobium, *Rhodospirillum and Sinorhizobium* genera are well known for the ability to improve plant development (Gonzalez LJ 2005, Emtiazi G 2007) as they excrete more than one hormone e.g. *Azotobacter* isolates synthesis *gibberellins, auxin and cytokinins* a focal point in promoting hydroponics. However, we are most likely the first ones to observe them in pond culture systems thus a pre-requisite for an in-depth understanding.

In sediment ecosystems, environmental conditions such as physical stratification and chemical gradients help to create and maintain high levels of diversity between and within bacterial communities (Lozupone and Knight 2007, Ye J 2012). This plays a vital role in shaping the abundance and spatial distribution of nitrogen fixation bacteria in the sediment. In this study, we observed 10 phyla with varying dominations and distribution in all the three ponds throughout the production period (Figure 1). The abundances of Phylum Chlorobi in P1 and P2 in the early months of culture could be attributed to its survival under limited organic carbon. As the accumulation of the content increases during the feeding process, the microbes fail to consume organic carbon for their growth (Bryant DA 2006) under monoculture systems. However P3 that practiced polyculture displayed all phyla at all stages of sampling, which could be attributed to the model of farming where the other stocked species could have facilitated the breakdown of the organic carbon although this requires further studies.

The TP, TN and TOC findings in comparison with different methodologies used during the experiment, revealed significant effects of environmental conditions in shaping bacterial community structures and diversity, that are in agreement with previous research works, (Dang 2013, Wang LP 2013, Wang LP 2014). With the exception of sediment pH and temperatures, that were within the microbial optimum growth range, as suggested in (Huijie L 2014), these findings showed that the diversity of the sediment bacterial community correlated mainly with the sediment TOC and TP, while the microbial abundances and diversity did correlate to pH and temperature. The relationship between environmental conditions and the bacterial community distribution indicated representation of abundant proportions of unclassified microbes found distributed at all taxa levels from domain to genera (Figures 1, 2 & Supplementary Figure SF1).

The microbes examined in the nutrient contents in correlation to physiochemical parameters revealed that TOC, TN and TP are the key factors that shaped the microbial community structure (Table 1). These factors could have had an influential function on the microbial community diversity and phylogeny differences. Effects of the above factors, on the environmental functional gene expression, revealed the major probable genera with higher relative abundances and key nitrogen fixers were genus *Geobacter*. These diverse taxa could break down free and inorganic nitrogen (N_2 & NH_3^+) into amino acids for uptake by target plants in ponds, aquaponics and hydroponic systems that eliminate or reduce pollution during production.

In PCR based community characterization as suggested by various authors, there are many potential biases including differential DNA extraction efficiencies (Hollister EB. 2010), hence the option of new characterization techniques as, Illumunia throughput, was adopted to increase the feasibility of dramatic numbers of sequences in a single study. The technique allowed a deeper coverage and provision of new insights regarding microbial communities and their environmental(Acosta-Martı´nez V 2008) interactions (Sogin 2006, Turnbaugh 2006, Acosta-Martı´nez 2008).

Although investigations on environmental *nifH* encoding genes have been done in different environments for decades, there is limited knowledge of cultural sediment especially diazotrophic microbial communities (Zehr JP 2003) and probably not all of the detected *nifH* encoding sequences come from active diazotrophic microbes (Dang 2013). Since, not all the *nifH* encoding sequences originally defined at phylum, class or genus levels of environmental samples are characterized, some may not to be involved in N_2 fixation (Raymond J 2004, Staples 2007.). Although the *nifH* database, are used to evaluate the diversity of *nifH* genes (Gaby JC 2011), in different environments and to evaluate PCR primers under environmental surveys of *nifH* diversity (Gaby JC 2012), functional gene analyses have limited primer designs,

conforming to insignificant numbers and diversity of *nifH* sequences available, (Gaby JC 2012). However to-date, its notable that as sequential numbers in public databases grow the PCR performance is evaluated through considering undesirable effects for high level primer degeneracy on universal primers (Gaby JC 2014). Evaluation on the primer combination, *nifH-1F and nifH-2R*; generated the projected coverage pair that produced the best performance for the empirical analysis within the diverse nitrogen-fixation strains which in turn produced lower coverage for each individual primer as suggested by (Ro¨sch 2005, Smith CJ 2006, Gaby JC 2014).

In comparison to Wang's works (Wang LP 2013, Wang LP 2014), the study conducted experiments to observe the abundances of *nifH*- encoding microbes through quantification of the *nifH* functional genes. It was observed that the systems sediment microbial communities harbored a wide variety of taxa with large proportions of individuals that had limited matches with existing molecular databases. However to our knowledge this study is the first to quantify *nifH* genes in freshwater intensive tilapia aquaculture ponds that identified communities of *Acetobacterium, Anaeromyxobacter, Azoarcus, Candidatus_Accumulibacter, Azospira, Azotobacter, Bradyrhizobium, Burkholderia, Chromatiales, Chroococcidiopsis, Cylindrospermopsis, Dechloromonas, Desulfobacca, Desulfobulbus, Desulfomicrobium, Desulfuromonadales, Desulfovibrio, Geobacter, Leptolyngbya, Lyngbya, Magnetospirillum, Methanosaeta, Methylobacter, Methylomonas, Polaromonas, Rhodomicrobium, Rhodospirillum, Sinorhizobium, Sulfuricurvum, Syntrophobacter, Thiocapsa, Tolumonas, unclassified_Opitutaceae Trichormus, and Zoogloea microbial* genera. The success of detection of the variety of novel *nifH* encoding sequences is attributed to the use of the primer designs (Dang 2013). P2 displayed distinctive genera of *Burkholderia and Tolumonas*, while *Azospira* was observed in P3 that practiced polyculture, with the highest fish stocking density and *nifH* gene abundance. The benthic Carps' activities, most likely explained the modification of the microbial environment that facilitated the breakdown of organic carbon in the decomposing waste feeds. The decrease in microbial abundances in deeper sediment of P2 and P1 in that respective order might have been due to a depletion of potential electron acceptors such as NO_3 and O_2 that influenced indirectly via rhizosphere nitrification in the case of nitrates (Thomas F 2014) or directly by oxygenation in free living form, Azotobacter (aerobic bacteria) (Affourtit J 2001), facilitated through aeration by the activities of the benthic feeders movements.

Another approach, characterizing functional genes revealed a unique Yi Xing pond environment with great diversity and novelty within the *OTU* sequences as represented in (Figure 3). The abundances of related micro-biota with similar OTU sequences observed across the three ponds signified the effects of the environmental structure towards the microbes, although several conditions might have played a role in this observation. In Wang's studies, (Wang LP 2014), various authors argued that environmental settings influenced the diverse microbial selection within the sediment such as habitat specificity (Hewson 2006) and similar environmental conditions (Hewson I 2007).

The heat-map, suggested that ponds harbored specific environmental microbes based on the complete linkage method (Elie J 2013), this revealed 5 community clusters with relative abundances obtained (Figure 4). Clusters 1 and 5, suggested that P1 and P3 microbes shared similar community and evolutionary structures, although these might be expressed differently at species levels. Cluster 2 observations revealed that the microbes in all the ponds may be closely linked or related. While cluster 3 and 4 revealed the existence of family members within P2 and P3 to be evolutionary closer at their genus levels

Rarefaction curves (Figure 5), displaying the expected OTU numbers against the number of tags or sequences in relation to the shape of the *nifH* curves suggested no further OTUs were to be expected if more clones were sequenced. However, the characterization of the bacterial taxa based on DNA extractions depended on the quality of the DNA retrieved, the DNA amplification by PCR and the primers used. Formation of flattened curves at the end of amplification cycle deduced the best results. However at this stage although most samples registered positive yields, all results of environmental samples analyzed yielded exclusively unknown sequences (Schloss PD 2004)

The effects of physiochemical parameters on the bacterial community analyzed using RDA, (Figure 6), furthermore clarified the different microbial genera, influenced by the stocking densities and the nutrient variables. Out of the identified 62 genera, this study focused on the dominant genera that were significantly influenced by the environmental conditions which included: *Candidatus_Accumulibacter, Dechloromonas, Desulfobulbus, Desulfomicrobium, Geobacter, Methylomonas, and unclassified_Opitutaceae,* affected by TP concentrations in P1, while TN concentrations greatly influenced genus *Geobacter, Candidatus_Accumulibacter, and Methylomonas* distributions in the same pond. In P3 *Geobacter, Candidatus_Accumulibacter, Methylomonas,* and *Desulfobulbus*, revealed a pattern of correlation influence by TOC levels. Similarly, the different genera observed in the monthly studies, revealed the distribution pattern in correlation to the environmental and nutrient variables. The first RDA axes explained the 19.14% total variations in the dominant phyla while the other axes explained the 0.6% of the cumulative variations in the dominant phyla - environment relationship. As the study revealed TOC and TP significantly correlated with RDA 1 and RDA 2 respectively, stocking densities under 1,800 fish per 667 sq. M didn't influence the microbial abundances although the Polyculture system (P3) registered significantly higher

abundances of microbial densities and correlated highly with the environmental factors as compared to the Monoculture systems in P1 & P2.

Previous studies reported the capacity of nitrogen fixation microbes in terrestrial farmlands to facilitate the breakdown of nitrates, NH_3, and other inorganic nitrogenous compounds through enzymatic actions for plant uptake and boost yields. To our knowledge, this is the first identification of specific bacterial groups of *nifH* encoding genes that would contribute to the N_2 – fixation in aquaculture systems to boost the fish cum horticulture pond production system. The present study complements the existing body of knowledge on the N_2 – fixers in aquaculture systems by providing lacking information in its effect of environmental conditions and microbial interactions within different pond setups, but sets stage for future investigations on enzymatic catalysis and oxidative part of the characterized microbes at species levels in higher stocking densities and in-situ experiments.

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Ethical or competing interests

This study never required specific permission, nor involved endangered or protected species and the study plan was reviewed and approved by the ethics committee of the Eco-environment department of the FFRC for the CAFs.

REFERENCES

Acosta-Martı´nez V, Dowd S, Sun Y, Allen V (2008). "Tagencoded pyrosequencing analysis of bacterial diversity in a single soil type as affected by management and land use." Soil Biol Biochem(40): 2762-2770.

Affourtit J, Zehr JP and Paerl HW (2001). "Distribution of Nitrogen-Fixing Microorganisms along the Neuse River Estuary, North Carolina." Microb Ecol 41(2): 114-123.

Avnimelech Y (2007). "Feeding with microbial flocs by tilapia in minimal discharge bio-flocs technology ponds." Aquaculture 264(1-4): 140-147.

Bagali S, Shridhar (2012). "Review: Nitrogen Fixing Microorganisms." International Journal of Microbiological Research 3(1): 46-52.

Baker GC, Smith JJ and Cowan DA (2003). "Review and re-analysis of domain-specific 16S primers." J Microbiol Methods 55(3): 541-555.

Bryant DA, Frigaard NU, (2006). "Prokaryotic photosynthesis and phototrophy illuminated." Trends Microbiol (14): 488-496.

Caporaso JG, J Kuczynski, J Stombaugh, K Bittinger, FD Bushman, EK Costello, N Fierer, AG Peña, JK Goodrich, JI Gordon, GA Huttley, ST Kelley, D Knights, JE Koenig, RE Ley, CA Lozupone, D McDonald, B Muegge D, M Pirrung, J Reeder, JR Sevinsky, PJ Turnbaugh, WA Walters, J Widmann, T Yatsunenko, J Zaneveld and R Knight (2010). "QIIME allows analysis of high-throughput community sequencing data." Nature Methods 7(5): 335-336.

Dang, H, Yang J, Li J, Luan X, Zhang Y, Gu G, Xue R, Zong M, Klotz MG (2013). "Environment-Dependent Distribution of the Sediment nifH-Harboring Microbiota in the Northern South China Sea." Appl Environ Microbiol: 121-132.

Dang HY, Chen RP, Wang L, Guo LZ, Chen PP, Tang ZW, Tian F (2010). "Environmental factors shape sediment anammox bacterial communities in hyper mutrified Jiaozhou Bay. China." Appl Environ Microbiol 76: 7036-7047.

Edgar RC (2010). "Search and clustering orders of magnitude faster than BLAST." Bioinformatics 26(19): 2460-2461.

Elie Jami , et al. (2013). " Exploring the bovine rumen bacterial community from birth to adulthood. ." ISME

Emtiazi G, Pooyan M and Shamalnasab M (2007). "Cellulase Activities in Nitrogen Fixing Paenibacillus Isolated from Soil in N-free Media." World Journal of Agricultural Sci. 3: 602-608.

Eren AM, JH Vineis, HG Morrison and ML Sogin (2013). "A filtering method to generate high quality short reads using illumina paired-end technology." PLoS One 8(6): e66643.

Gaby JC and DH Buckley (2011). "A global census of nitrogenase diversity." Environ Microbiol 13(7): 1790-1799.

Gaby JC and DH Buckley (2012). "A comprehensive evaluation of PCR primers to amplify the nifH gene of nitrogenase." PLoS One 7(7): e42149.

Gaby JC and DH Buckley (2014). "A comprehensive aligned nifH gene database: a multipurpose tool for studies of nitrogen-fixing bacteria." Database (Oxford) 2014: bau001.

Gonzalez LJ, Rodelas B, Pozo C, Salmeron V, Mart nez MV and Salmeron V (2005). "Liberation of amino acids by heterotrophic nitrogen fixing bacteria " Amino Acids 28: 363-367.

Hewson I, Fuhrman JA (2006). "Spatial and vertical biogeography of coral reef sediment bacterial and diazotroph communities. ." Mar. Ecol. Prog. Ser. 306: 79-86.

Hewson I, M E Jacobson Meyers and JA Fuhrman (2007). "Diversity and biogeography of bacterial assemblages in surface sediments across the San Pedro Basin, Southern California Borderlands." Environ Microbiol 9(4): 923-933.

Hollister EB, Engledow, Amanda, SA, Hammett JM, Provin TL, Wilkinson HH and Gentry TJ (2010). "Shifts in microbial community structure along an ecological gradient of hypersaline soils and sediments." The ISME Journal 4(4): 829–838.

Huijie L, C. K., Stensel D (2014). "Microbial ecology of denitrification in biological wastewater treatment." water research (6 4): 237 - 254.

Huse SM, Dethlefsen L., Huber JA, MarkWelch D, Welch DM, Relman DA et al (2008): "Exploring microbial diversity and taxonomy using SSU rRNA hypervariable tag sequencing." PLoSGenet. **4**: e1000255.

Johnson J, Bell J, De Young C (2013): Priority adaptations to climate change for Pacific fisheries and aquaculture; Reducing risks and capitalizing on opportunities. FAO FISHERIES AND AQUACULTURE PROCEEDINGS. Rome Italy, FAO.

Katherine RA (2013). "Habitat degradation impacts black howler monkey (Alouatta pigra) gastrointestinal microbiomes " The ISME Journal **7**: 1344–1353.

Lozupone CA. and R Knight (2007): "Global patterns in bacterial diversity." Proc Natl Acad Sci U S A **104**(27): 11436-11440.

McDonald D, Price MN, Goodrich J, Nawrocki EP, DeSantis TZ, Probst A, Andersen GL, Knight R, Hugenholtz P (2012): "An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea." ISME J **6**(3): 610-618.

Mergel A, Schmitz O, Mallmann T & Bothe H (2001): "Relative abundance of denitrifying and dinitrogen-fixing bacteria in layers of a forest soil." FEMS Microbiol Ecol **36**: 33-42.

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glockner FO (2013). "The SILVA ribosomal RNA gene database project: improved data processing and web-based tools." Nucleic Acids Res **41**(Database issue): D590-596.

Raymond J, Siefert JL, Staples CR, Blankenship RE (2004): "The natural history of nitrogen fixation." Mol Biol Evol **21**(3): 541-554.

Ro¨sch C, Bothe H (2005). "Improved assessment of denitrifying, N2 - fixing and total-community bacteria by terminal restriction fragment length polymorphism analysis using multiple restriction enzymes." Appl Environ Microbiol **71**: 2026-2035.

Rubio LM, L. P. (2002): "The gene products of the nif regulon. In: Leigh GJ, editor. Nitrogen fixation at the millennium" Elsevier Science B.V: 101-136.

Schloss PD, G. D., Westcott SL (2011): "Reducing the Effects of PCR Amplification and Sequencing Artifacts on 16S rRNA-Based Studies." PLoS ONE **6**(12): e27310.

Schloss PD, H. J. (2004): "Status of the microbial census." Microbiol Mol Biol Rev **68**(4): 686-691.

Schloss PD, W. S., Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF (2009): "Introducing mothur: opensource, platform-independent, community-supported software for describing and comparing microbial communities." Appl. Environ. Microbiol. **75**: 7537– 7541

Smith CJ, Nedwel DB, Dong LF, Osborn AM (2006). "Evaluation of quantitative polymerase chain reaction-based approaches for determining gene copy and gene transcript numbers in environmental samples." Environ Microbiol **8**(5): 804-815.

Sogin ML, Morrison HG, Huber JA, Welch DM, Huse SM, Neal PR (2006): "Microbial diversity in the deep sea and the underexplored 'rare biosphere'." Proc Natl Acad Sci **103**(103): 12115–12120.

Staples CR, Lahiri S, Raymond J, Von Herbulis L, Mukhophadhyay B, Blankenship RE (2007). "Expression and association of group IV nitrogenase NifD and NifH homologs in the non-nitrogen-fixing archaeon Methanocaldococcus jannaschii. ." J. Bacteriol. **189**: 7392-7398.

Thomas F, Giblin AE, Cardon ZG, Sievert SM (2014). "Rhizosphere heterogeneity shapes abundance and activity of sulfur-oxidizing bacteria in vegetated salt marsh sediments." Front Microbiol **5**(309): 309.

Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI (2006): "An obesity-associated gut microbiome with increased capacity for energy harvest." Nature **444**(444): 1027–1131.

Wang LP, Liu LS, Zheng BH, Zhu YZ, Wang X (2013): "Analysis of the bacterial community in the two typical intertidal sediments of Bohai Bay, China by pyrosequencing." Mar. Pollut. Bull. **72**(1): 181–187.

Wang LP, Liu LS, Zheng BH, Nan BX, Hu PL (2014): "Diversity of bacterial community and detection of nirS- and nirK- encoding denitrifying bacteria in sandy intertidal sediments along Laizhou Bay of Bohai Sea, China." Marine Pollution Bulletin.

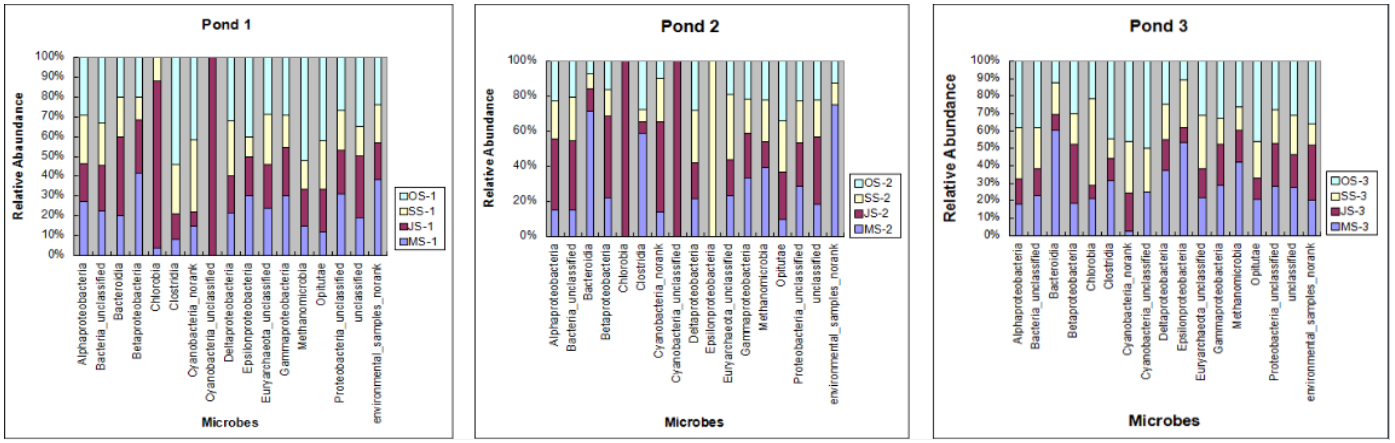
Wei FS, Ed. (2002): The state environmental protection administration, water and wastewater monitoring analysis methods of editorial committee of China environmental Science. Water and wastewater monitoring analysis methods Beijing, China environmental Science press

Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL (2012). "Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction." BMC Bioinformatics **13**(134).

Zehr JP, Jenkins BD, Short SM et al (2003): " Nitrogenase gene diversity and microbial community structure: a cross-system comparison." Environmental Microbiology **5**: 539–554.

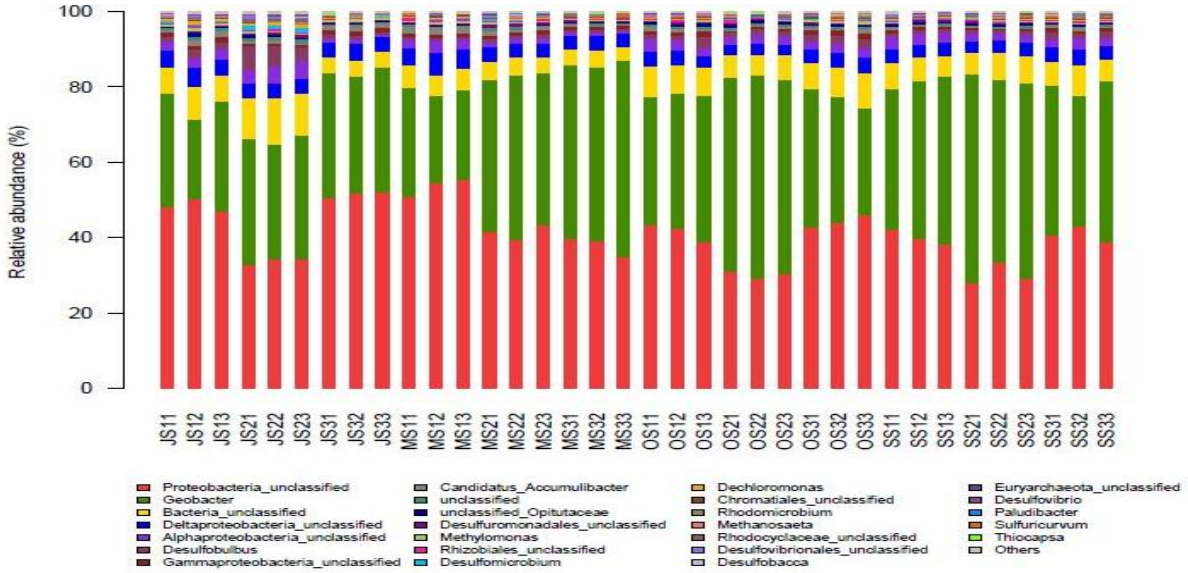
Supplementary Figure

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



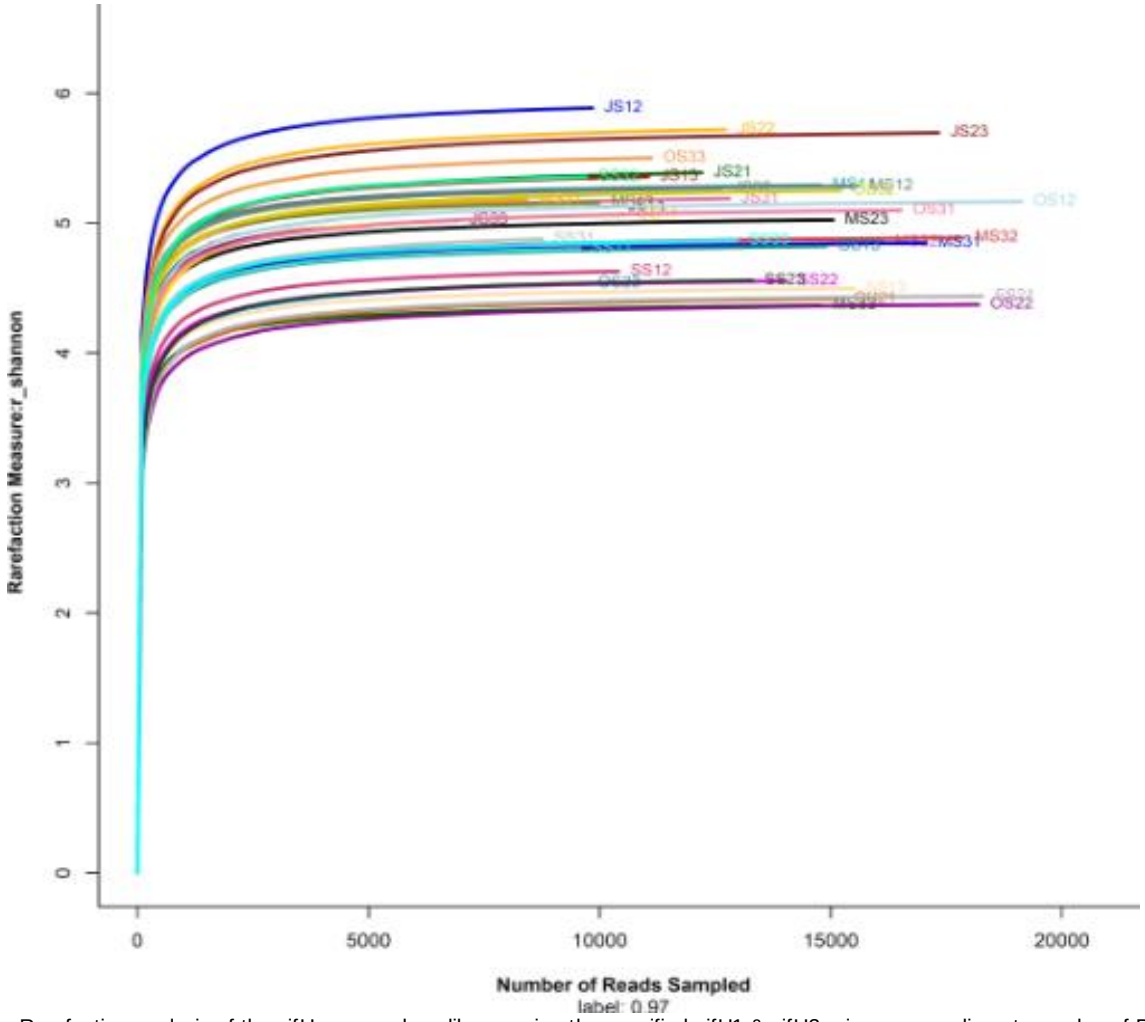
Bacterial communities class distributions in pond 1, 2 and 3 sediment samples. The abundances are presented in terms of taxon numbers affiliated to that class divided by the total effective bacteria taxa with percentage representation in the four months sediment samples (May –MS; July – JS; September – SS and October- OS).

SF2: Class Distribution Bar graphs for microbial communities in experimental sites in Ponds 1, 2 and 3 of Yi Xing city, China



Bacterial communities' distribution patterns in experimental sites in ponds 1, 2 & 3 sediment samples. The abundances are presented in terms of taxon numbers affiliated to that class divided by the total effective bacteria taxa for each sampled site.

SF 3 Showing Rare-fraction measures within the ponds analyzed with the r_Shannon index



Rarefaction analysis of the nifH – gene clone library using the specified nifH1 & nifH2 primers on sediment samples of Ponds 1, 2 & 3

SUPPLEMENTARY DATA

Supplementary Data SD1 Title: Supplementary data on SYBR Green standard curve and microbial communities in ponds 1, 2 and 3

Well	Sample Name		Target Name	Task	Reporter	Quencher	Cт	Cт Mean	Cт SD	Quantity	Quantity Mean	Automatic Ct Threshold	Ct Threshold		Automatic Baseline	Baseline Start	Baseline End	Tm 1	Comments	AMPNC	MTP
A1			STANDA		Non	13.59233	13.60067	0.011797			TRU	0.21576	TRU								
1		nifh	RD	SYBR	e	189	368	746	87000000		E	3948	E	3	8	87.06666565			N	N	
A1			STANDA		Non	13.60901	13.60067	0.011797			TRU	0.21576	TRU								
2		nifh	RD	SYBR	e	642	368	746	87000000		E	3948	E	3	8	87.06666565			N	N	
B1			STANDA		Non	17.55681	17.51744	0.055679			TRU	0.21576	TRU								
1		nifh	RD	SYBR	e	229	08	698	8700000		E	3948	E	3	12	87.2444458			N	N	
B1			STANDA		Non	17.47806	17.51744	0.055679			TRU	0.21576	TRU								
2		nifh	RD	SYBR	e	931	08	698	8700000		E	3948	E	3	12	87.06666565			N	N	
C1			STANDA		Non	21.20339	21.14596	0.081213			TRU	0.21576	TRU								
1		nifh	RD	SYBR	e	203	558	266	870000		E	3948	E	3	16	87.2444458			N	N	
C1			STANDA		Non	21.08853	21.14596	0.081213			TRU	0.21576	TRU								
2		nifh	RD	SYBR	e	912	558	266	870000		E	3948	E	3	16	87.06666565			N	N	
D1			STANDA		Non	24.95488	24.87327	0.115413			TRU	0.21576	TRU								
1		nifh	RD	SYBR	e	739	766	584	87000		E	3948	E	3	20	87.2444458			N	N	
D1			STANDA		Non	24.79166	24.87327	0.115413			TRU	0.21576	TRU								
2		nifh	RD	SYBR	e	794	766	584	87000		E	3948	E	3	19	87.06666565			N	N	
E1			STANDA		Non	28.23641	28.27843	0.059430			TRU	0.21576	TRU								
1		nifh	RD	SYBR	e	014	475	428	8700		E	3948	E	3	23	87.2444458			N	N	
E1			STANDA		Non	28.32045	28.27843	0.059430			TRU	0.21576	TRU								
2		nifh	RD	SYBR	e	746	475	428	8700		E	3948	E	3	23	87.06666565			N	N	
	JS1		UNKNO		Non	22.68772	22.81524	0.111701	318062.0	294101.1	TRU	0.18881	TRU								
C4	1	nifh	WN	SYBR	e	888	086	086	204	446	E	0387	E	3	17	90.45025635			N	N	
	JS1		UNKNO		Non	22.86218	22.81524	0.111701	285095.5	294101.1	TRU	0.18881	TRU								
C5	1	nifh	WN	SYBR	e	452	086	086	637	446	E	0387	E	3	18	90.61929321			N	N	
	JS1		UNKNO		Non	22.89580	22.81524	0.111701	279145.8	294101.1	TRU	0.18881	TRU								
C6	1	nifh	WN	SYBR	e	917	086	086	495	446	E	0387	E	3	18	90.61929321			N	Y	
	JS1		UNKNO		Non	22.46456	22.53614	0.075859	365848.3	350049.8	TRU	0.18881	TRU								
D4	2	nifh	WN	SYBR	e	528	235	129	62	048	E	0387	E	3	17	89.94314575			N	N	
	JS1		UNKNO		Non	22.52820	22.53614	0.075859	351531.7	350049.8	TRU	0.18881	TRU								
D5	2	nifh	WN	SYBR	e	969	235	129	129	048	E	0387	E	3	17	90.28121948			N	N	
	JS1		UNKNO		Non	22.61565	22.53614	0.075859	332769.3	350049.8	TRU	0.18881	TRU								
D6	2	nifh	WN	SYBR	e	971	235	129	396	048	E	0387	E	3	17	90.11218262			N	N	
	JS1		UNKNO		Non	22.87706	22.92260	0.039546	282446.9	274549.4	TRU	0.18881	TRU								
E4	3	nifh	WN	SYBR	e	566	933	371	37	124	E	0387	E	3	18	76.08223724			N	Y	
	JS1		UNKNO		Non	22.94825	22.92260	0.039546	270111.9	274549.4	TRU	0.18881	TRU								
E5	3	nifh	WN	SYBR	e	935	933	371	854	124	E	0387	E	3	18	90.61929321			N	Y	
	JS1		UNKNO		Non	22.94250	22.92260	0.039546	271089.3	274549.4	TRU	0.18881	TRU								
E6	3	nifh	WN	SYBR	e	107	933	371	149	124	E	0387	E	3	18	90.61929321			N	N	
	JS2		UNKNO		Non	21.45641	21.52130	0.063504	688524.0	661412.4	TRU	0.18881	TRU								
F4	1	nifh	WN	SYBR	e	518	699	368	335	787	E	0387	E	3	16	90.28121948			N	N	
	JS2		UNKNO		Non	21.52418	21.52130	0.063504	659872.0	661412.4	TRU	0.18881	TRU								
F5	1	nifh	WN	SYBR	e	137	699	368	527	787	E	0387	E	3	16	90.28121948			N	N	
	JS2		UNKNO		Non	21.58332	21.52130	0.063504	635841.3	661412.4	TRU	0.18881	TRU								
F6	1	nifh	WN	SYBR	e	634	699	368	499	787	E	0387	E	3	16	90.28121948			N	N	
	JS2		UNKNO		Non	22.05542	22.06027	0.005652	472878.2	471445.7	TRU	0.18881	TRU								
G4	2	nifh	WN	SYBR	e	755	222	84	944	179	E	0387	E	3	17	90.11218262			N	N	
	JS2		UNKNO		Non	22.06648	22.06027	0.005652	469610.7	471445.7	TRU	0.18881	TRU								
G5	2	nifh	WN	SYBR	e	254	222	84	425	179	E	0387	E	3	17	90.11218262			N	N	
	JS2		UNKNO		Non	22.05890	22.06027	0.005652	471848.1	471445.7	TRU	0.18881	TRU								
G6	2	nifh	WN	SYBR	e	465	222	84	168	179	E	0387	E	3	17	89.94314575			N	Y	
	JS2		UNKNO		Non	21.95537	21.86924	0.076219	503503.2	531853.7	TRU	0.18881	TRU								
H4	3	nifh	WN	SYBR	e	949	744	052	344	771	E	0387	E	3	17	90.11218262			N	N	
H5	JS2	nifh	UNKNO	SYBR	Non	21.84185	21.86924	0.076219	540662.6	531853.7	TRU	0.18881	TRU	3	17	90.11218262			N	N	

	3		WN		e	41	744	052	203	771	E	0387	E				
	JS2		UNKNO		Non	21.81051	21.86924	0.076219	551395.4	531853.7	TRU	0.18881	TRU				
H6	3	nifh	WN	SYBR	e	445	744	052	766	771	E	0387	E	3	17	90.11218262	N	N
	JS3		UNKNO		Non	22.11023	21.99867	0.103358	456898.6	490697.9	TRU	0.18881	TRU				
F7	1	nifh	WN	SYBR	e	521	249	731	624	03	E	0387	E	3	17	90.28121948	N	Y
	JS3		UNKNO		Non	21.97960	21.99867	0.103358	495909.3	490697.9	TRU	0.18881	TRU				
F8	1	nifh	WN	SYBR	e	854	249	731	789	03	E	0387	E	3	17	90.28121948	N	N
	JS3		UNKNO		Non	21.90617	21.99867	0.103358	519285.6	490697.9	TRU	0.18881	TRU				
F9	1	nifh	WN	SYBR	e	18	249	731	678	03	E	0387	E	3	17	90.28121948	N	N
	JS3		UNKNO		Non	22.20025	22.15276	0.074438	431816.6	445199.4	TRU	0.18881	TRU				
G7	2	nifh	WN	SYBR	e	253	146	989	226	805	E	0387	E	3	17	90.11218262	N	Y
	JS3		UNKNO		Non	22.06696	22.15276	0.074438	469467.5	445199.4	TRU	0.18881	TRU				
G8	2	nifh	WN	SYBR	e	892	146	989	034	805	E	0387	E	3	17	90.11218262	N	Y
	JS3		UNKNO		Non	22.19105	22.15276	0.074438	434314.3	445199.4	TRU	0.18881	TRU				
G9	2	nifh	WN	SYBR	e	721	146	989	155	805	E	0387	E	3	17	90.11218262	N	Y
	JS3		UNKNO		Non	21.46746	21.49351	0.055591	683769.6	672956.7	TRU	0.18881	TRU				
H7	3	nifh	WN	SYBR	e	254	692	609	584	956	E	0387	E	3	16	90.28121948	N	N
	JS3		UNKNO		Non	21.45573	21.49351	0.055591	688815.6	672956.7	TRU	0.18881	TRU				
H8	3	nifh	WN	SYBR	e	997	692	609	848	956	E	0387	E	3	16	90.11218262	N	N
	JS3		UNKNO		Non	21.55735	21.49351	0.055591	646285.0	672956.7	TRU	0.18881	TRU				
H9	3	nifh	WN	SYBR	e	207	692	609	436	956	E	0387	E	3	16	90.11218262	N	N
	MS1		UNKNO		Non	22.03431	22.06841	0.031639	479183.0	469104.0	TRU	0.13324	TRU				
B4	1	nifH	WN	SYBR	e	129	278	419	024	312	E	8295	E	3	18	90.53227234	N	N
	MS1		UNKNO		Non	22.07411	22.06841	0.031639	467368.3	469104.0	TRU	0.13324	TRU				
B5	1	nifH	WN	SYBR	e	385	278	419	214	312	E	8295	E	3	18	90.53227234	N	N
	MS1		UNKNO		Non	22.09681	22.06841	0.031639	460760.7	469104.0	TRU	0.13324	TRU				
B6	1	nifH	WN	SYBR	e	511	278	419	699	312	E	8295	E	3	18	90.53227234	N	N
	MS1		UNKNO		Non	20.85925	20.95641	0.107607	1001343.	943569.8	TRU	0.13324	TRU				
C4	2	nifH	WN	SYBR	e	865	327	223	998	989	E	8295	E	3	16	90.70952606	N	N
	MS1		UNKNO		Non	20.93790	20.95641	0.107607	953145.7	943569.8	TRU	0.13324	TRU				
C5	2	nifH	WN	SYBR	e	817	327	223	879	989	E	8295	E	3	16	90.88677979	N	N
	MS1		UNKNO		Non	21.07207	20.95641	0.107607	876219.9	943569.8	TRU	0.13324	TRU				
C6	2	nifH	WN	SYBR	e	298	327	223	113	989	E	8295	E	3	17	90.70952606	N	N
	MS1		UNKNO		Non	20.82027	21.05513	0.214970	1026129.	891026.2	TRU	0.13324	TRU				
D4	3	nifH	WN	SYBR	e	626	954	917	141	034	E	8295	E	3	16	90.70952606	N	N
	MS1		UNKNO		Non	21.10299	21.05513	0.214970	859389.5	891026.2	TRU	0.13324	TRU				
D5	3	nifH	WN	SYBR	e	492	954	917	074	034	E	8295	E	3	17	90.70952606	N	N
	MS1		UNKNO		Non	21.24215	21.05513	0.214970	787559.9	891026.2	TRU	0.13324	TRU				
D6	3	nifH	WN	SYBR	e	317	954	917	623	034	E	8295	E	3	17	90.70952606	N	N
	MS2		UNKNO		Non	19.97440	20.13139	0.142068	1744275.	1584944.	TRU	0.13324	TRU				
E4	1	nifH	WN	SYBR	e	72	153	818	151	863	E	8295	E	3	15	90.88677979	N	N
	MS2		UNKNO		Non	20.16864	20.13139	0.142068	1544206.	1584944.	TRU	0.13324	TRU				
E5	1	nifH	WN	SYBR	e	395	153	818	25	863	E	8295	E	3	16	90.88677979	N	N
	MS2		UNKNO		Non	20.25112	20.13139	0.142068	1466353.	1584944.	TRU	0.13324	TRU				
E6	1	nifH	WN	SYBR	e	152	153	818	188	863	E	8295	E	3	16	90.88677979	N	N
	MS2		UNKNO		Non	20.18614	20.28274	0.119911	1527343.	1440235.	TRU	0.13324	TRU				
F4	2	nifH	WN	SYBR	e	96	727	477	794	232	E	8295	E	3	16	90.88677979	N	N
	MS2		UNKNO		Non	20.24513	20.28274	0.119911	1471866.	1440235.	TRU	0.13324	TRU				
F5	2	nifH	WN	SYBR	e	817	727	477	567	232	E	8295	E	3	16	90.88677979	N	N
	MS2		UNKNO		Non	20.41695	20.28274	0.119911	1321495.	1440235.	TRU	0.13324	TRU				
F6	2	nifH	WN	SYBR	e	595	727	477	336	232	E	8295	E	3	16	90.88677979	N	N
	MS2		UNKNO		Non	20.92881	20.86369	0.074128	958596.9	999287.2	TRU	0.13324	TRU				
G4	3	nifH	WN	SYBR	e	584	133	173	99	959	E	8295	E	3	16	90.70952606	N	N
	MS2		UNKNO		Non	20.78302	20.86369	0.074128	1050387.	999287.2	TRU	0.13324	TRU				
G5	3	nifH	WN	SYBR	e	383	133	173	402	959	E	8295	E	3	16	90.70952606	N	N
	MS2		UNKNO		Non	20.87923	20.86369	0.074128	988877.4	999287.2	TRU	0.13324	TRU				
G6	3	nifH	WN	SYBR	e	241	133	173	865	959	E	8295	E	3	16	90.70952606	N	N
	MS3		UNKNO		Non	18.99343	18.98378	0.022595	3227233.	3247040.	TRU	0.13324	TRU				
H4	1	nifH	WN	SYBR	e	3	563	974	014	779	E	8295	E	3	14	90.53227234	N	N
	MS3		UNKNO		Non	18.95796	18.98378	0.022595	3299829.	3247040.	TRU	0.13324	TRU				
H5	1	nifH	WN	SYBR	e	585	563	974	502	779	E	8295	E	3	14	90.53227234	N	N
	MS3		UNKNO		Non	18.99995	18.98378	0.022595	3214059.	3247040.	TRU	0.13324	TRU				
H6	1	nifH	WN	SYBR	e	422	563	974	82	779	E	8295	E	3	14	90.53227234	N	N
	MS3		UNKNO		Non	19.68523	19.64855	0.031850	2091149.	2140095.	TRU	0.13324	TRU				
A7	2	nifH	WN	SYBR	e	407	766	085	833	282	E	8295	E	3	15	90.53227234	N	N

	MS3		UNKNO		Non	19.63258	19.64855	0.031850	2161356.	2140095.	TRU	0.13324	TRU					
A8	2	nifH	WN	SYBR	e	553	766	085	993	282	E	8295	E	3	15	90.53227234	N	N
	MS3		UNKNO		Non	19.62785	19.64855	0.031850	2167779.	2140095.	TRU	0.13324	TRU					
A9	2	nifH	WN	SYBR	e	53	766	085	02	282	E	8295	E	3	15	90.35502625	N	N
	MS3		UNKNO		Non	19.27704	19.11634	0.139175	2701314.	2995254.	TRU	0.13324	TRU					
B7	3	nifH	WN	SYBR	e	43	254	579	79	902	E	8295	E	3	15	90.70952606	N	N
	MS3		UNKNO		Non	19.03489	19.11634	0.139175	3144388.	2995254.	TRU	0.13324	TRU					
B8	3	nifH	WN	SYBR	e	494	254	579	334	902	E	8295	E	3	14	90.70952606	N	N
	MS3		UNKNO		Non	19.03709	19.11634	0.139175	3140061.	2995254.	TRU	0.13324	TRU					
B9	3	nifH	WN	SYBR	e	03	254	579	581	902	E	8295	E	3	15	90.53227234	N	N
D1	OS1		UNKNO		Non	21.08822	21.02297	0.084320	867387.1	904471.8	TRU	0.13324	TRU					
0	1	nifH	WN	SYBR	e	632	783	91	479	039	E	8295	E	3	17	90.88677979	N	N
D1	OS1		UNKNO		Non	21.05293	21.02297	0.084320	886800.3	904471.8	TRU	0.13324	TRU					
1	1	nifH	WN	SYBR	e	655	783	91	203	039	E	8295	E	3	17	90.70952606	N	N
D1	OS1		UNKNO		Non	20.92776	21.02297	0.084320	959227.9	904471.8	TRU	0.13324	TRU					
2	1	nifH	WN	SYBR	e	68	783	91	435	039	E	8295	E	3	17	90.53227234	N	N
E1	OS1		UNKNO		Non	20.20326	20.09232	0.102767	1511037.	1622151.	TRU	0.13324	TRU					
0	2	nifH	WN	SYBR	e	233	903	378	811	77	E	8295	E	3	16	90.70952606	N	N
E1	OS1		UNKNO		Non	20.00037	20.09232	0.102767	1716096.	1622151.	TRU	0.13324	TRU					
1	2	nifH	WN	SYBR	e	384	903	378	612	77	E	8295	E	3	15	90.70952606	N	N
E1	OS1		UNKNO		Non	20.07334	20.09232	0.102767	1639320.	1622151.	TRU	0.13324	TRU					
2	2	nifH	WN	SYBR	e	709	903	378	886	77	E	8295	E	3	15	90.53227234	N	N
F1	OS1		UNKNO		Non	20.74102	20.69242	0.051930	1078426.	1112200.	TRU	0.13324	TRU					
0	3	nifH	WN	SYBR	e	211	096	703	837	421	E	8295	E	3	16	90.70952606	N	N
F1	OS1		UNKNO		Non	20.69854	20.69242	0.051930	1107547.	1112200.	TRU	0.13324	TRU					
1	3	nifH	WN	SYBR	e	164	096	703	288	421	E	8295	E	3	16	90.53227234	N	N
F1	OS1		UNKNO		Non	20.63770	20.69242	0.051930	1150627.	1112200.	TRU	0.13324	TRU					
2	3	nifH	WN	SYBR	e	294	096	703	139	421	E	8295	E	3	16	90.35502625	N	N
G1	OS2		UNKNO		Non	19.78854	19.76786	0.020114	1959947.	1985643.	TRU	0.13324	TRU					
0	1	nifH	WN	SYBR	e	179	041	565	068	541	E	8295	E	3	15	90.70952606	N	N
G1	OS2		UNKNO		Non	19.74836	19.76786	0.020114	2009964.	1985643.	TRU	0.13324	TRU					
1	1	nifH	WN	SYBR	e	54	041	565	135	541	E	8295	E	3	15	90.70952606	N	N
G1	OS2		UNKNO		Non	19.76667	19.76786	0.020114	1987019.	1985643.	TRU	0.13324	TRU					
2	1	nifH	WN	SYBR	e	023	041	565	421	541	E	8295	E	3	15	90.53227234	N	N
	OS2		UNKNO		Non	undetermi	undetermi	undetermi	undetermi	undetermi	TRU	0.21576	TRU					
A1	2	nifh	WN	SYBR	e	ned	ned	ned	ned	ned	E	3948	E	3	32		N	N
	OS2		UNKNO		Non	20.64051	20.64843	0.011198	1148525.	1142849.	TRU	0.21576	TRU					
A2	2	nifh	WN	SYBR	e	819	75	249	125	25	E	3948	E	3	15	90.6222229	N	N
	OS2		UNKNO		Non	20.65635	20.64843	0.011198	1137173.	1142849.	TRU	0.21576	TRU					
A3	2	nifh	WN	SYBR	e	49	75	249	25	25	E	3948	E	3	15	90.80000305	N	N
	OS2		UNKNO		Non	20.41579	20.37003	0.044660		1361236.	TRU	0.21576	TRU					
B1	3	nifh	WN	SYBR	e	628	326	024	1322374	875	E	3948	E	3	15	90.80000305	N	N
	OS2		UNKNO		Non	20.36773	20.37003	0.044660	1362842.	1361236.	TRU	0.21576	TRU					
B2	3	nifh	WN	SYBR	e	682	326	024	25	875	E	3948	E	3	15	90.80000305	N	N
	OS2		UNKNO		Non	20.32656	20.37003	0.044660	1398494.	1361236.	TRU	0.21576	TRU					
B3	3	nifh	WN	SYBR	e	479	326	024	5	875	E	3948	E	3	15	90.9777832	N	N
	OS3		UNKNO		Non	21.40948	21.44355	0.058573	709049.6	694368.6	TRU	0.21576	TRU					
C1	1	nifh	WN	SYBR	e	105	202	261	25	25	E	3948	E	3	16	90.6222229	N	N
	OS3		UNKNO		Non	21.40998	21.44355	0.058573	708824.8	694368.6	TRU	0.21576	TRU					
C2	1	nifh	WN	SYBR	e	65	202	261	75	25	E	3948	E	3	16	90.6222229	N	N
	OS3		UNKNO		Non	21.51118	21.44355	0.058573	665231.4	694368.6	TRU	0.21576	TRU					
C3	1	nifh	WN	SYBR	e	469	202	261	375	25	E	3948	E	3	16	90.6222229	N	N
	OS3		UNKNO		Non	21.05420	20.97561	0.070886	886037.3	931420.8	TRU	0.21576	TRU					
D1	2	nifh	WN	SYBR	e	876	073	321	75	125	E	3948	E	3	16	90.80000305	N	N
	OS3		UNKNO		Non		20.97561	0.070886	942266.9	931420.8	TRU	0.21576	TRU					
D2	2	nifh	WN	SYBR	e	20.95611	073	321	375	125	E	3948	E	3	16	90.80000305	N	N
	OS3		UNKNO		Non	20.91651	20.97561	0.070886	965958.3	931420.8	TRU	0.21576	TRU					
D3	2	nifh	WN	SYBR	e	917	073	321	75	125	E	3948	E	3	16	90.80000305	N	N
	OS3		UNKNO		Non	21.18027	21.20173	0.047357	818676.6	807966.1	TRU	0.21576	TRU					
E1	3	nifh	WN	SYBR	e	306	645	09	25	875	E	3948	E	3	16	90.6222229	N	N
	OS3		UNKNO		Non	21.16890	21.20173	0.047357	824532.8	807966.1	TRU	0.21576	TRU					
E2	3	nifh	WN	SYBR	e	907	645	09	125	875	E	3948	E	3	16	90.80000305	N	N
	OS3		UNKNO		Non	21.25602	21.20173	0.047357	780689.1	807966.1	TRU	0.21576	TRU					
E3	3	nifh	WN	SYBR	e	341	645	09	875	875	E	3948	E	3	16	90.80000305	N	N
	SS1		UNKNO		Non	21.42035	21.42775	0.025205		701028.5	TRU	0.21576	TRU					
G4	1	nifh	WN	SYBR	e	294	154	947	704231	625	E	3948	E	3	16	90.80000305	N	N

	SS1		UNKNO		Non	21.40707	21.42775	0.025205	710120.9	701028.5	TRU	0.21576	TRU					
G5	1	nifh	WN	SYBR	e	397	154	947	375	625	E	3948	E	3	16	90.9777832	N	N
	SS1		UNKNO		Non	21.45582	21.42775	0.025205	688733.7	701028.5	TRU	0.21576	TRU					
G6	1	nifh	WN	SYBR	e	962	154	947	5	625	E	3948	E	3	16	90.9777832	N	N
	SS1		UNKNO		Non	20.68632	20.75764	0.110441	1115997.	1068860.	TRU	0.21576	TRU					
H4	2	nifh	WN	SYBR	e	317	465	163	75	375	E	3948	E	3	15	90.6222229	N	N
	SS1		UNKNO		Non	20.88485	20.75764	0.110441	985331.9	1068860.	TRU	0.21576	TRU					
H5	2	nifh	WN	SYBR	e	909	465	163	375	375	E	3948	E	3	15	90.6222229	N	N
	SS1		UNKNO		Non	20.70174	20.75764	0.110441	1105251.	1068860.	TRU	0.21576	TRU					
H6	2	nifh	WN	SYBR	e	98	465	163	625	375	E	3948	E	3	15	90.80000305	N	N
	SS1		UNKNO		Non	22.32091	22.16837	0.132967	400317.7		TRU	0.21576	TRU					
A7	3	nifh	WN	SYBR	e	141	502	71	813	441516	E	3948	E	3	17	90.80000305	N	N
	SS1		UNKNO		Non	22.10727	22.16837	0.132967	457719.0		TRU	0.21576	TRU					
A8	3	nifh	WN	SYBR	e	501	502	71	625	441516	E	3948	E	3	17	90.80000305	N	N
	SS1		UNKNO		Non	22.07694	22.16837	0.132967	466511.1		TRU	0.21576	TRU					
A9	3	nifh	WN	SYBR	e	054	502	71	563	441516	E	3948	E	3	16	90.6222229	N	N
	SS2		UNKNO		Non	21.28672	21.23371	0.047060	765801.8		TRU	0.21576	TRU					
B7	1	nifh	WN	SYBR	e	028	696	955	125	791917.5	E	3948	E	3	16	90.9777832	N	N
	SS2		UNKNO		Non	21.21759	21.23371	0.047060	799735.1		TRU	0.21576	TRU					
B8	1	nifh	WN	SYBR	e	415	696	955	875	791917.5	E	3948	E	3	16	90.80000305	N	N
	SS2		UNKNO		Non	21.19683	21.23371	0.047060			TRU	0.21576	TRU					
B9	1	nifh	WN	SYBR	e	647	696	955	810215.5	791917.5	E	3948	E	3	16	90.80000305	N	N
	SS2		UNKNO		Non	22.32366	22.06636	0.224288	399625.8	472638.9	TRU	0.21576	TRU					
C7	2	nifh	WN	SYBR	e	943	81	523	75	063	E	3948	E	3	17	90.9777832	N	N
	SS2		UNKNO		Non	21.91217	22.06636	0.224288	517301.2	472638.9	TRU	0.21576	TRU					
C8	2	nifh	WN	SYBR	e	613	81	523	5	063	E	3948	E	3	17	90.9777832	N	N
	SS2		UNKNO		Non	21.96325	22.06636	0.224288	500989.6	472638.9	TRU	0.21576	TRU					
C9	2	nifh	WN	SYBR	e	874	81	523	25	063	E	3948	E	3	17	90.9777832	N	N
	SS2		UNKNO		Non	21.89592	21.71105	0.165710	522601.5	588934.6	TRU	0.21576	TRU					
D7	3	nifh	WN	SYBR	e	361	957	762	313	875	E	3948	E	3	16	91.15555573	N	N
	SS2		UNKNO		Non	21.66140	21.71105	0.165710	605415.3	588934.6	TRU	0.21576	TRU					
D8	3	nifh	WN	SYBR	e	366	957	762	75	875	E	3948	E	3	16	90.9777832	N	N
	SS2		UNKNO		Non	21.57585	21.71105	0.165710	638787.0	588934.6	TRU	0.21576	TRU					
D9	3	nifh	WN	SYBR	e	716	957	762	625	875	E	3948	E	3	16	90.9777832	N	N
	SS3		UNKNO		Non	21.53512	21.30144	0.210601	655315.1		TRU	0.21576	TRU					
E7	1	nifh	WN	SYBR	e	955	691	285	875	763105.5	E	3948	E	3	16	90.9777832	N	N
	SS3		UNKNO		Non	21.24289	21.30144	0.210601	787146.0		TRU	0.21576	TRU					
E8	1	nifh	WN	SYBR	e	131	691	285	625	763105.5	E	3948	E	3	16	90.9777832	N	N
	SS3		UNKNO		Non	21.12631	21.30144	0.210601	846855.1		TRU	0.21576	TRU					
E9	1	nifh	WN	SYBR	e	989	691	285	25	763105.5	E	3948	E	3	16	90.80000305	N	N
	SS3		UNKNO		Non	21.61078	21.40060	0.191774		716403.6	TRU	0.21576	TRU					
F7	2	nifh	WN	SYBR	e	262	997	935	624946	875	E	3948	E	3	16	90.9777832	N	N
	SS3		UNKNO		Non	21.35591	21.40060	0.191774	733274.9	716403.6	TRU	0.21576	TRU					
F8	2	nifh	WN	SYBR	e	888	997	935	375	875	E	3948	E	3	16	90.80000305	N	N
	SS3		UNKNO		Non	21.23512	21.40060	0.191774	790989.9	716403.6	TRU	0.21576	TRU					
F9	2	nifh	WN	SYBR	e	459	997	935	375	875	E	3948	E	3	16	90.80000305	N	N
	SS3		UNKNO		Non	21.61553	21.55844	0.063161	623084.9	646141.7	TRU	0.21576	TRU					
G7	3	nifh	WN	SYBR	e	764	307	574	375	5	E	3948	E	3	16	90.80000305	N	N
	SS3		UNKNO		Non	21.56919	21.55844	0.063161		646141.7	TRU	0.21576	TRU					
G8	3	nifh	WN	SYBR	e	479	307	574	641462	5	E	3948	E	3	16	90.80000305	N	N
	SS3		UNKNO		Non	21.49059	21.55844	0.063161	673878.1	646141.7	TRU	0.21576	TRU					
G9	3	nifh	WN	SYBR	e	486	307	574	875	5	E	3948	E	3	16	90.6222229	N	N

Supplementary Data SD2 Title: Supplementary data on identified OTUs for microbial communities in ponds 1, 2 and 3

Sample Name	Quantity Mean	Vol DNA (µl)	Weight of sediment used (g)	Conc. (Copies/g)	Conc. x 10^6 (Copies/g)		Pond-1	Pond-2	Pond-3
JS11	294101.1446	20	0.5	11764045.78	11.76404578		12.24933816	22.19615965	21.45138906
JS11	294101.1446	20	0.5	11764045.78	11.76404578		30.71600178	53.65956521	111.7652128
JS11	294101.1446	20	0.5	11764045.78	11.76404578		48.51765327	61.63169874	32.450075
JS12	350049.8048	20	0.5	14001992.19	14.00199219		29.48539917	24.71321458	28.3420125
JS12	350049.8048	20	0.5	14001992.19	14.00199219		**30.24209809**	**40.55015955**	**48.50217235**
JS12	350049.8048	20	0.5	14001992.19	14.00199219				
JS13	274549.4124	20	0.5	10981976.5	10.9819765				
JS13	274549.4124	20	0.5	10981976.5	10.9819765				
JS13	274549.4124	20	0.5	10981976.5	10.9819765	12.24933816			
JS21	661412.4787	20	0.5	26456499.15	26.45649915				
JS21	661412.4787	20	0.5	26456499.15	26.45649915				
JS21	661412.4787	20	0.5	26456499.15	26.45649915				
JS22	471445.7179	20	0.5	18857828.72	18.85782872				
JS22	471445.7179	20	0.5	18857828.72	18.85782872				
JS22	471445.7179	20	0.5	18857828.72	18.85782872				
JS23	531853.7771	20	0.5	21274151.08	21.27415108				
JS23	531853.7771	20	0.5	21274151.08	21.27415108				
JS23	531853.7771	20	0.5	21274151.08	21.27415108	22.19615965			
JS31	490697.903	20	0.5	19627916.12	19.62791612				
JS31	490697.903	20	0.5	19627916.12	19.62791612				
JS31	490697.903	20	0.5	19627916.12	19.62791612				
JS32	445199.4805	20	0.5	17807979.22	17.80797922				
JS32	445199.4805	20	0.5	17807979.22	17.80797922				
JS32	445199.4805	20	0.5	17807979.22	17.80797922				
JS33	672956.7956	20	0.5	26918271.82	26.91827182				
JS33	672956.7956	20	0.5	26918271.82	26.91827182				
JS33	672956.7956	20	0.5	26918271.82	26.91827182	21.45138906			
MS11	469104.0312	20	0.5	18764161.25	18.76416125				
MS11	469104.0312	20	0.5	18764161.25	18.76416125				
MS11	469104.0312	20	0.5	18764161.25	18.76416125				
MS12	943569.8989	20	0.5	37742795.96	37.74279596				
MS12	943569.8989	20	0.5	37742795.96	37.74279596				
MS12	943569.8989	20	0.5	37742795.96	37.74279596				
MS13	891026.2034	20	0.5	35641048.14	35.64104814				

MS13	891026.2034	20	0.5	35641048.14	35.64104814	
MS13	891026.2034	20	0.5	35641048.14	35.64104814	30.71600178
MS21	1584944.863	20	0.5	63397794.52	63.39779452	
MS21	1584944.863	20	0.5	63397794.52	63.39779452	
MS21	1584944.863	20	0.5	63397794.52	63.39779452	
MS22	1440235.232	20	0.5	57609409.29	57.60940929	
MS22	1440235.232	20	0.5	57609409.29	57.60940929	
MS22	1440235.232	20	0.5	57609409.29	57.60940929	
MS23	999287.2959	20	0.5	39971491.84	39.97149184	
MS23	999287.2959	20	0.5	39971491.84	39.97149184	
MS23	999287.2959	20	0.5	39971491.84	39.97149184	53.65956521
MS31	3247040.779	20	0.5	129881631.1	129.8816311	
MS31	3247040.779	20	0.5	129881631.1	129.8816311	
MS31	3247040.779	20	0.5	129881631.1	129.8816311	
MS32	2140095.282	20	0.5	85603811.29	85.60381129	
MS32	2140095.282	20	0.5	85603811.29	85.60381129	
MS32	2140095.282	20	0.5	85603811.29	85.60381129	
MS33	2995254.902	20	0.5	119810196.1	119.8101961	
MS33	2995254.902	20	0.5	119810196.1	119.8101961	
MS33	2995254.902	20	0.5	119810196.1	119.8101961	111.7652128
OS11	904471.8039	20	0.5	36178872.16	36.17887216	
OS11	904471.8039	20	0.5	36178872.16	36.17887216	
OS11	904471.8039	20	0.5	36178872.16	36.17887216	
OS12	1622151.77	20	0.5	64886070.79	64.88607079	
OS12	1622151.77	20	0.5	64886070.79	64.88607079	
OS12	1622151.77	20	0.5	64886070.79	64.88607079	
OS13	1112200.421	20	0.5	44488016.84	44.48801684	
OS13	1112200.421	20	0.5	44488016.84	44.48801684	
OS13	1112200.421	20	0.5	44488016.84	44.48801684	48.51765327
OS21	1985643.541	20	0.5	79425741.65	79.42574165	
OS21	1985643.541	20	0.5	79425741.65	79.42574165	
OS21	1985643.541	20	0.5	79425741.65	79.42574165	
OS22	undetermined	20	0.5	#VALUE!		
OS22	1142849.25	20	0.5	45713970	45.71397	
OS22	1142849.25	20	0.5	45713970	45.71397	
OS23	1361236.875	20	0.5	54449475	54.449475	
OS23	1361236.875	20	0.5	54449475	54.449475	
OS23	1361236.875	20	0.5	54449475	54.449475	61.63169874
OS31	694368.625	20	0.5	27774745	27.774745	
OS31	694368.625	20	0.5	27774745	27.774745	
OS31	694368.625	20	0.5	27774745	27.774745	

OS32	931420.8125	20	0.5	37256832.5	37.2568325	
OS32	931420.8125	20	0.5	37256832.5	37.2568325	
OS32	931420.8125	20	0.5	37256832.5	37.2568325	
OS33	807966.1875	20	0.5	32318647.5	32.3186475	
OS33	807966.1875	20	0.5	32318647.5	32.3186475	
OS33	807966.1875	20	0.5	32318647.5	32.3186475	32.450075
SS11	701028.5625	20	0.5	28041142.5	28.0411425	
SS11	701028.5625	20	0.5	28041142.5	28.0411425	
SS11	701028.5625	20	0.5	28041142.5	28.0411425	
SS12	1068860.375	20	0.5	42754415	42.754415	
SS12	1068860.375	20	0.5	42754415	42.754415	
SS12	1068860.375	20	0.5	42754415	42.754415	
SS13	441516	20	0.5	17660640	17.66064	
SS13	441516	20	0.5	17660640	17.66064	
SS13	441516	20	0.5	17660640	17.66064	29.48539917
SS21	791917.5	20	0.5	31676700	31.6767	
SS21	791917.5	20	0.5	31676700	31.6767	
SS21	791917.5	20	0.5	31676700	31.6767	
SS22	472638.9063	20	0.5	18905556.25	18.90555625	
SS22	472638.9063	20	0.5	18905556.25	18.90555625	
SS22	472638.9063	20	0.5	18905556.25	18.90555625	
SS23	588934.6875	20	0.5	23557387.5	23.5573875	
SS23	588934.6875	20	0.5	23557387.5	23.5573875	
SS23	588934.6875	20	0.5	23557387.5	23.5573875	24.71321458
SS31	763105.5	20	0.5	30524220	30.52422	
SS31	763105.5	20	0.5	30524220	30.52422	
SS31	763105.5	20	0.5	30524220	30.52422	
SS32	716403.6875	20	0.5	28656147.5	28.6561475	
SS32	716403.6875	20	0.5	28656147.5	28.6561475	
SS32	716403.6875	20	0.5	28656147.5	28.6561475	
SS33	646141.75	20	0.5	25845670	25.84567	
SS33	646141.75	20	0.5	25845670	25.84567	
SS33	646141.75	20	0.5	25845670	25.84567	28.3420125