Microbial Diversity Assessment in Milkfish Culture Ponds

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Authors' contributions

This work was carried out in collaboration among all authors. Author LMMD collaborated on study design, performed molecular biological studies, conducted literature searches and wrote the first draft and for this manuscript. Authors BLR and RE collaborated on study design and organized field collections. Author IB was a key liason with SEADEC to gain access to aquaculture ponds. Author JMR assisted with field activities, data organization and transportation of samples. Author KVE assisted author LMMD with laboratory based molecular biological studies and data analysis. Authors EM and FG were key scientific liaisons in Iloilo between SEADEC, facilities at West Visayas State University and the University of the Philippines, Manila and also participated in field collections. Author MAK was principal investigator on both US NIH grants that provided partial support of these research activities. Also author MAK updated literature searches, edited and formatted this manuscript and data for final manuscript submission. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: To determine bacterial diversity in milkfish culture ponds that contain different life-cycle stages of the milkfish (pond A: fry, pond B: juveniles and pond C: adults) by DNA sequence analysis of organisms and compare that microbial diversity to organisms found in soil adjacent to the ponds.

Study Design: Comparative metagenomic study of aquatic and terrestrial biodiversity based on DNA sequence analysis of water and soil DNA.

Place and Duration of Study: SEADEC milkfish ponds in Tingnauan, Iloilo. Philippines. All water and soil samples were collected over a three-day period.

Methodology: DNA sequence analysis of nucleic acids extracted from water samples collected from the three types of milkfish ponds along with soil adjacent to the ponds. DNA was extracted and PCR was performed using the 11F-1492R primer pair to amplify 16S rRNA gene. Purified 16S rDNA amplicons were cloned in using the TOPO-TA cloning kit for DNA sequencing. 16s rRNA gene sequences were analyzed with the use of software tools at the National Center for Biotechnology Information website and imported into the ARB phylogenetic analysis software. Distance matrices were exported using the neighbor-joining algorithm in ARB, in the form of PHYLIP-formatted lower triangular matrices. The distance matrices were then used to calculate Shannon-Weaver and Simpson diversity indices to evaluate the richness and evenness of the sampled populations. Rarefaction curves were determined to evaluate sampling efficiency.

Results: Rarefaction curves indicated that the sampling effort was sufficient to reveal the majority of phyla present in the sample. Shannon-Weaver and Simpson indices suggested that the diversities of all the groups were statistically different from each other. It was observed that pond A was least diverse, followed by pond C and pond B. The soil was most diverse. DNA sequence analysis identified the various species of bacteria in soil and water.

Conclusion: All three pond communities were significantly different in diversity. This study did not identify any significant human pathogens such as *Vibrios*, *Salmonella* or *Shigella*. Bacterial diversity of sites decreased in the following order: soil > fry pond > fingerling pond > adult pond.

1. INTRODUCTION

The Philippines is one of the Worlds' most important aquaculture producers of milkfish (Bangus, *Chanos chanos*). The Philippines top producing regions are Regions 6, 3, 1 and 4A with Capiz, Iloilo, Bulacan (Panay, Iloilo), Negros Occidental, Pangasinan and Quezon. Milkfish can be raised in salt, fresh or brackish waters. Brackish water comprises the bulk of farmed milkfish production in Panay, Iloilo, Philippines. According to the Philippines Department of Science and Technology (2019) [1-7], an average rate of 2.62% increase were recorded for milkfish production from 2002-2011 where in the third quarter alone of 2019 saw 112,271 metric tons of production valued to P 30.2 million with an annual growth rate of 13.1 percent in 2019/2018 compared to 2017/2018.

The combined use of inorganic (e.g. phosphorus) and organic fertilizers (e.g. chicken manure or MASA, processed from agricultural waste) for growing natural food organisms within pond culture operations is popular throughout the Philippines. Ninety-one percent of milkfish ponds use inorganic fertilizers, 60% use organic fertilizers and 50% use both inorganic and organic fertilizers. However, these practices tend to be generalized without specific reference to critical soil or water parameters. In addition, regular application of manure over the culture period as a fertilizer and indirectly as a feed is well established in the pond culture. The benefits of manuring milkfish ponds have yet to be properly quantified and established [8-13].

A problem with manure loading in extensive milkfish culture is that the ponds are shallow, averaging 25-50 cm, capable of supporting only a maximum biomass load of 1,000-1,500 kg/ha. This is especially true during the summer months when salinity and temperature are high (resulting in low dissolved oxygen solubility, DO) and when spring tides are relatively low. As manure adds up to the DO demand of a pond, even moderate levels of manure application can cause water quality to deteriorate in milkfish ponds. This in turn exposes the animals to stressful and sometimes lethally low early morning DO levels. Beyond this, DO depletion, growth retardation, and fish kill becomes a serious problem;

Scientists engaged in milkfish culture however are concerned that current culture methods have improved production but may otherwise have a negative effect in the ecosystem processes in the aquaculture ponds. This may be due to the massive application of chemicals in the form of fertilizers and pesticides which are important for supplemental milkfish nutrition and for killing snail pests, respectively. How these practices affect the ecological balance is not fully understood.

One way of understanding how fertilizers, pesticides and environmental chemicals affect the baseline pond environment is to study the impact on microbial communities [14-17]. The concept that microorganisms are ubiquitous in distribution and can proliferate in any habitat that supports their growth has been a long-standing notion in microbial ecology. In general, patterns of microbial diversity are correlated with habitat conditions, owing to varying degrees of habitat preference, and adaptability of different groups of microorganisms. In particular, salinity is shown to be a major factor relating microbial communities [16]. As a result, inferences may be made by studying the composition of microbial communities, as these represent the combined consequence of abiotic conditions and biotic dynamics that generate environment-specific heterogeneity of communities. Changes in microbial communities reflect changes in the over-all aquatic ecosystem. Because of this, monitoring genotypic community changes overtime is very significant in assessing the effects and impacts of different disturbances in the environment. One approach to describe the microbial community structure in culture ponds is through metagenomics. In metagenomics, the 16S ribosomal RNA gene (16S rDNA) is used as it contains conserved and variable regions that can be utilized for microbial identification and phylogenetic analysis. An analysis of the 16S rRNA gene is conducted through: (1) isolation & extraction of genetic material from the source, (2) manipulation of the genetic material, such as through the amplification of the 16S rRNA gene, (3) library construction and (4) the analysis of genetic material in the metagenomic library. Several genomic approaches have greatly advanced understanding of the ecology and diversity of microbial communities in aquatic environments [14-17]. Together with polymerase chain reaction (PCR), fingerprinting methods like denaturing-gradient gel electrophoresis (DGGE), obtain a qualitative representation of the presence and abundance of different phylotypes in a sample. By profiling the composition and structure of microbial communities, these techniques are valuable for tracking genotypic community changes over time, as well as for comparative analysis of microbial community profiles inhabiting different environments. Therefore, this study applied such metagenomic methods to assess comparative microbial diversity in milkfish ponds.

2. METHODOLOGY

2.1 Sample Collection

Water samples (500 mL) were collected from points 10 feet from the edge of each of the three ponds in Iloilo which contains different milkfish life-cycle stages reared using various culture practices. Pond A contained *bangus* fingerlings only, Pond B contained fish in different stages of their life-cycle, and Pond C contained adult (ready-to-harvest) fish. Sample of the soil one meter from the edge of the pond were also collected. The samples were transported to the laboratory in cold storage within 6 hours from collection time.

2.2 Bacterial DNA Isolation

The samples were centrifuged to collect the bacterial cells. The Soil DNA Extraction Kit (MoBio, USA) was used for DNA extraction. DNA isolation begins with the filtration of a water sample onto a filter membrane. The membrane is then added to a 5 ml bead beating tube containing a unique bead mix. Rapid lysis occurs through vortex mixing in a lysis buffer that enhances the isolation of microorganisms from filter membranes. After protein and inhibitor removal steps, total genomic DNA is captured on a silica spin column. High quality DNA is then washed and eluted.

2.3 Amplification of Bacterial 16S rDNA

PCR was performed in a standard thermocycler (MJ Research PTC-2000) using the 11F-1492R primer pair. The PCR products were detected by agarose gel electrophoresis and were purified using the QIAgen PCR purification kit.

2.4 Cloning and Sequencing of Bacterial 16S rDNA

The purified 16S rDNA amplicons were cloned in chemically-competent *E. coli* using the TOPO-TA
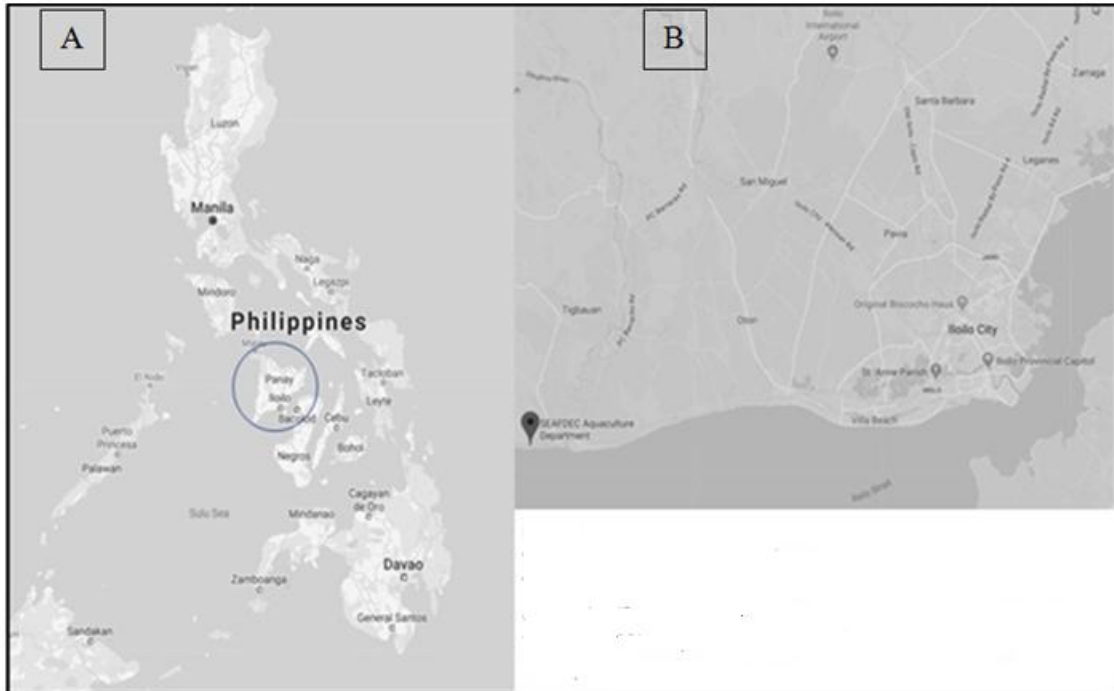
Fig. 1. Map of Panay island and Iloilo city

cloning kit (Invitrogen). The preserved clones were sent to Michigan State University Macromolecular Facility for DNA sequencing. DNA sequence of each close was used to identify the various microbes.

2.5 Phylogenetic Analysis

16s rRNA gene sequences from the obtained isolates were encoded in FASTA file format, then analyzed with the use of the BLASTn tool at the National Center for Biotechnology Information (NCBI) [18] Results were retrieved as a comma-separated-variable file containing the hits for each query sequence, in the form of NCBI reference numbers. Species descriptions were retrieved using the Batch Entrez utility on the NCBI website. Sequences were identified presumptively using the identity of the best match for each query sequence with respect to MaxScore and percent coverage.

The identified sequences were then imported into the ARB [19] phylogenetic analysis software. Sequences were aligned using the ClustalW algorithm while utilizing a positional tree server which was updated using the Green Greenes 16s rRNA library. Phylogenetic trees were constructed using the neighbor-joining algorithm of ARB.

2.6 Diversity Analysis

Distance matrices were exported using the neighbor-joining algorithm in ARB, in the form of PHYLIP-formatted lower triangular matrices. The distance matrices were then run through DOTUR (Distance based OTU and Richness determination) [20] version 1.53. DOTUR was used to calculate Shannon-Weaver and Simpson diversity indices to evaluate the richness and evenness of the sampled populations. DOTUR was also used to calculate rarefaction curves to evaluate sampling efficiency as a function of evolutionary distance, using a random sampling without replacement algorithm.

3. RESULTS AND DISCUSSION

3.1 Rarefaction Analysis

Rarefaction curves constructed at the estimated phylum level (distance = 0.21) showed that all libraries satisfactorily exhibited a plateau, indicating that the sampling effort was sufficient to reveal most phyla present in the sample. However, none of the libraries were shown to have been sampled sufficiently to plateau at the species level (distance=0.03). All three pond communities were observed to plateau at the

estimated Phylum level of similarity (Fig. 2), as a result, the 80% level of similarity was used in further analyses.

3.2 Evaluation of Microbial Diversity

Microbial diversity was evaluated on the basis of the Shannon-Weaver and Simpson indices calculated from the distance matrices exported from ARB. DOTUR also computes for the confidence interval (α=0.05) for the indices computed at each level of evolutionary distance. It was observed that for both Shannon-Weaver and Simpson indices, none of the indices for all groups fell within the 95% confidence interval of any other. As a result, it is said that the diversities of all the groups are statistically different from each other. It was observed that Pond A was least diverse, followed by Pond C and Pond B. The soil population was most diverse. While the soil community did not exhibit a stabilization of either estimators, it was nevertheless observed that the final calculated estimate of richness for soil far exceeded that of all the pond communities. Using both Chao1 and ACE as estimators of community richness, it was seen that Pond B had the highest richness, followed by Pond A, then Pond C (Figs. 3 and 4).
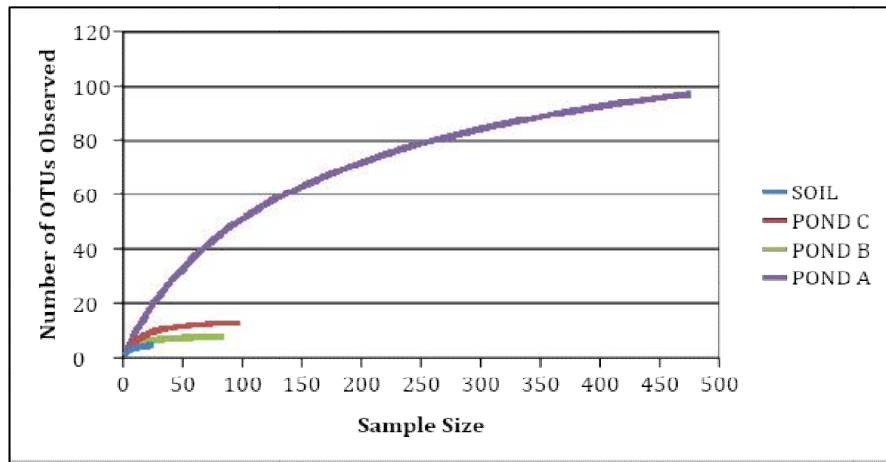

Fig. 2. Rarefaction curves for 16s rRNA clone libraries isolated from pond A, B, C and soil sites at 80% similarity cutoff
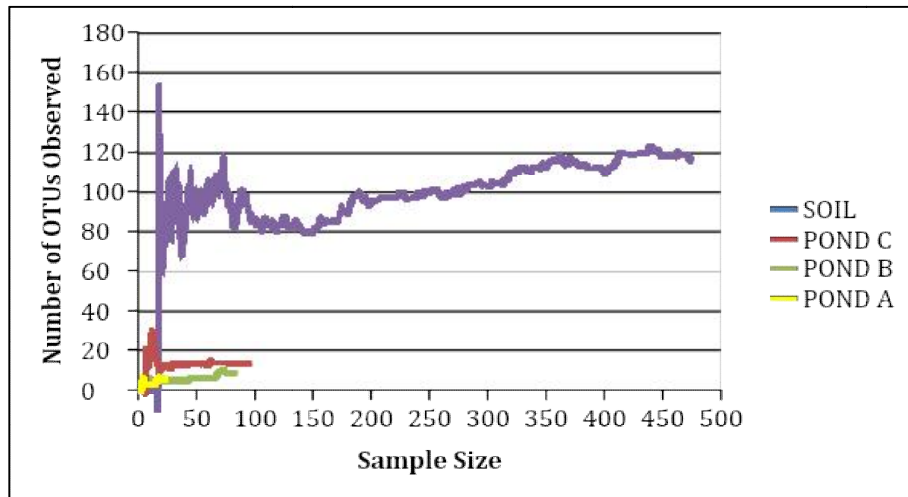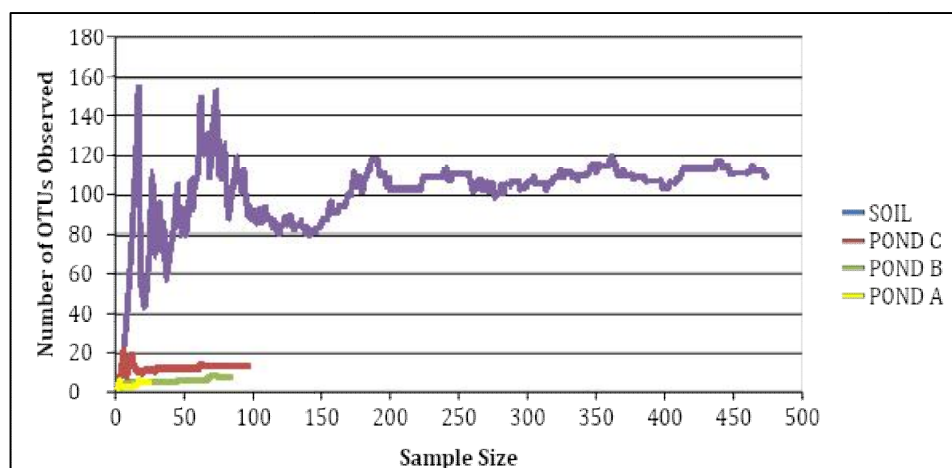

Fig. 3. Abundance-based Coverage Estimator (ACE) richness estimate collector's curve using 16s rRNA clone libraries isolated from Pond A, B, C and soil sites at 95% OTU (Operational Taxonomic Units) similarity cutoff

Fig. 4. Chao1 richness estimate collector's curve using 16s rRNA clone libraries isolated from Pond A, B, C and soil sites at 95% OTU (Operational Taxonomic Unites) similarity cutoff

The computation of the Simpsons and Shannon indices revealed that at the estimated phylum level of similarity, all communities were significantly different from each other (Figs. 5 and 6). Similarly, the most ecologically rich pond community was found to be Pond B, followed by Pond A, then Pond C. The same trend applies to community evenness, as seen in the computed Shannon indices.

3.3 Phylogenetic Analysis

The composition of clones is shown in Tables 1 and 2. Clones isolated from Pond A consisted mainly of principally marine or halotolerant microorganisms, *Maricaulis salignorans* strain MCS 18 (33.33%), *Marinimicrobium koreense* strain M9 (11.11%), Phaselicystis flava strain SBKo001 (7.41%), *Pirellula staleyi* strain Michigan (3.70%). Soil myxobacteria have also been detected: *Phaselicystis flava* strain SBKo001, *Cystobacter miniatus* strain DSM 14712, *Singulisphaera acidiphila* DSM 18658 strain: ATCC BAA-1392=MOB10. The detection of *Desulfonatronum lacustre* DSM 10312 strain DSM 10312 (3.70%) alludes to the presence of sulfate and alkaline environment, owing to the alkalaphilic and sulfate-reducing nature of this organism. *Blastobacter denitrificans* strain IFAM 1005; LMG 8443 (3.70%), an aquatic, nitrogen-fixing bacterium normally found in symbiosis with the tree, *Aeschynomene indica*, was also detected.

At the simulated phylum level of phylogenetic similarity, pond B was demonstrated to have the highest richness, according to the computed Abundance-based coverage estimator, Simpson's index and Chao1 richness estimator, and the highest evenness, according to the Shannon-Weaver index, among the three pond ecosystems. Samples from pond B contained the same halotolerant microbiota found in pond A, but with the presence of alkaliphilic organisms, *Desulfonatronum lacustre DSM 10312* strain DSM 10312, *Nitrincola lacisaponensis* strain 4CA, and *Desulfuromonas alkaliphilus* strain Z-0531. The presence of sulfur-reducing bacteria, *Desulfuromonas alkaliphilus* strain Z-0531, *Desulfuromonas svalbardensis* strain 112, *Desulfovibrio oxamicus* strain DSM 1925 suggest the presence of sulfur or sulfate products in the ecosystem. Also, the presence of *Desulfovibrio oxamicus,* a nitrogen reducing bacteria, suggests the presence of denitrifying activity in the community.

In the sampling of the different microbial ecosystems, only 4 clones were seen to be exclusively site-specific, all of which were found in Site B alone. These were *Haliangium tepidum* strain SMP-10, *Sorangium cellulosum* strain DSM14627, *Iamia majanohamensis* strain NBRC 102561, and *Conexibacter woesei* DSM 14684 strain ID131577. *Sorangium cellulosum* and *Haliangium tepidum* are myxobacteria, the latter was isolated from coastal environments and has been shown to be moderately halophilic [21]. *Conexibacter woesei* [22] and *Iamia majanohamensis* [23] both belong to class Actinobacter and have been isolated from the epidermis of Holothurians. The latter has been shown to favor acidic, sulphur- or mineral sulphide-rich environments. In pond C,

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Desulfuromonas alkaliphilus strain Z-0531, sulfur-reducing alkalophile consisted the majority of sequence hits for the processed sample (68.24%). *Nitrincola lacisaponensis* strain 4CA, another alkalophile, was also detected. Similar to Pond B, the nitrogen-reducing bacteria *Desulfovibrio oxamicus* was also detected.

The bacterium *Aquincola tertiaricarbonis* strain L10 was the only organism found present in all three pond ecosystems and soil samples,

particularly comprising 14.81% of hits for Pond A and 24.24% in Pond B. This organism has been uniquely identified in its ability to utilize the carcinogenic fuel oxygenate, methyl tert-butyl ether, and its subsequent degradation intermediate, tert-butyl-alcohol and has been identified as an agent of their biodegradation [24]. The organism *Methylibium petroleiphilum* PM1 strain PM1, detected in pond B and soil samples, has also been demonstrated to degrade and utilize MTBE [25].
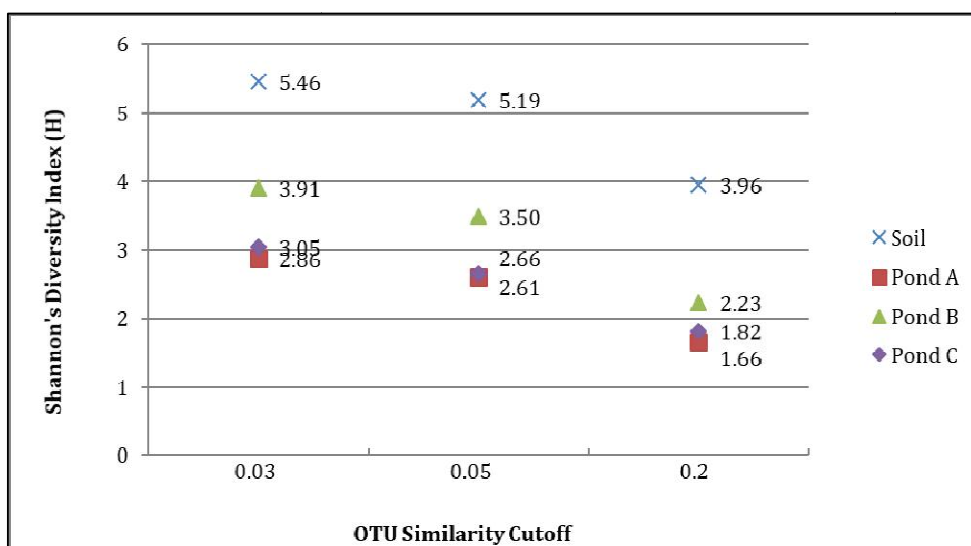

Fig. 5. Computed values for Shannon-Weaver diversity index for soil and ponds A, B and C communities at 97%, 95% and 80% OTU similarity cutoff

Table 1. Summary of relevant bacteria detected in sampling sites, grouped by preferred habitat

Saline	A	B	C	Soil
Maricaulis salignorans strain MCS 18	x	x		x
Marinimicrobium koreense strain M9	x	x		x
Pirellula staleyi strain Michigan	x	x		x
Nitrincola lacisaponensis strain 4CA		x		x
Haliangium tepidum strain SMP-10		x		x
Geothermobacter ehrlichii strain SS015		x		x
Alkaline				
Desulfonatronum lacustre *DSM 10312* strain DSM 10312	x	x		x
Nitrincola lacisaponensis strain 4CA		x	x	x
Desulfuromonas alkaliphilus strain Z-0531		x	x	x
Acidic				
Geothermobacter ehrlichii strain SS015	x			x
Iamia majanohamensis strain NBRC 102561	x			
Sulfur-reducing				
Desulfonatronum lacustre DSM 10312 strain DSM 10312	x	x		x
Desulfuromonas alkaliphilus strain Z-0531		x	x	x
Desulfuromonas svalbardensis strain 112		x		x
Desulfovibrio oxamicus strain DSM 1925		x		x
Iamia majanohamensis strain NBRC 102561		x		

Also, *Pseudomonas mendocina* strain NCIB 1054, detected in pond C, is known to co-metabolize MTBE [26]. The presence of these organisms may indicate the presence of petroleum products as contaminants in the lake environment. The definitive source of such pollutants is disputable. However, it is known that, in comparison to other gasoline contaminants, MTBE is highly water soluble and does not adhere to organic compounds as easily. As a result, groundwater contamination and transfer due to surface runoff and subsurface effluents, remains highly possible. In the identification of clonal identities, it was found that all clones identified from the sampling of the three pond ecosystems were also represented in soil samples, with the exception of 4 species, which belonged exclusively to pond B.

Table 2. Percentage of hits generated per clone in each site. Eleven strains highlighted in bold font indicate genera that could contain human pathogens if additional species level data were available. However, there were no DNA matches with pathogenic *Vibrios, Salmonella* or *Shigella*. Seventy eight of the 126 species identified (62%) were only found in soil

Bacteria	Pond A	Pond B	Pond C	Soil
Maricaulis salignorans strain MCS 18	33.33%	7.07%	0.00%	0.28%
Aquincola tertiaricarbonis strain L10	14.81%	24.24%	4.71%	1.11%
Marinimicrobium koreense strain M9	11.11%	1.01%	0.00%	0.28%
Phaselicystis flava strain SBKo001	7.41%	2.02%	0.00%	0.83%
Pirellula staleyi strain Michigan	3.70%	4.04%	0.00%	0.83%
Cystobacter miniatus strain DSM 14712	3.70%	2.02%	0.00%	0.83%
Desulfonatronum lacustre *DSM 10312* strain DSM 10312	3.70%	2.02%	0.00%	0.28%
Curvibacter delicatus strain 146	3.70%	1.01%	0.00%	0.28%
Singulisphaera acidiphila *DSM 18658* strain :ATCC BAA-1392=MOB10	3.70%	1.01%	0.00%	0.28%
***Burkholderia soli* strain GP25-8**	3.70%	0.00%	0.00%	1.11%
Blastobacter denitrificans strain IFAM 1005; LMG 8443	3.70%	0.00%	0.00%	0.28%
***Propionivibrio pelophilus* strain asp 66**	3.70%	0.00%	0.00%	0.28%
***Bordetella holmesii* strain CDC F5101**	3.70%	0.00%	0.00%	0.28%
Lysobacter niastensis strain GH41-7	0.00%	6.06%	2.35%	0.56%
Geopsychrobacter electrodiphilus strain A1	0.00%	5.05%	0.00%	0.56%
Nitrincola lacisaponensis strain 4CA	0.00%	4.04%	1.18%	0.28%
Planifilum fulgidum strain 500275	0.00%	4.04%	0.00%	0.56%
Desulfuromonas alkaliphilus strain Z-0531	0.00%	3.03%	68.24%	1.11%
Zavarzinella formosa strain : A10	0.00%	3.03%	0.00%	0.83%
Propionivibrio limicola strain GolChi1	0.00%	3.03%	0.00%	0.28%
Cystobacter ferrugineus strain Cb fe18	0.00%	3.03%	0.00%	0.28%
Burkholderia *phytofirmans* PsJN strain PsJN	0.00%	3.03%	0.00%	0.28%
Haliangium tepidum strain SMP-10	0.00%	3.03%	0.00%	0.00%
Ideonella dechloratans strain Anox B	0.00%	2.02%	0.00%	0.28%
***Legionella beliardensis* strain Montbeliard A1**	0.00%	2.02%	0.00%	0.28%
Bradyrhizobium liaoningense strain 2281; USDA 3622	0.00%	2.02%	0.00%	0.28%
Geothermobacter ehrlichii strain SS015	0.00%	1.01%	0.00%	1.11%
Leptothrix mobilis strain Feox-1	0.00%	1.01%	0.00%	0.56%
***Streptomyces aureofaciens* strain KACC 20180**	0.00%	1.01%	0.00%	0.28%
Desulfuromonas svalbardensis strain 112	0.00%	1.01%	0.00%	0.28%
Carboxydothermus siderophilus strain 1315	0.00%	1.01%	0.00%	0.28%
Aquabacterium fontiphilum strain CS-6	0.00%	1.01%	0.00%	0.28%
Methylibium *petroleiphilum PM1* strain PM1	0.00%	1.01%	0.00%	0.28%
Arenimonas malthae strain CC-JY-1	0.00%	1.01%	0.00%	0.28%
Desulfovibrio oxamicus strain DSM 1925	0.00%	1.01%	0.00%	0.28%
Sorangium cellulosum strain DSM14627	0.00%	1.01%	0.00%	0.00%
Iamia majanohamensis strain NBRC 102561	0.00%	1.01%	0.00%	0.00%
Conexibacter woesei DSM 14684 strain ID131577	0.00%	1.01%	0.00%	0.00%
Blastochloris viridis strain DSM 133	0.00%	0.00%	4.71%	0.83%

Bacteria	Pond A	Pond B	Pond C	Soil
Cupriavidus taiwanensis strain LMG 19424	0.00%	0.00%	4.71%	0.83%
Thermoleophilum album strain HS-5	0.00%	0.00%	3.53%	0.28%
Methylibium subsaxonicum strain : BF49	0.00%	0.00%	2.35%	0.28%
***Pseudomonas mendocina* strain NCIB 10541**	0.00%	0.00%	2.35%	0.28%
***Burkholderia endofungorum* strain : HKI 456**	0.00%	0.00%	1.18%	0.83%
Pelobacter acidigallici strain DSM 2377	0.00%	0.00%	1.18%	0.56%
Byssovorax cruenta strain : By c2 = DSM 14553	0.00%	0.00%	1.18%	0.56%
Terrimonas ferruginea strain : DSM 30193	0.00%	0.00%	1.18%	0.28%
Geobacter pickeringii strain G13	0.00%	0.00%	1.18%	0.28%
Schlegelella thermodepolymerans strain K14	0.00%	0.00%	0.00%	2.78%
Gemmata obscuriglobus strain UQM 2246	0.00%	0.00%	0.00%	2.22%
Prochlorococcus marinus subsp. pastoris str. PCC 9511	0.00%	0.00%	0.00%	1.39%
Geobacter metallireducens strain GS-15	0.00%	0.00%	0.00%	1.39%
Derxia gummosa strain IAM 13946	0.00%	0.00%	0.00%	1.39%
Nitrospira moscoviensis strain NSP M-1	0.00%	0.00%	0.00%	1.11%
Terrimonas lutea strain DY	0.00%	0.00%	0.00%	1.11%
Gemmatimonas aurantiaca strain T-27	0.00%	0.00%	0.00%	1.11%
Pelobacter acetylenicus strain WoAcy1	0.00%	0.00%	0.00%	1.11%
***Helicobacter cetorum* strain MIT 99-5656**	0.00%	0.00%	0.00%	1.11%
Acanthopleuribacter pedis strain NBRC 101209	0.00%	0.00%	0.00%	0.83%
Pseudolabrys taiwanensis strain CC-BB4	0.00%	0.00%	0.00%	0.83%
Cellvibrio japonicus strain Ueda107	0.00%	0.00%	0.00%	0.83%
Herbaspirillum lusitanum strain P6-12	0.00%	0.00%	0.00%	0.83%
Rhodoplanes serenus strain TUT3530	0.00%	0.00%	0.00%	0.83%
Steroidobacter denitrificans strain FS	0.00%	0.00%	0.00%	0.83%
Wautersia numazuensis strain TE26	0.00%	0.00%	0.00%	0.56%
Thiobacillus thiophilus strain D24TN	0.00%	0.00%	0.00%	0.56%
Ammonifex thiophilus strain SR	0.00%	0.00%	0.00%	0.56%
Cupriavidus respiraculi strain AU3313	0.00%	0.00%	0.00%	0.56%
Solirubrobacter soli strain Gsoil 355	0.00%	0.00%	0.00%	0.56%
Desulforhabdus amnigena strain ASRB1	0.00%	0.00%	0.00%	0.56%
Variovorax soli strain GH9-3	0.00%	0.00%	0.00%	0.56%
Clostridium argentinense strain ATCC 27322	0.00%	0.00%	0.00%	0.56%
Bradyrhizobium japonicum strain 3I1b6	0.00%	0.00%	0.00%	0.56%
Prosthecomicrobium consociatum strain 11	0.00%	0.00%	0.00%	0.56%
Planctomyces limnophilus DSM 3776 strain Mu 290	0.00%	0.00%	0.00%	0.56%
Sphingomonas kaistensis strain PB56	0.00%	0.00%	0.00%	0.28%
Methylobacterium fujisawaense strain DSM 5686	0.00%	0.00%	0.00%	0.28%
Balneimonas flocculans strain TFB	0.00%	0.00%	0.00%	0.28%
Thermaerobacter subterraneus strain C21	0.00%	0.00%	0.00%	0.28%
Aspromonas composti strain : TR7-09	0.00%	0.00%	0.00%	0.28%
Azospira restricta strain SUA2	0.00%	0.00%	0.00%	0.28%
Afipia broomeae strain F186	0.00%	0.00%	0.00%	0.28%
Actinomadura hibisca strain IMSNU 22185	0.00%	0.00%	0.00%	0.28%
Terriglobus roseus strain KBS 63	0.00%	0.00%	0.00%	0.28%
Saccharopolyspora hirsuta strain ATCC 27875	0.00%	0.00%	0.00%	0.28%
Thermovibrio guaymasensis strain SL19	0.00%	0.00%	0.00%	0.28%
Fibrobacter succinogenes subsp. succinogenes S85 strain ATCC 19169	0.00%	0.00%	0.00%	0.28%
Brevundimonas lenta strain DS-18	0.00%	0.00%	0.00%	0.28%
Desulfofrigus fragile strain LSv21	0.00%	0.00%	0.00%	0.28%
Ilumatobacter fluminis strain YM22-133	0.00%	0.00%	0.00%	0.28%
Peredibacter starrii strain A3.12	0.00%	0.00%	0.00%	0.28%
Desulfomicrobium macestii strain M-9	0.00%	0.00%	0.00%	0.28%
Desulfomicrobium thermophilum strain P6.2	0.00%	0.00%	0.00%	0.28%

| Bacteria	Pond A	Pond B	Pond C	Soil
Desulfovibrio burkinensis strain HDv	0.00%	0.00%	0.00%	0.28%
Ramlibacter henchirensis strain TMB834	0.00%	0.00%	0.00%	0.28%
Thiocapsa roseopersicina strain 5811	0.00%	0.00%	0.00%	0.28%
Pseudaminobacter salicylatoxidans strain BN12	0.00%	0.00%	0.00%	0.28%
Bradyrhizobium sp. BTA-1 strain BTA-1	0.00%	0.00%	0.00%	0.28%
Rhodopseudomonas rhenobacensis strain Klemme Rb	0.00%	0.00%	0.00%	0.28%
Stigmatella erecta strain : DSM 16858	0.00%	0.00%	0.00%	0.28%
Microbacterium imperiale strain DSM 20530	0.00%	0.00%	0.00%	0.28%
Desulfotomaculum solfataricum strain V21	0.00%	0.00%	0.00%	0.28%
Nannocystis exedens strain DSM71	0.00%	0.00%	0.00%	0.28%
Novosphingobium indicum strain H25	0.00%	0.00%	0.00%	0.28%
Desulfovibrio vulgaris strain DSM 644	0.00%	0.00%	0.00%	0.28%
Desulfovibrio alcoholovorans strain SPSN	0.00%	0.00%	0.00%	0.28%
Planosporangium flavigriseum strain : YIM 46034 = CCTCC AA 205013	0.00%	0.00%	0.00%	0.28%
Hyphomicrobium hollandicum strain IFAM KB-677	0.00%	0.00%	0.00%	0.28%
Acidisphaera rubrifaciens strain HS-AP3	0.00%	0.00%	0.00%	0.28%
Thiobacter subterraneus strain C55	0.00%	0.00%	0.00%	0.28%
Corallococcus coralloides strain : DSM 2259	0.00%	0.00%	0.00%	0.28%
Desulfocapsa sulfexigens strain SB164P1	0.00%	0.00%	0.00%	0.28%
Burkholderia rhizoxinica HKI 454 strain : HKI 454	0.00%	0.00%	0.00%	0.28%
Heliobacillus mobilis strain DSM 6151	0.00%	0.00%	0.00%	0.28%
Pseudorhodoferax soli strain TBEA3	0.00%	0.00%	0.00%	0.28%
Opitutus terrae PB90-1	0.00%	0.00%	0.00%	0.28%
Pedomicrobium australicum strain IFAM ST1306	0.00%	0.00%	0.00%	0.28%
Herbaspirillum autotrophicum strain IAM 14942	0.00%	0.00%	0.00%	0.28%
Halothiobacillus halophilus strain DSM 6132	0.00%	0.00%	0.00%	0.28%
Hyalangium minutum strain DSM 14724	0.00%	0.00%	0.00%	0.28%
Stella humosa strain DSM 5900	0.00%	0.00%	0.00%	0.28%
Pedobacter insulae strain DS-39	0.00%	0.00%	0.00%	0.28%
Prosthecobacter fusiformis strain FC4	0.00%	0.00%	0.00%	0.28%
Bradyrhizobium jicamae strain PAC68	0.00%	0.00%	0.00%	0.28%
Humicoccus flavidus strain DS-52	0.00%	0.00%	0.00%	0.28%
Ferrimicrobium acidiphilum strain T23	0.00%	0.00%	0.00%	0.28%
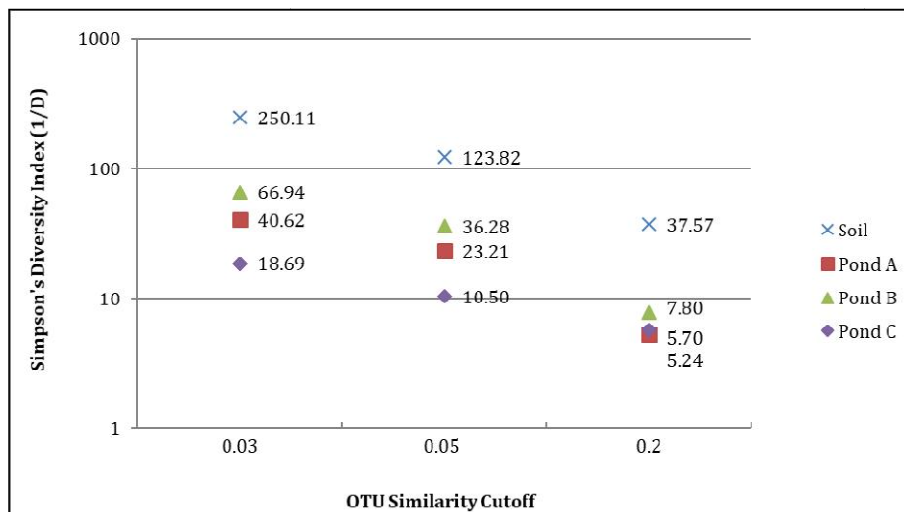
Fig. 6. Computed values for Simpson's diversity index for soil and ponds A, B and C communities at 97%, 95% and 80% OTU similarity cutoff

4. CONCLUSION

All three pond communities were observed to be significantly different in diversity, with respect to community richness and evenness. Bacterial diversity was seen to be highest in the pond containing fry of various stages, followed by the pond containing only fingerlings, then finally by the pond containing adults. Soil was more diverse than the ponds. One explanation for the lower diversity in adult pond would be the possible stabilizing effect on microbial communities due to larger fish biology, longer duration in culture or the effects of other microbes. Microbial communities were similar in all three ponds and did not reveal any significant human pathogens, such as *Vibrios, Salmonella or Shigella*. The role of specific environmental chemicals including residual antibiotics or heavy metals, variations in dissolved oxygen content, nitrates, nitrites, ammonia and phosphates were not included in this study and thus their impact on diversity could not be assessed.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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