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RESEARCH LETTER

Bacterial profiling in brine samples of the Emalahleni Water Reclamation Plant, South Africa, using 454-pyrosequencing method

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halophilic bacteria; brine; acid mine drainage.

Abstract

A metagenomic approach was applied using 454-pyrosequencing data analysis for the profiling of bacterial communities in the brine samples of the water reclamation plant. Some physicochemical characteristics of brine samples were also determined using standard methods. Samples ranged from being lightly alkaline to highly alkaline (pH 7.40–10.91) throughout the various treatment stages, with the salinity ranging from 1.62 to 4.53 g L^{-1} and dissolved oxygen concentrations ranging from 7.47 to 9.12 mg L^{-1}. Phenotypic switching was found to occur due to these physicochemical parameters. Microbial diversities increased from those present in Stage I reactor (six taxonomic groups) to those in Reverse Osmosis (RO) stage I (17 taxonomic groups), whereas in the second phase of the treatment, it increased in Stage II clarifier (14 taxonomic groups) followed by a decrease in RO stage II (seven taxonomic groups). Overall, seven phyla were detected, apart from many bacterial sequences that were unclassified at the phylum level. The most dominant phylum found was *Proteobacteria* accounting for 59% of the total sequences. A BLASTN sequence similarity search showed that the majority of the sequences (56%) were homologous to the uncultured bacterial species, underlining the vast untapped bacterial diversity.

Introduction

The discharge of industrial wastewater is one of the main causes of water pollution in South Africa. It is exacerbated by the fact that wastewater that has been for the most part untreated or only partially treated is being discharged into natural water bodies, which are intake water sources for facilities that supply drinking water (Shiklomanov, 1998). Acid mine drainage (AMD) is one of the typical pollutants of South Africa's freshwater environment. It represents an environmental problem as it contributes to both groundwater and surface water pollution with elevated levels of sulphate in the water (Hutton *et al.*, 2009). In order to deal with the AMD problem, a public–private initiative was launched by South Africa's biggest mining companies (Anglo Coal and BECSA) and the Emalahleni Local Municipality and the Emalahleni Water Reclamation Plant (EWRP) was built and commissioned to recover potable water from AMD from several mines in the Witbank area (Hutton *et al.*, 2009). In terms of a bulk supply agreement with Emalahleni Local Municipality, the treated water is pumped into the reservoirs of water-stressed Emalahleni Local Council, and some of the mines also use the treated water for mining activities such as dust suppression. Reverse osmosis (RO) membranes are often selected for the treatment of AMD from EWRP. RO is a pressure-driven process, which drives fresh water through a membrane (Cath, 2010). However, this treatment results in a concentrated brine reject stream which also has to be managed. It is estimated that 0.2 ML of brine is produced every day while processing 25 ML of AMD. Brine is also generated by water-desalination processes, oil and gas production, evaporative cooling in power stations and process industries, coal and gold mine drainage (Yalala *et al.*, 2009). In the process of biological treatment of brine, halophilic microorganisms could play a major role in the removal of dissolved minerals in brine (Woolard & Irvine, 1994; Kargi & Uygur, 1996; Shahalam, 2009).

Metagenomics enables the genomic study of uncultured microorganisms; this discipline was developed as a consequence of the discovery that prokaryotic diversity was much greater than previously realised and that the prokaryotic population was a significant resource for biotechnology and environmental applications. In the past, much microbial diversity was missed due to the limitations of traditional culture-based investigations, and the application of metagenomic techniques has led to major advances in prokaryote ecology and biotechnology (Steele & Streit, 2005). Metagenomics can be used to address the challenge of studying prokaryotes directly in almost any environment, circumventing the need for isolation and laboratory cultivation of each species, and these as-yet-unculturable organisms represent more than 99% (Amann *et al.*, 1990).

With the advent of metagenomics, particularly next-generation sequencing, a greater number of bacterial symbiotic relationships are being discovered as more systems (from marine invertebrates to those found in the human gut) are characterised (Handelsman *et al.*, 1998; Kennedy *et al.*, 2007; Simon & Daniel, 2011). Although pyrosequencing has been applied to study wastewater treatment systems (Schlüter *et al.*, 2008; Sanapareddy *et al.*, 2009; Kim *et al.*, 2011; Hu *et al.*, 2012), previous investigators mostly targeted any specific microbial community or plasmids. Hence, it is interesting to study bacterial community structures present in the different stages of wastewater treatment plants. There is good potential for employing the halophiles for the treatment of the brine reject stream produced when AMD is treated. In addition, only limited literature is available on the isolation and utilisation of borderline halophiles for the biodigestion of proteins in saline waste streams. This study focused on the complete microbial profiling of the different stages in a water reclamation plant using the metagenomics approach.

Materials and methods

Description of the site and sampling

Brine samples were collected from EWRP at Emalahleni (Witbank), which is located in the Mpumalanga Province, South Africa (S 25°56′41.4, E 29°11′67.0). The study was conducted on the 14 different stages in the treatment process, such as in Stage I reactor, Stage II reactor, Stage III reactor, Stage I clarifier, Stage II clarifier, Stage III clarifier, Brine dam, Reactor A, Reactor B, Dewatering A, Dewatering B, RO stage I, RO stage II, RO stage III (Fig. 1). All samples collected in sterile bottles were kept in a cooler box and transported to Tshwane University of Technology Water Research Group Laboratory where they were analysed within 2 h after collection.

Physicochemical characteristics of the brine samples

The profile of the brine samples in terms of physicochemical parameters such as dissolved oxygen (DO), pH and salinity g L^{-1} was determined. The pH, DO and conductivity were immediately recorded on site using a Hach Portable Meter (Model No. HQ40d, CO).

Molecular characterisation

DNA extraction

The 14 samples were filtered using filter paper with a pore size of 0.45 μm (Whatman Filter Paper No. 1), and the residues containing microorganisms were mixed with PBS 1×. The metagenomic DNA was extracted independently for all the samples with ZR fungal/bacterial DNA Kit (Zymo Research), as per manufacture's instruction. Finally, the DNA was eluted in 30 μL of ultrapure water. The integration of the metagenomic DNA was assessed on the 0.8% agarose gel. The DNA quantity, quality and purity were measured using a NanoDrop ND-1000 spectrophotometer (PeqLab, Germany).

PCR amplification

The PCR was performed on the extracted DNA samples using the universal primer 27F (Kamika & Momba, 2014) and 518R (Lee *et al.*, 2008). These primers amplify roughly the 500 bp of the 16S rRNA gene sequence. Each PCR contained DreamTaqTM Green DNA polymerase (dNTPs and 4 mM $MgCl_2$) Master Mix (2X) (25 μL), nuclease-free water (22 μL), forward primer (1 μL) (0.2 μM) and reverse primer (1 μL) (0.2 μM), and metagenomic DNA (50–100 ng) up to the final reaction volume of 50 μL. The following cycling parameters were used: initial denaturation step at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 30 s and 72 °C for 1 min 30 s, with a final 10 min extension at 72 °C that was then followed by a cooling temperature of 4 °C. The PCR products were loaded onto 1.5% agarose gel and then visualised with an ultraviolet (UV) transilluminator (InGenius Bio Imaging System, Syngene, Cambridge, UK). The DNA was subsequently re-amplified with two sets of primers. The pooled samples were sequenced on the GS-FLX-Titanium series 454/Roche by Inqaba Biotechnology, South Africa.

Analysis of sequences

A total of 72 276 reads of 16S rRNA gene sequences were generated from 14 brine samples. These sequences were

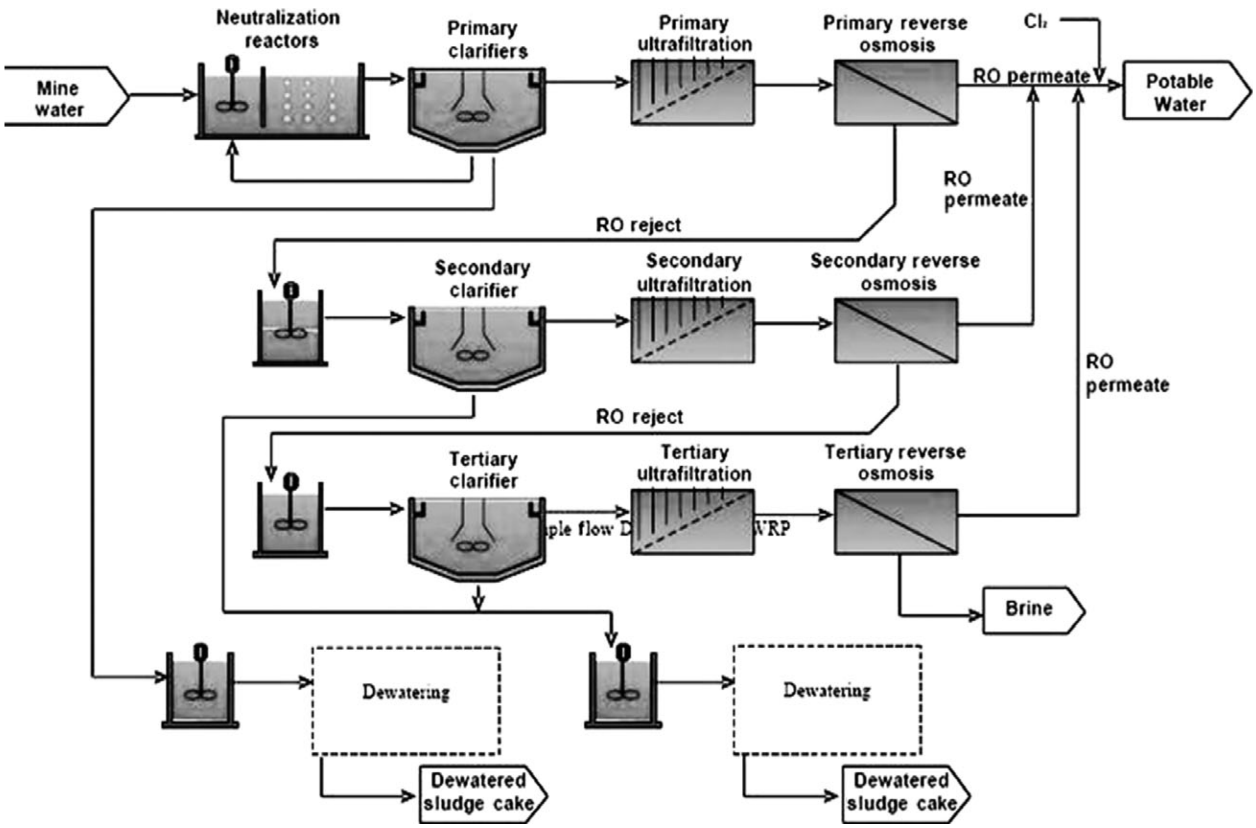

Fig. 1. Sampling collection detail at EWRP (adapted from Hutton *et al.*, 2009).

filtered for read length with a minimum cut-off length of 350 bp, and reads of < 350 bp were excluded from further analysis. The sequences with more than 97% similarity were clustered within the same operational taxonomic unit (OTU). The Shannon diversity index and the Chao1 richness estimator were calculated using the online tool FastGroup II (Yu *et al.*, 2006). The putative phylogenetic affiliations of 16S rRNA gene sequences were determined with 95% confidence (http://rdp.cme.msu.edu) using the CLASSIFIER program (Wang *et al.*, 2007) provided by the RDP-II database (Cole *et al.*, 2009), which uses the naive Bayesian rRNA classifier method (Version 10.0) for assigning the sequence to a taxonomic hierarchy. Sequence were also assembled using *de novo* assembling and BLASTN search was performed through NCBI using the CLC genomics work version 6.0.5.

Results and discussion

Physicochemical analysis of the brine samples

Brine samples ranged from being slightly alkaline to highly alkaline (pH 7.40–10.91) throughout the various stages of treatment, with the salinity ranging from 1.62 to 4.53 g L^{-1} and DO concentrations ranging from 7.47 to 9.12 mg L^{-1} as shown in Table 1. The pH values, DO concentration and salinity (similar to conductivity) increased as the brine was subjected to treatment in Stage I reactor (pH: 9.56, DO: 8.15 mg L^{-1} and salinity: 1.94 g L^{-1}) to Stage I clarifier (pH: 9.64, DO: 9.12 mg L^{-1} and salinity: 1.99 g L^{-1}) followed by a decrease after passing through the RO stage I (pH: 7.89, DO: 7.69 mg L^{-1} and salinity 1.78 g L^{-1}). Similar observations were also noted during the secondary and tertiary stages. The only exception was found in the secondary step where salinity decreased regardless of the increase in pH in the clarifier.

Metagenomic, DNA isolation and PCR amplification

The integrity of metagenomic DNA was visualised on 0.8% agarose gel, and purity was measured using the ratio of absorbance at 260 and 280 nm (range 1.72–1.86). The amplified product showed a band of *c.* 500 bp.

Phylogenetic analysis

After removing the reads lower than 350 bp from raw sequences, a total of 57 871 sequences from all the 14

Table 1. Physiochemical parameters for brine samples

S. no	Sample collection site	pH	DO (mg L^{-1})	Conductivity (mS cm^{-1})	Salinity (g L^{-1})
1.	Clarifier A	9.83	7.94	3.24	1.62
2.	RO I	7.89	7.69	3.55	1.78
3.	RO II	7.40	7.63	4.63	2.32
4.	RO III	7.72	7.85	6.68	3.34
5.	Clarifier B	9.67	7.92	4.55	2.28
6.	Brine dam	7.61	7.58	9.06	4.53
7.	Stage I reactor	9.56	8.15	3.87	1.94
8.	Stage II reactor	10.65	7.66	4.34	2.17
9.	Stage III reactor	10.88	7.96	6.66	3.33
10.	Stage I clarifier	9.64	9.12	3.98	1.99
11.	Stage II clarifier	10.69	7.99	4.24	2.12
12.	Stage III clarifier	10.91	7.66	7.68	3.84
13.	Reactor A	9.92	7.47	3.49	1.75
14.	Reactor B	9.77	7.53	4.89	2.45

DO, dissolved oxygen; RO, reverse osmosis.

samples were obtained. The number of valid sequences varied from 662 to 13 881 sequences for the various samples. The putative classification of these samples represented the members belonging to seven different phyla (*Firmicutes*, *Actinobacteria*, *Bacteroidetes*, *Proteobacteria*, *Deinococcus-Thermus*, *Cyanobacteria* and *Planctomycetes*) along with the unclassified bacteria after assigning sequences to a taxonomic lineage using the RDP Bayesian classifier. The relative abundance of the phylotypes varied among the samples collected at different stages of brine treatment (Fig. 2). Microbial populations increased from six taxonomic groups observed in Stage I reactor to 17 taxonomic groups present in RO stage I, whereas in the Stage II reactor it increased to 14 taxonomic groups in the Stage II clarifier followed by a decrease observed in RO stage II (seven taxonomic groups). A similar observation was found in the Stage III clarifier where the decrease was followed in RO stage III. The rapid decrease in microbial populations was linked to the decrease in pH and conductivity during the different stages of brine treatment (Table 1). The members representing the phyla *Firmicutes*, *Actinobacteria* and *Proteobacteria* were observed in each treatment stage, whereas no members of the *Bacteroidetes* phylum were recorded in one of the stages, that is in the Stage I clarifier. The phylum *Proteobacteria* was represented by the maximum number of sequences and the phyla *Gammaproteobacteria* and *Betaproteobacteria* accounted for an overall average of 59.63% of the assigned sequences. The observed dominance of the *Proteobacteria* was consistent with diversity estimates reported in earlier studies of wastewater systems (Wong *et al.*, 2005; Sanapareddy *et al.*, 2009; Hu *et al.*, 2012). Sequences corresponding to the *Proteobacteria* dominated in every stage of the plant except in the Stage III clarifier where Cyanobacterial sequences were dominant. The predominance of Cyanobacterial sequences in Stage III could be due to the excess of light and nutrients on this stage as these microorganisms obtain their energy through photosynthesis. The *Firmicutes* was the second major phylum with an average of 19.27% of sequence abundance, followed by *Actinobacteria* (averaging 11.22%), *Cyanobacteria* (averaging 5.88%) and *Bacteroidetes* (averaging 1.21%) in the Stage III clarifier. The phyla *Deinococcus-Thermus* and *Planctomycetes* were detected in a negligible percentage (≤ 0.02%) of the assigned sequences. The bacterial sequences which could not be classified to a phylum level accounted for an average of 2.74% of the total sequences (ranging between 1.52% and 3.84%); this finding represents a hitherto unidentified diversity in the brine samples and offers remarkable insights into the microbial ecology of the wastewater reclamation plant.

Based on the analysis of BLASTN sequence similarity searches for the closest homologs of the sequences after the *de novo* assembly, it was found that all 14 samples represented substantial percentages of uncultured bacteria (Fig. 3). The percentages of uncultured bacteria accounted for overall 56.1% and varied from 36.6% to 92% among different samples (Fig. 4a–c). The presence of a high number of sequences related to previously uncultured bacteria further indicated the possibility of isolating and identifying novel species from these brine samples which may be biotechnologically beneficial. The phylogenetic resemblances suggest a rich diversity of bacteria containing a large proportion of the microbial population from these brines that has never been cultured. It is noteworthy that only a few of the sequences had 100% sequence identity when compared to sequences contained in the database. This finding suggests that these brine samples contained different specific lineages of bacteria that have adapted to or prefer the harsh physicochemical conditions (especially high pH and salinity), suggesting that they represented novel species. This

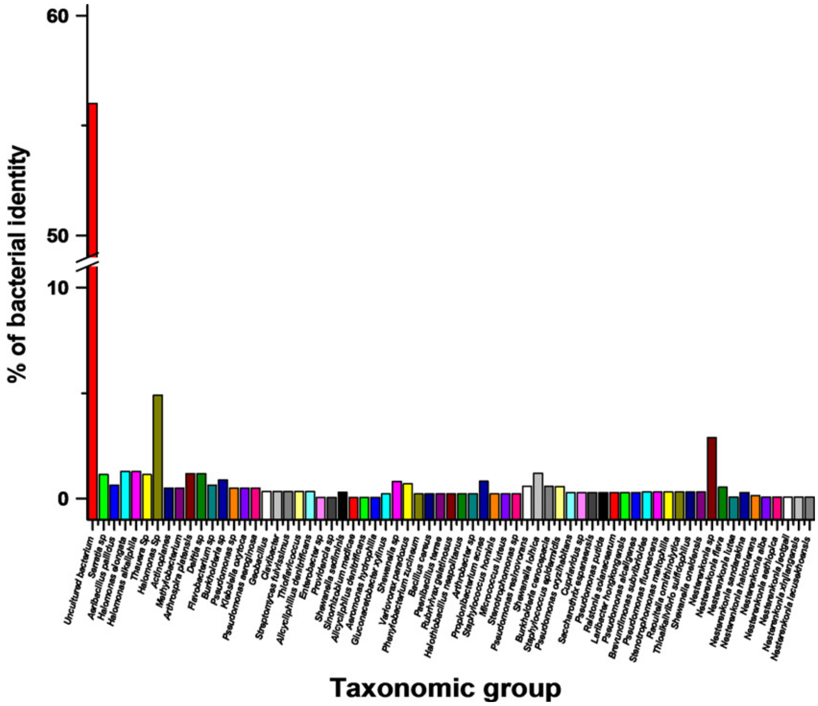

Fig. 2. Relative abundances of different bacterial phylogenetic groups in 16S rRNA gene sequences. Analysis of amplified 16S rRNA gene sequences was performed in comparison with the RDP-II database at the 80% confidence. The percentages of the phylogenetically classified sequences are plotted on *y*-axis.

demonstrates the existence of a complex bacterial community in these brine samples. It is of great interest to understand physiological properties, roles in biogeochemical cycling and the impacts on ecosystems of such potentially novel bacteria that inhabit these brine samples.

The cultured species found in all the brine samples were mostly halotolerant or halophilic (Fig. 3). The following species were identified: *Serratia* spp. (2%), *Aeribacillus pallidus* (1%), *Halomonas elongata* (1%), *Halomonas alkaliphila* (1%), *Thauera* spp. (6%), *Halomonas* spp. (6%), *Actinoplanes* spp. (1%), *Methylobacterium* spp. (1%), *Arthrospira platensis* (2%), *Delftia* spp. (2%), *Flavobacterium* spp. (1%), *Burkholderia* spp. (2%), *Pseudomonas* spp. (1%), *Klebsiella oxytoca* (1%), *Pseudomonas aeruginosa* (1%), *Geobacillus* spp. (1%), *Clavibacter* spp. (1%), *Streptomyces fulvissimus* (1%), *Thioflavicoccus* spp. (1%), *Alicycliphilus denitrificans* (1%), *Enterobacter* spp. (1%), *Providencia* spp. (1%), *Shewanella sediminis* (2%), *Sinorhizobium medicae* (1%), *Aeromonas hydrophila* (1%), *Gluconacetobacter xylinus* (1%), *Shewanella* spp. (3%), *Variovorax paradoxus* (2%), *Phenylobacterium zucineum* (1%), *Bacillus cereus* (1%), *Paenibacillus terrae* (1%), *Rubrivivax gelatinosus* (1%), *Halothiobacillus neapolitanus* (1%), *Arthrobacter* spp. (1%), *Propionibacterium acnes* (3%), *Staphylococcus hominis* (1%), *Micrococcus luteus* (1%), *Stenotrophomonas* spp. (1%), *Pseudomonas resinovorans* (1%), *Shewanella loihica* (3%), *Burkholderia cenocepacia* (1%), *Staphylococcus epidermidis* (2%), *Pseudomonas oryzihabitans* (1%), *Cupriavidus* spp. (1%), *Saccharothrix espanaensis* (1%), *Pseudomonas putida* (1%), *Ralstonia solanacearum* (1%), *Laribacter hongkongensis* (1%), *Pseudomonas alcaligenes* (1%), *Brevundimonas subvibrioides* (1%), *Pseudomonas fluorescens* (1%), *Stenotrophomonas maltophilia* (1%), *Raoultella ornithinolytica* (1%), *Thioalkalivibrio sulfidophilus* (1%), *Shewanella oneidensis* (1%), *Nesterenkonia* spp. (2%), *Nesterenkonia lutea* (2%), *Nesterenkonia sandarakina* (1%), *Nesterenkonia halotolerans* (1%), *Nesterenkonia halobia* (1%), *Nesterenkonia alba* (1%), *Nesterenkonia aethiopica* (1%), *Nesterenkonia jeotgali* (1%), *Nesterenkonia xinjiangensis* (1%) and *Nesterenkonia lacusekhoensis* (1%).

The microbial ecology of the wastewater treatment plants has been studied elsewhere, using various molecular tools, viz. fluorescence *in situ* hybridisation (FISH; Egli *et al.*, 2003), denaturing gradient gel electrophoresis (Stamper *et al.*, 2003), terminal restriction fragment length polymorphism (T-RFLP; Wang *et al.*, 2011) and clone library (Figuerola & Erijman, 2007). Each of these methods has its own limitations; however, in terms of performance, cost and read lengths, the 454-pyrosequencing technology stands out, because of its speed, cost-effectiveness and longer read length (*c.* 400 bp; Shendure & Ji, 2008).

Species richness and diversity indices

Estimation of species richness (Chao1) and diversity (Shannon index) were calculated for the 14 samples (Table 2). At the species level, defined as OTUs at 3% genetic difference,

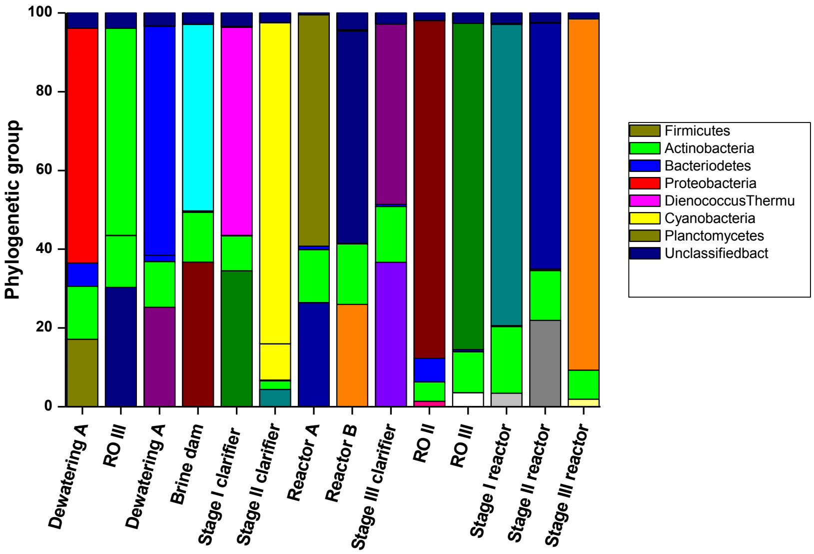

Fig. 3. Abundance of bacterial taxonomic group based on BLASTN similarity search after *de novo* assembly of the 16S rRNA gene sequences.

12 069 species or OTUs are estimated to be part of the brine community. The nonparametric richness estimator Chao1 provides a statistical estimate of the true species richness of a community including unobserved species, based on the observed sample data. The utility of this estimator lies in its potential to approximate asymptotic species richness from relatively small sample sizes. Chao1 is an estimator of the minimum species richness and is based on the number of rare OTUs (singletons and doublets) within a sample (Chao, 1984). In this study, the total number of cultured species observed was found to be considerably lower than the actual species richness found in all the brine samples when using the Chao1 estimator. This would imply that an even greater diversity of life forms lies undiscovered. Ecological diversity measured by Shannon index indicate that bacterial diversity was highest in Stage I clarifier brine and lowest in Stage II reactor brine. This could be due to the increase in salinity from Stage I clarifier (1.99 g L^{-1}) to Stage II clarifier (2.12 g L^{-1}). The Shannon diversity index is the most widely used index for comparing diversity between various habitats (Clarke & Warwick, 2001) which combines estimates of richness (total number of OTUs) and evenness (relative abundance). These results cannot be fully addressed within the scope of this study but provide important insights for future research.

Conclusion

An understanding of the complex microbial populations is the tool we need to allow us to in rapid decision-making when it comes to dealing with the environmental challenges we face in South Africa. The results of this study have shown that there are a number of uncultured bacteria which might be new species. Further research will now be undertaken on the as-yet-uncultured species, in order to gain insight into their genome structure, physiology and biochemistry. Once identified and characterised, these novel species could then be used in the targeted environment. The traditional culture techniques are widely used but are unable to capture the full spectrum of microbial diversity and are not the best way to elucidate the bacterial populations within the EWRP. The culture-independent approach used in this study contributes to our knowledge of bacterial diversity and provides valuable information on the existence of many novel genera in the brine samples of the wastewater reclamation plant. The high degree of phylogenetic novelty observed during this study highlights the fact that a great deal remains to be understood about the bacterial diversity. The findings of this study provide the scientific background and technology platform for further research and utilisation of bacterial species inhabiting brine samples of the EWRP. There are significant benefits in building capacities for developing high-level scientific research in metagenomics in developing countries.

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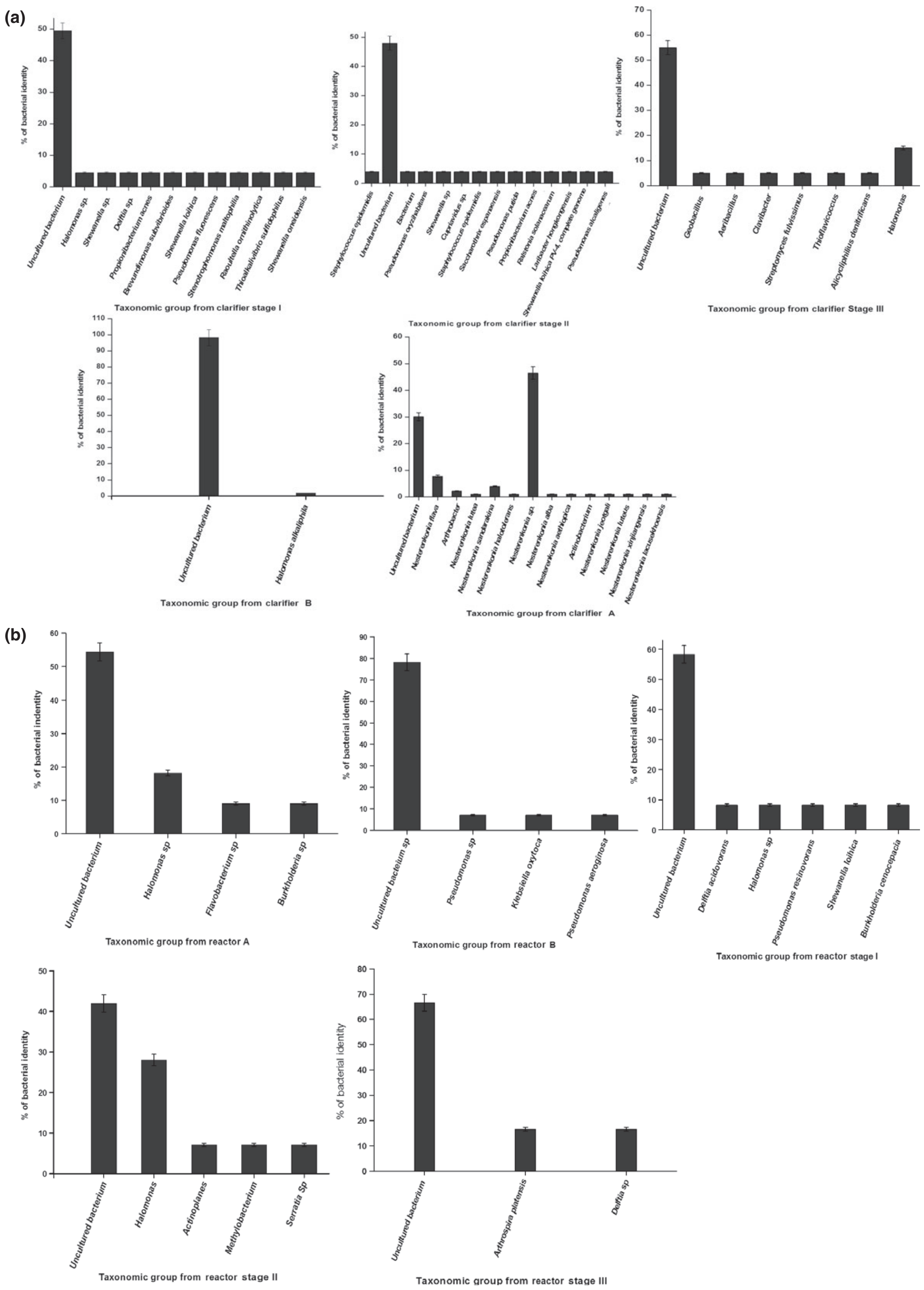

Fig. 4. Composition of the bacterial taxonomy detected in the brine from (a) clarifier, (b) reactors and (c) RO and dam of the wastewater reclamation plant.

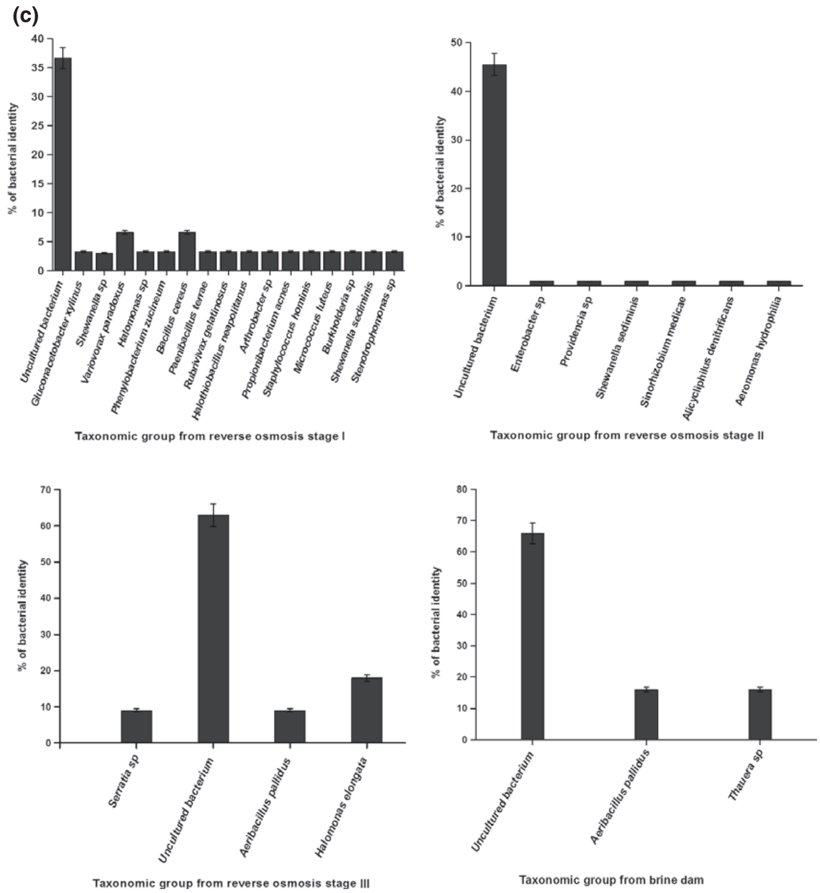

Fig. 4. (Continued).

Table 2. Sampling depth and diversity found by 16S rRNA gene amplicon 454 pyrosequencing from brine samples at the different stages of water reclamation plant

Sampling points	No. of reads	No. of valid sequences at length cut-off 350 bp	OTUs (3%)	Shannon index	Chao1
Clarifier A	1636	1624	563	5.824	4660.03
RO III	2003	1838	485	5.665	3599.22
Clarifier B	2502	2489	689	5.873	4660.5
Brine dam	1609	1410	308	4.983	1701.92
Stage II reactor	2902	2639	453	3.729	2649.15
Stage III reactor	2879	2621	620	5.411	3469.26
Reactor A	801	662	195	4.923	995.735
Reactor B	2312	2087	693	5.807	5225.01
Stage III clarifier	3556	3527	830	5.823	4902.53
RO II	3242	3208	763	6.346	4595.64
RO I	14 270	7783	563	5.826	4516.27
Stage I reactor	9950	9826	2347	7.51	17517.9
Stage II reactor	10 537	4276	857	6.598	5746.29
Stage I clarifier	14 077	13 881	2703	7.511	17725.7
Total	72 276	57 871	12 069		

RO, reverse osmosis.

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