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COMPARACIÓN DE LA COMUNIDAD MICROBIANA EN FOTOBIORREACTORES TRATANDO PURINES DE CERDO

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COMPARISON OF THE MICROBIAL COMMUNITY IN PHOTOBIOREACTORS TREATING SWINE MANURE

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RESUMEN

En este trabajo se utilizó la técnica DGGE (electroforesis en gel desnaturalizante en gradiente) para comparar la comunidad microbiana en 4 fotobiorreactores tratando purines de cerdo, en diferentes concentraciones (1:10, 1:20) y condiciones de luz y temperatura (indoor, outdoor). Tras la extracción del ADN genómico y amplificación por PCR de las regiones V6-V8 del ARNr bacteriano, se analizaron los amplicones mediante DGGE, obteniendo el perfil electroforético de cada muestra.

Según el índice de Diversidad de Shannon-Wiener, la diversidad bacteriana fue media (2.6 en 3 reactores) o baja (2.1 en el reactor 1:20 outdoor). El índice de similaridad mostró poca variabilidad entre las muestras, siendo las más semejantes entre sí las de los reactores indoor (83.4%). Las bacterias identificadas a partir de las bandas más significativas de cada perfil electroforético, utilizando las bases de datos RDP y BLAST, pertenecen mayoritariamente al filo *Proteobacteria*, siendo especialmente representativo el género *Psychrobacter*.

Palabras Clave

Identificación de microorganismos; Biología molecular; Valorización de residuos; Purines de cerdo; Microalgas.

ABSTRACT

The DDGE technique was used to compare the microbiological community in the inoculum and 4 photobioreactors treating piggery wastewater. The conditions tested in the photobioreactors were: concentration of swine manure (1:10 or 1:20), and light exposure indoor (12 h light; led) and 12 h dark while outdoor was a natural photoperiod. Temperature was maintained at 20 – 35 ˚C. Samples were collected from the photobioreactors and genomic DNA was extracted from these samples to be used for PCR amplification of the V6-V8 regions of the bacterial rRNA. The amplicons were analysed using DGGE to obtain the electrophoretic profile of each sample. According to the Shannon-Weiner diversity index,

bacterial diversity was found to be averaged (2.6 in 3 reactors and inoculum) and low (2.1 in R4; 1:20). The similarity index showed that there was little variability among the samples; the most similar among the indoor reactors was 83.4%. Finally, the most significant bacterial phylum group identified using the RDP and BLAST was *Proteobacteria,* mainly, to the genera *Psychrobacter.*

Keywords

Identification of microorganisms, Molecular Biology; Valorization of Waste Biomass; Swine manure; Microalgae

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1.0 INTRODUCTION

Biological treatment processes are capable of converting the organic matter in the waste/wastewater by living organisms such as bacteria, thereby, resulting in the formation of carbon dioxide and other compounds such as ammonia and phosphorous which can be used as fertilizers. These processes are used in wastewater treatment plants were biological reactors are engineered to boost biochemical degradation under carefully controlled conditions, thereby enhancing the removal of pollutants and the stabilization of sludge (Samer, 2015, Salama et al., 2017). Metcalf and Eddy (1991) grouped biological systems into five groups namely: aerobic, anoxic, anaerobic, combine and pond processes, and as well these processes are further subdivided, depending on whether the treatment takes place in a suspended-growth system or an attached-growth system or a combination of both.

Biological systems have shown to be effective in the treatment of wastewater at relatively minimal cost; the use of microalgae has further increase this platform. The potential offered by microalgae to treat wastewater can be categorised into three main approaches; (1) the efficiency of microalgae-based high-rate algal ponds (HRAPs) treating urban wastewater; (2) the ability of microalgae to treat specific wastewaters (agricultural or industrial); and (3) the ability of microalgae to treat a specific pollutant (Delrue et al., 2016).

The use of microalgae has presented advantages in wastewater treatment due to the fact that they require large amounts of nitrogen and phosphorus for their growth and as such high nitrogen and phosphorus removal of 80 – 100% has been reported from wastewater treatment of different sources (Gonçalves et al., 2017). Cultivation of microalgae (a general term which is used to refer to photosynthetic microorganisms, such as eukaryotic microalgae and prokaryotic cyanobacteria) can take place either in open or closed systems such as open ponds and high-rate algal ponds (HRAP), photobioreactors (PBRs) or attached microalgae cultivation (Delrue et al., 2016). The diversity of these microalgae is quite much and hence

the composition has a large effect on both their treatment capabilities and the biomass production. Microalgae comprise of a phylogenetically heterogeneous group of prokaryotic and eukaryotic microorganisms (Gonçalves et al., 2017). They all use oxygenic photosynthesis thereby converting atmospheric CO_2 to biomass (Krohn-Molt et al., 2013).

Open ponds are usually easy to design, construct and operate, however, there are substantially restricted in their operations due to; water evaporation, large space requirement, contamination of algal cultures, and lack of control over operating parameters (Salama et al., 2017). PBRs are designed to tackle the issues encountered with open pond culturing process. They can provide improved photosynthetic efficiency and reduced footprint. However, they also have their limitations which can be; poor settleability, biomass washout, harvesting limitations (Muñoz et al., 2009). However, Muñoz et al. (2009) and Gonçalves et al. (2017) have suggested biomass immobilization as a means to overcome these challenges faced by PBRs.

The synergistic relationship between microalgae and bacteria is significant during the treatment of wastewater in PBRs. The synergistic relationship between the microalgae and bacteria could be competitive and this could have some negative effects on the organism performance. For example, the excretion of microalgae metabolites could present a bactericidal effect and similarly bacteria can excrete metabolites thereby presenting an algicidal effect. However, a symbiotic relationship could be observed in their interactions such that during photosynthesis, microalgae could release organic compounds that can be used by bacteria as carbon and energy source and O_2 that is used for the oxidation of organic matter while bacteria release the CO_2 required for photosynthetic reactions (Gonçalves et al., 2017, Muñoz and Guieysse, 2006). Therefore, photosynthetic oxygenation, together with microalgae heterotrophic metabolism, can boost the biodegradation of organic pollutants present in wastewater (Muñoz and Guieysse, 2006, Alcantara et al., 2015).

Factors that affect the performance of microalgae could be biotic or abiotic. The presence of pathogens such as bacteria, fungi and viruses, and the competition by other microalgae are categorised as biotic and abiotic factors include light (quality and quantity), temperature, pH, salinity, nutrient qualitative and quantitative profiles, dissolved oxygen concentration and the presence of toxic compounds. Similarly, other parameters such as hydraulic retention time, organic loading rate, temperature, pH, mixing, harvesting rates, gas transfer and mixing, shear rates and light exposure are additional factors that affect their performance (Gonçalves et al., 2017). Therefore the performance of each system using microalgae depends on the ability to operate the system at the right conditions that will enable the optimum performance of the microorganisms.

Different microbial consortium has been reported for different biological systems treating different types of wastewater. For example, in the study by García et al. (2017) for the evaluation of wastewater treatment in a novel anoxic–aerobic algal–bacterial photobioreactor with biomass recycling through carbon and nitrogen mass balances, they revealed that C. *vulgaris* and *Pseudanabaena* sp. were the dominant microalgae species, while *Proteobacteria* was the main phylum according to bacterial phylogenetic analyses. There is a variation to which microbial community exists and this is affected as earlier indicated by various factors. Understanding the microbial population in any treatment process therefore is paramount.

The performance of a biological wastewater treatment plant strongly depends on the activities and interactions of its microbial community. Thus, information on the identity of microorganisms responsible for specific activities, of interactions between cells of the same or different populations and information on the influence of changing environmental conditions are important for optimizing these processes (Delrue et al., 2016). The possibility of identifying specific populations of microorganisms without the need to isolate them has revolutionized microbial ecology and has given rise to various new applications in

numerous research fields. Using traditional and conventional techniques to understand the microbial populations has proved to be much more difficult and ineffective a few decades ago. Characteristics of the organisms such as species composition, structure and bacterial distribution as well as the spatial activity were not well defined.

Improvement in technology and the emergence of methods such as cloning and the creation of a gene library, denaturant gradient gel electrophoresis (DGGE) and fluorescent *in situ* hybridization with DNA probes (FISH) stands out. These techniques are frequently used to detect and characterize bacteria in natural environments. The DGGE is a rapid and easier method that provides distinctive band patterns for different samples thereby allowing quick sample reporting, yet, still maintaining the possibility of a more through genetic analysis of each band (Delrue et al., 2016).

OBJECTIVE

The study presented here is aimed to determine the microbial community in 4 photobioreactors and in inoculum, treating swine manure, using the denaturing gradient gel electrophoresis (DGGE) molecular technique.

2.0 MATERIALS AND METHODS

2.1 Photobioreactors operation and samples collection

Photobioreactors were fed with swine manure, diluted to the ratios of 1:10 or 1: 20. The operating parameters for the photobioreactors are shown in Table 1. The pH was maintained at 8 while temperature was 20 – 25℃ for indoor operation and 20 – 35℃ for the outdoor operation.

Samples for analysis were collected from the four different photobioreactors. The letter R in the table denotes reactor. Therefore R4 was the inoculum while RA – RD were feed with different dilutions of the manure. The reactors were semi-continuously fed as shown in Table 1.

Table 1: Operating parameters for the photobioreactors

Photobioreactors	**R4**	**RA**	**RB**	**RC**	**RD**
Swine manure dilution	Inoculum	1:10	1:20	1:10	1:20
Location		Indoor	Indoor	Outdoor	Outdoor
pH		8	8	8	8
Temperature		20-25˚C	20-25˚C	20-35˚C	20-35˚C
Photoperiod		12L:12D	12L:12D	Natural	Natural
Semi Continuous feeding		12 hours	12 hours	12 hours	12 hours
Evaporation		27%	27%	44%	44%

2.2 DNA Extraction

DNA was extracted from the samples taken from the photobioreactors using the FastDNA spin kit for soil according to the manufacturer's protocol (MP Biomedicals, LLC) handbook. The integrity of extracted DNA was checked using 1.6% (w/v) agarose gel (Anex: Figure A). The samples were immediately used for PCR, or stored at -20 ˚C for further analyses.

2.3 PCR and DGGE analysis

The protocols for the PCR and the DGGE analysis were preformed according to García et al. (2017) and Akmirza et al. (2017). The genomic DNA was amplified by PCR at three different dilutions (undiluted, 1:10, 1:25, and 1:50) to determine the optimal conditions for the DGGE. Two primers, 0968-F-GC forward (5'CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGAACGCGAAGAAC CTTAC3') and 1401-R reversed (5'CGG TGT GTA CAA GAC CC3') (Sigma-Aldrich, St. Louis, MO, USA) were used to amplify the V6-V8 region of eubacterial 16S ribosomal DNA (rDNA). PCR mixtures (50 µL) contained 25 µL of BIOMIX ready-to use with 2 reaction mixture (Bioline, Ecogen), 2 µL of each primer 0968-F-GC and 1401-R, 2 µL of diluted DNA and 19 µL of sterile DNase/RNase Free distilled water.

Amplification of 16S rDNA was carried out using a thermocycler (iCyclerTM Bio-Rad, US). There were three steps involved; the denaturation, annealing and extension. The set program was: 5 min of predenaturation at 94 ˚C, 32 cycles of denaturation at 94 ˚C for 30 s, annealing at 56 ˚C for 30 s and elongations at 72 ˚C for 60 s and a final elongation at 72 ˚C for 10 min. A negative control without DNA was included in the amplification set. Aliquots of 5 µl were used to analyse the size and yield of PCR products on a 1.6% (w/v) agarose gel (Akmirza et al., 2017) (Anex: Figure B). Dilutions with higher amplification for each sample were reamplified to be used in the DGGE analyses.

The DGGE analysis of the amplicons was performed with a D-Code Universal Mutation Detection System (Bio Rad Laboratories) using 10% (w/v) polyacrylamide gels with a urea/formamide denaturing gradient of 45 to 65%. DGGE running conditions were applied according to (Akmirza et al., 2017). The gels were stained with GelRed Nucleic Acid Gel Stain (biotium) for 1 h at room temperature.

2.4 Sequencing analysis

Bands from the gel were excised using a sterile blade and were then eluted into 50 µL of sterile distilled water at 60 ˚C for 1 h. Thereafter, 5 µL of the eluted DNA were reamplified using the original primer set. The thermocycler was programed for 5 mins of pre-denaturation at 94 ˚C, 30 cycles of denaturation, annealing and elongation (94 ˚C for 30 s, 56 ˚C for 30 s and 72 ˚C for 60 s, respectively) and a final 72 ˚C for 10 min last extension. The PCR reamplified products were purified with the Gen Elute ™ PCR Clean-Up Kit (Sigma–Aldrich ™, USA) and quantified by agarose gel electrophoresis using a 50–2000 bp DNA molecular weight marker. Purified PCR products were sent for sequencing.

2.5 Data analysis

The 16S rRNA gene fragments were phylogenetically assigned according to their best matches to sequences based upon BLASTn against GenBank and a curated 16S database derived from high-quality 16S sequences in RDPII database. The 16S rRNA sequences were aligned and clustered in RDPII database (Wang et al., 2007).

DGGE profiles were compared using the GelCompar IITM software (Applied Maths BVBA, Sint-Martens-Latem, Belgium). After image normalization, bands were defined for each sample using the bands search algorithm within the program. The peak heights in the densitometric curves were also used to determine the diversity indices based on the Shannon–Wiener diversity index (H), calculated as follows:

$$H=-\sum [Pi \ln (Pi)] \quad \text{..... (1)}$$

Where, H is the diversity index and Pi is the importance probability of the bands in a lane (Pi = ni/n, and ni is the height of an individual peak and n is the sum of all peak heights in the densitometric curves). Therefore, this index reflects both the sample richness (relative number of DGGE bands) and evenness (relative intensity

of every band) (Akmirza et al., 2017). Usually, it ranges from 1.5 to 3.5 (low and high species evenness and richness, respectively) (Akmirza et al., 2017).
Similarity indices were calculated from the densitometric curves of the scanned DGGE profiles by using the Pearson product–moment correlation coefficient. The taxonomic position of the sequenced DGGE bands was obtained using the RDP classifier tool (50% confidence level). The closest cultured and uncultured relatives to each band were obtained using the BLAST search tool at the NCBI (National Centre for Biotechnology Information).

2.6 Cluster analysis

Similarities between the samples were displayed graphically as a dendrogram. The clustering algorithm used to calculate the dendrograms was the software Gel compare II.

3.0 RESULTS AND DISCUSSION

3.1 Occurrence and comparative sequence analysis of DGGE bands

There are 23 bands from the DGGE gel as shown in Table 2 and Figure 1 from the sequences results. Four different phyla were retrieved from the Ribosomal Database Project (RDP) namely: *Proteobacteria* (15 bands, 59%), *Firmicutes* (2 bands, 7%), *Cyanobacteria/Chloroplas*t (1 band, 3%) and *Bacteroidetes* (4 bands, 14%). *Proteobacteria* were the most in abundance in all the bioreactors. The *Bacteroidetes* were the second most abundant, followed by the *Firmicutes* while the least was the C*yanobacteria/Chloroplast*. The presence of the various classes of phyla indicated the diversity of the microbial community that was in the various sample reactors. The inoculum which was used to inoculate the other reactors showed a more diversified classification, having all the 3 of phyla; *Proteobacteria, Firmicutes and Cyanobacteria/Chloroplast.* The *Bacteroidetes* were not seen in R4. It has been reported that monoalgal-bacterial cultures are not common during the treatment of wastewaters in photobioreactors, despite a microalgae strain can eventually be predominant during a certain period of time. The bacterial community in this study were found to be diversed. The most prominent class in the *Proteobacteria* were the *Gammaproteobacteria* and *Betaproteobacteria*. For the *Cyanobacteria/Chloroplast*, the *Chloroplast* was the main organism. For the *Bacteroidetes*, the class found was the *Cytophagia*. Finally, the *Firmcutes* had the *Clostridia*. The most abundant genus was the *Pschyrobacter* observed in bands 3, 4, 5, 6, 7 and 8. The *Alphaproteobacteria* (band 14), *Betaproteobacteria* (bands 9, 10 and 11) and *Gammaproteobacteria* (band 2) were also present in the reactors. *Pseudomonadales, Rhodocyclales, Rhizobiales,* and *Clostridiales* were the order found. Others such as the *Thauera* were found and *Chlorophyta* were also detected in the samples. The complete list of the bacterial community that was analysed can be further checked in Table 2.

Comparing the microbial community in the inoculum (R4) and those in other reactors, it was observed that R4 had the only three phylum in it (*Proteobacteria,*

Firmicutes and *Chloroplast*) while the rest of the reactors had a more variety. The *Firmicutes* were only found in R4 and RD. Therefore it could be said that the *Firmicutes* disappeared in the other photobioreactors. As a result the *Proteobacteria* and the *Bacteroidetes* were the major phyla found in those reactors.

Chinnasamy et al. (2010) reported that the response of the same species to similar nutrient concentration varied among studies. The reasons for the such change in the characteristics of the microorganisms is not well understood, but have however been linked to variables such as the organic load of the receiving wastewater, species interaction, seasonal environmental conditions, competition and interaction within the microcosms (Riaño et al., 2012).

The abundance of the *Proteobacteria* in the reactors conforms to that which was carried out by García et al. (2017), that study was the enhancement of carbon, nitrogen and phosphorus removal from domestic wastewater in a novel anoxic-aerobic photobioreactor coupled with biogas upgrading. They used DGGE analysis to evaluate the bacterial community in the photobioreactor and it was observed that of the 33 bands, there were 10 phyla and *Proteobacteria* was the dominant phylum, occurring in 17 bound of the 33 (García et al., 2017). Similarly, *Firmicutes* were another phylum group identified in that study. The bacteria from the phyla *Proteobacteria* and *Firmictutes* were likely responsible for the degradation of the organic matter in the photobioreactors. Bacteria from these types of phyla are typically found in activated sludge wastewater treatment plant (WWTP), autotrophic nitrifying and denitrifying bioreactors and HRAPs.

Other studies carried by other authors have identified the bacterial community in microalgae batch degradation tests of swine slurry. These authors found three genera belonging to the *Proteobacteria* phylum (*Acinetobacte*r spp., *Alcaligenes* spp. and *Achromobacter* spp.), two genera from the *Firmicutes* phylum (*Streptococcaceae* ssp. and *Bacillus* ssp.), two from the *Actinobacteria* phylum (*Corynebacterium* ssp. and *Micrococcaceae* ssp.) and one genus belonging to the *Bacteroidetes* phylum (*Flavobacterium* sp.). Even though there are similar

bacterial community identified in this present work, variations still exists from the other studies, this could have been due to techniques applied for isolation and molecular methods and as well the operating conditions of the photobioreactors (Ferrero et al., 2012; Christenson and Sims, 2011).

These findings however, differ from those reported by Ferrero et al. (2012) on the molecular characterization of bacterial communities in algal–bacterial photobioreactors treating piggery wastewaters. In that study, twenty-four bands from the DGGE profile were successfully amplified and sequenced and they were able to identified three bands relating to the *Proteobacteria* phylum (*Gammaproteobacteria*) and two bands were closely affiliated with the *Deinococcus-Thermus* and *Chlamydiae* phyla, respectively. Finally, one band was found to be affiliated with an unidentified bacterium from an environmental sample.

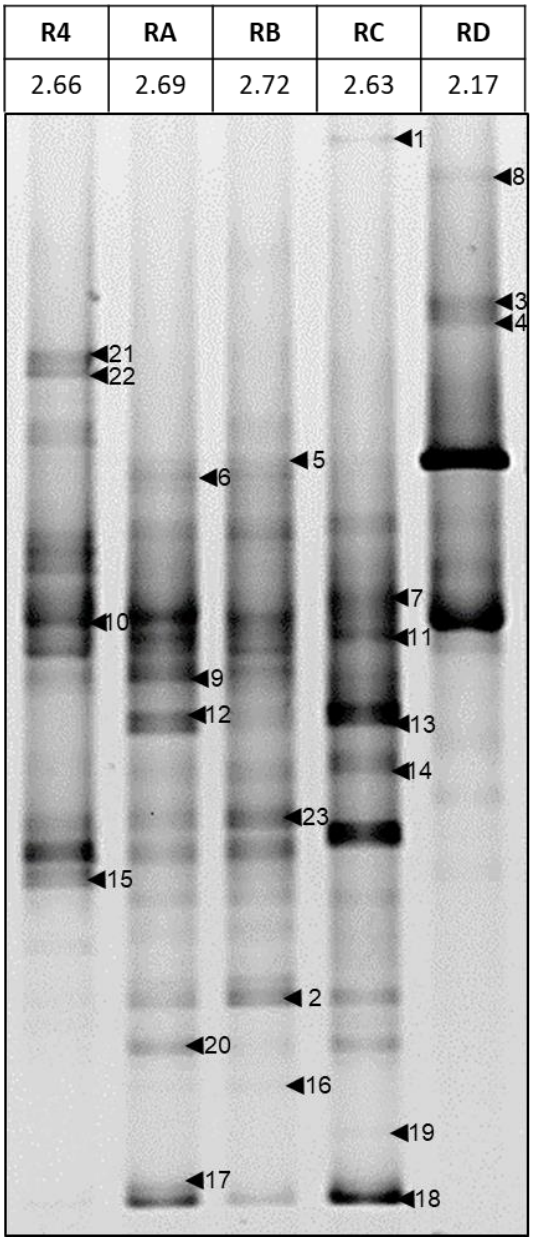

Figure 1: DGGE profile of the bacterial communities present in the inoculum (R4) and RA, RB, RC and RD biomass from the PBR. The Shannon-Wiener diversity indexes are indicated in the upper part of the gel. The sequenced bands are indicated by arrows and the corresponding number of each band.

The Shannon–Wiener diversity index takes into account both the sample richness (relative number of DGGE bands) and evenness (relative intensity of every band) of the species present in a microbial community, with low and high typical values of 1.5 and 3.5, respectively (Akmirza et al., 2017). Figure 1 shows the Shannon index to be 2.66 for the R4 which was the inoculum. The photobioreactor inoculum sample exhibited a low bacterial diversity index. Similarly, low diversity was observed from the other reactors as; RA; 2.69, RB; 2.72, RC; 2.63 and finally RD; 2.17.

The Shannon–Wiener diversity indexes observed in this study are relatively lower than those obtained in similar studies of this kind. HRAPs treating wastewater

typically exhibit H indexes ranging from 3.0 to 3.5, which confirm the high robustness and functionality of the microbiology present in algal-bacterial processes.

Cluster analysis (Figure 2) was performed to assess the degree of similarity in the microbial community structure of the reactor samples. A high similarity between the inoculum and RD (65%) was observed and similarly between RA and RB (83.4%).

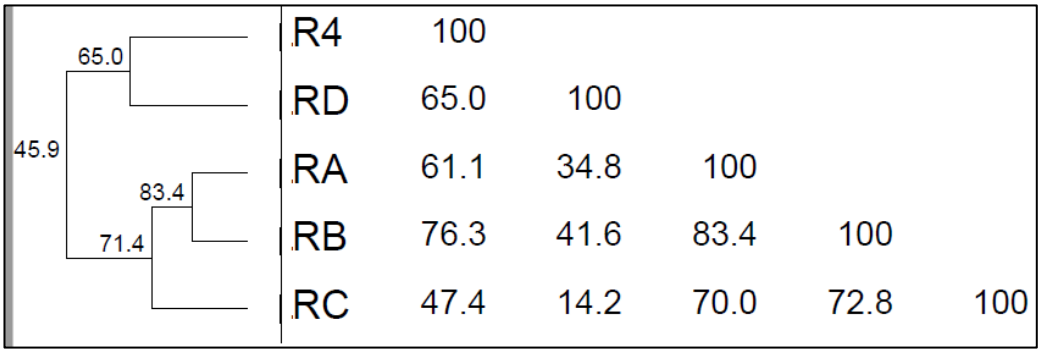

Figure 2: Cluster analysis of the microorganisms.

Table 2: Taxonomic position of the sequenced DGGE bands (RDP classifier tool; 50% confidence level) and corresponding closest relatives in GenBank (BLAST search tool at the NCBI) with their similarity percentages, and environments from which they were retrieved. Presence or absence of the bands in each sample are represented by (X) or (-), respectively. Number of (X), represent intensity of the band in the DGGE gel (X: Intensity ≤ 20; XX: 20 < Intensity ≤ 80; XXX: Intensity > 80).

Taxonomic placement (50% confidence level)	**Band nº**	**R4**	**RA**	**RB**	**RC**	**RD**	**Closest relatives in Blast Name (Accession number)**	**Similarity (%)**	**Source of origin**
Phylum *Proteobacteria*	1	-	-	-	X	-	Uncultured bacterium (JQ320097)	87%	Soil polluted with BDE209 and Cd
Class *Gammaproteobacteria*	2	-	XX	XX	XX	-	Uncultured bacterium (JQ300186)	90%	Soil
Order *Pseudomonadales*									
Family *Moraxellaceae*									
Genus *Psychrobacter*	3	-	-	-	-	XX	Uncultured proteobacterium (JQ218906)	96%	Marine macro-alga
							Psychrobacter sp. Bsw21512 (GQ358937)	96%	Seawater
							Uncultured bacterium (JF332609)	96%	Duodenal biopsy
	4	-	-	-	-	XX	Uncultured Psychrobacter sp. (JQ999390)	97%	Lake Vostok accretion ice (Antarctica)

Taxonomic placement (50% confidence level)	Band nº	R4	RA	RB	RC	RD	Closest relatives in Blast Name (Accession number)	Similarity (%)	Source of origin
							Uncultured bacterium (JF332609)	97%	Duodenal biopsy
							Psychrobacter sp. (KY406049)	96%	Soil sample from penguin breeding colony
	5	-	XX	XX	X	XXX	*Psychrobacter piscatorii* (NR_112807)	99%	Waste water
							Psychrobacter psychrophilus (DQ337513)	99%	Swine effluent holding pit
	6	-	XX	XX	X	XXX	Psychrobacter sp. Mixed culture X14-2 (KR029412)	99%	Bioaerosol emitted from wastewater treatment plant
							Uncultured bacterium (JF332609)	99%	Duodenal biopsy
							Uncultured bacterium (KR514346)	99%	Bovine reproductive tract
	7	-	-	-	XXX	-	Uncultured Psychrobacter sp. (JQ999390)	100%	Lake Vostok accretion ice (Antarctica)
							Psychrobacter sp. Mixed culture X14-2 (KR029412)	100%	Bioaerosol emitted from wastewater treatment plant

Taxonomic placement (50% confidence level)	Band nº	R4	RA	RB	RC	RD	Closest relatives in Blast Name (Accession number)	Similarity (%)	Source of origin
							Psychrobacter sp. KHH8 (KT368953)	100%	-
	8	-	-	-	-	XX	Psychrobacter sp. Mixed culture X14-2 (KR029412)	97%	Bioaerosol emitted from wastewater treatment plant
							Uncultured Psychrobacter sp. (JQ999390)	97%	Lake Vostok accretion ice (Antarctica)
Class *Betaproteobacteria*	9	XX	XXX	XX	XXX	-	Uncultured bacterium (GU390196)	86%	Anaerobic digester treating feedstock
	10	XXX	XXX	XXX	-	XXX	Acinetobacter sp. HPC497 (AY854128)	89%	Wastewater from dye industry
	11	XXX	XXX	-	XXX	-	Uncultured bacterium (KU991981)	87%	Anoxic removal of BTEX compounds
Order *Rhodocyclales*									
Family *Rhodocyclaceae*									
Genus *Thauera*	12	-	XX	XX	XXX	-	Uncultured bacterium (HG380609)	97%	Wastewater
							Uncultured beta proteobacterium (AF450463)	97%	Full-scale aerated-anoxic wastewater treatment plant
							Uncultured Thauera sp. (KX914731)	97%	Activated sludge

Taxonomic placement (50% confidence level)	Band nº	R4	RA	RB	RC	RD	Closest relatives in Blast Name (Accession number)	Similarity (%)	Source of origin
	13	-	XX	-	XXX	-	Thauera sp. (MF155554)	98%	Waste water treatment plant
							Uncultured bacterium (HG380609)	98%	Wastewater
							Uncultured beta proteobacterium (AF450463)	98%	Full-scale aerated-anoxic wastewater treatment plant
Class *Alphaproteobacteria*	14	X	XX	XX	XX	-	Uncultured bacterium (KT200337)	86%	Algal-bacterial biomass from an air-lift bioreactor treating toluene, inoculated with activated sludge, Pseudomona Putida and Chlorella Sorokiniana
Order *Rhizobiales*	15	XX	-	-	-	X	Iron-reducing bacterium enrichment culture clone fec_1_F2 (FJ802355)	89%	Danube River sediment
Phylum *Bacteroidetes*	16	-	-	X	-	-	Uncultured bacterium (KU991981)	90%	Anoxic removal of BTEX compounds
							Uncultured bacterium (KU650792)	90%	Anaerobic full-scale reactors
							Uncultured bacterium (JN087868)	90%	Nitrifying bioreactor under inorganic carbon limitation

Taxonomic placement (50% confidence level)	Band nº	R4	RA	RB	RC	RD	Closest relatives in Blast Name (Accession number)	Similarity (%)	Source of origin
	17	-	XX	-	XX	-	Uncultured bacterium (HQ640531)	98%	Artial nitrifying-ANAMMOX municipal wastewater reactor
							Uncultured bacterium (KU650792)	98%	Anaerobic full-scale reactors
							Uncultured bacterium (JN087868)	98%	Nitrifying bioreactor under inorganic carbon limitation
	18	-	XXX	XX	XXX	-	Uncultured bacterium (HQ640531)	98%	Artial nitrifying-ANAMMOX municipal wastewater reactor
							Uncultured bacterium (KU650792)	98%	Anaerobic full-scale reactors
							Uncultured bacterium (JN087868)	98%	Nitrifying bioreactor under inorganic carbon limitation
Class *Cytophagia*									
Order *Cytophagales*	19	-	-	-	X	-	Uncultured bacterium (HQ640531)	100%	Artial nitrifying-ANAMMOX municipal wastewater reactor
							Uncultured bacterium (KU650792)	99%	Anaerobic full-scale reactors
Class *Sphingobacteria*									
Order *Sphingobacteriales*	20	-	XX	X	XX	-	Uncultured bacterium (KU650792)	99%	Anaerobic full-scale reactors
							Uncultured bacterium (JN087868)	99%	Nitrifying bioreactor under inorganic carbon limitation

Taxonomic placement (50% confidence level)	Band nº	R4	RA	RB	RC	RD	Closest relatives in Blast Name (Accession number)	Similarity (%)	Source of origin
							Uncultured bacterium (KU991981)	99%	Anoxic removal of BTEX compounds
Phylum *Firmicutes*									
Class *Clostridia*									
Order *Clostridiales*	21	XX	-	-	-	-	Uncultured bacterium (GQ132773)	86%	SBR reactor treating swine waste; reactor 2, day 809; temperature: 35 deg C; ammonia: 1,800 mg N/L; solids loading rate: 2.2 g VS/L/day
	22	XX	-	-	-	XX	Uncultured bacterium (KP797907)	87%	Microalgae from HRAP treating diluted vinasse with wastewater treatment plant activated sludge
Phylum *Cyanobacteria/Chloroplast*									
Class *Chloroplast*									
Family *Chloroplast*									
Genus *Chlorophyta*	23	XX	XX	XXX	XXX	-	Uncultured bacterium (KT200344)	98%	Algal-bacterial biomass from an air-lift bioreactor treating toluene, inoculated with activated sludge,

Taxonomic placement (50% confidence level)	Band nº	R4	RA	RB	RC	RD	Closest relatives in Blast Name (Accession number)	Similarity (%)	Source of origin
									Pseudomona Putida and Chlorella Sorokiniana
							Plastid Chlorella sp. UMPCCC 1110 (KM218897)	98%	Water
							Uncultured Chlorella (KC994689)	96%	Microalgae photobioreactor

3.2 Effects of light on microorganism

The availability of light is one of the major limiting factors in photobioreactor design. It is the main medium through which energy is added to the algae in the system so parameters such as light wavelength, intensity, penetration, regime etc. must be considered. In photosynthetic cultures, the amount of light energy received and stored by the cells has a direct relationship with the carbon fixation capacity, consequently determining the productivity in biomass and cell growth rate (Jacob-Lopes et al., 2009). Generally algal growth activity increases with light intensity until around 200-400 µmol/m^2s (Muñoz and Guieysse, 2006). However, one major challenge with lighting a photobioreactor is light penetration. This is particularly a problem when the photobioreactor uses raw wastewater which is dark in coloration and can have a high amount of particulate matter (Christenson and Sims, 2011). Wastewaters of animal sources are characterised by high organics, hence the presence of these organic particulates could impede the maximum penetration of light into the reactors.

The reactors RA and RB were exposed to a 12 hour light (LED) and a 12 hour non light. Similarly, reactors RC and RD were exposed to a 12 hour natural light. Both reactors were operated under the same pH, however with regards to temperature, reactor RA and RB were operated at 20 – 25 ˚C while RC and RD were at 20 – 35 ˚C. The 12h light and 12h dark chosen in this study were in accordance to other studies. According to Christenson and Sims (2011) the 12h light and 12h dark photoperiod has been chosen to be the best for the achievement of a higher cell density. Noteworthy is the evaporation that occurred in the reactors. Reactors that were operated in the open had a higher evaporation rate of 44% while the indoors were at 27%. Again, relating to the behaviours of the microorganisms to the photoperiods used in this shows that there was variations in each reactor. It has been reported that higher diversity is typically found in outdoor cultures during summer as a result of the higher temperatures and light irradiances favouring microalgae-bacterial growth (Posadas et al; 2015).

However, in this study, higher diversity was found in the indoor reactors RA and RB (2.69 and 2.72) while lower diversity was seen in reactor RC and RD (2.63 and 2.17). The reason for the low diversity could have been due to the influence of environmental conditions which limited the proper maintenance of operating parameters. Studies by De Godos et al (2009) however showed that higher microalgae diversity is typically found in outdoors cultures during summer as a result of the higher temperatures and light irradiances favouring microalgae growth. Similarly, Brennan and Owende (2010) reported that low biomass growth in open photobioreactors could be affected by other factors, including evaporation losses, inefficient mixing and light limitation. This was the case of the RC and RD. They were operated at a higher evaporation rate of 44% as compared to RA and RB which were at 27%.

Jacob-Lopes et al (2009) on the study of the effect of light cycles (night/day) on CO_2 fixation and biomass production by microalgae in photobioreactors reported that the cultures grown under photoperiods of 2:22 (night: day) showed characteristics similar to those grown with a continuous supply of light energy, whilst those grown in the absence of light showed evidence of limited carbon source for cell growth. They attributed that to the fact that *Cyanobacteria* are unable to use inorganic carbon sources in the absence of light, and the organic carbon concentrations in the culture medium were insufficient for the energy maintenance of respiratory metabolism. The influence of the light cycles has been reported as a determinant factor in photosynthetic activity and in the growth rates of microalga-bacterial in photobioreactors. Jacob-Lopes et al (2009) further stated that light is a limiting substrate in reacting systems, which are affected by light/dark zones that depend primarily on the configuration, agitation and mixture in the reactor, associated with the possibility of cultures with discontinuous periods of light energy supply. Additionally, cell concentration is another parameter which determines the availability of light in photobioreactors. As a result of the mutual shading occurring at high cell densities, therefore, the light intensity within the reactor becomes a function of the biomass

concentration and due to the fact that the cells are exposed to different light intensities, with a considerable effect on system performance.

3.3 Effect of influent dilution on microorganisms

Wastewaters from animal sources contain high organic substances such as ammonia and other substances which can limit the performances of the microorganisms thus causing their inhibition. Therefore to limit such inhibit, dilution of feed stock is recommended. Also to allow the penetration of light into the reactors, it was deemed suitable to dilute the feed. The influent feeds were diluted to ratios of 1:10 and 1:20. Averagely, the reactors with higher concentration appeared to have more abundancy of the microorganisms in them as can be seen in Table 2. Reactors RA and RC had a lower dilutions of 1:10 while RB and RD had higher dilutions of 1:20. This suggested that the population of the microorganisms increased with a higher feed influent this could be due to a higher carbon and nutrients (N and P) available in the feed. Studies have reported that variables such as the organic load of the receiving wastewater, the seasonal environmental conditions, or the potential interactions within the microorganisms have shown to influence the composition of the microalgal-bacterial population (De Godos et al., 2009). In their study of the long-term operation of high rate algal ponds for the bioremediation of piggery wastewaters at high loading rates, De Godos et al (2009) found out that the combination of moderate temperatures/irradiances and nutrient concentrations supported higher microalgae.

A similar study carried out by Riaño et al. (2012) on the microalgal-based systems for wastewater treatment: effect of applied organic and nutrient loading rate on biomass using two different photobioreactors for comparison found out that the biomass productivity increased concurrently with higher loading rates in both photobioreactors and they ascribed this to higher carbon and nutrients (N and P) availability in the initial feed to the reactors.

Again, Posadas et al. (2015) reported that DGGE analyses have been consistently carried out in algal-bacterial photobioreactors in order to determine the richness and composition of the bacterial community supporting wastewater treatment. In their study, they recorded a high bacterial diversity of 2.8-3.3 during the treatment of diluted anaerobically digested vinasse in a 180 L HRAP with simultaneous biogas upgrading based on the Shannon-Wiener diversity index, which indicated low and high bacterial diversity for 1.5 and 3.5, respectively. In the same note, Alcántara et al. (2015) found a high microbial diversity (Shannon-Wiener indices of 2.6-3.5) during the evaluation of WW treatment in a novel anoxic-aerobic algal-bacterial photobioreactor.

3.4 Effect of different operating conditions on microorganisms

The composition of the algae and bacteria community has a large effect on both the treatment capabilities of a photobioreactor and the biomass production. Several factors could influence the type of microalga-bacterial that will be found in a system. Some of these factors could be operating parameters or environmental conditions. Some authors have reported chance and random immigration within the reacting vessels. All these factors have been shown to be of importance for the dynamics of microbial communities. Therefore this means that at certain conditions, different types of microorganisms could be seen. This is the case for the reactors (RA-RD) used in the study. For instance, the inoculum (R4) which was used to activate other reactors (RA-RD) showed different variability of microalga-bacterial distribution. Further still RA and RB were operated at (12 h day) and feed concentration diluted to 1:10 and 1:20 and Reactors RC and RD at (12 h natural light) and feed concentration of 1:10 and 1:20 show disparity in the distribution of the microalga-bacterial community. This study conforms to the fact that variations in characteristics of the incoming wastewater into a treatment plant/unit can affect the organic community that will be found in the reacting vessel.

Novo et al. (2013) reported that the bacterial community composition in an incoming wastewater, which is the pool from where immigrating species are drawn, is expected to be dynamic and the dynamism patterns could be independent of the processes that dominate the assembly of the microbial community in the reacting vessels. However, it is worthy to note that in most cases the communities are often reported to be highly dynamic while the process performances could remain stable.

Other studies have described the hydraulic retention time (HRT) as the most important operating parameter in wastewater treatment using microalgae and both temperature and solar radiation are regarded as crucial environmental factors for the performance of outdoor PBRs (Maza-Márquez et al., 2017).

According to Ramanan et al. (2016) algae, cyanobacteria and bacteria coexist in a wide range of extreme habitats and fight unfavourable environmental conditions by an array of mutualistic mechanisms. Muñoz and Guieysse (2006) reported that the efficiency of microalgae-based treatments is known to decrease at lower temperatures due to slowing down of biological activities; however, they recommended that the effect can be overcome by using cold adapted photosynthetic strains.

Maza-Márquez et al (2017) found a strong correlation between the relative abundance of the dominance of bacterial and microalgae detected in the PBR biofilms and the environmental/operational parameters during their studies. They further observed that the taxa that displayed the highest relative abundances in the PBR appeared to play crucial functions on the removal of pollutants from the treated effluent. They concluded that it was worth saying that few populations became dominant in PBR and they correlate strongly with the removal of pollutants.

The metabolism of organisms can cause them to strive in some systems and not the others. For example the *Proteobacteria* (*Alphaproteobacteria, Betaproteobacteria* etc.*)* grow at very low levels of nutrients and, the *Alphaproteobacteria* group can utilize nutrients in a variety of ways and are good

nitrogen fixating bacteria. This attribute of the *Proteobacteria* has made them possible to be used in wastewater treatment plants. Similarly, the *Firmicutes* produce endospores, which are resistant to desiccation and can survive extreme conditions, again, another reason they are found in most WWTPs.

Microorganisms adapt to different water treatment processes, for instance, there are the anaerobic ammonia oxidation (anammox) bacteria, the methanogenic bacteria (digestion of activated sludge to produce biogas through methanogenesis) (Zhou et al., 2011).

4.0 CONCLUSION

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APPENDIX

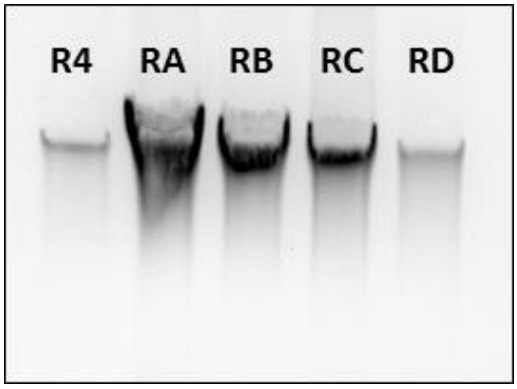

Figure A: Genomic DNA after electrophoresis in 1.6% (w/v) agarose gel. R4 correspond to the inoculum, and RA, RB, RC and RD correspond to the samples of each photobioreactor.

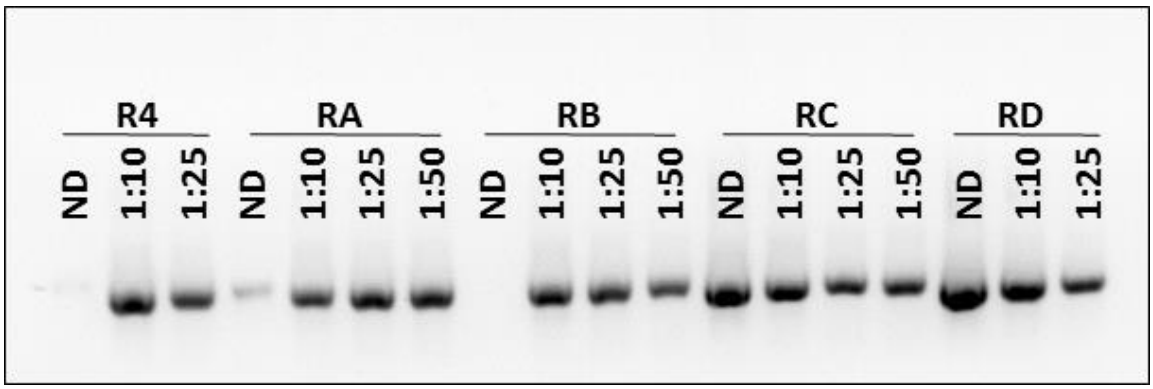

Figure B: Amplicons of the V6-V8 region of eubacterial 16S ribosomal DNA (rDNA), after electrophoresis in 1.6% (w/v) agarose gel. The genomic DNA extracted from the inoculum (R4), and from the 4 photobioreactors (RA, RB, RC, RD) were amplified by PCR at three different dilutions (ND: undiluted, 1:10, 1:25, and 1:50)