Waste and Biomass Valorization
https://doi.org/10.1007/s12649-022-01759-0

ORIGINAL PAPER

Characterization of Bacterial and Archaeal Communities by DGGE and Next Generation Sequencing (NGS) of Nitrification Bioreactors Using Two Different Intermediate Landfill Leachates as Ammonium Substrate

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Received: 29 September 2021 / Accepted: 27 March 2022
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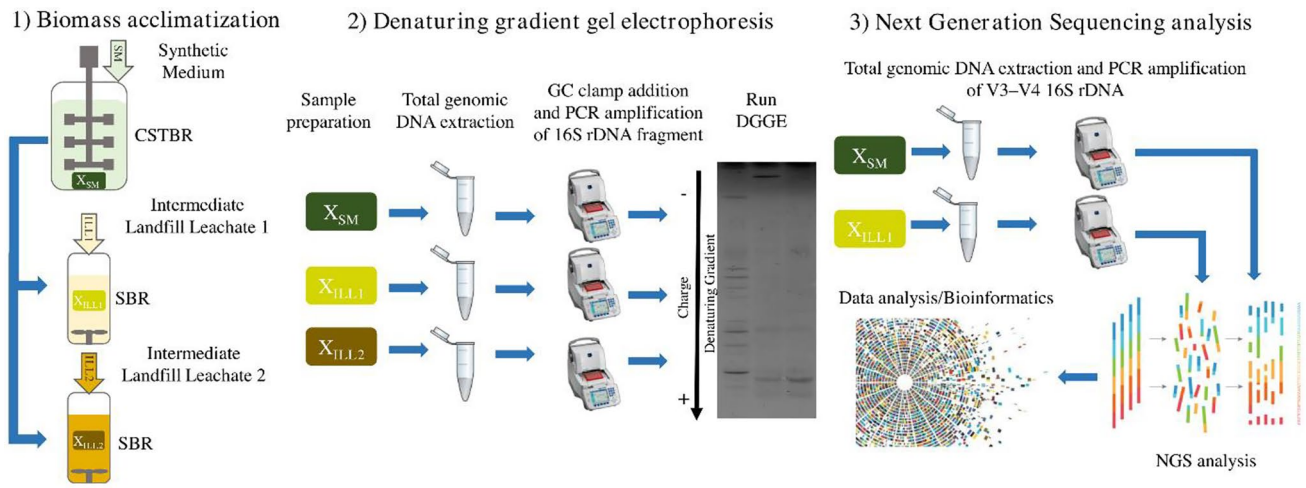
Abstract

Nitrification–denitrification is an environmentally friendly and cost-effective way to treat landfill leachates. Special attention has been given to the nitrification step, usually the limiting one due to its special sensitivity to environmental factors. Here, the effect of the acclimatization of the nitrifying biomass to two different intermediate landfill leachates with different salt concentrations, COD and BOD_5 has been studied. Despite the complete nitrification being successfully performed, the specific nitritation rates were reduced after the biomass adaptation to both landfill leachates caused by the presence of heavy metals and the high salt concentration. NGS analysis of the biomass samples revealed that *Proteobacteria* (48.5%), *Actinobacteriota* (14.4%) and *Chloroflexi* (9.5%) were the dominant phyla in the non-adapted biomass. The leachate feeding led to a decrease in OTU diversity and favored the growth of the phyla *Bacteroidetes* (27.2%), *Euryarchaeota* (26.6%) and *Proteobacteria* (20.0%) accounting for more than 70% of relative abundance. Several OTUs capable of performing the nitritation belong to the *Xanthobacteraceae* and the *Xanthomonadaceae* families, the *Saccharimonadales* order, and the genus *Nitrosomonas*, *Nitrosospira* and *Paracoccus*. In the nitratation process, the *Xanthobacteraceae* family and *Lautropia* and *Nitrolancea* genera were found.

Graphical Abstract



Keywords Nitrification · Landfill leachate · Denaturing gradient gel electrophoresis (DGGE) · Next generation sequencing (NGS)

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Published online: 29 April 2022

Statement of Novelty

Landfill leachates are highly polluted effluents whose treatment is difficult. The use of microorganisms can achieve its treatment efficiently. In the present work, the effect of feeding landfill leachate to unadapted microorganisms is studied from a microbiological point of view. In this way, the process can be better understood and potential improvements can be applied in full-scale plants.

Introduction

The rapid economic and population growth lead to a remarkable increase in the generation of municipal solid waste (MSW) over the past decades [1]. Landfilling is the most common means of disposing of this MSW due to its low cost and effectiveness [2]. However, its uncontrolled operation can cause significant health and environmental risks such as the pollution of air, soil and groundwater [3]. The ground-water pollution generated by the infiltrations of landfill leachates is a challenging issue to overcome. Landfill leachates are highly polluting liquids that result from water percolation through the waste deposits and their generation has been expected to increase [4]. These effluents are mainly characterized by containing high concentrations of ammonium and organic matter as well as toxic metals and chlorinated salts [5]. Biological treatment of these aqueous effluents is preferred over physicochemical methods from an economic and environmental point of view [6]. A commonly accepted indicator to determine and classify the biodegradability of landfill leachate is the ratio of biological oxygen demand (BOD_5) to chemical oxygen demand (COD). This BOD_5/COD ratio mainly depends on landfill age and can vary from 0.4 in young (< 5 years old) to 0.1 in mature (> 10 years old) leachates [7, 8]. Therefore, young landfill leachates are usually better treated by biological methods compared to mature leachates in which the presence of recalcitrant compounds becomes dominant [9].

During biological treatment, in the first stage, the ammonium is removed by nitrification which is carried out by ammonium-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). This stage is usually followed by a denitrification step, in which the microbial reduction of nitrate or nitrite takes place at the same time that the organic matter, inherent or supplemented, is degraded [10]. Due to the nitrification stage being usually considered the limiting step, the research on this topic has been usually focused on this first step [11]. These AOB and NOB communities are well-known to be highly sensitive to changes in environmental factors such as salinity, pH, temperature or dissolved oxygen [12]. Therefore, it is essential to assess the diversity of the microbial communities present during landfill leachate treatment in order to understand the chemical transformations that occur during the process and to optimize their operational conditions in bioreactors.

Traditionally, the microbial communities present in activated sludge used for wastewater treatment have been described employing different molecular biology techniques such as PCR- denaturing gradient gel electrophoresis (DGGE) based on the 16S RNA ribosomal gene [13–15], fluorescence in situ hybridization (FISH) [12] or sequencing of clone libraries of 16S rRNA genes [16]. However, due to the limited information provided by these techniques, new methods such as Next Generation Sequencing (NGS) based on 16S rRNA gene sequencing have been developed as faster and more efficient techniques for microbial community analysis compared to traditional methods. NGS allows a higher resolution and sensitivity for relative quantification of operational taxonomic units (OTUs) including those with very low abundances compared to DGGE or the clone library sequencing techniques.

Despite the characterization of nitrifying communities having been widely reported in the literature, the study of changes in the microbial community composition, that takes place during its acclimatization to intermediate landfill leachates, has not been performed so far.

For this reason, the present study aims to elucidate the effect of acclimatization to landfill leachates on the microbial community composition. Three different ammonia sources consisting of synthetic medium (SM) and two different intermediate landfill leachates (ILL1 and ILL2) were used. After acclimatization, three different nitrifying consortia were generated: (i) Biomass adapted to synthetic medium (X_{SM}); (ii) Biomass adapted to intermediate landfill leachate 1 (X_{ILL1}) and (iii) Biomass adapted to intermediate landfill leachate 2 (X_{ILL2}) whose microbial composition and shifts were assessed using a DGGE and NGS analysis.

Materials and Methods

Landfill Leachate Collection and Characterization

In March and July 2017 two types of landfill leachates were obtained from a municipal solid waste treatment plant located in Cadiz (*Complejo Medio Ambiental de Miramundo*, situated in: 36° 28′ 42.5″ N 6° 00′ 56.1″ W). The ILL1 was obtained from an active cell (12 years of operational life) and the ILL2 was collected from a closed landfill cell (running for 19 years). The methodology used to determine the main physico-chemical parameters, ions and

heavy metals of both landfill leachates is summarized in Gonzalez-Cortes et al. [17].

Activated Sludge Growth and Acclimatization

Primary activated sludge from a conventional wastewater treatment plant (*El Torno*, Cadiz, Spain) was used to inoculate a continuous stirred tank bioreactor (CSTBR) of 5 L (Applikon Biotechnology BV, The Netherlands) with a settler (1.3 L) for biomass recirculation. The ammonium source used to feed the CSTBR was synthetic wastewater composed of NH_4Cl to a concentration of 1.1 g N–NH_4^+ L^{-1} and nutrients coming from a commercial NPK fertilizer 6-4-6 (Infertosa, Spain) (5 g L^{-1}). The ammonium inlet load (IL) applied to this bioreactor was 0.7 kg N-NH_4^+ m^{-3} day^{-1}. Dissolved oxygen (DO), pH and temperature were measured with a digital multiparametric (Multimeter 44, Hach Lange, Spain). $NaHCO_3$ (50 g L^{-1}) was used as inorganic carbon source for the nitrifying biomass and to control the pH. The system was monitored and controlled using the LabVIEW™ platform (National Instruments™, USA) [18]. The bioreactor was operated for 1 year at room temperature of 24 ± 2 °C, pH of 8 and DO > 2 mg O_2 L^{-1}, which led to the development of nitrifying biomass X_{SM} which was inoculated into two different 1 L sequential batch reactors (SBRs) (Multifors 2, Infors HT, Switzerland). The first SBR was fed only with ILL1 while the other one was provided with ILL2. Landfill leachates were fed in 72 h cycles. For the period of 72 h, the unit was aerated for 70.5 h, 1 h was settling, and 30 min was needed for decanting and feeding. The ammonium IL of both SBRs was 0.2 kg N-NH_4^+ m^{-3} day^{-1}. Ammonium accumulation was avoided by monitoring ammonium uptake. The acclimatization was considered to be completed when the ammonium concentration in the effluent was stabilized after three months of operation at the same conditions (24 ± 2 °C, pH 7.8). The alkalinity present in the leachates required the addition of H_3PO_4 (2 N), besides $NaHCO_3$, to adjust the pH to 7.8.

Determination of Nitrifying Activity by a Respirometric Essay

A glass bioreactor (Multifors 2, Infors HT, Switzerland) of 1 L was used as a respirometer, in which temperature, pH (EasyFerm Plus PHI Arc 225, Hamilton Iberia S.L.U, Spain) and DO (InPro 6050, Mettler Toledo, USA), were monitored. The nitritation rates of the three different microbial populations (X_{SM}, X_{ILL1} and X_{ILL2}) were evaluated by monitoring the oxygen uptake rate (OUR) after the addition of NH_4Cl. Firstly, endogenous respiration was established by keeping the system without substrate for 5 h. Then, the oxygen uptake rate (OUR) was determined (Eq. 1) as follows [19]:

$$OUR = d([O_2]^{sat} - [O_2]^{obs})/dt \quad (1)$$

For OUR measurement the system was aerated until the oxygen saturation concentration was reached in the medium. Then, the aeration was stopped and a certain amount of the concentrated NH_4Cl solution was added to the inoculated respirometer to an ammonium concentration of 100 mg N-NH_4^+ L^{-1}.

After substrate addition, the OUR was calculated considering the total biomass concentration in the bioreactor in triplicate. Selective inhibitors of NOB as $KClO_3$ (6 mg L^{-1}) and AOB as allylthiourea (ATU) (15 mg L^{-1}) were added to the respirometer to distinguish between the OUR corresponding to AOB, NOB and total heterotrophic bacteria (THB) [20]. Firstly, without inhibitors in the respirometer, the oxygen consumption was the total of the oxygen consumed by AOB, NOB and THB at the same time. Then, taking into consideration the complete oxidation of ammonium to nitrate, 75% of oxygen uptake is attributable to the ammonium oxidation to nitrite (Eq. 2) while 25% is caused by the nitrite oxidation to nitrate (Eq. 3) once deducted the oxygen consumed by THB and the endogenous respiration of all bacteria can be determined.

$$NH_4^+ + 3/2\ O_2 \rightarrow NO_2^- + 2H^+ + H_2O \quad (2)$$

$$NO_2^- + 1/2\ O_2 \rightarrow NO_3^- \quad (3)$$

When $KClO_3$ was added to the respirometer, the oxygen consumption was assignable to the activity of NOB and THB. In contrast, the oxygen consumption can be related only to the THB action when ATU and $KClO_3$ were supplied. The oxygen consumption corresponding to the ammonium oxidation to nitrite was obtained deducing from the OUR measurements when $KClO_3$ was added to the respirometer the oxygen consumed by the THB ($KClO_3$ + ATU) and the endogenous respiration. Equation (2) was used to determine the total ammonium oxidation from the oxygen consumption. Ammonium, nitrate and nitrite were determined by ion chromatography (Metrohm, 930 Compact IC Flex, Switzerland) on a system equipped with a conductivity detector. Anions and cations were measured using Metrosep A Supp 5-250/4.0 and Metrosep C 6-250/4.0 columns (Metrohm, Switzerland), respectively. Nitrogen gas was determined through mass balance by subtraction.

DNA Extraction and PCR-DGGE

The three biological samples (X_{SM}, X_{ILL1} and X_{ILL2}) were taken from the bioreactors in which the acclimatization period took place (92 days). A volume of 50 mL was harvested from the bioreactors and centrifuged at 9000×*g* for 10 min. Then, samples were processed for total genomic

DNA extraction using UltraClean™ Soil DNA Isolation Kit (MoBio Laboratories Inc., USA). The total extracted DNA was used for amplification of the region V3–V5 of 16S rRNA gene by PCR using the universal primer forward GC-338-F (5′ CGCCCGCCGCGC GCGGCGGGCGGGGCG GGGGCACGGGGGGCCTACGGGAGGCAGCAG-3′) and the reverse primer 907-R (5′-CCGTCAATTCCTTTGAGT TT-3′) following the procedure as previously described [21].

The denaturing gradient gel electrophoresis (DGGE) was performed using a 6% (w/v) polyacrylamide gel in 1X TAE buffer (40 mM Tris–acetate 1 mM EDTA, pH 8.3) with a denaturing gradient from 30 to 60%, and 1 µg of DNA was loaded onto DGGE using the DCode™ system (*Bio-rad, USA*). Electrophoresis was performed at 60 °C for 20 h at a constant voltage of 75 V. The gel was stained with ethidium bromide and DNA bands were visualized using an ultraviolet transilluminator (GE Healthcare ImageQuant, Germany). The similarities among the microbial populations along with the diversity and dominance indexes were calculated form DGGE profiles using Bionumerics software as described by Brito et al. [22].

Next Generation Sequencing (NGS)

To study the microbial communities further, the X_{SM} gDNA and X_{ILL1} V3−V4 16S rRNA region samples were used as input material for library constructing and sequencing of 16S rRNA V3–V4 region gene using a NGS platform (STAB Vida, Portugal). The library construction was performed using the Illumina 16S Metagenomic Sequencing Library preparation protocol and the generated DNA fragments were sequenced with MiSeq Reagent Kit v3 in the Illumina MiSeq platform, using 300 bp paired-end sequencing reads. The resulting reads were denoised using the Deblur plugin and were searched in SILVA (release 138 QIIME2 v2020.8) [23] database with a clustering threshold of 97% similarity and organized in features so-called OTUs as units of observation and then classified by taxon using a fitted classifier. For classification purposes, only OTUs containing at least 10 sequence reads, were considered significant. The number of reads of each OTU is represented as relative abundance, with the total reads being 100%.

Results and Discussion

Characterization of Landfill Leachates

The characteristic properties of landfill leachates are mainly dependent on the production site conditions like temperature, available oxygen, moisture, the landfill age as well as solid waste composition [24]. With the purpose of gaining insights into the composition of the two landfill leachates used for the acclimatization of the nitrifying biomasses, the main physicochemical parameters were obtained. An adaptation of the previously published data highlighting the most important features of both landfill leachates is shown in Table 1. The values of most parameters are in concordance with those characterized by several authors [24–26]. The conductivity of the ILL2 (51.5 mS cm^{-1}), which is related to the salinity concentration of the effluent, is one of the parameters that must be stressed. The application of a conversion factor [27] to this high conductivity allowed the total dissolved solids concentration of ~ 36 g L^{-1} (comparable to seawater [28]) to be obtained. In view of the results, this salinity can be mainly attributed to chloride, sodium and potassium ions. It is also important to note the higher COD, BOD, alkalinity and solid content of the ILL2 compared to the ILL1 which could be explained by the differences in the generation of these two effluents.

Table 1 Summary of main physicochemical parameters and heavy metal concentration in the landfill leachates used for biomass adaptation (Adapted from [17])

Test parameter	ILL1	ILL2
pH	7.96	8.31
Conductivity (mS cm^{-1})	15.8	51.5
COD (mg L^{-1})	1,881.8	9,570.2
BOD_5 (mg L^{-1})	560	2,120
BOD_5/COD ratio	0.3	0.2
N-NO_3^- (mg N L^{-1})	74.5	144.7
N-NO_2^- (mg N L^{-1})	2.2	< 0.2
N-NH_4^+ (mg N L^{-1})	803.7	1,996.7
Cl^- (mg L^{-1})	1,777.7	12,728.1
Na^+ (mg L^{-1})	1,018.4	6,192.1
K^+ (mg L^{-1})	828.5	3,470.67
Ca^{2+} (mg L^{-1})	81.6	76.4
Heavy metals (mg L^{-1})	ILL1	ILL2
Cu	0.400 ± 0.04	0.170 ± 0.01
Ni	0.350 ± 0.010	1.20 ± 0.01
Zn	2.36 ± 0.12	1.04 ± 0.05

Broadly speaking, the heavy metal concentration of both landfill leachates is comparable to those values obtained by other studies. Only some heavy metals such as Cu, Ni and Zn can be found in the inhibition range for nitrifying biomass as was stated by Hu et al. [29], Kapoor et al. [30] and Tang et al. [31].

Nitritation Activity of the Nitrifying Sludge

In order to quantify and verify the nitrifying activity of the different biomasses, respirometric assays were performed before the biomass harvesting. Figure 1a represents the q_s obtained by the three different biomass samples. The highest

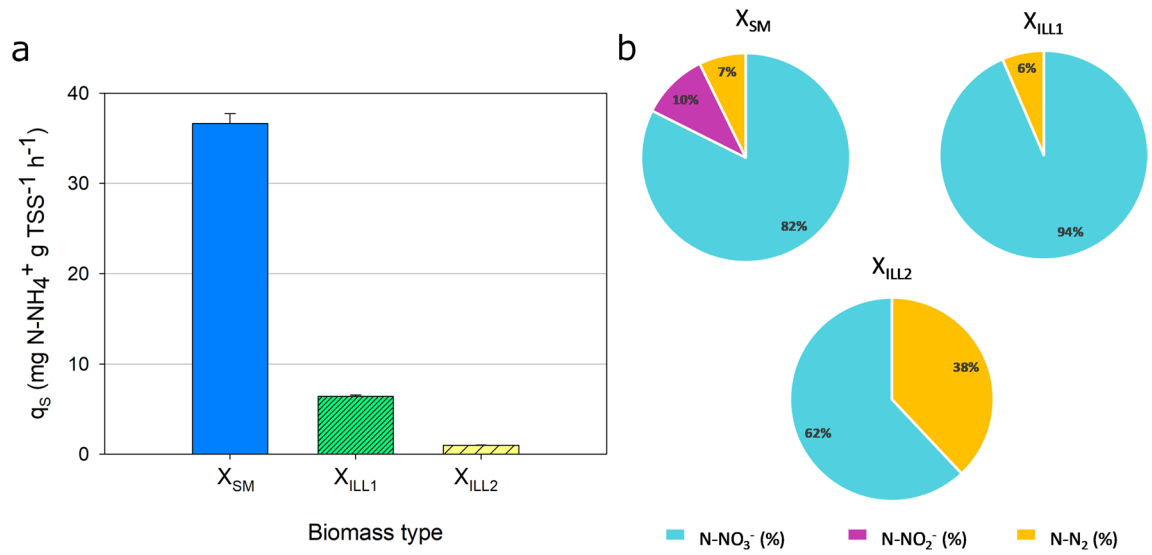

Fig. 1 (**a**) Specific ammonium oxidation rate of the three different types of biomasses at an ammonium concentration of 100 mg N–NH_4^+ L^{-1}. (**b**) The fate of nitrogen species in the different bioreactors after biomass acclimatization

q_s was obtained by the X_{SM} (36.64 ± 1.10 mg N-NH_4^+ g TSS^{-1} h^{-1}), followed by the X_{ILL1} (6.40 ± 0.17 mg N-NH_4^+ g TSS^{-1} h^{-1}) and finally the X_{ILL2} (0.98 ± 0.4 mg N-NH_4^+ g TSS^{-1} h^{-1}). The differences among these samples can be explained by the composition of the substrates used to feed the bioreactors. While the CSTBR inoculated with X_{SM} was only fed with the synthetic medium whose composition lacks inhibitors, the other two SBRs where X_{ILL1} and X_{ILL2} were present, were fed with landfill leachates. The composition of these landfill leachates includes several toxic compounds for the nitrifying biomass such as heavy metals or a high salt concentration among others. Therefore, the use of these substrates caused a strong inhibition of the nitritation activity of X_{ILL1} and X_{ILL2} which was most likely caused by the heavy metal internalization of X_{ILL1} and the osmotic shock caused by the high salt concentration of the ILL2 [17].

The q_s shown by our different biomasses agree with data reported by other authors using synthetic medium and real effluents. Rongsayamanont et al. [32] found a maximum q_s of 30.8 mg N-NH_4^+ g TSS^{-1} h^{-1} using synthetic medium and an inoculum enriched in nitrifiers coming from activated sludge from wastewater treatment facilities. Also a similar maximum q_s of 37.3 mg N-NH_4^+ g TSS^{-1} h^{-1} was obtained by Carrera et al. [33], who operated a suspended biomass system using again synthetic medium as ammonium substrate. On the other hand, using real ammonium-rich effluents these q_s values are normally lower. Nhat et al. [34] obtained a maximum q_s of 6.3 mg N-NH_4^+ g TSS^{-1} h^{-1} using a lab-scale SBR fed with high ammonium strength old municipal landfill leachate. Other authors such as Whang et al. [35] also obtained lower q_s (4.7 mg N-NH_4^+ g TSS^{-1} h^{-1}) operating a full scale wastewater treatment plant fed with wastewater.

Figure 1b shows the nitrogen species present in the bioreactors before the respirometric assays and the subsequent biomass harvesting took place. It can be highlighted that nitrate was the predominant nitrogen species in the bioreactor indicating that the three bioreactors were successfully operated to favor the complete nitrification. Nitrite was only found in the CSTBR, probably due to the higher nitrogen load applied to this bioreactor (0.7 kg N-NH_4^+ m^{-3} d^{-1}) in comparison with the SBRs (0.2 kg N-NH_4^+ m^{-3} day^{-1}). Also, the existence of anoxic zones in the bioreactor led to the development of denitrifying bacteria which was noticed by the production of nitrogen gas (N_2) during the operation. The highest N_2 production was found in the bioreactor fed with the effluent with the highest organic matter (ILL2) which probably led to the development of heterotrophic denitrifiers.

Therefore, taking into consideration the above data it can be concluded that the three biomasses were enriched in active nitrifying bacteria performing complete nitrification. However, the biological treatment of ILL2 seems not to be feasible due to the low q_s value of the X_{ILL2}, probably related to the biomass inhibition by the high salinity of the effluent.

DGGE Fingerprint Analysis of the Different Biomasses

A large group of bacteria is involved in ammonium removal from landfill leachates. Gaining knowledge in the composition and diversity of this consortium is essential to develop and design strategies for the treatment of this effluent. Therefore, a preliminary microbial diversity analysis of the three

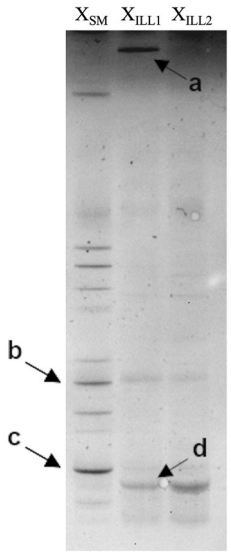

Fig. 2 DGGE band profiles of amplified 16S rDNA from the three biomass samples adapted under different ammonium substrates and similarity coefficients (%) – Pearson correlation for Bacteria Domain

Table 2 Diversity indexes of the different nitrifying biomass samples

Sample	Simpson		Shannon–Wiener	
	DGGE	NGS	DGGE	NGS
Non-adapted biomass (X_{SM})	0.8896	0.9631	2.435	4.665
Biomass adapted to ILL1 (X_{ILL1})	0.7159	0.9218	1.550	4.686
Biomass adapted to ILL2 (X_{ILL2})	0.6723	–	1.479	–

acclimatized biomass samples (X_{SM}, X_{ILL1} and X_{ILL2}) was assessed by 16S rDNA-DGGE band profiles (Fig. 2). Arrows were used to highlight the most representative OTUs. It is noticeable that the bacterial diversity was sharply reduced when the nitrifying biomass was fed with intermediate landfill leachate. While the first lane showed a higher diversity of OTUs, the second and the third lane, which correspond to biomass fed with ILL1 and ILL2, respectively, showed a lower amount of OTUs (Fig. 2). The bacterial OTUs richness and evenness were evaluated using Simpson and Shannon–Wiener indexes (Table 2). The decrease of the Simpson index and the increase in the Shannon–Wiener index highlighted that the adaptation of X_{SM} to the ILL1 and ILL2 led to a decrease in the richness of the bacterial OTUs present in the bioreactor.

From the DGGE image analysis, several bands can be highlighted in terms of intensity and appearance in the three samples. Firstly, the band 'a' corresponded to an OTU that appeared after the feeding of X_{SM} with ILL1 (Fig. 2). However, it did not appear when the same consortium was fed with ILL2. These findings may suggest that any compound present in the ILL1 and missing in the SM, most likely organic matter, may have promoted the differential growth of bacteria. Even though ILL2 also had organic matter, once it was fed the microbes were not able to grow, probably due to their sensitivity to high salt concentrations or other compounds present in ILL2 and missing in ILL1. Other OTUs whose growth was promoted by the addition of landfill leachates were the bands labeled as 'd'. Unlike the OTU/s corresponding to the band 'a', band 'd' was able to grow under both landfill leachates showing its resilience to different types of landfill leachates.

On the other hand, there were also some OTUs that persisted after the change in the ammonium substrates. The bacterial OTUs corresponding to bands 'b' and 'c' remained present after the change of the ammonium substrate from the synthetic medium to either of the other two landfill leachates. From an operational point of view, this type of microorganism is the most interesting due to its apparent ability to degrade pollutants from different substrates. Therefore, it can be concluded that the presence of some OTUs remains in all samples but the landfill leachate feeding led to the loss of several OTUs and the appearance of new ones.

DGGE can be considered a useful tool to get an initial profile of the microbial diversity present in several samples that can help to understand de dynamic and structure of the microbial community in response to environmental perturbation. However, this method has several technical bias problems which are important to highlight. These problems can be due to the inherent limitations in 16S rRNA gene interspecies heterogeneity, template annealing in the amplification of 16S rRNA genes, single DGGE bands not always representing single bacterial species, the presence of intraspecific polymorphisms of 16S rRNA genes, differential gene amplification and problems related to the PCR conditions such as annealing temperature, primer mismatch or PCR cycle numbers affecting the optimal amplification of the 16S rRNA gene [36].

Next Generation Sequencing (NGS) Analysis

To further characterize the microbial communities at the taxonomic level, NGS analysis was then performed. Due to the similarities of the DGGE band profiles for samples X_{ILL1} and X_{ILL2} and the low specific ammonium consumption rate obtained by X_{ILL2}, the study of the relative abundances of OTUs with N-related metabolism was only performed for X_{SM} and X_{ILL1} samples (Fig. 2). The quality of the samples to identify the taxa was previously analyzed and the number of generated reads was considered sufficient by the Alpha rarefaction curve (Fig. S1). X_{SM} and X_{ILL1} samples generated 323,138 and 150,838 raw sequence reads respectively which are in accordance with the expected output (around 100,000 sequence reads). After denoising, a total of 182 OTUs in X_{SM} and 191 OTUs in X_{ILL1} samples were found, which are listed in Tables S1 and S2 respectively.

NGS results again show lower microbial richness in the biomass adapted to ILL1 compared to X_{SM} which concur well with the DGGE band patterns. However, calculating the diversity indexes using the results of the NGS analysis, lower differences between samples are found in diversity terms compared to calculations made using the DGGE results (Table 2). These differences can be mainly attributed to the different lengths of the analyzed 16S rRNA gene segment and potentially different efficiency in the amplification of the 16S rRNA gene.

In order to facilitate the presentation and discussion of results, the relative abundance of phyla for the samples corresponding to non-adapted biomass and biomass adapted to intermediate landfill leachate 1 is shown in Fig. 3.

The comparison of distribution and diversity of phyla of both samples are plotted in Fig. 3. In sample X_{SM} it can be seen that phyla *Proteobacteria* (48.5%), *Actinobacteriota* (14.4%) and *Chloroflexi* (9.5%) accounted for more than 70% of the relative abundance (Fig. 3). Besides, the significant presence of *Gemmatimonadetes* (7.3%), *Planctomycetota* (7.2%), and *Deinococcus* (7.1%) was found. The feeding of ILL1 led to several changes in the presence and distribution of the phyla in the sample. In X_{ILL1}, it can be observed again that phyla *Bacteroidetes* (27.2%), *Euryarchaeota* (26.6%) and *Proteobacteria* (20.0%) dominated the consortium (> 70%). With lower relative abundance other phylum

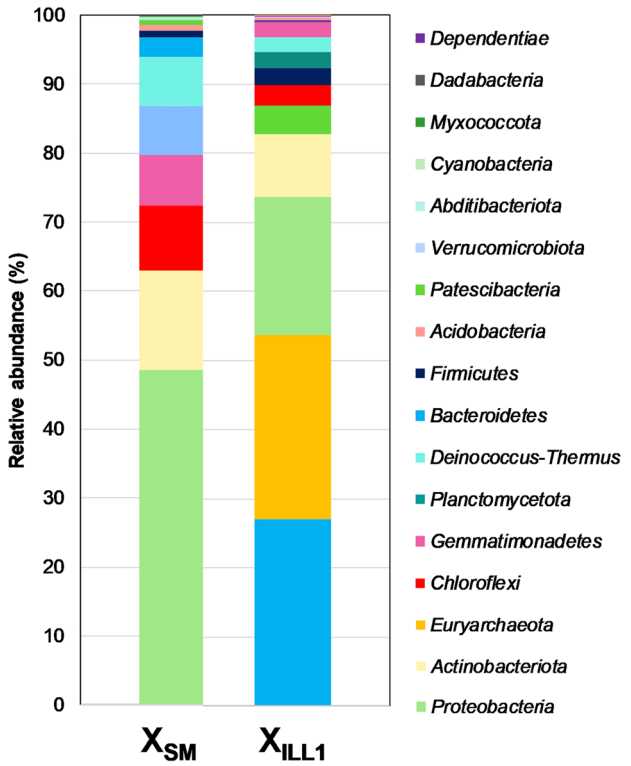

Fig. 3 Relative abundance of phyla for non-adapted biomass (X_{SM}) and biomass adapted to intermediate landfill leachate 1 (X_{ILL1})

containing OTUs with relevance in the nitrification process were found such as *Actinobacteriota* (9.0%), *Patescibacteria* (4.1%), *Chloroflexi* (3.0%), *Firmicutes* (2.5%), *Planctomycetota* (2.3%), *Deinococcus-Thermus* (2.1%) and *Gemmatimonadetes* (2.1%).

The presence and dominance of *Proteobacteria* phylum in nitrification bioreactors have been widely referenced in the literature [5, 37]. It is important to note that the presence of this phylum decreased its relative abundance from 48.5 to 20.0% when the X_{SM} was fed with ILL1. This ubiquitously distributed phylum is known for its diverse metabolic types, including phototrophs [38], autotrophs [39], and heterotrophs [40]. Among the *Proteobacteria* phylum, the family *Xanthobacteraceae* stands as the most abundant in both samples (12.24 and 2.56% in X_{SM} and X_{ILL1}, respectively). This family is widely known to have N_2 fixing capacity under heterotrophic or chemolithoautotrophic growth conditions [41]. However, this family also includes genera capable of performing nitratation (oxidation of NO_2^- to NO_3^-) like the genus *Nitrobacter* as NOB. Despite not being identified at the genus level, this OTU is most likely corresponding to this genus considering the prevalence of NO_3^- to NO_2^- in the medium. *Xanthomonadaceae* was the second most abundant family in the X_{SM} (8.3%) belonging to the *Proteobacteria* phylum but disappeared when ILL1 was fed to the biomass (Table S1 and Table 2). This family has been recently reported to have a high number of genes involved in nitrogen pathways including those involved in NH_4^+ oxidation to NO_2^- (*hao* genes) and NO_2^- oxidation to NO_3^- (*norB* genes) [42]. Therefore, it can be highlighted as a candidate taxon capable to perform the complete oxidation of NH_4^+ to NO_3^-. Taking into consideration its significant abundance in the X_{SM}, the disappearance in the X_{ILL1} and the ~ fivefold reduction in the nitritation rate of X_{ILL1} compared with X_{SM}, the *Xanthomonadaceae* genus could be assigned as one of the main drivers of the ammonium oxidation performed by X_{SM}. In addition to the families stated above, other families common in both samples and belonging to the *Proteobacteria* phylum such as *Rhodobacteraceae* and *Rhizobiaceae* (Table 3) performed different roles non-related to the nitrification like the heterotrophic denitrification [43] and nitrogen fixation [44], respectively.

Actinobacteriota was the second most abundant phylum in X_{SM} with a relative abundance of 14.4% and this percentage remained similar in X_{ILL1} (9.0%), indicating the resilience of this phylum to different ammonium substrates. This phylum is mostly aerobic and is ubiquitously distributed in both terrestrial and aquatic ecosystems [45]. On the other hand, the *Microbacteriaceae* family was the most abundant member of this family in X_{SM} (8.4%) and X_{ILL1} (0.98%) (Table 3). Even though its presence has been reported in nitrification bioreactors [46], in aerobic granular sludge systems treating municipal wastewater [47], and in biofilters

Table 3 Relative abundance, taxonomic classification and nitrogen metabolism of different identified OTUs with abundance > 0.02% for samples X_{SM} and X_{ILL1}

	Phylum	Class	Order	Family	Genus	Species	X_{SM}	X_{ILL1}
Ammonium oxidizing bacteria (AOB)	*Proteobacteria*	*γ-proteobacteria*	*Xanthomonadales*	***Xanthomonadaceae***	*Uncultured*	*uncultured Xanthomonas*	**8.30**	**n.d**
	Patescibacteria	*Saccharimonadia*	***Saccharimonadales***	—	—	—	**0.63**	**0.37**
	Proteobacteria	*γ-proteobacteria*	*Burkholderiales*	*Nitrosomonadaceae*	*Nitrosomonas*	***Nitrosomonas eutropha***	**1.12**	**n.d**
	Proteobacteria	*γ-proteobacteria*	*Burkholderiales*	*Nitrosomonadaceae*	*Nitrosomonas*	*Nitrosomonas mobilis*	**0.33**	**n.d**
	Proteobacteria	*γ-proteobacteria*	*Burkholderiales*	*Nitrosomonadaceae*	***Nitrosospira***	—	**0.07**	**n.d**
	Proteobacteria	*γ-proteobacteria*	*Burkholderiales*	*Nitrosomonadaceae*	***Nitrosomonas***	—	**0.02**	**n.d**
	Proteobacteria	*α-proteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	***Paracoccus***	—	**n.d**	**0.66**
Nitrite oxidizing bacteria (NOB)	*Proteobacteria*	*α -proteobacteria*	*Rhizobiales*	***Xanthobacteraceae***	—	—	**12.24**	**2.56**
	Chloroflexi	*JG30-KF-CM66*	*uncultured bacterium*	*uncultured organism*	*uncultured bacterium*	*uncultured bacterium*	**n.d**	**0.82**
	Proteobacteria	*γ-proteobacteria*	*Betaproteobacteriales*	*Burkholderiaceae*	*Lautropia*	—	**n.d**	**0.07**
	Chloroflexi	*Chloroflexia*	*Thermomicrobiales*	*Thermomicrobiaceae*	***Nitrolancea***	*uncultured bacterium*	**n.d**	**0.07**
Found in N-removal bioreactors	*Chloroflexi*	*Chloroflexia*	*Thermomicrobiales*	*AKYG1722*	***Uncultured Chloroflexi***		**2.23**	**0.33**
	Chloroflexi	*Chloroflexia*	*Thermomicrobiales*	*JG30-KF-CM45*	***Uncultured anaerobic ammonium-oxidizing bacterium***	*uncultured anaerobic ammonium-oxidizing bacterium*	**1.65**	**n.d**

The taxa used for searching in the literature the metabolism characteristics is denoted in bold

n.d. not determined

treating a gas effluent containing ammonia among other pollutants [48], the role in these systems has not yet been clearly defined. Zakhia et al. [49] reported the presence of a *nifH*-like gene in *Microbacterium*, a genus belonging to this family, confirming the N_2 fixing capacity of this family. This capacity, usually observed in legume-nodulating bacteria, could potentially benefit the nitrification consortium.

Another important aspect to discuss is the increase in the dominance of the *Bacteroidetes* phylum, whose presence increased from 2.8% in X_{SM} to become dominant in X_{ILL1} with 27.2% (Fig. 3). The growth promotion of this phylum by the addition of landfill leachate may be caused by the relation between the high organic matter content of the landfill leachate and its function. Several microorganisms included in this phylum have been reported to degrade high-molecular weight organic matter to acetic and propionic acids [50]. Their presence has been found in systems performing the anaerobic digestion of different substrates like organic residues/waste or sludge [51, 52]. The dominance of this phylum when ILL1 was fed to the system was mainly caused by the growth of the *Saprospiraceae* family, which was present at 14.1% in X_{ILL1} while it was absent in X_{SM} (Table S1 and S2). The presence of this family has been reported in bioreactors treating landfill leachates [53, 54] playing an important role in degrading complex organic compounds and denitrification [55]. *Flavobacteriales* is another order whose presence remained more or less constant between X_{SM} (1.7%) and X_{ILL1} (1.9%) (Table 3). This order has been described as one of the dominant denitrifiers in activated sludge [56] and the presence of denitrifying genes in their genome has been confirmed [57].

It is also important to highlight that the most abundant OTU in X_{ILL1} was the genus *Methanosarcina* (24.8%) which belongs to the *Archaea* domain (Table S2). This OTU, which was absent in X_{SM}, is a well-known acetoclastic methanogen that can resist the high ammonium concentrations found

Table 4 Relative abundance of different identified OTUs with abundance > 0.5% found in both X_{SM} and X_{ILL1} samples

Phylum	Class	Order	Family	Genus	Species	XSM	XILL1
Proteobacteria	*α-proteobacteria*	*Rhizobiales*	***Xanthobacteraceae***	—	—	12.24	2.56
Actinobacteriota	*Actinobacteria*	*Micrococcales*	***Microbacteriaceae***	—	—	8.37	0.98
Deinococcus-Thermus	*Deinococci*	*Deinococcales*	*Trueperaceae*	***Truepera***	*uncultured bacterium*	7.06	2.00
Proteobacteria	*α-proteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*uncultured*	***Rhodobacteraceae bacterium***	3.22	1.51
Proteobacteria	*α-proteobacteria*	*Rhizobiales*	*Rhizobiaceae*	—	—	2.72	2.72
Bacteroidetes	*Bacteroidia*	***Flavobacteriales***	*NS9 marine group*	*metagenome*	*metagenome*	1.67	1.87

The taxa used for searching in the literature the metabolism characteristics is denoted in bold

in landfill leachates [58, 59]. The large input of complex organic matter associated with landfill leachate led to the growth of *Bacteroidetes* phylum which generated acetate. The accumulation of acetate may promote the growth of *Archaea* belonging to the *Methanosarcina* genus which converted this metabolite into methane and carbon dioxide.

The relative abundances obtained through NGS analysis of the nitrifying (AOB and NOB) bacteria and other bacteria usually found in bioreactors treating ammonium-rich effluents are summarized in Table 4. Given the results, it can be seen that feeding with landfill leachate significantly affected the AOB population reducing its relative abundance from 10.5% to 1.0%. This reduction was expected due to the well-known sensitivities of AOB to salinity, the presence of toxic organic compounds, pH changes, etc. [60]. *Sacchari-monadales* order can be highlighted as one of the AOB that remained present in the nitrifying consortium after the substrate change. Shi et al. [61] recently reported the presence of the *amoA* gene in this order whose presence allows the holder microbes to oxidize ammonium to NO_2^-. The presence of the family *Nitrosomonadaceae* was also detected, specifically, the widely known genera *Nitrosomonas* and *Nitrosospira.* Its relative abundance is modest in X_{SM} while it was undetectable in X_{ILL1}. Considering the data shown in Fig. 1 where the complete oxidation of NH_4^+ to NO_3^- was demonstrated, it can be concluded that other microbes not belonging to the family *Nitrosomonadaceae* also performed the ammonium oxidation. The low concentration of these families is something frequently seen in nitrification bioreactors. For example, Zhang et al. [62] recently reported a relative abundance of *Nitrosomonas* and *Nitrosospira* genera of 0.98% y 1.73%, respectively, in biomass adapted to *Para*-nitrophenol. Since this biomass did not lose nitrification activity, they also propose that other genera present in our biomasses such as *Brevundimonas* (0.2% in X_{SM} and 0.27% in X_{ILL1}) play roles in maintaining good nitrification. Zeng et al. [63] also reported a similar relative abundance of these genera. In a process treating wastewater, the total AOB present in the sludge kept stable at 1.8% of relative abundance, while the *Nitrosomonas* genus accounted for 81.6% of the *amoA* genes present in the consortium. The scarce presence of these genera in aerobic bioreactors treating landfill leachate is also common. Xie et al. [64] detected a modest relative abundance of the genera *Nitrosomonas*, *Nitrosospira*, and *Nitrosococcus* ranging from 0.49% to 1.78%. Diaz et al. [5] only found *Nitrosomonas* and *Nitrosococcus* in one out of four bioreactors performing the nitrification of landfill leachates, whereas they did not identify *Nitrosospira*, *Nitrosovibrio*, and *Nitrosolobus*.

It is also interesting to highlight the appearance of the *Paracoccus* genus in X_{ILL1}. This genus has been traditionally considered as a heterotrophic denitrifying microorganism [65]. However, recent evidence indicates that, in the

presence of organic matter, this genus is capable of performing complete nitrification (NH_4^+ to NO_3^-) and then, reducing the NO_3^- to N_2 in the presence of organic matter [66].

Among the NOB, it can be considered that the *Xanthobacteraceae* family, most likely represented by the *Nitrobacter* genus as discussed above, played the main role in the oxidation of NO_2^- to NO_3^- in X_{SM} (12.24%) and X_{ILL1} (2.56%). However, it should be noted that the genus *Nitrolancea* has also been described as NOB although it was only found in X_{ILL1} with a very low relative abundance (0.07%) (Table 4). The OTU identified in this sample which belongs to the *Nitrolancea* genus could be the species *Nitrolancea hollandicus*, first described from a nitrifying bioreactor and capable of performing the nitrite oxidation [67]. It is also important to remark that apart from *Nitrosomonas* and *Nitrosospira* genus as representative of AOB found in the X_{SM} sample it is most likely that the *Paracoccus* genus also helped to maintain the nitratation activity in X_{ILL1} (Table 3).

Finally, the presence of other OTUs frequently found in bioreactors treating ammonium-rich effluents is worth noting (Table 3). Despite not finding any OTU classified as anammox (*Anammoxoglobus*, *Brocadia*, *Jettenia*, *Kuenenia*, *Scalindua* and *Anammoximicrobium*) [68], other OTUs which have been previously found in bioreactors treating ammonium rich wastewater through the anammox process have been found. For example, Park et al. [69] found *Chloroflexi* JG30-KF-CM45 and *Limnobacter* in a bioreactor performing the anammox process. Another phylum such as *Planctomyces* has been detected in a relative abundance ranging from 3.19 to 5.73% in aerobic bioreactors treating landfill leachate [64]. Other authors such as Li et al. [70] found *Patescibacteria* (9.9–13.2%) and *Chloroflexi* (10.5–23.1%) in a full-scale membrane bioreactor treating mixed landfill leachates.

Table 4 shows the common OTUs in both samples with a relative abundance higher than 0.5%. The persistence of these bacteria is interesting because they showed resistance/tolerance to the toxic matrix of the landfill leachates. Therefore, they stand as potential candidates to be used in bioaugmentation strategies in bioreactors performing the nitrification of landfill leachates.

The reported function of *Xanthobacteraceae* and *Microbacteriaceae* families and *Flavobacteriales* order has been discussed above so we will focus our attention on the remaining OTUs. Firstly, we had the *Truepera* genus whose presence was significant in X_{SM} (7.1%) and X_{ILL} (2.0%). The function of some bacteria belonging to this genus have been mainly related to nitrogen removal via autotrophic denitrification [71], nitrification bioreactors treating landfill leachate [72] and industrial wastewater [71]. Another OTU that persisted in both samples was *Rhodobacteraceae*. This family has been reported to show an anoxygenic photoautotrophic lifestyle but also has potential capabilities including aerobic heterotrophy and utilization of complex organic compounds [43, 73].

Despite being widely used, NGS provides only limited information on the physiology of microorganisms in the samples studied. While these techniques can measure quantitative changes over time, they cannot assess the viability cross-sectionally. PCR cannot differentiate between DNA from viable cells, DNA from inactivated ones and free DNA fragments. This limitation can be addressed by other techniques such as (i) the use of a viability PCR which requires the incubation of microbes with a membrane-impermeative reagent such as propidium monoazide (PMA) [74] or (ii) the measurement of bacterial groups or species by quantification of reference genes related to a specific activity by real time quantitative PCR (RT-qPCR) [75]. This last technique was used by Casado Muñoz et al. [76] who studied the resistance of lactic acid bacteria to several biocides.

Conclusions

The acclimatization of a nitrifying consortium to landfill leachate led to a reduction in its ammonium oxidation capacity most likely caused by the high salinity present in the medium. The DGGE-PCR technique allowed the identification of significant differences between biomass profiles fed with SM and different landfill leachates. The NGS analysis was consistent with the results obtained by DGGE-PCR. *Nitrosomonas* and *Nitrosospira* genera were present in non-adapted biomass at 1.47% and 0.07% respectively but were not detected in X_{ILL1} indicating that the genera *Brevundimonas* or *Paracoccus* among others present in the biomass, play the role of maintaining the nitrification using landfill leachate as substrate. Denitrifying bacteria were also present in both bioreactors despite being continuously aerated, indicating the presence of anoxic zones in the bioreactor.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12649-022-01759-0.

Author contributions JJGC: Investigation, Formal analysis, Writing—original draft preparation. AV Conceptualization, Methodology, Supervision Writing- Reviewing and Editing. MR: Conceptualization, Methodology, Supervision, Project administration, Funding acquisition, Writing- Reviewing and Editing. DC: Project administration, Funding acquisition, Reviewing and Editing.

Funding Open Access funding provided thanks to the CRUE-CSIC agreement with Springer Nature. The Spanish Government (Ministry of Economy and Competitiveness) and the Vice-rectorate for Research of the University of Cadiz provided financial support through the project CTM2016-79089-R "Enhancement of landfill gas by an integrated biological system (Biointegra3)" and UCA/REC01VI/2017 (Universidad de Cadiz) respectively

Data Availability Data available within the article or its supplementary materials.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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