Contents lists available at ScienceDirect

Waste Management

journal homepage: http://ees.elsevier.com

Intermittent aeration of landfill simulation bioreactors: Effects on emissions and microbial community

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ARTICLE INFO

Article history:
Received 7 May 2020
Received in revised form 4 August 2020
Accepted 5 August 2020
Available online xxx

Keywords
Landfill remediation
Intermittent waste aeration
Leachate characterization
Metagenomic microbiome assessment

ABSTRACT

Landfill simulation experiments were run at lab-scale to compare the effects of intermittent and continuous aeration on the evolution of leachate composition and biogas production. The experiments were carried out using six reactors; two of them under continuous aeration, two under intermitted aeration and two anaerobic as a control. Different aeration regimes produced different effects on reactors. As expected, carbon discharge via biogas was higher in reactors under continuous aeration than under intermittent aeration. The evolution of leachate quality was affected by the aeration regimes; however, at test end very similar concentration were ascertained for relevant leachate parameters in all aerated reactors. A comprehensive description of the aerobic and anaerobic landfill microbiome is provided, using a metagenomic approach focused on the microbial genome reconstruction. A time course investigation evidenced the modification of the microbiome and revealed taxa and specific microbes more strictly connected to the environmental parameters of the reactors. *Methanoculleus*, *Syntrophomonas* and *Parabacteroides* were identified as the genera more strictly connected to biogas production, while numerous species belonging to *Thiomonas*, *Nitrosomonas*, Xanthomonadaceae, Myxococcales and Alcaligenaceae were found to be connected with NH_4^+ oxidation.

1. Introduction

Landfill aeration is aimed at enhancing waste stabilization, reducing landfill emissions and shortening post-closure care (Raga and Cossu, 2014; Ritzkowski et al., 2006).

The results of numerous lab-scale projects are available, providing insights into the effects of process parameters on the evolution of emissions and waste quality (Berge et al, 2005; Raga and Cossu, 2013; Brandstätter et al., 2015).

Field scale applications were run and results enabled the evaluation of carbon discharge via process gas and leachate extraction and the comparison with results of lab-scale simulations (Hrad and Huber-Humer, 2016; Matsuto et al., 2015; Raga and Cossu, 2014; Ritzkowski et al., 2016; Ritzkowski and Stegmann, 2012).

In situ aeration coupled with enhanced leachate extraction was applied at full scale for landfill conditioning before and during Landfill Mining (Raga et al., 2015), providing a significant reduction of biogas emission during excavation and improved biological stability of excavated waste.

Full scale applications of landfill aeration have been carried out according to different protocols, but in many cases intermittent running of the aeration plant was experienced for various technical reasons. Regular plant switch-offs are necessary for maintenance and repair or for limiting temperature increase in the landfill body. Moreover, especially in those cases where oxygen consumption rates are low, aerobic conditions might endure for some time in the landfill body after air injection switch off, making continuous aeration unnecessary. For this reason, intermittent running might provide significant energy savings.

Alternating aerobic and anaerobic conditions caused by intermittent running of aeration plants might be beneficial for waste stabilization process. Some lab-scale experiments have been carried out so far to compare the effects of continuous and intermittent aeration, proving that both conditions result in significant improvement of emissions (He and Shen, 2006; Nag et al., 2015; Sang et al., 2009; Shao et al., 2008). According to (Nag et al., 2015) continuous aeration showed better results in terms of greenhouse gas (GHG) emission reduction and improvement of leachate quality. During intermittent aeration, short aerobic/anaerobic cycles (6 h/day of aeration) resulted more effective than longer cycles (3 days/week) in improving leachate quality and GHG emission reduction, with a 75% reduction of the energy costs required by continuous aeration. Xu et al. (2016) studied the effects of reducing aeration frequency over time (from 4 times to twice per day,

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https://doi.org/10.1016/j.wasman.2020.08.010
0956-053/

2-hour aeration each cycle) based on observation of reduced oxygen consumption rates. Reduced frequency enabled the enhancement of carbon transfer into biogas and the reduction of COD and VFA concentration in leachate.

This research is aimed at studying the influence of intermittent aeration on emissions and microbial community in comparison to continuous aeration, using longer aerobic/anaerobic cycles than other tests, to better simulate what is expected in full scale applications, where aeration stops can last several days to some weeks, and to provide enough time for new milieu conditions to establish and for microbial populations to adapt to them.

A comprehensive description of the aerobic and anaerobic landfill microbiome was performed, using a metagenomic approach focused on the microbial genome reconstruction, in order to show the modification of the microbiome and reveal taxa and specific microbes more strictly connected to process parameters in the reactors. This is among the first studies where a predictive functional analysis of the microbial species was performed in order to provide details on the biochemical pathways involved in degradation processes in leachate under intermittent waste aeration.

2. Materials and methods

2.1. Equipment and waste sample

The experiment was carried out using six lab-scale landfill simulation methacrylate reactors (Raga and Cossu, 2013), internal height of 106 cm and diameter of 24 cm, equipped with valves for leachate and process gas extraction as well as for the injection of water and recirculated leachate. A PVC ring for liquid distribution was installed in the head space of reactors. A 5-cm gravel layer was placed above the waste to homogenize water/leachate inlet and at the bottom of reactors, for drainage purposes.

Reactors Ca and Cb were run with continuous aeration; reactors Ia and Ib were run with intermittent aeration, reactors Aa and Ab were maintained anaerobic as a control.

Leachate recirculation was carried out by means of a Heidolph PD 5001 peristaltic pump; air injection through a vertical, slotted PVC tube was provided with Prodac Air Professional pump 360 and Maxima-R air pumps. The airflow was regulated by means of Sho-Rate GT1135 and Cole-Parmer flow-meters. Temperature monitoring was performed using Thermo Systems TS100 temperature probes inserted in the center of each reactor and connected to a Endress-Hauser data logger. Reactors were run at 36 ± 2 °C; temperature control was carried out by means of a thermo-regulated insulation system installed in reactors.

Each reactor was filled with 18 kg of municipal solid waste, collected right before landfill disposal at a local waste management plant, where it had undergone mechanical–biological pretreatment involving grinding to less than 60 mm and forced aeration in windrows for 3 weeks.

Before reactor filling, larger objects were removed and waste was thoroughly mixed to enhance homogeneity. Thickness of the waste layer at test start was equal to 0.75 m in each reactor; initial emplacement density was equal to 530 kg/m^3. 4 L of deionized water were added into each reactor, to saturate field capacity and to produce approximately 2 L of process water (leachate), which was collected into a leachate tank and made available for sampling and recirculation purposes.

2.2. Methodology

Simulation of landfill disposal (Landfill Simulation, LS) was carried out, followed by the simulation of landfill remediation by means of in situ aeration (Landfill Remediation, LR).

Aerobic conditions naturally occur in the first stages after landfill disposal, especially if thin waste layers are laid. This is beneficial to accelerate following methanogenesis when anaerobic conditions are established (Cossu et al, 2016). For this reason, at test start (LS phase) aeration was provided for the first two weeks and then switched off; anaerobic degradation processes developed over the following eight weeks. Subsequently (LR phase), the effects of continuous and intermittent aeration were assessed and compared to traditional landfilling under anaerobic conditions (Fig. 1).

During LS phase, leachate recirculation was carried out in all six reactors (2 L/d; 0.174 L/kg dry mass (DM)/d); aeration was performed with flow rate of 5.2 NL/kgDM/d) during the first 2 weeks of LS.

In order to enhance contact between injected air and leachate, during LR phase recirculation flow rate was increased in all reactors to 10 L/d (0.87 L/kgDM/d). Aeration conditions in reactors were the following:

- reactors Ca and Cb, continuous aeration, air flow rate of 57.6 L/d (5.0 NL/kgDM/d);
- reactors Ia and Ib, intermittent aeration, same air flow rate of 57.6 L/d, aeration/no aeration cycles of 18/20, 20/22 and 26/36 days;
- reactors Aa and Ab, anaerobic conditions.

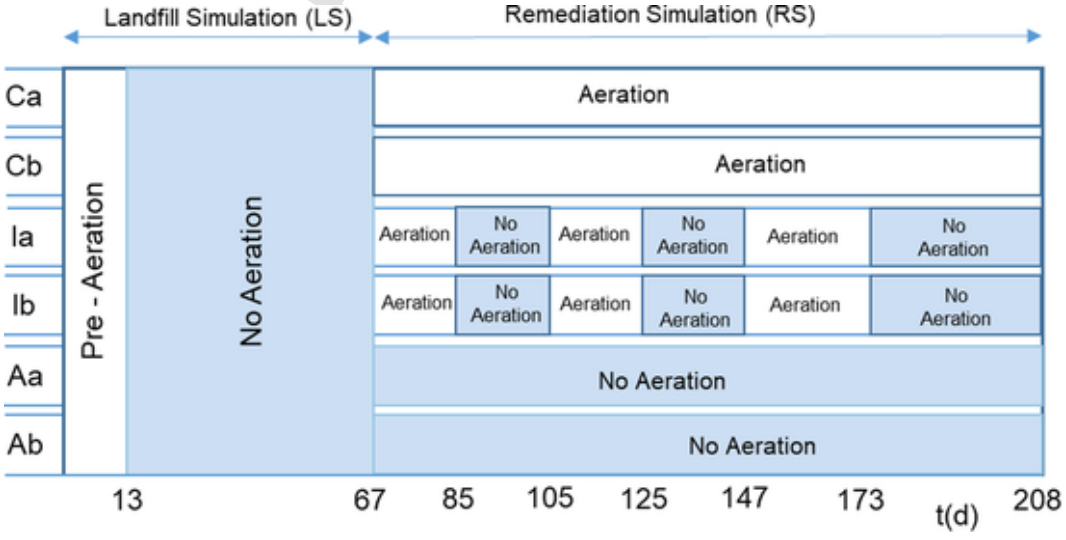

Fig. 1. Overview of the sequence of aeration regimes in reactors.

2.3. Sampling and analytical methods

International standard methods were used for the analysis of solid samples, leachate and gas.

Respiration index (RI_4) has been determined by means of a respirometer (Sapromat, H + P Labortechnik, Germany) according to pertinent German regulations for AT4 (Anonymous, 2001)

During anaerobic phases of intermittently aerated reactors and in anaerobic reactors biogas was stored in 25 L Tedlar and Restek bags, then volume and composition were ascertained. During aeration, gas samples were collected from the top of each reactor at least three times per week for composition analysis (O_2, CO_2 and CH_4), by means of IR-analyser model LFG20. The composition analysis was possible only on process gas present in the upper part of the reactors since no lateral ports were available. For this reasons, it was not possible to collect gas samples within the waste body after aeration switch off.

Leachate was collected for characterization at least once per week, from the bottom valve of each reactor. In total, at the end of the experiment, 39 samples were extracted from each reactor, for a total volume of 6.5 L of leachate per reactor. Deionized water was injected in reactors after sampling, to replace the extracted liquid phase.

Leachate samples were analyses for pH, BOD_5, COD, TKN, $N\text{-}NH_4^+$, $N\text{-}NO_3^-$, $N\text{-}NO_2^-$, TOC, VFA, $SO_4^=$. A comprehensive characterization for the description of landfill microbiome was carried out for leachate sampled at day 59, 84, 104 and 116 from aerated reactors (leachate from anaerobic reactors was analysed at day 59 and 116 only).

2.4. Samples collection, DNA extraction and sequencing

In order to describe the aerobic and anaerobic landfill microbiome, 10 ml leachate samples were collected and centrifuged at 16000 g for 10 min. Supernatant was discarded and DNA was extracted from pellet using PowerSoil Extraction Kit (MOBIO Laboratories, Carlsbad, CA) with some minor modifications (Campanaro et al., 2018b). Genomic DNA quality and quantity were determined using NanoDrop (ThermoFisher Scientific, Waltham, MA) and Qubit fluorimeter (Life Technologies, Carlsbad, CA); absence of degradation was verified using agarose gel electrophoresis. Nextera XT library preparation kit (Illumina, San Diego, CA) was used for library construction, while random shotgun DNA sequencing was performed with NextSeq 500 2x150 bp High Output at the Ramaciotti centre for functional genomics (Sydney, Australia).

2.5. Bioinformatics analysis

Paired-end reads were quality-filtered and adaptors were removed using Trimmomatic software (v0.33) (Bolger et al., 2014) and a number of reads ranging from ~7 to ~6 million reads were obtained for each sample. Shotgun reads were analysed using Metagenomic Phylogenetic Analysis (MetaPhlAn) tool (Segata et al., 2012) to obtain a general overview of the taxonomic composition. The minimum relative abundance value was set to 0.01% and the similarity calculation was performed using Bray Curtis distance. Correlation between abundance of taxa and reactors parameters was performed using Pearson in CoStat v 6 software (CoHort Software, Birmingham UK).

Filtered reads were assembled using CLC Genomics workbench (v5.1) (CLC Bio, Aarhus, DK, USA), using a kmer of 63, a bubble size of 60 and a minimum scaffold length of 500 bp. QUAST software was used to calculate the assembly metrics (Gurevich et al., 2013). Scaffolds obtained were used as input for predicting protein encoding genes using Prodigal (v2.6.2) run in metagenomic mode (Hyatt et al., 2012). Genes were annotated with reverse-position specific BLAST, using RPSBLAST database as reference (Galperin et al., 2015), filtering the output and discarding values higher than 1e-5. GhostKOALA software (Kanehisa et al., 2016) was used to annotate genes according to KEGG. Binning of scaffolds into Metagenome Assembled Genomes (MAGs) was performed with MetaBAT (v0.25.4) (parameters --specific, -m 1500) (Kang et al., 2015) collecting 356 MAGs. Coverage profile of the scaffolds was checked to capture additional MAGs with the "hierarchical clustering followed by canopy profile selection" procedure (Campanaro et al., 2016). Other 20 MAGs were captured with this procedure and compared to those previously extracted using the "bin-compare" module of checkM (Parks et al., 2015). Additional verification was performed using checkM to remove scaffolds assigned to multiple MAGs. After this verification, "completeness" and "contamination" parameters of all the MAGs were calculated using checkM. KEGG IDs were assigned to each gene using FOAM software; results were analysed to identify metabolic pathways for each MAG using self-written perl scripts (Campanaro et al., 2018a). Taxonomic assignment for each MAG was obtained combining four different methods: (1) taxonomic informative genes were analysed using Phylophlan (v0.99) (Segata et al., 2013) and with (2) checkM; (3) 16S rRNA sequences were extracted from each MAG using self-written perl scripts based on hmm of RNAmmer (Lagesen et al., 2007) and analysed using RDP classifier trained on GreenGenes clustered at 97% similarity; (4) Average Nucleotide Identity (ANI) was calculated comparing MAGs to all the genomes in the NCBI microbial genome database with a procedure previously described (Campanaro et al., 2020) and using 95% ANI and more than 50% genes in common as thresholds for assignment (Varghese et al., 2015). Coverage of scaffolds was used to estimate relative abundance values and were calculated from the alignment results obtained with Bowtie2 (v2.2.4) (Langmead and Salzberg, 2012). Alignments were converted into coverage values with the BEDTools package (v2.17.0) and were normalized considering as reference the sample with the lowest number of aligned reads. The matrix of coverage values obtained for all the MAGs in the different conditions was analysed and clustered with MeV software (Saeed et al., 2003). The R function of VEGAN package (Dixon, 2003) was used to calculate the Canonical Correspondence Analysis (CCA), while the Correspondence Analysis (CA) based on Pearson correlation was calculated with R as previously described (Torondel et al., 2016). Shotgun Illumina sequences were submitted to the NCBI Sequence Read Archive (SRA) with accession number BioProject ID PRJNA453670. Additional resources, including genome and protein sequences of MAGs, annotation, and the global metagenomic assembly are available in figshare database with doi numbers 10.6084/m9.figshare.12595175; 10.6084/m9.figshare.12595175; 10.6084/m9.figshare.12596984 and included in the project "Intermittent aeration of landfill simulation bioreactors: effects on emissions and microbial community".

3. Results and discussion

Results of waste characterization at start and at different stages of the experiment are reported in Table 1.

Landfill simulation phase enabled a significant improvement of waste biological stability; respiration index dropped by 75% over two months. Settlements in Ca and Ab resulted significantly higher than in other reactors, causing higher waste compaction and probably affecting air flow in Ca. Values of initial TOC and of carbon emissions via gas and leachate at test end (Table 1) enable the estimation of gasification ratio, which is highest in Cb (5%) and lowest in Aa (less than 1%).

The evolution of biogas composition and production is reported in Fig. 2. Aerobic conditions established in reactors and maintained for the first 2 weeks had a beneficial effect on biogas production in the following anaerobic phase, as expected and reported in previous studies (Cossu et al., 2016; Sang et al., 2009). Indeed, methane concentration peaked to values close to 60% in all reactors 5 days after aeration was switched off.

Table 1
Waste characterization at different stages of the test and carbon emissions via gas and leachate. The results of waste analyses are reported as average (three replicates) and standard deviation. n.a.: not ascertained.

	Initial	End of LS phase	End of test					
			Ca	Cb	Ia	Ib	Aa	Ab
Waste characterization								
Mass (kg)	18.0	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Humidity (%)	36.1 ± 1.5	50.8 ± 4.1	62.6 ± 0.5	60.6 ± 2	58.4 ± 2.5	62.6 ± 1.8	56.5 ± 2.2	56.5 ± 3.0
Waste density (kg/m ^{3})	530	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Thickness of waste layer (m)	0.75	0.70	0.57	0.68	0.65	0.65	0.65	0.60
Waste settlements at test end (m)	–	–	0.18	0.07	0.10	0.10	0.10	0.15
RI_4 (mg O_2/g DM)	23.7 ± 3	5.7 ± 0.8	3.8 ± 0.3	3.4 ± 1	3.5 ± 0.5	3.7 ± 0.2	4.8 ± 0.3	5.1 ± 0.7
TKN (g N/kg DM)	6.7 ± 0.9	n.a.	3.7 ± 0.3	2.5 ± 0.6	3.3 ± 0.1	3.2 ± 0.4	3.4 ± 0.5	4.0 ± 0.7
TOC (g C/kg DM)	217 ± 25	n.a.	189 ± 23	176 ± 31	185 ± 14	198 ± 20	162 ± 18	180 ± 22
Carbon emissions								
C_{gas} (g C/kg DM)	–	–	9.53	10.68	6.58	7.25	1.88	4.47
$C_{leachate}$ (g C/kg DM)	–	–	1.99	2.01	2.08	2.11	2.73	2.64

Biogas production under anaerobic conditions (Aa and Ab) became negligible after day 80, as visible in Fig. 2c. Despite the efforts to guarantee gastight conditions for Aa and Ab, some of the biogas samples analysed showed a low but not negligible presence of oxygen, between 0.1 and 2%.

In continuously aerated reactors, O_2 concentration in the extracted gas was higher in Ca compared to Cb until day 170. Accordingly, CO_2 was lower in Ca than Cb (Fig. 2a). This is probably due to the higher compaction in Ca than in other reactors, that might have hampered oxygen homogeneous distribution and utilization in the reactor.

During no aeration periods in intermittently aerated reactors, no biogas was observed in the collection bags connected with reactors. After aeration switch off, oxygen trapped into waste porosity was consumed and its concentration dropped to zero after a couple of days in the head space above the waste, from which sample of gas composition monitoring were taken. However, as already mentioned, no lateral sampling ports in reactors were available and therefore oxygen consumption rates in the waste body could not be assessed.

As shown in Fig. 2, carbon discharge via the gas phase was the highest in continuously aerated reactors.

Some results of leachate characterization during the experiment are shown in Table 2.

Values measured for pH, BOD_5, COD, TKN, N-NH_4^+, TOC, VFA, $SO_4^=$ are reported for leachate samples that were extracted at day 59, 84, 104 and 116 and used for microbial characterization also.

Composition at day four, after three days of aeration, is typical of a young landfill leachate, with BOD_5/COD equal or above 0.5; BOD_5 above 25,000 mg/L and ammonia nitrogen approximately equal to 2000 mg/L.

At day 59, one week before the end of LS phase, BOD_5 values were one order of magnitude lower than at test start. As expected, much lower was the influence of landfill simulation phase under anaerobic conditions on ammonia nitrogen concentration, which ranged between 1480 mg/L and 1780 mg/L at day 59, still close to initial values (Table 2).

During continuous and intermittent aeration of RS phase, the evolution of different parameters determined in the reactors was very different. In Fig. 3, the concentration of N-NH_4^+, N-NO_3^- and N-NO_2^- in leachate extracted from reactors during the test is reported.

In anaerobic reactors, ammonia nitrogen concentration in leachate at test end accounted for 50% of the initial value. The decrease is mainly due to the dilution caused by the weekly extraction of leachate samples and the following re-fill performed with deionized water. The previously mentioned unexpected and uncontrolled oxygen inlet in anaerobic reactors might have enabled oxidation processes and thus contributed to the observed decrease of ammonia concentration. Nevertheless, neither NO_3^- nor NO_2^- production was observed, although this can be caused by denitrification processes often observed in similar cases (Berge et al., 2005).

As visible in Fig. 3, continuously aerated reactors Ca and Cb showed a significantly different behavior. Ammonia nitrogen concentration decreased in both reactors with a similar trend until day 90. Subsequently, N-NH_4^+ concentration in Cb dropped significantly from 1500 mg/L to below 80 mg/L in a month (day 120). On the contrary, the decreasing trend in Ca maintained the same rate as before day 90; N-NH_4^+ concentration dropped below 80 mg/L at day 167 only. The higher efficiency in ammonia removal in Cb might be due to the more effective aeration (oxygen utilization) compared to Ca, as mentioned earlier. Following to the rapid decrease of N-NH_4^+ concentration in reactor Cb, a sudden increase of NO_2^- concentration was recorded (day 100), followed by NO_3^- (Fig. 3). Another peak of NO_2^- production was observed at day 135, when ammonia concentration was already low and steady around 80 mg/L, followed by another peak of NO_3^- concentration. Conversely, nitrate was always below detection limit in Ca, where nitrite first appeared much later and in lower concentration than Cb.

In the same Fig. 3, evolution of ammonia, nitrous and nitric nitrogen is reported for intermittent reactors. Ammonia concentration in Ia showed a very slow decrease in the first months, the first aeration period of RS phase produced no visible effects. First significant decrease of ammonia concentration was observed during second aeration of RS phase (day 105–125); another drop occurred during third aeration (from day 147). Accordingly, nitrite and nitrate appearance was observed. Ammonia nitrogen concentration in Ib maintained constantly around 1500 mg/L until start of third aeration (day 147), then a drop similar to what observed for Ia and the related appearance of nitrous and nitric nitrogen were recorded.

Both in continuously and intermittently aerated reactors, rapid nitrate depletion suggests that nitrification was followed by denitrification. Evidence of the possible co-existence of nitrification and denitrification processes in aerated reactors had already been observed in previous experiments (Berge et al., 2006; Raga and Cossu, 2013). In some cases, nitrate decrease was associated to an increase in sulphate concentration. According to Berge et al. (2006) this might suggest that nitrate removal may be partly attributed to autotrophic denitrification, which occurs in case of limited availability of biodegradables and

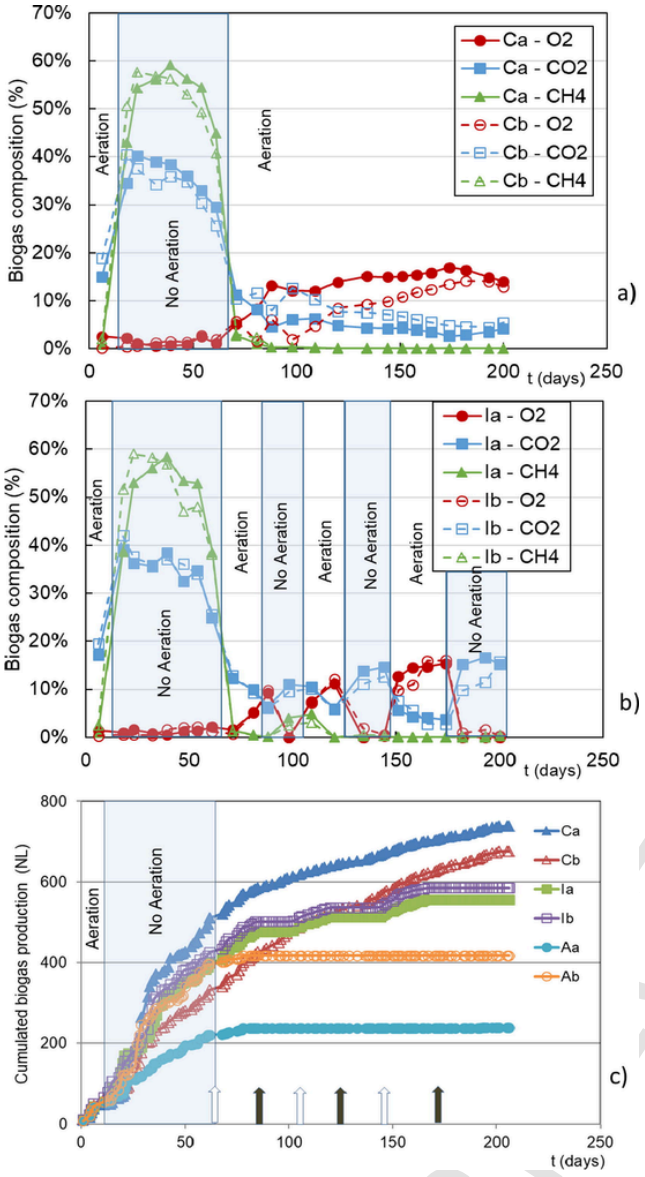

Fig. 2. Evolution of biogas composition in continuous (a) and intermittent (b) reactors. (c) shows the cumulative biogas production in reactors; for Ia and Ib only, white arrows at aeration start; black arrows at aeration switch off.

in presence of inorganic sulphur compounds which are oxidized to sulphate.

Fig. 4 shows the evolution of TOC concentration in leachate extracted from reactors. As happened to Ammonia nitrogen, TOC concentration decreased in Cb faster than in Ca. In reactors Ia and Ib, TOC concentration decreased rapidly during aeration phases with the exception of second aeration (day 105 – 125) in Ib, where TOC decrease was negligible.

As shown in Figs. 3 and 4, a rebound was observed for concentration of N-NH_4^+ and TOC after the end of third aeration phase in reactors Ia and Ib. This effect was expected and should be taken into account when evaluating long term effects of aeration of municipal solid waste landfills. Biodegradation processes are enhanced in leachate percolating through an aerated media and very often relevant parameters rapidly reach concentrations below detection limits in lab-scale tests. However, leachate quality in such cases does not necessarily reflect waste quality and further accumulation in leachate of elements/compounds from the solid matrix is likely to occur and cause the abovementioned rebound after aeration switch-off.

The same effect, to a far greater extent, is expected in full scale applications.

3.1. Microbial analysis

Microbial composition in the six reactors was analysed at different time points in order to determine changes during time and to identify correlations between the microbial composition, reactors performance, process parameters and leachate chemical parameters. Reactors Ca, Cb, Ia, Ib were sampled at four time points: 59, 84, 104 and 116 days after the beginning of the experiment. Reactors Aa and Ab were sampled only twice: at 59 and 116 days. The time points were chosen by taking into account the aeration process operated in different reactors. The first time point (day 59) was common to all reactors and was operated when the CH_4 production started to significantly decrease, a few days before the end of the anaerobic LS phase. The second time point (day 84) was at the end of the first aeration period and was performed both in continuously aerated reactors (Ca, Cb) and in those subjected to intermittent aeration (Ia, Ib). After this time point aeration was interrupted in reactors Ia and Ib until day 104, when sampling was performed again in reactors Ca, Cb, Ia, Ib. Finally, aeration was restarted in reactors Ia, Ib and samples were collected again in all the six reactors to have a picture of the final microbial composition after 116 days of reactor operation.

In order to have a detailed comprehension of the taxonomic composition of the microbiome and to identify its biochemical potential, two different approaches were used. The first approach was a "taxonomic investigation" based on the classification of the shotgun DNA sequences on a database of nearly 13,500 bacteria and archaea species (Segata et al., 2012). Thanks to the high number of sequences obtained for each sample, this analysis generated a detailed catalogue of the microbiome and the identification of the species even if present at very low abundance (the so called "rare" microbiome). The second approach was a "genome centric" investigation performed to identify microbes belonging to underexplored taxa, which are not included in public databases. More importantly, this second approach was used to have an overview of the functional capabilities of microbes.

The two complementary approaches used evidenced that the microbial community involved in waste degradation is extremely complex. The "taxonomic investigation" identified 35 different phyla, corresponding to 672 different genera. Most of the genera were extremely rare and only 65 were present at relative abundance higher than 1% in at least one of the samples analysed (Supplementary File S1 Worksheet MetaPhlAn). The "genome centric" approach identified 376 MAGs belonging to 24 different phyla, with a particularly high abundance of Firmicutes (91 MAGs), Bacteroidetes (52 MAGs), Betaproteobacteria (34 MAGs) and Gammaproteobacteria (33 MAGs). Taxonomic assignment was not possible for 52 MAGs (Supplementary File S1 Worksheet MAG_genomes_and_cover). Differences in the number of taxonomic groups identified between the two approaches can be explained by the impossibility to recover the extremely low abundant species with the "genome-centric" approach. The huge complexity of the microbiome was presumably due to two main effects: the shift from anaerobic to aerobic conditions and the modification of the microbiome during time. Introduction of oxygen through the aeration process can have an adverse effect on strictly anaerobic and microaerophilic species which, under this condition, decreased in abundance. However, aeration can have a positive effect on aerobic and facultative microbes that increased during time. The combined effect of the aeration process and the depletion of the fermentable organic matter, had adverse effect on the strictly anaerobic microbes that, during the first anaerobic period, were involved in the Anaerobic Degradation

Table 2
Some results of leachate analysis. Values in samples extracted at day 59, 84, 104 and 116 are shown. Samples extracted at the same days were analysed for microbial characterization also. n.a.: not ascertained.

		Ca	Cb	Ia	Ib	Aa	Ab
Day 4 (after 3 days of pre-aeration)	pH	6.7	6.6	6.5	6.8	6.8	7.0
	TKN (mgN/L)	2440	3070	2950	2930	3550	2910
	$N\text{-}NH_4^+$ (mgN/L)	1910	2090	1900	2050	1940	2030
	TOC (mgC/L)	16,600	21,150	19,500	18,550	20,450	19,050
	COD (mgO_2/L)	52,350	61,150	62,800	61,560	60,260	55,980
	BOD_5 (mgO_2/L)	26,620	39,860	39,840	37,180	38,430	30,200
	VFA (mg CH_3COOH/L)	6060	7200	6450	6820	6350	6590
	$SO_4^=$	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Day 59	pH	7.9	7.9	8.0	8.1	8.1	8.0
	TKN (mgN/L)	2050	2330	2130	2370	2570	2360
	$N\text{-}NH_4^+$ (mgN/L)	1650	1730	1480	1590	1780	1520
	TOC (mgC/L)	3500	3680	3280	3620	4100	4040
	COD (mgO_2/L)	14,870	14,380	13,300	16,560	15,740	14,690
	BOD_5 (mgO_2/L)	2530	1540	1970	2520	1820	3100
	VFA (mg CH_3COOH/L)	650	710	590	660	730	710
	$SO_4^=$	25	28	25	84	93	28
Day 84	pH	7.9	8.0	7.9	8.0	8.0	7.9
	TKN (mgN/L)	1640	2090	1750	1830	2370	2290
	$N\text{-}NH_4^+$ (mgN/L)	1090	1590	1250	1490	1750	1500
	TOC (mgC/L)	2720	2710	2460	2250	3750	3490
	COD (mgO_2/L)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	BOD_5 (mgO_2/L)	71	71	140	70	n.a.	n.a.
	VFA (mg CH_3COOH/L)	620	600	510	500	750	700
	$SO_4^=$	600	1490	1440	1950	85	35
Day 104	pH	8.0	7.5	7.8	8.0	7.9	7.9
	TKN (mgN/L)	1230	780	1620	1940	2070	2020
	$N\text{-}NH_4^+$ (mgN/L)	900	470	1180	1520	1470	1600
	TOC (mgC/L)	2060	1820	2350	2550	4880	4040
	COD (mgO_2/L)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	BOD_5 (mgO_2/L)	55	47	56	57	n.a.	n.a.
	VFA (mg CH_3COOH/L)	500	270	620	690	680	640
	$SO_4^=$	583	2880	46	38	90	30
Day 116	pH	7.9	7.3	7.8	8.1	8.2	8.1
	TKN (mgN/L)	1350	240	970	2030	1720	2240
	$N\text{-}NH_4^+$ (mgN/L)	670	80	700	1470	1390	1450
	TOC (mgC/L)	1450	1110	2010	2480	2720	3660
	COD (mgO_2/L)	5240	2580	4620	7980	9540	9200

Table 2 (*Continued*)

		Ca	Cb	Ia	Ib	Aa	Ab
Day 171 (end of third aeration)	BOD_5 (mgO_2/L)	41	35	45	47	94	94
	VFA (mg CH_3COOH/L)	410	120	340	500	580	540
	$SO_4^{=}$	430	3220	2220	1440	115	32
	pH	8.0	8.0	8.5	8.1	8.4	8.5
	TKN (mgN/L)	139	150	112	54	1250	1200
	$N\text{-}NH_4^{+}$ (mgN/L)	57	14	23	27	1180	1060
	TOC (mgC/L)	710	460	640	770	2600	1750
	COD (mgO_2/L)	2170	1240	1820	2300	8700	6850
	BOD_5 (mgO_2/L)	<10	<10	<10	<10	36	71
	VFA (mg CH_3COOH/L)	73	50	50	50	434	415
	$SO_4^{=}$	2030	2710	2840	3320	95	250

(AD) process. This led to a deep modification of the microbiome from the first to the second sampling point as clearly evidenced by the hierarchical clustering and the PCoA performed on the results obtained from the "taxonomic investigation" (Fig. 5 A-B). Both the "taxonomic" and the "genome-centric" investigations evidenced that most of the microbes involved in the AD process during the landfill simulation process tend to decrease in relative abundance during the simulated remediation process. This could be interpreted as mainly due to the decrease of the degradable organic matter and, at a lower extent, to the aeration. In fact, the members of this anaerobic microbial community decrease during time both in aerated reactors and in those maintained in anaerobic conditions (Supplementary File S1 worksheet MetaPhlAn; Supplementary Fig. 1). The "landfill AD microbiome" is responsible for the degradation of the organic matter present in the waste by following the four steps of the process, hydrolysis, acidogenesis, acetogenesis and methanogenesis. Interestingly, all the three classes of methanogenic archaea are represented, hydrogenothrophic of the *Methanoculleus* genus (*Methanoculleus* sp. UNIPD-la0258, *Methanoculleus* sp. UNIPD-la0344), acetogenic of the *Methanosarcina* genus (*Methanosarcina* sp. UNIPD-la0225 and *Methanosarcina thermophila* UNIPD-la0139) and methylothrophic (*Methanomassiliicoccales* archaeon UNIPD-la0020 and *Methanomassiliicoccales* archaeon UNIPD-la0021). The presence of ten different archaeal MAGs suggests that the archaeal microbiome is more complex that those present in biogas reactors (Treu et al., 2016b) and possibly related to the heterogeneity of the organic matter present in the municipal solid waste. It is also noticeable the heterogeneity in the archaeal abundance and composition among different reactors; for example, hydrogenothrophic and methylothrophic methanogens are abundant in Ia, while acetoclastic methanogens of the *Methanosarcina* genus are very abundant in Ib (Fig. 5C). In the organic matter degradation, hydrolytic bacteria have an extremely important role, and both members of Firmicutes (particularly Clostridiales) and Bacteroidetes phyla are extremely active in this process (Treu et al., 2016a). Two anaerobic MAGs having high abundance at the first time point and a high number of genes involved in polysaccharides degradation are Firmicutes sp. UNIPD-la0354 and Methylococcaceae sp. UNIPD-la0074. Other relevant members of the AD process, but present at lower abundance, are acetogenic microbes of the Synthrophomonadaceae family (e.g. Syntrophomonadaceae sp. UNIPD-la0200), which are usually involved in fatty acids oxidation. This functional property is also evidenced by the high number of genes present in the core pathway of fatty acid beta-oxidation II in Syntrophomonadaceae sp. UNIPD-la0036 (Supplementary File S1 worksheet KEGG).

In reactors Ia and Ib some members of the AD microbiome (e.g. *Methanoculleus* spp.) slightly increased their abundance during the second anaerobic phase (Fig. 5C; Supplementary Fig. 1), suggesting that the duration of the anaerobic period was long enough to allow them to recover after the aeration period. The mild increment evidenced can be ascribed to different effects, such as the low abundance of residual fermentable organic matter after 104 days, but also to the difficulty in recovering their activity after the aeration process.

It has also to be considered that when comparing communities at different time points with DNA-based methods the results are based on relative abundance. Therefore, the growth of any microbe (e.g. aerobes), determines obviously a reduction of the relative abundance of others (e.g. the anaerobes present at the first time point) when those do not grow at equal or higher pace. This means that one given group could have been increasing, but its share in the sum could appear as if it had instead decreased if a different group has grown faster. This consideration, that applies inevitably for all metagenomics studies, should be kept in mind for all kinds of interpretations about increases and decreases which could be either real or apparent (when driven by a stronger change of a different group).

Analysis of the Pearson correlation between environmental parameters of the reactors and species abundance, together with the CCA, allowed the identification of taxa and MAGs more strictly connected to the environmental and the operational parameters (Supplementary Figure S2; Supplementary Figure S3). CCA evidenced that *Methanoculleus*, *Syntrophomonas* and *Parabacteroides* genera were strictly connected to biogas production. The obligately anaerobic, non-spore-forming, non-motile, *Parabacteroides* was also positively correlated (Pearson correlation $P < 0.001$) to BOD; this latter parameter was also positively linked to *Blautia*, a genus known as a degrader of complex polysaccharides to short chain fatty acids including acetate, butyrate, and propionate (Biddle et al., 2013) Other methanogenic archaea belonging to *Methanosarcina* and *Methanomassillicoccus* were less relevant to this process. The same result was confirmed by the Pearson correlation analysis and also by the results obtained for *Methanoculleus* sp. UNIPD-la0258 and *Methanoculleus* sp. UNIPD-la0344, which were strictly connected with biogas production ($P < 0.001$). Another noticeable finding was the correlation of some members of the Firmicutes (e.g. *Syntrophomonas*, *Ruminococcus*) and Bacteroidetes (*Proteiniphilum*) with biogas production. These results

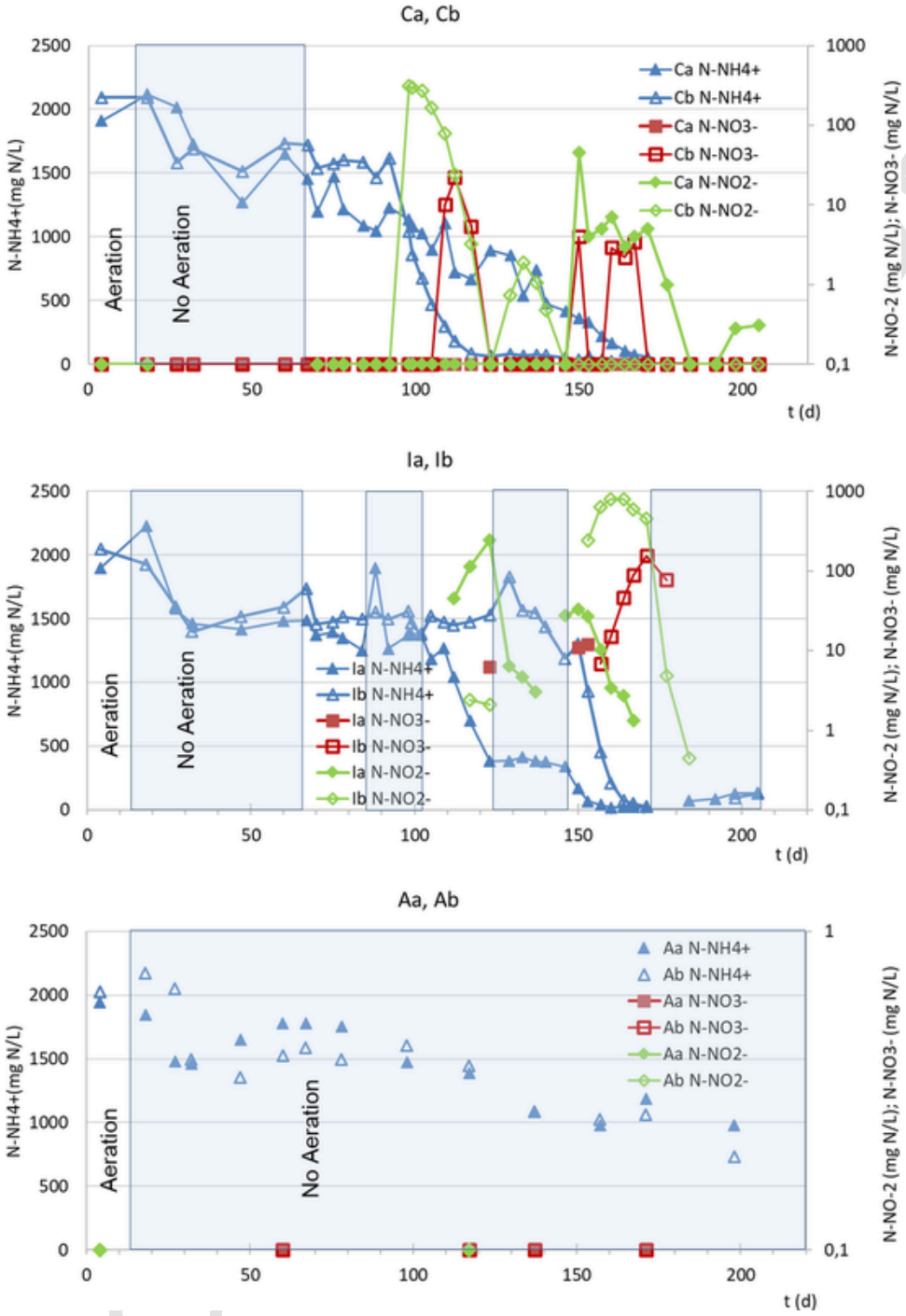

Fig. 3. Evolution of $N\text{-}NH_4^+$, $N\text{-}NO_3^-$ and $N\text{-}NO_2^-$ in leachate extracted from reactors. Shading indicates periods when aeration was switched off in reactors.

were confirmed by the CCA performed on MAGs, which revealed that some Firmicutes such as Firmicutes sp. UNIPD-la0354 (involved in hydrolysis of complex polysaccharides) or *Fermentimonas caenicola* UNIPD-la0360 (involved in acetogenesis with the production of acetic and propionic acids) (Hahnke et al., 2016) were correlated with biogas production (Supplementary Figure S2). *Chlorobium,* a photolitotrophic oxidizer, was positively correlated with biogas production, TOC, and BOD (Pearson correlation $P < 0.001$), while the denitrifyer *Alicycliphilus* was inversely correlated.

During the incubation, in all the reactors the microbial community underwent a strong modification, suggesting that the progressive increase during time of genera such as *Alcanivorax*, *Thioalkalivibrio* or *Azoarcus* is due to the progressive decrease of the organic matter and the modification of the chemical composition of the leachate, more than the aeration. However, effect of intermittent aeration can explain the fluctuation in abundance of some genera such as *Spiribacter* which revealed an increased abundance during aeration in reactors Ia and Ib. Moving from the first sampling to the next ones the points in the PCoA plot tend to cluster together, suggesting that the microbial community become more similar in different reactors (Fig. 5 B). The deep modification of the microbiome was evidenced by the increased abundance of some genera which were very rare at the first sampling point, such as *Marinobacter*, *Caulobacter*, *Alicycliphilus*, *Halomonas*, *Thiomonas*, *Bordetella*, *Pusillimonas* and *Deinococcus* (Supplementary Figure S1).

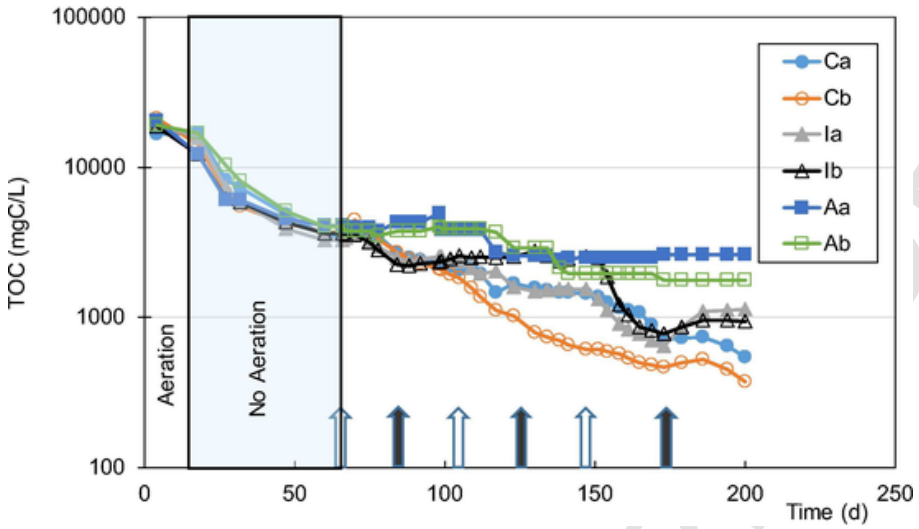

Fig. 4. Evolution of TOC during the experiment. For Ia and Ib only, white arrows at aeration start; black arrows at aeration switch off.

Regarding the activity of the microbial community, an important parameter to consider is the ability to utilize or to efficiently oxidize ammonia. It has already been reported that the intermittent aeration in landfill bioreactors can stimulate nitrifiers and denitrifiers, resulting in an effective nitrogen removal (He et al., 2007; Shao et al., 2008). The increase in ammonia-oxidizing bacteria and fungi in an aerated landfill bioreactor can remove nitrogen from solid waste and leachate (Sang et al., 2008). Since in the six reactors the efficiency of ammonia conversion was strongly variable, it is expected that this reflected diversity in microbial composition.

Investigation of the results obtained from CCA and Pearson correlation evidenced that some species can have an important role in nitrogen cycle. By taking into account the biochemical pathways present in different microbes, it is possible to obtain an overview of their functional role.

Regarding ammonia oxidation, biochemical parameters such as NO_2^- concentration evidenced that this process was highly different in the six reactors considered, it started earlier in the continuously aerated Cb, followed by the intermittently aerated Ia (Fig. 3). Analysis of MAGs abundance, their correlation with biochemical parameters (CCA and Pearson correlation) and the presence in their genomes of enzymes belonging to the nitrogen cycle allowed the identification of the most relevant species involved in this process.

As regards the Pearson correlation analysis between reactor chemical parameters and specific genera, *Thiomonas* (Arsene-Ploetze et al., 2010), a facultative chemolithoautotroph able to grow in mixotrophic media containing reduced inorganic sulphur compounds, resulted positively correlated with SO_4^{2-} and nitrite but negatively with VFA ($P < 0.001$). Among the MAGs more strictly connected with NO_3^- concentration, *Nitrosomonas* sp. UNIPD-lă, Xanthomonadaceae sp. UNIPD-la0118 and Myxococcales sp. UNIPD-la0135 are of particular interest (Supplementary Figure S2). Ammonia-oxidizing species belonging to the *Nitrosomonas* genus were previously identified in reactors performing nitrification at low-dissolved oxygen (Fitzgerald et al., 2015). Despite the difficulty in defining the functional role of *Nitrosomonas* sp. UNIPD-lă due to the low genome completeness (33%), the presence of some key genes (*norC*, *hao*) indicates its involvement in ammonia oxidation. Members of Xanthomonadaceae family were also previously identified in reactors capable of autotrophic and heterotrophic ammonia utilization, albeit without stoichiometric accumulation of NO_2^- or NO_3^- (Fitzgerald et al., 2015). Xanthomonadaceae sp. UNIPD-la0118 (96% genome completeness) is particularly interesting, since it is one of the MAGs with the highest number of genes involved in nitrogen pathways (Supplementary File S1 worksheet nitrogen_pathways). Enzymes encoded in this MAG were mainly assigned to denitrification (12 *nirK* genes) (NO_2^- to NO), ammonification (*nasA*, *nasB*) and 2,4-nitrification (NO_2^- to NO_3^-) (*norB*,*C*). These three MAGs (UNIPD-lă, UNIPD-la0118, UNIPD-la0135) display a quite different pattern of abundance in the reactors examined, but all of them tend to accumulate in Cb at day 116, which is the reactor having the highest NH_4^+ removal. Their accumulation in Ab at day 116 indicate that their growth is possible even when the residual amount of O_2 is very low, however the limited NH_4^+ removal indicates that in the absence or at very low concentrations of oxygen, their efficiency in ammonia oxidation is very low.

Regarding the MAGs correlated with nitrite concentration in the reactors, the more interesting in terms of gene content are Xanthomonadaceae sp. UNIPD-la0346 and Alcaligenaceae sp. UNIPD-la0152 (Supplementary Figure S2). Both of them are present at high abundance in Cb, were they tend to accumulate at days 104 and 116. Interestingly they show a higher abundance in Ia in comparison to Ib suggesting that they are involved in the faster NH_4^+ oxidation happening in Ia after 100 days. Considering the total number of genes ($n = 43$) involved in nitrogen cycle, Xanthomonadaceae sp. UNIPD-la0346 (genome completeness 82%) is ranked second among the 376 MAGs identified. In particular, it encodes 21 *nirK* genes (involved in 4-denitrification NO_2^- to NO), but also one *norB* (2,4-nitrification NO_2^- to NO_3^-), three *norC* (2-nitrification NO_2^- to NO_3^-) and 3 *hao* genes (1-nitrification NH_2OH to NO_2^-). It encodes also three *nosZ* genes involved in 4-denitrification from N_2O to N_2. The presence of numerous *nirK* genes ($n = 12$) is also common to Alcaligenaceae sp. UNIPD-la0152 and to other MAGs identified in the study (Supplementary file S1 worksheet nitrogen_pathways). The presence in the same Alcaligenaceae species of genes involved in nitrification and denitrification capabilities has also been previously demonstrated (Velusamy and Krishnani, 2013) (REF). This apparently contradicting feature was associated with the ability to perform heterotrophic nitrification of NH_4^+ into NO_2^- via formation of NH_2OH, which is then oxidized to NO using oxygen or NO_2^- as electron acceptor. Alcaligenaceae sp. UNIPD-la0152 accumulates at high level in Cb particularly at 104 days and it is influenced by the aeration, particularly in Ib where it accumulated at days 84 and 116. There are also other MAGs showing a weak correlation with NO_2^- concentration ($P < 0.05$), among these, *Parcubacteria* sp. UNIPD-la0063 is of particular interest. Despite its low genome completeness (23%) its possible involvement in nitrogen cycle is relevant, particularly for its association with the poorly characterized candidate Phyla Radiation (CPR). This ability has been also recently suggested by other authors (Castelle et

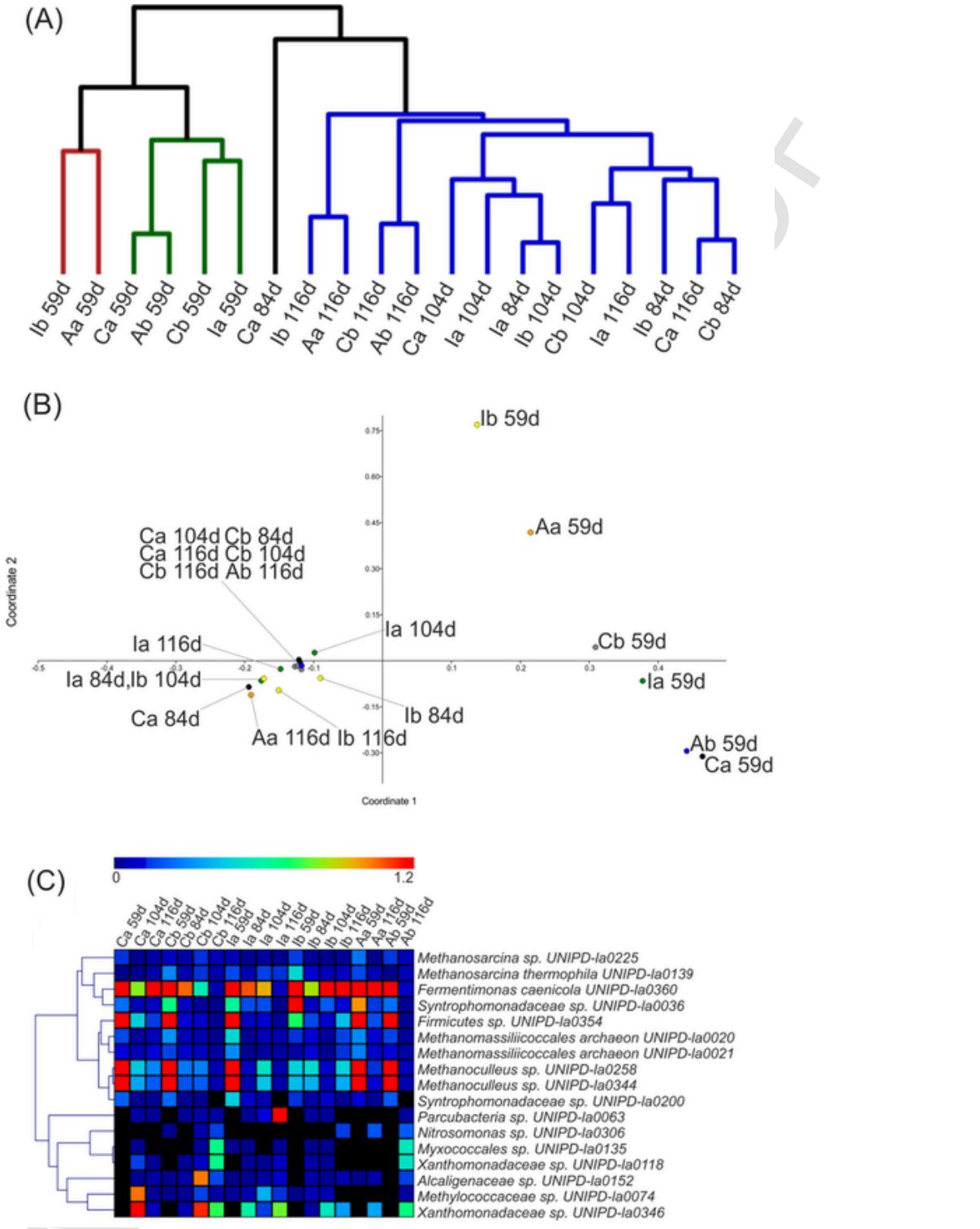

Fig. 5. Results obtained from metagenomic analyses. (A) Similarity between different samples was determined considering the coverage profiles calculated for all the genera identified from shotgun reads analysis. Three clusters of samples highly supported are evidenced in color. Names of the samples are reported along with the number of days passed from the beginning of the landfill simulation process. The complete figure including the heatmap for each genus is reported as an Additional Information file. (B) PCoA performed considering the abundance values reported in figure (A). Samples collected from the same reactor at different time points are highlighted with the same color. (C) Abundance values (coverage) of the MAGs described in the main text are represented as a heatmap. MAGs with similar coverage profiles are adjacent in the three, which was calculated measuring the Person correlation coefficient. The coverage values are reported in color and a scale is reported on top of the image. In this figure the sample "Ca 84d" is not represented because of the strongly difference obtained in comparison to the other samples.

al., 2017) and it was also confirmed by the presence of HAO and *amoA,B,C* in its genome (Supplementary file S1 worksheet nitrogen_pathways).

4. Conclusions

Emissions and microbial community were monitored during landfill simulation experiments in reactors run under continuous aeration, intermittent aeration and under anaerobic conditions as a control.

Different aeration regimes had different effects on emissions from different reactors: leachate nitrification started a few days earlier and carbon discharge via biogas was higher in reactors under continuous aeration than under intermittent aeration. Concerning leachate quality at day 171 (end of third aeration period), concentrations of relevant parameters (BOD_5, ammonia nitrogen, TOC, VFA) were pretty similar in all aerated reactors, no matter the aeration regime, and significantly lower than what observed in anaerobic reactors. Although concentration of some parameters (i.e. Ammonia nitrogen and BOD_5 in this case) rapidly reach very low values in leachate during aeration tests, a rebound effect is likely to be observed over time after aeration phase is over. Indeed, the results of leachate quality monitoring during such aeration experiments may be misleading if used for purposes beyond the scope of the investigation: the estimation of potential emissions from waste and of long term leachate quality should be evaluated through much longer landfill simulation tests.

Results revealed that metagenomics is able to provide a detailed reconstruction of the landfill microbiome both in aerobic and anaerobic conditions without the need of using techniques based on microbial cultivation.

The combined analysis of the microbial abundance, their correlation with biochemical parameters and the identification of enzymes belonging to specific steps of the nitrogen cycle allowed the identification of the most relevant species.

Results revealed also that twenty days in anaerobic conditions are enough for most of the species to recover after the aeration process. Analysis of functional pathways revealed that microbes associated to the $N\text{-}NH_4^+$ removal, such as Xanthomonadaceae sp. UNIPD-la0118, accumulated at higher level in the reactor with the fastest $N\text{-}NH_4^+$ decrease (Cb), particularly after 100 days of incubation.

This study represents a first attempt to use genome-centric metagenomics to perform a global survey in landfill simulation experiments, to disclose the role of poorly characterized microbial species and to reveal their efficiency in terms of $N\text{-}NH_4^+$ and organic carbon removal during intermittent aeration.

Declaration of Competing 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.

Acknowledgements

The authors wish to acknowledge the cooperation of Ms. Elisabetta Priante and Ms. Ilaria Negrin for the management of reactors and Ms. Annalisa Sandon, laboratory of environmental sanitary engineering of the University of Padova. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.wasman.2020.08.010.

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