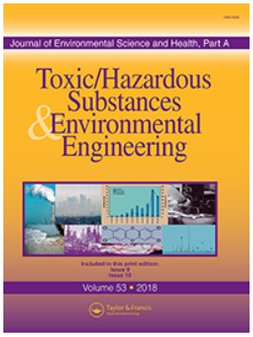

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Journal of Environmental Science and Health, Part A
Toxic/Hazardous Substances and Environmental Engineering

ISSN: 1093-4529 (Print) 1532-4117 (Online) Journal homepage: http://www.tandfonline.com/loi/lesa20

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To cite this article: Corrado Amodeo, Adriano Sofo, Maria Teresa Tito, Antonio Scopa, Salvatore Masi, Raffaella Pascale, Ignazio M. Mancini & Donatella Caniani (2018) Environmental factors influencing landfill gas biofiltration: Lab scale study on methanotrophic bacteria growth, Journal of Environmental Science and Health, Part A, 53:9, 825-831, DOI: 10.1080/10934529.2018.1455342

To link to this article: https://doi.org/10.1080/10934529.2018.1455342

Published online: 29 Mar 2018.

JOURNAL OF ENVIRONMENTAL SCIENCE AND HEALTH, PART A
2018, VOL. 53, NO. 9, 825–831
https://doi.org/10.1080/10934529.2018.1455342

Environmental factors influencing landfill gas biofiltration: Lab scale study on methanotrophic bacteria growth

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ABSTRACT

The post-management of landfills represents an important challenge for landfill gas treatment. Traditional systems (energy recovery, flares, etc.) present technical problems in treating flow with low methane (CH_4) concentrations. The objective of this study was to isolate methanotrophic bacteria from a field-scale biofilter in order to study the bacteria in laboratories and evaluate the environmental factors that mostly influence Microbial Aerobic Methane Oxidation (MAMO). The soil considered was sampled from the biofilter located in the landfill of Venosa (Basilicata Region, Italy) and it was mainly composed of wood chips and compost. The results showed that methanotrophic microorganisms are mainly characterized by a slow growth and a significant sensitivity to CH_4 levels. Temperature and nitrogen (N) also have a very important role on their development. On the basis of the results, biofilters for biological CH_4 oxidation can be considered a viable alternative to mitigate CH_4 emissions from landfills.

ARTICLE HISTORY
Received 7 December 2017
Accepted 20 February 2018

KEYWORDS
Biofiltration; Biolog®; methane oxidation; methanotrophic bacteria; landfill methane emissions

Introduction

The anaerobic decomposition of urban solid waste in landfills causes the production of landfill gas (LFG). LFG composition is a mixture of different gases that changes according to different conditions. Methane (CH_4) and carbon dioxide (CO_2) are the prevalent gases in the LFG flow, but nitrogen (N_2) and other trace gases, such as volatile organic hydrocarbons (VOCs), may be present in the flow.[1]

Both, CH_4 and CO_2 are considered strong greenhouse gases (GHGs) due to their capacity in absorbing and emitting infrared radiation. However, CH_4 has a global warming potential (GWP) 25 times stronger than CO_2.[2] Tropospheric concentrations of methane increased from 722 part per billion (ppb) in the preindustrial age to 1834 ppb in 2016.[3] CH_4 emitted from landfills has historically been considered the largest source of GHG emissions from the waste sector [2]: the global CH_4 emissions from landfills are estimated to be 500–800 Mt CO_2-eq year-^{1}.[4–6]

The implementation of vertical wells or horizontal collectors for active LFG extraction linked to energy-recovery plants is common in landfills in developed countries and represents the most important mitigation measure to reduce GHG emissions.[2] Nevertheless, energy recovery plants need to have a high LFG flow and CH_4 concentration to produce energy. In these situations, LFG combustion by flares represents the most common technical solution.[7] However, increasing the landfill post management period, LFG flow and CH_4 concentration decrease, and flares are not always capable to oxidize CH_4 by combustion; normally, the minimum flow must be 50 $Nm^3\ h^{-1}$ and the minimum CH_4 concentration 30% (v v^{-1}).[8] The same technical problems can be observed in small landfills, where LFG flow and CH_4 concentration are not enough for traditional LFG treatment systems.[9]

In the abovementioned circumstances, Microbial Aerobic Methane Oxidation (MAMO) represents a viable alternative to traditional systems for LFG treatment.[10] The MAMO process is due to methanotrophic bacteria (also called "methanotrophs"), a subset of a physiological group of bacteria known as methylotrophs.[11] Methanotrophs have the capacity of oxidizing CH_4 in aerobic conditions.[12] Methanotrophs have CH_4 as a carbon and energy source, and use the enzyme methane-monooxygenases (MMOs) to catalyse the oxidation of CH_4 to methanol (CH_3OH), followed by oxidation of methanol to formaldehyde (CHOH) and the subsequent oxidation of formaldehyde to formate (CHOOH), before the final conversion to CO_2.[13]

The biochemical reaction ($\Delta°G = -780$ kJ mol^{-1} CH_4, $\Delta°H = -890$ kJ mol^{-1}) is the following [14,15]:

$$CH_4 + (2-x)\,O_2 \rightarrow (1-x)\,CO_2 + (2-x)\,H_2O + \text{heat}$$

The kinetics of this process was described by Scheutz et al.,[13] Abichou et al.[16] and Ng et al.[15] as:

$$r = \frac{V_{\max} \cdot C_{CH_4}}{K_m^{CH_4} + C_{CH_4}} \cdot \frac{C_{o_2}}{K_m^{O_2} + C_{o_2}}$$

where r = CH_4 oxidation rate [mol $(m^3\ h)^{-1}$], V_{max} = maximum CH_4 oxidation rate [mol $(m^3\ h)^{-1}$], K_m = Michaelis–

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Menten or half-saturation constant [mol m^{-3}], and C = concentration [mol m^{-3}].

The biofiltration is a biological process in which MAMO occurs. The biofilter is an aerobic reactor, generally a container with standard sizes (easier to set up), filled with packing material. The packing material is very important as it is the core of the process. Many researchers focused their studies on the choice of the best packing material to improve MAMO efficiency.[17–20] Wood chips have been detected as an excellent packing material due to their capacity to support and sustain the development of a methanotrophic biofilm.[21] In landfills, the collectors of active LFG extraction lead the gas to the base of the biofilter through a piping system. In this way, LFG is conducted to the packing material by natural trend. An empty space is placed at the bottom of the system to avoid preferential pathways and to allow an LFG homogeneous diffusion over the entire oxidation surface.[8]

Several environmental parameters influence the MAMO efficiency, as with all biological processes. Among them, the most influential, as it is expected for biological processes, is the temperature. Generally, methanotrophs are mesophile bacteria and the perfect range for their development is between 25 and 35°C.[22–24] The moisture of the packing material also represents an important parameter to facilitate the nutrient supply and the optimal range is 10–20% (w w^{-1}) [25]: 5% is the threshold below which the bacteria activity dramatically drops.[26–28] The optimal pH lies between 5.5 and 8.5.[13]

The aim of this study was to isolate methanotrophic bacteria from a field-scale biofilter in order to evaluate the environmental factors that most influence MAMO. The soil was sampled from the biofilter located in the landfill of Venosa (Basilicata Region, Italy) and it was mainly composed of wood chips and compost.

Materials and methods

Full-scale biofilter

In 2014, a biofilter for the LFG treatment was installed in the Venosa landfill. It was designed and built by a collaboration between the Sanitary Engineering research group of the Basilicata University and the private company Entsorga Italia Spa (Tortona, AL, Italy). The biofilter was implemented in a 20 ft (feet) standard container (external measurements: length 6.058 m, width 2.438 m, height 2.591 m). The packing material was leaned on an internal base, leaving 55 cm of empty space at the bottom of the container. In this way, the LFG could be uniformly distributed over the entire oxidation surface avoiding preferential pathways. According to most scientific studies,[13,21,29] a mix of wood chips and compost was selected as the packing material (the main characteristics are reported in Table 1).

Approximately 19 m^3 of packing material were introduced in the container (1.4 m height). About 20 cm of empty space was left on top, between the packing material and a geomembrane located under the closing system. The geomembrane has the task of retaining the moisture in the system, letting the treated LFG pass. Furthermore, a wet system linked to a water tank was set up on the final closure to ensure constant moisture. All the treated LFG comes out of a chimney located at the top of the container. A discharge valve was also installed at the bottom to unload the leachate. A blower (7.5 kW, 2900 rpm) was implemented and linked to the landfill pipelines (already connected to the landfill vertical wells) to bring LFG to the biofilter. Figure 1 shows the biofilter design.

Table 1. Packing material characteristics.

Parameter	*Value*	*U.M.*
pH	8.04	—
Moisture	48.66	% t.q.
Dry matter	51.34	% t.q.
Organic matter	78.10	% s.s.
N tot	1.35	% s.s.
CIM	190	% s.s.
Bulk density	0.38	g cm^{-3}
Density	1.55	g cm^{-3}
Total Porosity	75.48	%
Dynamic Respiration Index	615	mg_{O2} kg_{SS}^{-1} h^{-1}

The biofilter was was put in operation in 2015. The test site and the measurements of CH_4 oxidation efficiency of the biofilter are shown in Figure 2 and Table 2, respectively.

Methanotrophs growth

Three replicates of 10 g-subsamples (dry weight equivalent) of the biofilter packing material (sampled from the top of the packing material) were suspended in 90 mL sterile 0.18% sodium pyrophosphate-one quarter strength Ringer solution (NaCl 2.25 g L^{-1}, KCl 0.105 g L^{-1}, $CaCl_2$ 0.045 g L^{-1}, $NaHCO_3$ 0.05 g L^{-1}, and citric acid 0.034 g L^{-1}), sonicated for 2 min to disperse particles, and left at 4°C for 15 min to disperse microbial cells. Ten-fold serial dilutions of the supernatants were made in sterile one-quarter strength Ringer solution. Aliquots (100 μL) of the 10^{-1}, 10^{-3}, and 10^{-5} dilutions were spread plated in duplicate in Petri dishes on a medium (M1) for the growth of methanotrophs having the following characteristics [30]: K_2HPO_4 0.5 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 0.2 g L^{-1}, $CaCl_2$ 0.015 g L^{-1}, 0.001 g L^{-1} $FeSO_4 \cdot 2H_2O$, 0.001 g L^{-1} $Na_2MoO_4 \cdot 2H_2O$, bacteriological agar 12.5 g L^{-1}, pH 6.8. A second medium (M2) was prepared using the same concentrations as described above and adding 1.43 mL of NH_3 solution (70% w w^{-1}), for a final concentration of 20 mM NH_3. The final pH of medium M2 was 9.8 and it was corrected to 6.8 by adding drops of ultrapure HNO_3 for a final solution volume of 1 L. All the chemicals used were purchased from Sigma-Aldrich (Trace-SELECT®; St. Louis, MO, USA). A total of 144 Petri dishes were prepared: 72 containing M1 and 72 containing M2. The dishes were placed in special polyethylene sterile bags with a hermetic seal (Sigma Aldrich AtmosBag two-hand, size S, Z118370). Each bag (4 in total) contained 36 dishes: 18 filled with medium M1 (three sample replicates with three dilutions in duplicate) and 18 filled with medium M2 (three sample replicates with three dilutions in duplicate). Two bags were incubated at 20°C and the remaining two at 35°C. For every incubation temperature, the two bags were filled with different proportions of air and biogas, as described in the following paragraph. The biogas used was taken from the biogas recovery system installed in the landfill described above. Bacterial counting took place throughout a period of 100 days from plating (5 May 2016).

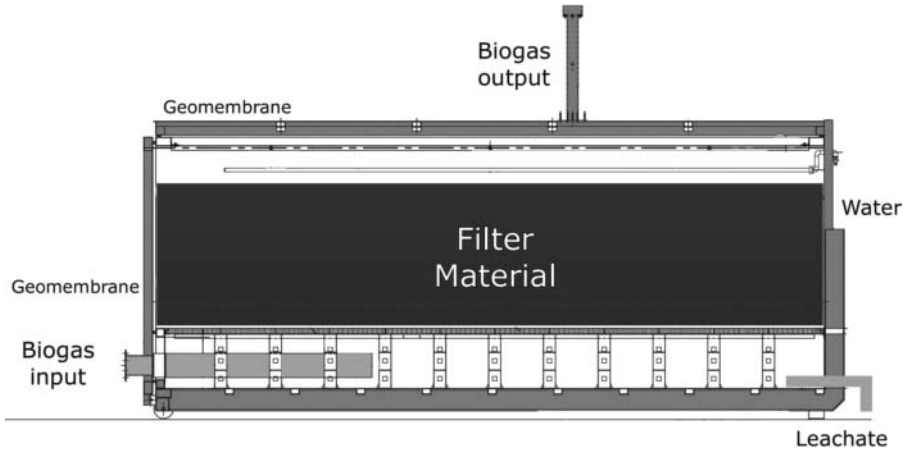

Figure 1. Cross-sectional details of the biofilter.

Biogas analysis

Before filling the bags with air/biogas, biogas composition was analyzed in several dates. CH_4 and CO_2 gas concentrations were determined in a Shimadzu 2010 gas chromatograph-barrier ionization discharge detector (GC-BID) (Shimadzu, Kyoto, Japan) equipped with a Restek ShinCarbon ST micropacked column (2 m × 1 mm i.d.) (Restek Corporation, Bellefonte, USA). Such GC-BID system was previously described by Pascale et al.[31] Briefly, the oven temperature was set to 30°C for 5 min and was increased to 120°C at a rate of 10°C/min. The injector and detector temperatures were maintained at 150°C and 250°C, respectively. Helium with a 6.0 purity (SIAD Corporation, Bergamo, Italy) was used as the carrier gas at 15 mL/min during chromatographic separation and as the BID discharge gas at 80 mL/min. All injections were made in the direct mode by using a gastight syringe (injection volume 250 ul). For GC-BID quantitative analyses, three calibration gas standards of CO_2 and CH_4 were prepared over the range of desired concentrations (50–1000 ppm_v) by diluting the pure CH_4 (99.995%) and CO_2 (99.995%) (Sigma Aldrich, Milano, Italy) with ambient air. 10 mL, 1 mL, 250 μL and 100 μL gas-tight syringes (Sigma Aldrich

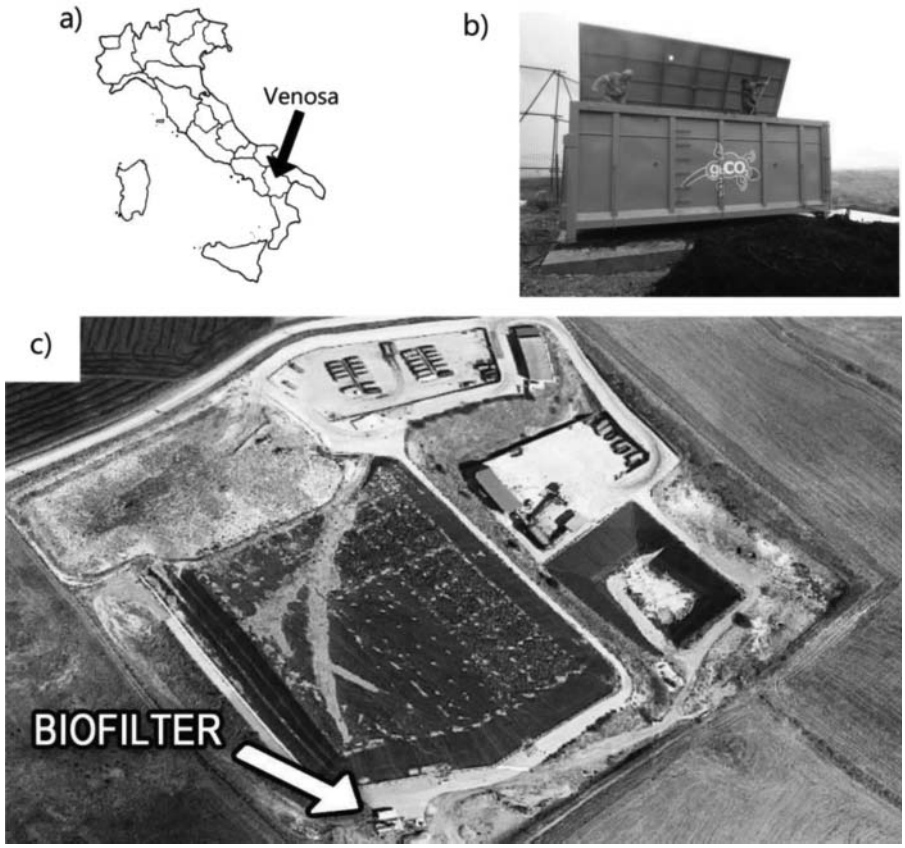

Figure 2. The test site. (a) Location of Venosa; (b) Picture of Biofilter; (c) Satellite Image of the Venosa landfill.

Table 2. Methane oxidation efficiency considering the percentage of methane in the biogas flow in input $CH_{4\ IN}$ and output of the Venosa biofilter.

Date	Biogas Flow ($Nm^3\ h^{-1}$)	$CH_{4\ IN}$ (% vol.)	$CH_{4\ OUT}$ (% vol.)	CH4 oxidation efficiency (%)
08/02/2016	24.0	1.15	0.65	43.48
13/05/2016	12.6	3.4	1.9	44.12
27/05/2016	10.2	8.3	4.04	51.32

Milano, Italy) were used for preparing calibration standards at different concentrations (50, 500, 1000 ppm_v) in 1 and 10 L Tedlar sampling bags equipped with PTFE fittings (Zefon International, Ocala, USA) via a Gilian GilAirSampling Pump (Sensidyne, St. Petersburg, USA). The results revealed the following average composition: 54% [v v^{-1}] CH_4, 31% CO_2, 13% N_2, 1.6% O_2. Two bags were filled with 30 L of atmospheric air: biogas in the proportion of 1:1 (v v^{-1}) by means of an electronic pump (Gilian GilAir Sampling Pump – Sensidyne, St. Petersburg, USA) with a volumetric counter, resulting in an approximated final composition of 27% (v v^{-1}) CH_4, 15% CO_2, 46% N_2, 11% O_2 (CH_4:O_2 ratio = 1:0.4). The other two bags were filled with 30 L of atmospheric air: biogas in the proportion of 72:28 (v v^{-1}), resulting in an approximated final composition of 15% (v v^{-1}) CH_4, 9% CO_2, 60% N_2, 15% O_2 (CH_4:O_2 ratio = 1:1).

Soil physicochemical parameters and Biolog® analysis

The measurements of pH, electric conductivity, total organic matter, total nitrogen (N), potassium (K) and phosphorus (Olsen) (P) of the biofilter packing material were determined in triplicate according to the official methods of the Italian Society of Soil Science (SISS, 2000).

Carbon source utilization pattern of the bacterial communities, also called community-level physiological profiles (CLPPs), of landfill soil was assessed in triplicate using the Biolog® 96-well Eco-MicroDishes™ (AES Laboratoire, France), containing 31 different carbon sources, following the manufacturer's instructions. For each well of the Biolog® MicroDishes™, an aliquot of 100 μL of the same landfill soil at 10^{-3} dilution used for the bacterial count was used for the Biolog® assay. The microdishes were incubated at 25°C in the dark. Color development, due to the utilization of carbon sources by bacteria, was measured every 24 h over a 144 h-period using a Microplate E-Max Reader (Bio-Rad; Hercules, CA USA) with an E590-nm wavelength filter.

Data were analyzed to determine metabolic diversity indices, according to Zak et al.[32] and Xu et al.[33] Average well color development (*AWCD*), that provides a measure of the total cultural bacterial activity, was calculated as follows:

$$AWCD = \sum \frac{(c_i - R)}{W}$$

where c_i is the OD_{590nm} in each well; R is the OD_{590nm} in the control well; and W is the number of all the wells. The carbon substrates of the Biolog® dishes were divided into eight main groups of compounds, respectively, and the *AWCD* value for each group was calculated.

Statistical analysis

Statistical analysis of data was carried out using STATISTICA v6.0 (StatSoft Inc.; Vigonza, PD, Italy). The values of bacterial microbial counts and Biolog® *AWCD* (three independent replicates for each treatment; $n = 9$) were examined by using a variance analysis (ANOVA). Means were separated according to Duncan's multiple comparison test at $P \leq 0.05$.

Results and discussion

Methanotrophic microorganisms

The biological analysis of the biofilter packing material was aimed to test different combinations of N content, CH_4 levels (and consequent CH_4:O_2 ratios) and temperature for the growth of methanotrophs in the examined substrate. The choice of the highly selective cultural medium [30] was successful, as it allowed the isolation of methanotrophs due to the lack of carbon sources other than CH_4 and CO_2 in the air mixture which was blown in. The presence of O_2 was necessary because methanotrophs are aerobic bacteria. Whereas, for the required N, methanotrophs can use both inorganic N sources or can fix atmospheric N_2. The choice of adding NH_3 (and HNO_3 for the pH correction) was done in order to avoid the addition of other nutrients in the culture medium, which could have influenced the microbial growth in an unpredictable way.

The microbial counts obtained from our experiments are reported in Table 3.

According to what was reported in literature,[11,34,35] methanotrophic microorganisms are mainly characterized by a slow growth. Numerous manuals of microbiology indicate that it is necessary to wait for at least 2–3 weeks for the appearance of the first colonies of significant size. Indeed, after five days of incubation, no colonies were identified in the dishes, while, after 10 days of incubation, the first colonies appeared at the lowest dilution (10^{-1}) only in the dishes without N.

After 13 days of incubation at 20°C, colonies appeared also at the highest dilution (10^{-3} and 10^{-5}). Interestingly, the microbial growth was higher in the dishes without N and in the bags with 1:0.4 CH_4:O_2 ratio. Therefore, it seems that the addition of N strongly inhibited microbial growth. In the bags with 1:1 CH_4:O_2 ratio, the inhibition due to N also occurred, but colonies were significantly lower than in the bags with a high CH_4 level (3000 vs 3500 UFC). This demonstrates the positive correlation between CH_4 concentration and microbial growth. The same microbial counts were carried out after 13 days in the dishes incubated at 35°C, where the growth of methanotrophs was lower, compared to the 20°C incubation. This is in agreement with researchers that report methanotrophs as mesophilic bacteria, with an optimum growth rate between 20 and 25°C.[13,36,37]

Table 3. Microbial counts of methanotrophic bacteria. Values represent means (n = 3) ± standard deviation. Values with different letters in the same column are statistically different at P ≤ 0.05, according to Duncan's multiple comparison test.

			Days of incubation Microbial counts (CFU/g soil FW)				
Medium	Temp. (°C)	CH_4:O_2 ratio	5	10	13	21	30
M1 (- N)	20	1.1	0 ± 0 a	120 ± 25 b	3000 ± 80 b	2100 ± 160 b	2300 ± 350 b
		1:0.4	0 ± 0 a	250 ± 25 a	3500 ± 95 a	2700 ± 250 a	3000 ± 220 a
	35	1.1	0 ± 0 a	10 ± 5 d	1000 ± 120 d	800 ± 180 d	1000 ± 150 c
		1:0.4	0 ± 0 a	30 ± 10 c	1600 ± 235 c	1200 ± 350 c	1200 ± 190 c
M2 (+ N)	20	1.1	0 ± 0 a	0 ± 0 e	140 ± 20 f	150 ± 25 f	300 ± 50 e
		1:0.4	0 ± 0 a	0 ± 0 e	300 ± 30 e	400 ± 30 e	600 ± 50 d
	35	1.1	0 ± 0 a	0 ± 0 e	0 ± 0 g	0 ± 0 g	0 ± 0 f
		1:0.4	0 ± 0 a	0 ± 0 e	0 ± 0 g	0 ± 0 g	0 ± 0 f

After 21 days of incubation, microbial growth slowed down (2700 UFC compared to 3500 UFC taken from the previous reading at 20°C incubation in the bags with high CH_4 levels). This was likely due to a growth block due to the consumption of the CH_4 in the bags, considering that CH_4 was the only carbon source available in the medium. Indeed, in the bags with low CH_4, the growth was completely inhibited, demonstrating the sensitivity of the microorganisms to CH_4 levels. In order to avoid this problem, bags were deflated with the electronic pump and the air replaced with a new insufflation at the same CH_4:O_2 ratio previously used.

Finally, after one month of incubation (8 June 2016), all four bags were opened and the final microbial counts were carried out. Again, the microbial growth was significantly higher in the dishes without N addition and in those incubated in the atmosphere containing more CH_4. As reported in Figure 3, the comparison of the two incubation temperatures shows that the number and the diameter of the colonies incubated at 20°C was significantly higher than that of the colonies at 35°C. The inhibitory effect of N addition was significant also in the dishes incubated at 35°C where microbial growth was lower. This could explain the controversy about the growth of N and methanotrophs observed by some authors,[11,34,36] which also seems to be temperature-dependent.

The results demonstrated that, at the concentration used in this study (20 mM NH_3), N is still not enough to completely inhibit microbial growth. This suggests that an eventual addition of nutrients to the biofilter should be carried out adding a fertilizer containing P, K and N, but maintaining N under a certain threshold in order not to inhibit bacterial growth.

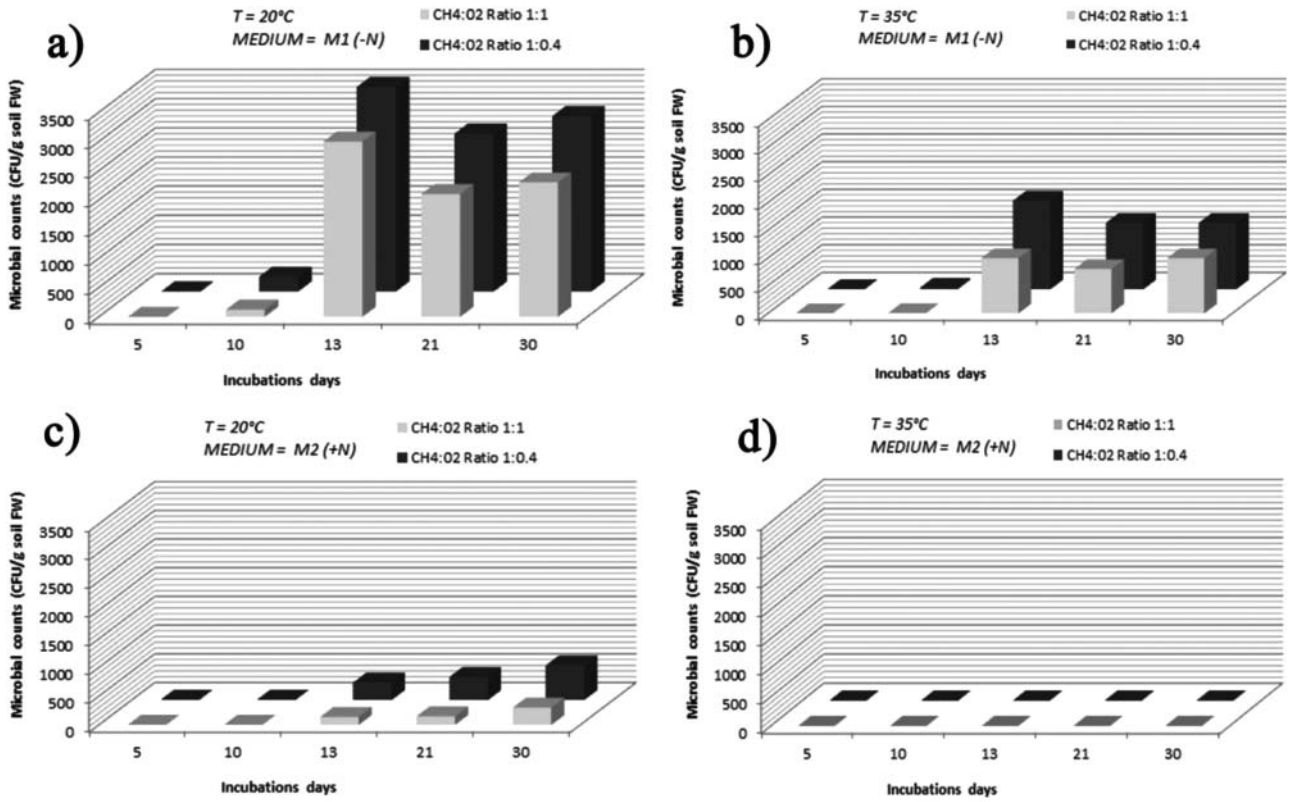

Figure 3. Microbial counts of methanotrophic bacteria at different ratio CH_4:O_2. (a) T° = 20°C Medium = M1 (-N); (b) T = 35°C Medium = M1 (-N); (c) T° = 20°C Medium = M2 (+N); (d) T° = 35°C Medium = M2 (+N).

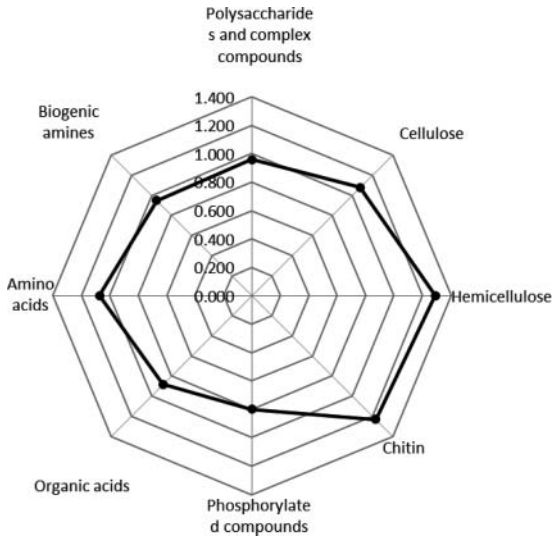

Figure 4. Radar diagrams of bacterial *AWCD* of all the principal classes of carbon substrates, identified by Biolog® 96-well Eco-Microdishes™, from landfill soil. Values represent means ($n = 9$).

Soil physicochemical parameters and Biolog® analysis

The average value of pH ($n = 6$) of the biofilter landfill material resulted to be 7.10 ± 0.13 (SD), a value comparable to that considered optimal for the growth of methanotrophs (6.8–7.0). Soil salinity of the same samples ($n = 6$) was 5.24 ± 0.46 (SD) mS cm^{-1}, which means a non-saline soil and, therefore, suitable for microbial growth. Regarding the analysis of the main nutrients, the soil ($n = 6$) had values of total N = 2.6 g kg^{-1}, K = 165 g kg^{-1}, and P-Olsen = 14 g kg^{-1} that can be considered as high, medium and medium-high, respectively. In particular, total N content is of key importance as it can affect the growth of methanotrophs.

Biolog® assay has been used to assess carbohydrate use and to determine metabolic profiling of microorganisms extracted from different matrixes.[38] The utilization of these specific dishes allows us to determine the microbial catabolic profiles and metabolic diversity indices referring to the number, variety and variability of microorganisms, including diversity within and between groups.[31,32] Among these parameters, total average well color development (*AWCD*) is particularly important, as it is a measure of the overall metabolic activity of microbial communities. Unfortunately, total *AWCD* does not necessarily reflect the composition of the bacterial communities as microbial communities can have similar *AWCD* values but utilize different substrates. In our case, Biolog® absorbance values demonstrated that the *AWCD* values of all the principal classes of bacterial carbon substrates were ≥0.8 (≥1.0 for six of eight classes) (Fig. 4). This can be considered a much equilibrated situation, where all the microbial groups present in the analyzed material exert their catabolic activity on different carbon sources.[39]

Conclusions

The aim of the study was to examine the growth of methanotrophic bacteria, that have a basic role in MAMO systems. Considering the field-scale biofilter located in Venosa (Basilicata Region, Italy), operative since 2015, samples of biofilter packing material were analysed in laboratory in order to evaluate the environmental factors that most influence the growth of methanotrophic bacteria.

Methanotrophs were plated in selective medium, with and without N inputs, and incubated at different temperatures in sterile bags filled with air-biogas mixtures. An overall chemical characterization of the packing material and the measurement of metabolic microbial activities (community-level physiological profiles – CLPPs) were carried out by means of chemical methods and Biolog® assay [39], respectively. The results clearly showed that the best temperature range for the growth of methanotrophic bacteria was 20°C and that added N inhibited their growth.

All these factors have to be considered for field-scale biofilters, in order to improve knowledge on some operational issues of MAMO systems and to further examine the development and improvement of currently available industrial installations. The applicative purpose of this study was to provide an original contribution to the LFG treatment challenge.

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