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TÍTULO:	EXPERIMENTAL IMPROVEMENT CAPABILITIES IN METHANE DEGRADATION BIOTECHNOLOGIES
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Dña. RAQUEL LEBRERO FERNÁNDEZ y D. RAÚL MUÑOZ TORRE, profesores del Departamento de Ingeniería Química y Tecnología del Medio Ambiente de la Universidad de Valladolid, CERTIFICAN:

Que D. IIRO LEHTINEN ha realizado bajo nuestra dirección el trabajo titulado "Experimental Improvement Capabilities in Methane Degradation Biotechnologies", con una dedicación de 33 ECTS (460 h Practical Training + 15 ECTS Bachelors Thesis).

Valladolid, 26 de Julio de 2013.

Fdo. Raquel Lebrero Fernández

Fdo. Raúl Muñoz Torre

Reunido el Tribunal designado en Junta de Sección para la evaluación de Trabajos de Investigación, y después de atender a la defensa del trabajo "Experimental Improvement Capabilities in Methane Degradation Biotechnologies", presentada por el alumno D. IIRO LEHTINEN, con una dedicación de 865 horas, y realizada bajo la dirección del profesor Dña. Raquel Lebrero Fernández y D. Raúl Muñoz Torre, del Dpto. de Ingeniería Química y Tecnología del Medio Ambiente, decidió otorgarle la calificación de _____.

Valladolid, 26 de Julio de 2013

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ABSTRACT

Within the last decades, biological treatment methods have emerged as a viable solution to mitigate methane´s adverse effects on climate change, treating low CH_4 concentration emissions far more cost-efficiently than conventional physical-chemical methods. However, the high stability and low aqueous solubility of methane still pose key constraints for a cost-effective biological removal. For this purpose, alternative approaches to increase the mass transfer of methane to the microorganisms were investigated in a biofilter and a biotrickling filter configuration.

A novel fungal biofilter was operated at an empty bed residence time (EBRT) of 4 minutes at a CH_4 inlet concentration of 15 ± 0.9 gCH_4/m^3 for 58 days in order to achieve an enhanced methane elimination supported by the low-moisture content biofilter conditions and the aerial growth of fungi. Moreover, batch biodegradation tests using CH_4 as the sole carbon source and a pure strain of the fungus *Graphium* sp. were performed. Additionally, a two phase partitioning biotrickling filter using silicone oil as a non-aqueous phase and *Methylosinus sporium* as CH_4 degrader was operated at 4 minutes of EBRTs and 14.3 ± 0.5 gCH_4/m^3 for 45 days in an attempt to increase CH_4 mass transfer.

An unstable and poor CH_4 removal performance was observed in the fungal biofilter throughout the complete operating period with average removal efficiencies of 0.5 ± 2.4 %. This poor CH_4 abatement performance was attributed to the low EBRT used in the bioreactor. *Graphium* sp. was not able to use methane as a sole carbon and energy source, CH_4 being only degraded co-metabolically with methanol. On the other hand, the two-phase biotrickling filter performed consistently, reaching a maximum steady elimination capacity of 49.5 ± 6.0 $g/m^3/h$.

The results achieved in this work can assist in the development of full-scale applications of innovative biotechnologies supporting an enhanced CH_4 mass transfer, with two-phase-biotrickling filtration as the most promising technology.

1 Introduction

1.1 Environmental Impact of Greenhouse Gases: Methane

The ongoing trend of increase in global warming is considerably due to anthropogenic emissions. From preindustrial levels at the end of 18th century, the atmospheric concentration of greenhouse gases (GHG) has risen at an unnatural rate, CO_2 concentration increasing from 280 ppm to 380 ppm, CH_4 from 800 ppb to 1,8 ppm, and N_2O from 270 ppb to 320 ppb (Figure 1) (IPCC 2007). The IPCC 2007 report estimated that the average land temperature of the earth will increase 0.6 - 4 ºC by the end of the 21st century depending on the model scenario and the reduction in anthropogenic GHG emissions achieved in this century. This increase in temperature might dramatically affect both natural ecosystems and anthropogenic environments (Dodman D. 2009).

CH_4 is ranked as the second most pernicious GHG in terms of global warming potential, after CO_2, with a global warming potential 21 times that of CO_2 and an atmospheric lifetime of approximately 9 years (Wuebbles et al. 2002, Bocka et al. 2012). As mentioned before, the atmospheric concentration of CH_4 has increased by a factor of 2.5 over the past millennium, and nowadays accounts for approximately 16% of the total GHG emissions (Karakurt et al. 2012). During 1990´s the emission rate slightly decreased and stabilized, probably due to the increased efforts to control CH_4 emissions, but in the last decade the emission rate showed a rapid increase from 3 to 8 ppb/year (Wuebbles et al. 2002, Dlugokencky et al. 2003, Dlugokencky et al. 2009). Therefore, there is a need for the development of cost-efficient and environmentally friendly CH_4 abatement methods in the upcoming century.

CH_4 is currently being emitted by anthropogenic (60%) and natural (40%) sources, accounting for estimated total global emissions of 540 $TgCH_4$/year (Montzka et al. 2011). Agricultural activities (53%), energy production (28%) and waste handling (19%) are the main sectors contributing to the total anthropogenic CH_4 emissions with domestic ruminants, fossil fuel production, waste decomposition and rice cultivation as the main CH_4 source (Wuebbles et al. 2002, Yusuf et al. 2012). The major natural contribution originates from wetlands (72%), with the rest being mainly emitted through termites and oceans (Wuebbles et al. 2002).

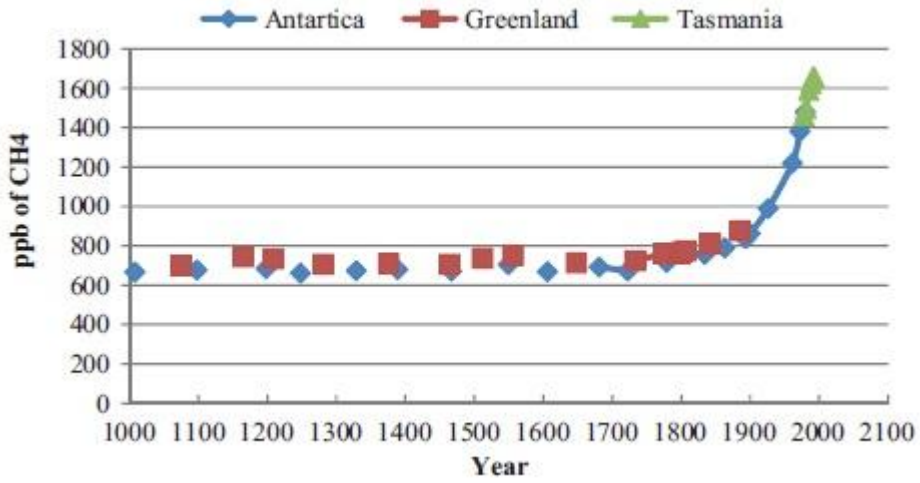

Figure 1 Time course of the atmospheric CH_4 concentration (Yusuf et al. 2012)

Most of the above mentioned anthropogenic emissions are point sources, which facilitates the implementation of end-of-the-pipe treatment methods to control these emissions (Annual inventory of GHG source study of the European Environmental Agency 2012). Moreover, these emissions are increasingly characterized by their low CH_4 concentrations, typically at concentrations ranging 0-3.5 $gCH_4/m^2/h$ (old landfills), 0-20 $mgCH_4/m^3$ (compost piles) or 0.7-7 g/m^3 (coal mines) (Scheutz et al. 2009, Ahn et al. 2011, Limbri et al. 2013). By the year 2016, it is expected that the enforcement of the European Commission's waste legislation (1999/31 directive) will reduce the permitted

amount of biodegradable waste to landfills, which will result in even lower, but still relevant from an environmental viewpoint, concentrations of CH_4 in the gas emissions (Gebert et al. 2009, Internet 1).

1. 2 Greenhouse Gas Treatment: Biological Technologies

End-of-pipe CH_4 treatment technologies rely on physical, chemical or biological principles (Estrada et al. 2012). Physical and chemical methods, including activated carbon adsorption, thermal combustion and flaring, or chemical oxidation have been traditionally preferred over biological technologies due to their high efficiency and robustness (Melse et al. 2005, Estrada et al. 2011, Limbri et al. 2013). In the particular case of CH_4, when the emissions have high flow rates and high CH_4 concentrations, such as those from young landfills (50 m^3/h and 30-100 $gCH_4/m^3_{\text{air mixture}}$ respectively), the emission can be used for energy recovery by combustion (Melse et al. 2005, Rocha-Rios et al. 2009). Nevertheless, in addition to the high cost associated to these physical-chemical technologies, they often use hazardous reagents. For instance, activated carbon filters need periodical adsorbent regeneration, and if incomplete, incineration might result in toxic by-product formation (NO_X, SO_X and PCB´s) depending on the fuel composition and efficiency of combustion (Limbri et al. 2013).

Biological methods for the abatement of volatile organic compounds (VOCs) have shown their ability to overcome physical/chemical methods in terms of process economics, environmental impact and robustness (Estrada et al 2012). Methods such as biofiltration (BF), biotrickling filtration (BTF) and bioscrubbing utilize the natural oxidizing capability of the microorganisms to degrade the gaseous pollutants into CO_2, H_2O and

biomass (Figure 2). A successful performance of the bioreactor requires the optimization of several operating parameters. Thus, parameters such as pH, humidity/moisture, mass flow rate, empty bed residence time (EBRT), packing material and inoculation play a critical role in determining the performance of the biodegradation process (Mudliar et al. 2010). So far biological treatment methods have been employed in the treatment of a wide range of VOCs in both industrial and malodorous emissions. However, biological processes still lack from a significant research and understanding, which has resulted in unexpected problems during their operation at full-scale

Microorganisms + Carbon Source (Pollutant) + Nutrients → CO_2 + H_2O + Biomass

Figure 2 General component balance for microbial oxidation.

1.2.1 Biofiltration

Biofiltration is by far the most commonly implemented biotechnology in full scale applications, due to its relatively low cost, simple implementation and operation, and broad VOC applicability (Estrada et al. 2011, Kraakman et al. 2011). Biofiltration uses a simple configuration to efficiently pass the pre-humidified polluted air stream through the biofilm, where gas pollutant oxidation occurs (Figure 3a). The biofilm of the reactor consists of a consortium of microorganisms attached onto the packing material that are able to biodegrade the target pollutants. The platform for the growth of the microorganisms is provided by the packing bed material, which can vary according to the required properties: organic (compost, soil, woodchips, etc.) and inorganic (polyurethane foam, rocks, Kaldness rings, etc.) packing materials. Essential water is provided by intermittent irrigation depending on the prevailing conditions and should result in a

moisture content of 30-60% on a weight basis (Mudliar et al. 2010). In this context, important properties of biofilters packing material also include a high specific surface area, good water retention capacity, high porosity, low compaction, structural stability, a diverse indigenous microbial community and good nutrient content (Nikiema et al. 2010, Limbri et al. 2013). For the treatment of large volumes of air with a low concentration of pollutant, biofilters have been reported as the most suitable biotechnology (Mudliar et al. 2010). Furthermore, biofilters using compost as packing material offer a high nutrient content combined with an indigenous population of degrading microorganisms and low operating costs (Mudliar et al. 2010, Nikiema et al. 2010).

However, one of the biofilters major drawback is the difficulty to control pH and moisture inside the filter bed. Besides, organic filter bed exhaustion due to a rapid degradation of the material and nutrients depletion is also observed after short operating periods in biofilters, thus requiring a frequent packing material replacement which entails higher costs. In addition, problems related to the use of compost include a high compaction rate and uneven channeling of the gas flow, which have promoted an intensive research on inorganic packing materials due to their improved physical properties (porosity, surface area, etc.) and lesser degradation. These inorganic packing materials are often applied in BTFs (Ortiz et al. 2003, Nikiema et al. 2010).

1.2.2 Biotrickling Filter

Among the available biotechnologies, BTFs have emerged as the preferred technology in terms of cost-efficiency (Estrada et al. 2012). Their operation is similar to that of biofilters but modified for higher controllability (Mudliar et al. 2010).The pollutant stream is also contacted with the biofilm, where the microorganisms catalyze the degradation of the gas

pollutants (Figure 3b). The biofilm grows on an inorganic filter bed that is under constant nutrient solution irrigation via liquid recycling. This continuous recirculation of the liquid allows for a better control of the BTF humidity and pH levels, together with the removal of possible toxic metabolites. Furthermore, the inorganic packing materials used in BTFs are degradation resistant and can be selected to meet specific requirements such as void space or surface area. BTFs generally operate at lower EBRT compared to BFs as the continuous liquid flow creates additional turbulence to the gas flow, thus increasing the contact time. EBRTs from several minutes to hours in BFs and higher than 1 minute in BTFs have been reported for hydrophobic pollutants (Nikiema et al. 2007, Kennes et al. 2009).

On the other hand, due to the inorganic nature of the packing material, the inoculation of the packed bed is necessary and entails a lengthened startup period. Additionally, an external nutrient supply has to be constantly maintained. In the case of toxic metabolites formation, the leachate will require pretreatment before disposal. Excess biomass accumulation has been reported to result in significant performance decrease in the long term operation (Mudliar et al. 2010). Moreover, the presence of a continuous water layer over the biofilm reduces the removal performance for hydrophobic pollutants due to mass transfer limitations from the gas to the aqueous phase.

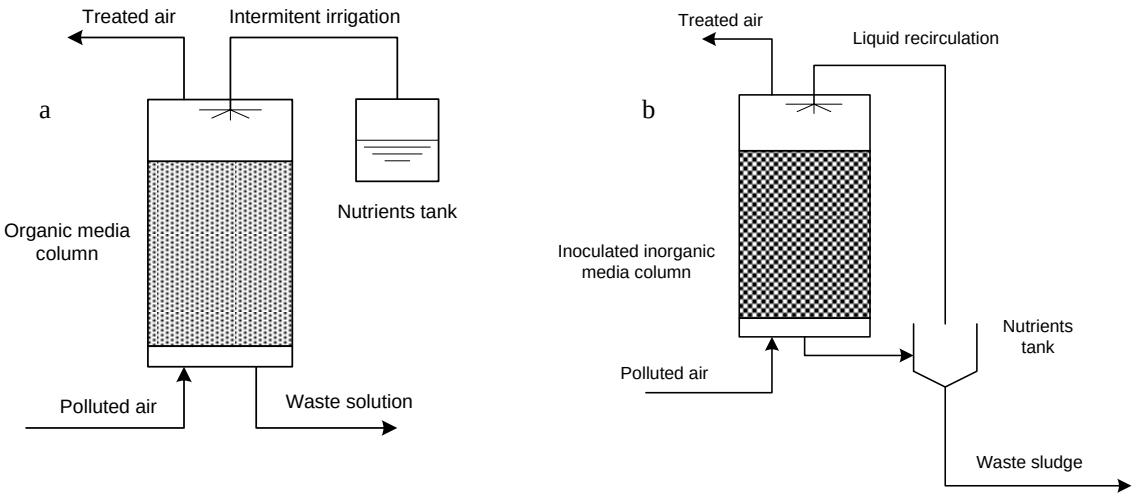

Figure 3 Schematic of (a) a biofilter unit and (b) a biotrickling filter (Lebrero et al. 2011).

1.3 Overcoming Mass Transfer Limitations

Although BFs and BTFs are the most studied biological treatment technologies, many operating problems such as mass transfer limitations, high pressure drops (due to clogging of the packed bed and compaction) and the need for long EBRT still need to be addressed (Kraakman et al. 2011, Limbri et al. 2013, Mudliar et al. 2013). Hydrophobic alkane compounds such as CH_4 or hexane are poorly removed in BFs and BTFs due to their low mass transfer rates from the gas phase to the aqueous phase surrounding the biofilm (Figure 4a) (Kraakman et al. 2011). In this regard, the poor CH_4 solubility in water (dimensionless Henry´s constant H = 30) has been so far compensated by increasing the EBRT of the bioreactors.

For a better understanding of the complexity of pollutant mass transfer, mathematical models have been developed to describe the different aspects of mass transfer. The simplified volumetric mass transfer rate R ($g/m^3/s$) (Eq. 1) can be expressed as the

concentration gradient created between the gas (C_G) and the liquid (C_L) phases multiplied by the overall mass transfer coefficient K_La (Koch 1990). K_La is an empirical constant, which itself is a function of the physical-chemical properties of the pollutant, the reactor characteristics and the operating parameters, with *a* representing the specific surface area of the packing material (Kraakman et al. 2011).

$$R = K_L a \times \left(\frac{C_G - C_L}{H}\right) \quad (1)$$

Recently, it has been suggested that the use of fungi in biofilters might improve the mass transfer of the hydrophobic compounds due to their large interfacial area and hydrophobic fungal cells, thus increasing the biofilter removal efficiency and reducing their large footprint. Alternatively, increases in the mass transfer rates have been achieved by the addition of a hydrophobic non-aqueous phase (NAP) able to increase pollutant mass transfer towards the microorganisms due to its high affinity for the target compound (Figure 4b) (Kraakman et al. 2011).

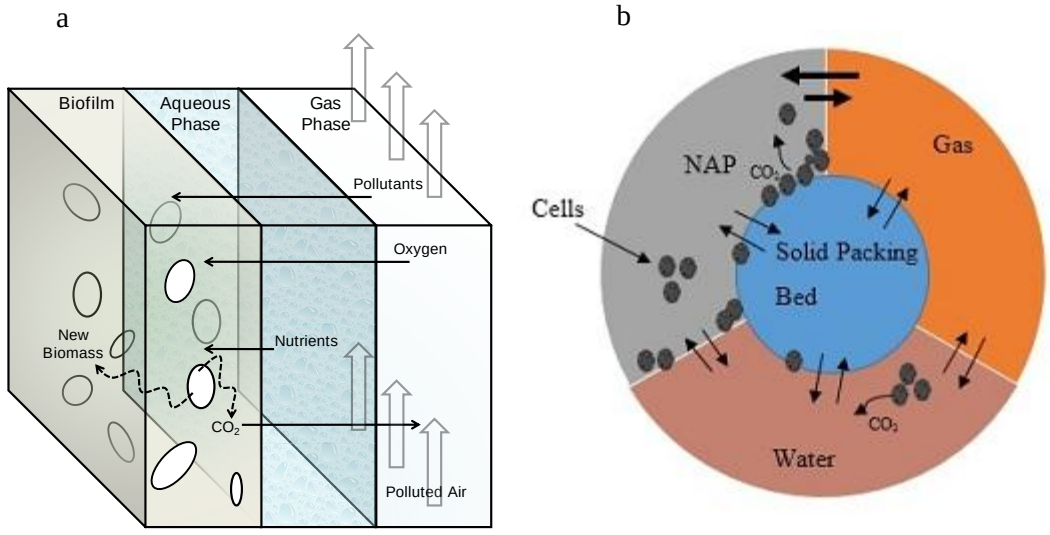

Figure 4 Mass transfer pathways of (a) 1-phase and (b) two-phase bioreactor ((a) Estrada et al. 2013 and (b) adapted from Quijano et al. 2009)

1.3.1 Fungal Biofilters

Studies on the performance of fungi in the field of bioremediation are still quite recent, although during the past few decades' laboratory scale studies have already shown efficient degradation of VOC emissions (Kennes et al. 2004). In 1969, Zajic et al. isolated a strain of fungi that was able to grow with natural gas (90.5% CH_4) as a substrate, which was identified as *Graphium* sp. This strain and four other yeast-like-fungi identified by Wolf and Hanson in 1979 growing under 70% v/v CH_4 constitute nowadays the only identified fungi able to thrive in CH_4 containing conditions. However, studies have continued with a variety of fungal strains and have shown that fungi can generally perform well under harsh environmental condition compared to bacterial bioreactors devoted to VOC abatement (Kennes et al. 2004, Aizpuru et al. 2005).

In low water content scenarios, conditions commonly found in full scale biofilters, a rapid growth of aerial mycelium is promoted, which has been hypothesized to increase the uptake and degradation of pollutants due to a direct gas phase – microorganism contact. The absence of a water layer covering the microbial biofilm results in a mass transfer enhancement for the hydrophobic pollutants (Kennes et al. 2004, Groenestijn et al. 2005). In addition, in the hydrophilic-hydrophobic interface, fungi produce amphipathic proteins named hydrophobins. These proteins possess both hydrophobic and hydrophilic properties, and increase the hydrophobicity of the fungi. For example, as the fungal mycelium grows in aerial form, the hydrophobins form an encapsulating layer around the hyphae that lowers the surface tension of the interface (Bayry et al. 2012). In addition, fungi can also tolerate and perform well at lower pH values (pH 4-7) compared to many bacteria, although some bacterial strains have been also reported to thrive under similar conditions (Rousk et al. 2009, Lebrero et al. 2011). Structurally, fungi pose the advantage

of having a high surface area to mass ratio with their hyphae and mycelium, which increases the possible contact area. In spite of these advantages, major challenges with fungal biofilters include the higher pressure drop caused by the rapid mycelium growth, which in turn requires the increase of energy for compression and increased operating costs, as well as the longer startup period (Estrada et al. 2010, Estrada et al. 2013).

1.3.2 Two-Phase Partitioning Bioreactors

In the traditional biological treatment methods described earlier, the existing aqueous phase surrounding the biofilm often limits the mass transfer of the pollutants towards the biofilm. The addition of a non-aqueous phase (NAP) to the liquid aqueous phase of these bioreactors, creating a two-phase liquid, has been reported to enhance pollutant mass transfer to the biofilm, which in turn will result in increased removal efficiencies and robustness. The improvement in removal efficiency is mainly caused by the higher affinity towards the target pollutant. For instance, the solubility of CH_4 in silicone oil is ~10 times higher than in water (reported partition coefficient C_G/C_L of 1.9 in silicon oil where C_G and C_L represent the CH_4 concentration is the gas and liquid phase, respectively) (Rocha-Rios et al. 2011). Besides, two-phase bioreactors (TPBs) have shown an improved robustness, especially in full scale applications where the pollutant concentration can fluctuate from low to toxic or inhibiting concentrations rapidly, since the NAPs can absorb the high load surges and act as a pollutant reservoir preventing the inhibition of microorganisms (Daugulis A. 2001). The most common NAPs investigated are large branched alkanes, plastic polymers and silicon oils, with silicon oil considered to be the most suitable. NAPs suitability in TPBs is evaluated based on certain characteristics such as immiscibility in water, high affinity for the target compound, non-

biodegradable, non-hazardous, low viscosity etc. (Quijano et al. 2009). On the downside, many NAPs like silicon oil have set backs such as high cost and high energy demand combined with some controversial performance results and unclear mass transfer improvements (Quijano et al. 2009). Thus more research is required to properly determine if two-phase partitioning bioreactors (TPPBs) are superior to conventional single-phase reactors.

1.3.3 Hydrophobic *Methylosinus Sporium*

The bacteria *Methylosinus Sporium* belong to the CH_4 oxidizing type II Methanotroph group (Bowman. J 2006). These bacteria utilize CH_4 in the presence of oxygen as their sole energy source to enzymatically assimilate it into biomass (serine pathway), resulting in CH_4 degradation to CO_2 and H_2O (Figure 5). The rates at which methanotrophs are able to oxidize CH_4 critically depend on the dissolved oxygen content, pollutant concentration, humidity, pH, nitrogen source and copper levels (Lopez et al. 2013). Depending on the available copper content, type II methanotrophs utilize either the particulate methane monooxygenase (pMMO) or soluble methane monooxygenase (sMMO) on the first step for oxidizing CH_4 to methanol (Glass et al. 2012). Furthermore, the hydrophobicity of microorganisms has been recently associated to an improved performance of TPPBs in a study that determined the relevance of confining microbial activity in the NAP (Hernandez et al. 2012). Thus, only by ensuring the complete hydrophobicity of the microorganisms and the maintenance of certain parameters, such as sufficient Cu^{2+} supply, maximal CH_4 removal efficiencies are expected.

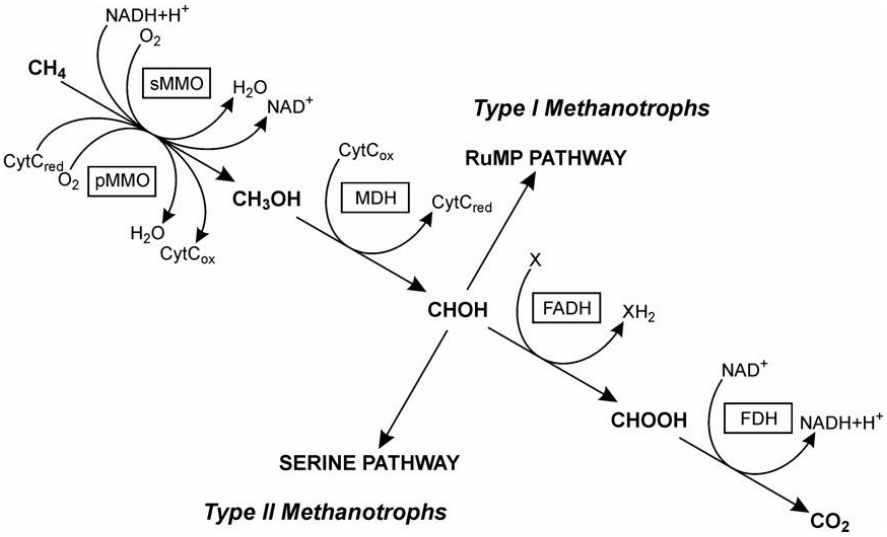

Figure 5. Enzymatic pathway of Methanotrophic bacteria (Scheuzt et al. 2009).

The present study focused on testing two different approaches to increase CH_4 mass transfer and the overall elimination capacity of biological treatment technologies. The first approach was a fungal BF treating low concentration of CH_4 which, to the best of our knowledge, has never been studied before. In addition, a BTF with 25% NAP was operated with CH_4 as the sole pollutant. In both the BF and BTF, the CH_4 concentration was maintained much below the required concentration for energy recovery by combustion. Finally, a pure culture batch biodegradation test was performed to determine the CH_4 oxidizing capability of the fungus *Graphium* sp., which has never been reported to be able to utilize CH_4 as a sole carbon and energy source.

2 Objectives

♠ To evaluate the performance of a novel fungal biofilter treating CH_4 at low concentrations.

♠ To evaluate the performance of a two-phase partitioning biotrickling filter combined with a facultative hydrophobic methanotroph culture of *Methylosinus Sporium*, treating low concentration CH_4 emissions.

♠ To evaluate the capability of CH_4 degradation by *Graphium* sp.

3 Materials and Methods

3.1 Cultivation of Methane Degrading *Graphium* Sp.

3.1.1 Nutrients and Chemicals

A modified Bruner mineral salt medium (MSM 1) was used for irrigation and nutrients in the batch biodegradation test (Lebrero et al. 2011): $Na_2HPO_4 \cdot 12\ H_2O$ (6.15 g/L), KH_2PO_4 (1.52 g/L), $(NH_4)_2SO_4$ (1.0 g/L), $MgSO_4 \cdot 7\ H_2O$ (0.2 g/L) and $CaCl_2 \cdot 2\ H_2O$ (0.038 g/L) and 10 mL/L of a trace elements solution. Methane was supplied from a 50 L pressurized bottle (Abello-Linde, Spain) with purity > 99.95%.

3.1.2 Set-up and Operation

A pure strain of *Graphium* sp. NRRL 3915 (ATCC 58400, USA) was cultivated on agar plates consisting of a potato glucose agar gel (Sigma-Aldrich, USA) with 1 mL/L of lactic acid (10%) to inhibit bacterial growth, for 6 days until clear growth was observed. Under sterile conditions, samples of these cultures were suspended into two identical 125 mL serological bottles containing 30 mL of sterile MSM 1. The bottles were sealed with rubber butyl stoppers and CH_4 was injected in the headspace in order to achieve a final concentration of ~20% v/v. The bottles were incubated in an orbital incubator (MaxQ 4000, Thermo Scientific, USA) at 150 rpm and 25°C for 33 days. At day 8, 1 mL of a 24 g/L potato starch solution containing was injected into the *Graphium* sp. cultures. At days 25 and 29, 4 and 8 µL of methanol, respectively, was added into the bottles with a 5 µL

syringe (Hamilton Co., USA) to achieve a concentration of 100 and 200 mg/L, respectively, in the aqueous phase.

3.1.3 Analytical Methods

The CH_4 and CO_2 concentration in the headspace of the bottles was periodically measured in a GC-TCD (Bruker, Germany) in order to determine the CH_4 biodegradation rates. All samples were injected with a gas tight syringe (Hamilton Co., USA) of 100 µL volume. Each measurement was done in duplicate. The GC oven, injector and detector temperatures were 45, 150 and 200 °C, respectively. The carrier gas was helium at 6 mL/min with a 24 mL/min of make-up flow and 30 mL/min of total flow. The GC was equipped with two capillary columns: a BR-Molesieve 5A (15 m × 0.53 mm diameter, USA) and a CP-Porabond Capillary Column (25 m × 0.53 mm diameter, USA).

3.2 Fungal Biofilter

3.2.1 Nutrients and Chemicals

A modified Bruner MSM (MSM 2) was used for nutrient supply during the operation of the fungal biofilter (Lebrero et al. 2011): $Na_2HPO_4 \cdot 12\ H_2O$ (6.15 g/L), KH_2PO_4 (1.52 g/L), $NaNO_3$ (2.44 g/L), $MgSO_4 \cdot 7\ H_2O$ (0.8 g/L) and $CaCl_2 \cdot 2\ H_2O$ (0.2 g/L) and 40 mL/L of a trace elements solution. Additionally 2.4 mL/L of a Cu^{2+} stock solution (1 g/L) was added to achieve an initial Cu^{2+} concentration of 2.4 mg/L. To promote fungal growth over possible competing bacterial strains, 0.02 g/L of Chloramphenicol antibiotic (Sigma-Aldrich, USA) was added, and the final pH of the MSM solution was lowered with H_2SO_4

to 4. Methane was supplied from a 50 L pressurized bottle (Abello-Linde, Spain) with a purity > 99.95%.

3.2.2 Set-up and Operation

A jacketed PVC filter column (1 m height and 0.08 m of inner diameter) was filled with approximately 4 L of 1 cm^3 Polyurethane foam (PUF) cubes (Filtren TM 25280, Recticel Iberica S.L.) (Figure 6), with a final height of 0.80 m. The PUF was characterized according to standard methods (TMECC, 2002).

The BF was operated for 2 days under abiotic conditions in order to rule out a possible CH_4 removal due to adsorption or photolysis. Then, the packing bed material was mixed with the inoculum composed of 50 mL of leachate from a fungal biofilter treating hexane, 100 mL of compost, 30 mL of woodchips and 50 mL of a mixed microbial sludge consisting of anaerobic sludge (Valladolid WWTP, Spain), lagoon digestate from manure treatment (Valladolid, Spain) and soil samples from a landfill cover (Almazán, Spain). The BF was initially irrigated with 200 mL of MSM 2 and the leachate was daily recirculated for three days to prevent inoculum loss. From day 4 onwards, an intermittent irrigation with 200 mL of MSM 2 was performed every three days.

A CH_4 gas flow of 34.0 ± 0.5 mL/min, accurately regulated with a mass flow controller (Aalborg, USA), was mixed with pre-humidified air (sparked through a 8.5 L water column) resulting in a CH_4 concentration of 15 ± 0.9 g/m^3. The polluted stream was fed to the BF in an up flow configuration at 1 L/min (flow controller, Aalborg, USA) resulting in an EBRT of 4 minutes. The temperature of the inlet gas was maintained at 25 ± 1.0°C by an air conditioning system.

At day 16 the BF was re-inoculated with 75 mL of a previously isolated CH_4 degrading fungal culture cultivated for 36 days batch wise at pH 4, in the presence of antibiotics, 2-3% CH_4 and silicon oil as the NAP (33% NAP/MSM 3). This inoculum was re-circulated for 3 days.

At day 37, 200 mL of a pure *Graphium* sp. were added to the reactor. The inoculum was previously cultivated according to ATCC 58400 guidelines in potato starch broth with MSM 1 for 10 days.

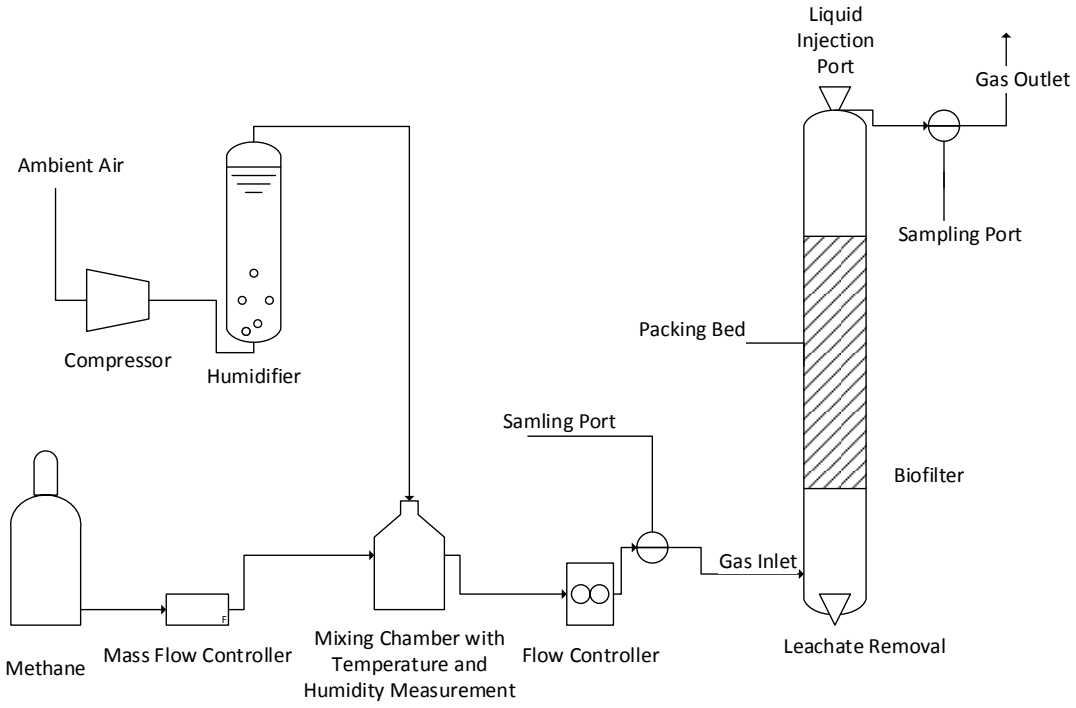

Figure 6 Schematic of the biofilter set-up

3.2.3 Analytical Methods

Inlet and outlet gas concentrations of CH_4 and CO_2 were determined by GC-TCD as previously described. Temperature and humidity from the inlet air flow were determined using a Testo 605-H1 detector (Testo AG, Germany). Pressure drop was monitored by means of a differential pressure meter using water as the manometric fluid. The pH of the leachate was measured after each irrigation with a pH meter Basic 20 (Crison, Spain). Compaction of the packed bed was also periodically recorded. Total organic carbon (TOC) and total nitrogen (TN) were analyzed from the leachate periodically with a total organic carbon analyzer (Shimadzu, Japan) coupled with a total nitrogen module (Shimadzu, Japan).

3.3 Two-Phase Partitioning Biotrickling Filter

3.3.1 Nutrients and Chemicals

A modified Bruner MSM (MSM 3) was used for irrigation and nutrient replenishment during BTF operation (Lebrero et al. 2011): $Na_2HPO_4 \cdot 12\ H_2O$ (61.5 g/L), KH_2PO_4 (15.2 g/L), $NaNO_3$ (6.1 g/L), $MgSO_4 \cdot 7\ H_2O$ (2.0 g/L) and $CaCl_2 \cdot 2\ H_2O$ (0.5 g/L) and 50 mL/L of a trace element solution. Since low concentrations of copper have been shown to inhibit methanotroph enzymatic functions, 6.0 mL/L of a Cu^{2+} stock solution (1 g/L) were added to the MSM to achieve an initial Cu^{2+} concentration of 6 mg/L (Lopez et al. 2013). Methane was supplied from a calibration bottle (Abello-Linde, Spain) with purity > 99.95%.

3.3.2 Set-up and Operation

The BTF setup was similar to the BF with the addition of a peristaltic pump Watson Marlow 520S (Watson-Marlow, UK) operating at 0.5 L/min recycling rate and an external 1-L MSM reservoir magnetically stirred (Agimatic S., P. Selecta, Spain) at 300 rpm (Figure 7). The BTF was also packed with PUF at 0.80 m of initial height.

A 3 day abiotic test was performed initially to assess the absence of CH_4 absorption or degradation. The inoculum used was a methanotrophic pure strain *Methylosinus Sporium* (17706, Type strain) purchased from DSMZ (Germany). Prior to inoculation, this methanotrophic inoculum was incubated in a 1.2 L gas tight bottle containing 100 mL of silicon oil (VWR International Ltd., UK) and 300 mL of MSM 3 (1:10 dilution) for three days in an orbital incubator (MaxQ 4000, Thermo Scientific, USA) at 150 rpm, 25ºC under an initial CH_4 concentration of ~11%. The *Methylosinu*s was capable of growing inside the silicon oil (NAP) or at the interphase of the NAP/aqueous phase. At inoculation, the NAP containing the *Methylosinus* culture was separated from the MSM and used as the inoculum.

The BTF recycling liquid consisted of silicon oil and MSM 3 at a ratio 1:4 v/v. Silicon oil exhibited a viscosity of 200 cSt (VWR International Ltd., UK). The total volume of the recycling liquid phase was maintained at 1.8 L throughout the experiment. In order to remove the accumulated metabolites and dead biomass and to replenish the nutrients consumed due to microbial growth, 300 mL of the recycling media were daily replaced with 300 mL of fresh MSM 3 (1:3 diluted to maintain optimal nitrate supply). NAP recovery from the withdrawn liquid was performed to maintain a constant NAP/MSM ratio.

A CH_4 inlet concentration of 14.3 ± 0.5 g/m^3 was continuously fed to the BTF by mixing a pure CH_4 stream (mass flow controller, Aalborg, USA) with a pre humidified air flow resulting in 1 L/min gas flow rate (flow controller, Aalborg USA), which corresponded to an EBRT of 4 minutes. Temperature of the experimental system remained at 25 ± 1°C throughout the experiment.

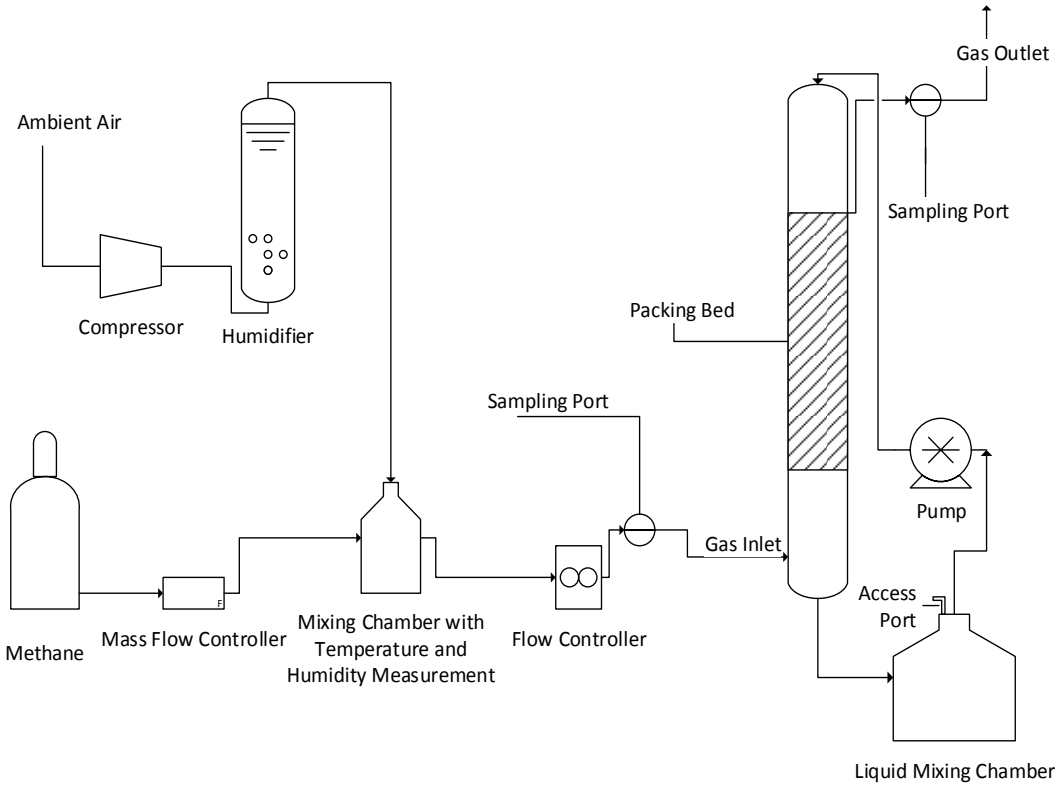

Figure 7 Schematic of the biotrickling filter set up

3.3.3 Analytical Methods

Temperature, humidity, pressure drop and CH_4 / CO_2 concentrations were determined using identical methods and equipment to those used in section 3.2.3. TOC and TN values were daily measured from the exchanged liquid using identical equipment than in section 3.2.3

3.4 Data Processing and Process Performance Evaluation

The results from the experiments were analyzed in order to determine the elimination capacity (g/m^3/h) (EC), the removal efficiency (RE), the CO_2 production rate (g/m^3/h) (CO_2PR) and the inlet load of pollutant (g/m^3/h) (IL):

$$EC = \frac{Q(C_{in} - C_{out})}{V} \quad (2)$$

$$RE = \frac{(C_{in} - C_{out})}{C_{in}} \times 100 \quad (3)$$

$$CO_2PR = \frac{Q(CO_{2,out} - CO_{2,in})}{V} \quad (4)$$

$$IL = \frac{Q(C_{,in})}{V} \quad (5)$$

Where Q is the polluted gas flow rate (m^3/h), C_{in} and C_{out} are the CH_4 inlet and outlet concentrations (g/m^3), and V the volume of the reactor (m^3) (Kennes et al. 2009).

4 Results and Discussion

4.1 *Graphium* Sp.

No visible growth was detected in the bottles from the inoculation to day 8 (Figure 8). Additionally, neither CH_4 degradation nor CO_2 production occurred. Following the re-inoculation of the bottles with potato starch broth at day 8, a rapid CO_2 production was detected until day 15, concomitant with rapid growth of both suspended spherical mycelia and bottom attached mycelium (Picture 1). The potato starch addition was performed in order to induce CH_4 degradation through co-metabolism, phenomenon that was previously demonstrated for *Graphium* sp. by Volesky et al. 1970 with ethane as the primary carbon source. However, despite the rapid CO_2 production resulting from the sole utilization of potato starch, degradation of CH_4 was not observed.

Picture 1 Growth of *Graphium* sp. at day 16 of cultivation

The lack of CH_4 degradation was likely due to the high stability of CH_4 which could be due to either the absence of the enzymatic machinery to perform methane oxidation or

the poor induction of monooxygenases at this CH_4 concentration. The first addition of 4 µL of methanol by day 25 resulted in further CO_2 production, confirming the capability of *Graphium* sp. to utilize methanol. (Figure 8). Similarly to the starch addition, this co-metabolic assay was performed to induce CH_4 co-metabolism, hypothesizing that enzyme production for methanol usage would result in CH_4 biodegradation. A decrease in CH_4 concentration of 13.1% was recorded, after the stabilization of CO_2 production. The subsequent 8 µL methanol addition resulted in CH_4 decrease of 17.6 %, calculated from the previous steady state to the end of operation. Both methanol additions resulted in significant and consistent CH_4 degradation, which supported the hypothesis of co-metabolic CH_4 biodegradation.

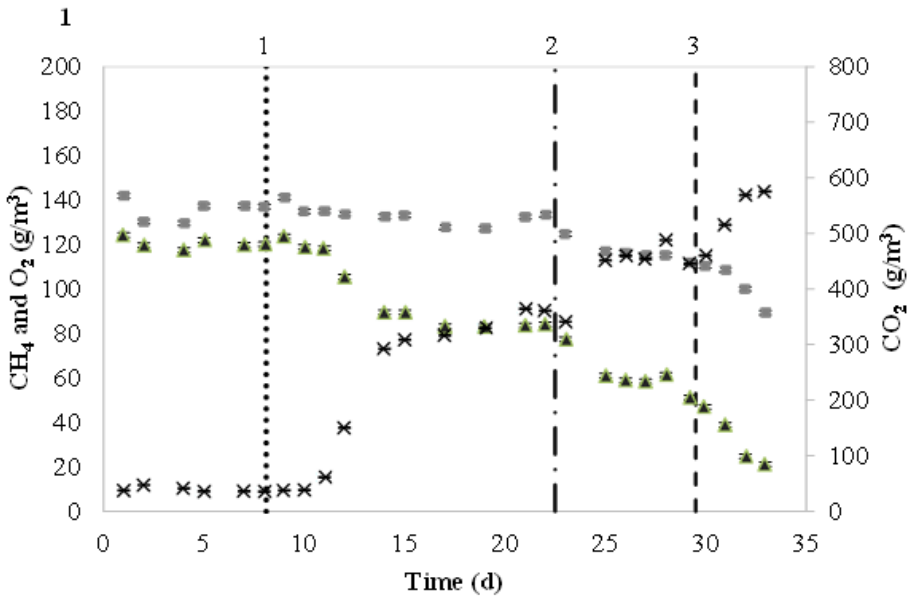

Figure 8 Time course in bottle **1** of CH_4 concentration (■), CO_2 production (X) and O_2 consumption (▲) in CH_4 biodegradation batch test containing *Graphium* sp. Vertical lines represent the addition of potato starch broth (1), 4 µL of CH_3OH (2) and 8 µL of CH_3OH (3)

4.2 Fungal Biofilter

The characterization of the packing bed material revealed that the PUF exhibited a void volume of 95.6%, a density of 0.025 g/mL as received, a pH of 5.80 ± 0.01, a water holding capacity of 12% (volume basis) and a surface area of 1000 m^2/m^3 (data provided by the manufacturer).

Following inoculation, the BF biodegradation performance was characterized by an EC of 3.8 ± 3.1 $g/m^3/h$, but with very unstable EC and CO_2 productions (Figure 9). This type of unstable start-up periods have been reported to precede stable operation. For example, Henckel et al. (2000) recorded start-up times of 19 days for methanotrophic microorganisms operated at low CH_4 concentrations (1000 ppm). A maximum EC of 9.35 ± 0.36 $g/m^3/h$ was achieved at day 14 followed by a decrease in the degradation rate to ~0. Throughout the experiment the IL was maintained constant at 244 ± 14 $g/m^3/h$ and the pressure drop remained at 0-4.9 Pa/m_{bed}.

The first re-inoculation at day 16 allowed for a rapid increase in the EC to a value of 12.5 ± 1.0 $g/m^3/h$ by day 20, which was the maximum EC achieved during the whole operating period. Nevertheless, after 4 days of operation the EC decreased to 4.1 ± 3.3 $g/m^3/h$ by day 26. The change in the environmental conditions of the microbial communities from batch suspended cultivation with a constant nutrient supply to a BF with intermittent irrigation and nutrient supply might have caused the reduction in the biodegradation capacity. The second re-inoculation at day 37 with *Graphium* sp. did not result in an improvement of the EC but in a severe deterioration of the CH_4 biodegradation capacity of the system, occasionally increasing to higher ECs. This unstable behavior was maintained until the end of the BF operating period at day 58 and steady state removal performance was not achieved. The correlation between the fluctuating ECs and steady

CO_2 productions by the microorganisms suggests that a biofilm existed where CH_4 degradation occurred under mass transfer limitation. This possibly resulted in a poor CH_4 degradation to metabolites, which were utilized as a carbon and energy source. Factors causing this mass transfer limitation, besides the inherently high Henry`s law constant of CH_4, might have been the low EBRT of the operation. A decrease in EBRT has been shown to reduce the removal performance in biofilters, especially for highly hydrophobic VOCs such as CH_4 which require large gas contact times for degradation to occur (Kennes et al. 2009, Kraakman et al. 2011). In addition, as the CH_4 polluted stream in the porous packing material was able to flow without large turbulence, this reduced gas-microorganism contact might have also creased additional mass transfer limitations.

CH_4 degradation in conventional biofilters has been mostly investigated using bacterial methanotrophs as the biodegrading microorganisms. Average CH_4 degradation rates ranging from 13 to 25 $g/m^3/h$ have been reported for conventional bacterial biofilters, while for third generation BFs equipped with inorganic packing materials higher maximum ECs of 29-50 $g/m^3/h$ were recorded (du Plessis et al. 2005, Nikiema et al. 2005, Haubrichs and Widman 2006, Nikiema and Heitz 2010). These values clearly exceeded the ECs achieved in our BF, although the typical EBRTs found in literature are 5 to 100 times higher. The EBRT is a parameter which clearly impacts the performance of BFs, generally requiring values of 0.3-20 h in laboratory scale studies to achieve successful CH_4 degradation (Kennes et al. 2012, Lopez et al. 2013).

The EC achieved here was much lower compared with fungal biofilters treating other hydrophobic pollutants such as hexane. For instance, Arriaga et al. (2005) reported maximum ECs of 50 $g/m^3/h$ and 40 $g/m^3/h$ for *Fusarium* sp. and *Cladosporium* sp., respectively, in a perlite-packed bed biofilter, while common fungal BFs can achieve maximum ECs of 70-90 $g/m^3/h$ for slightly hydrophobic compounds (Kennes et al. 2004).

Initially, CO_2 production increased gradually to 2.9 ± 2.2 g/m^3/h until day 16 when the CO_2 production dropped to negligible values, which correlated with the decrease in EC (Figure 9). This suggested that the microorganisms present in the bioreactor were unable to oxidize CH_4 to sustain their growth. After the second and third re-inoculations, the CO_2 production increased rapidly to stable values of 6.4 ± 1.5 g/m^3/h which remained roughly constant until the end of the experimentation period

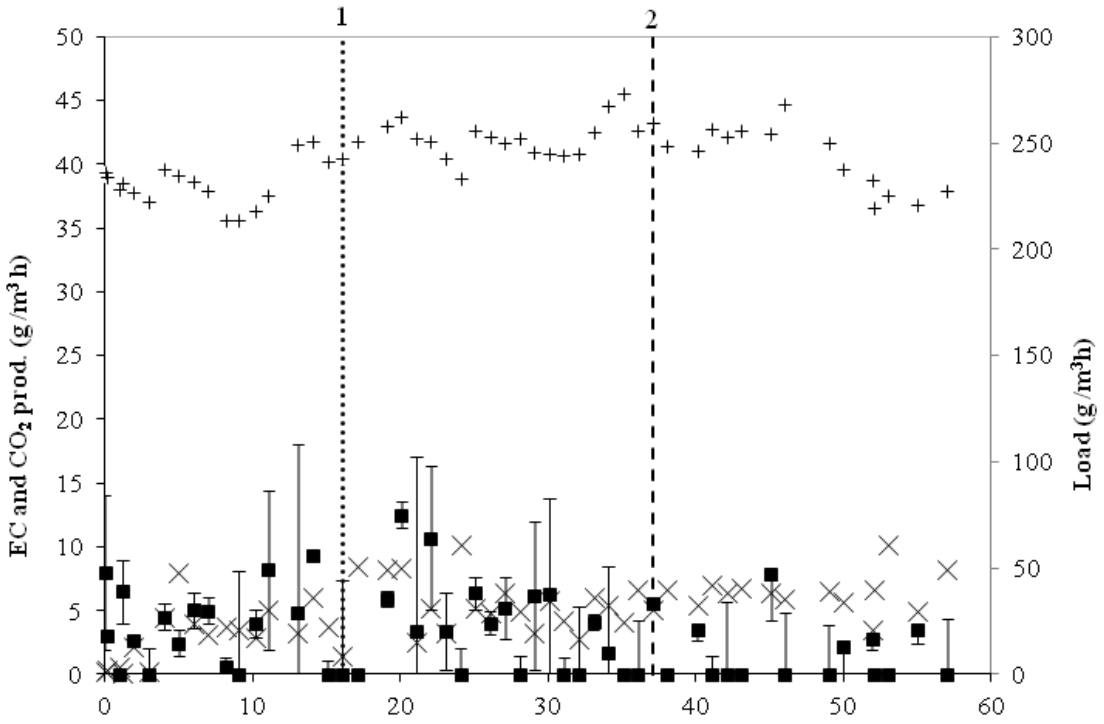

Figure 9 Time course of the BF loading rate (+), EC (■) and CO_2 (X) production. Vertical lines represent the first (1) and the second re-inoculation (2)

TOC and TN measurements showed a steady content of organic carbon and nitrogen in the leachate. (Figure 10a). The specific TOC increases correlated with the days of BF re-inoculation, thus showing the increase of TOC derived from the medium in which the inoculum was provided. The pH values measured from the leachate remained between 5.5 and 6.5 (Figure 10b). A difference of 1.5-2.5 in pH was thus observed between the

irrigation MSM 2 (pH= 4) and the leachate, which should not be inhibitory as pH values of 3-7 are suitable for fungal growth (Kennes et al. 2004). This difference suggested an excretion of basic CH_4 biodegradation metabolites.

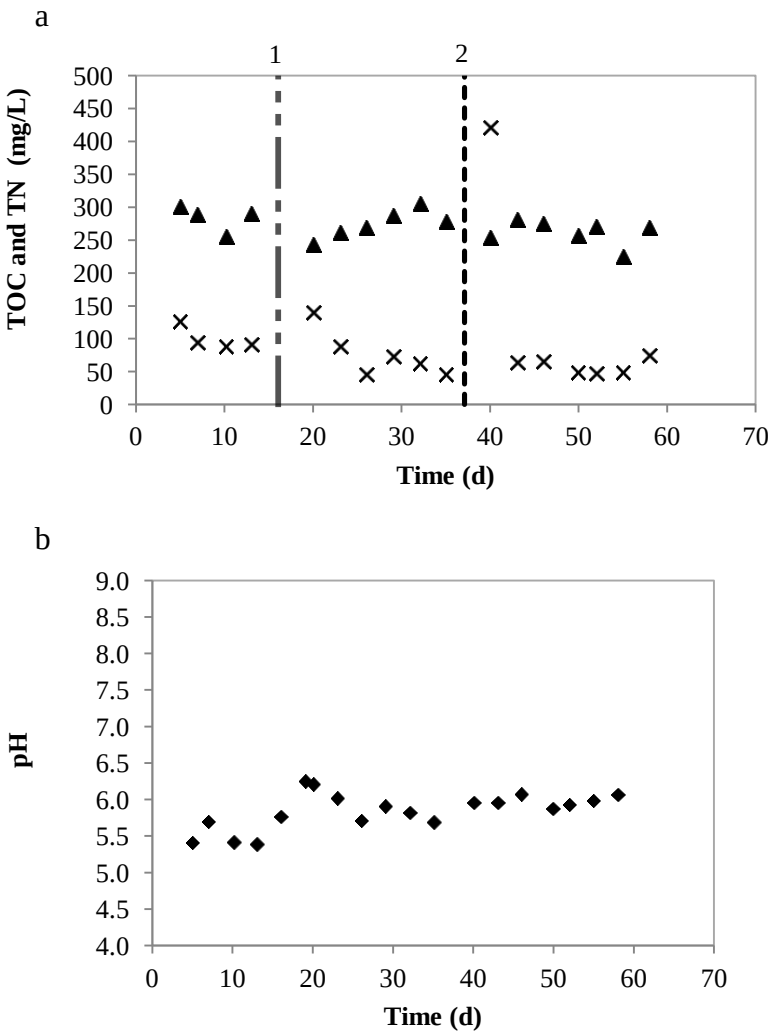

Figure 10 Time course of TOC (x) and TN (▲) concentrations (a) and pH (◆) (b) values recorded in the BF leachate. Vertical lines represent the first (1) and the second re-inoculation (2)

4.3 Two-Phase Partitioning Biotrickling Filter

Following BTF inoculation, the elimination capacity rapidly increased and stabilized at ECs of 18.1 ± 9.0 $g/m^3/h$ from day 4 to 15, experiencing however high fluctuations until

day 16 (Figure 11). During this initial start-up period (~ 10 days) no clear biomass growth was observed. From day 16, the EC steadily increased up to 60.1 ± 4.6 $g/m^3/h$ by day 34, the highest EC reached in the BTF over the entire operational period. This period was concomitant with an intense accumulation of biomass in the packed bed and the upper part of the reactor wall, which was not filled with packing material. During the last 10 days of BTF operation, the EC stabilized (variations of the EC lower than 10% in 5 consecutive days) at 49.5 ± 6.0 $g/m^3/h$.

The compaction of the BTF was daily monitored, and a 49% compaction compared to the original 0.8 m height was observed, which was attributed to the gravity forces mediated by the accumulation of silicone oil in the bed. However, this compaction had no effect on the pressure drop, which remained at a negligible 1.9 Pa/m_{bed} on average throughout the experiment. Thus, the calculations of IL, EC and CO_2PR (equations 2-5) were re-adjusted to a packing height of 0.42 m from day 8 onwards. Considering the steady EC achieved and the IL, which was maintained at 391± 30 $g/m^3/h$ during the complete operating period, the BTF achieved a RE of 12.0 ± 0.5% during steady state operation. These results are in accordance to other studies of two-phase partitioning biotrickling filters (TPPBTFs) treating CH_4. For instance, Avalos et al. (2012) reported ECs ranging from 3.9 to 21 $g/m^3/h$ in a BTF operated with a non-ionic surfactant, while an EC of 51 $g/m^3/h$ was recorded by Rocha-Rios et al. (2009) with a similar TPPBTF reactor configuration than that used in our experiment. Comparative studies in varied bioreactor configurations operating with and without a NAP, reported improvements in the CH_4 removal performance of up to 102-131% due to the presence of a NAP (Rocha-Rios et al. 2009, Avalos et al. 2012). Thus, the high EC achieved in our reactor can be attributed to the mass transfer increase brought by the addition of a NAP.

CO_2 production showed a steady increase throughout the experiment, with values of 108.3 ± 4.3 $g/m^3/h$ by the end of the experiment (Figure 11). The CO_2 production exhibited a steady increase regardless of EC fluctuations as a result of the steady growth of biomass. The stabilization of the EC from day 25 onwards, together with a continuous increase in CO_2 production, suggested the occurrence of higher CH_4 mineralization rates, which were supported by an increase of 33.5% in CO_2 mineralization between days 25 and 45. The stabilization of CO_2 production from day 39 onwards concomitantly with the stable EC further suggested the occurrence of mass transfer limitations, as the limited availability of CH_4 prevented further CO_2 production or biomass growth. However, considering the relationship between microbial activity limitation and mass transfer limitation, Kraakman et al. (2011) noted that commonly a laminar flow bioreactor such as BTFs is operating under a combination of both microbial activity and mass transfer limitations. This originates from the large heterogeneities often encountered, such as uneven distribution and thickness of the biofilm and convectional liquid and gas flows.

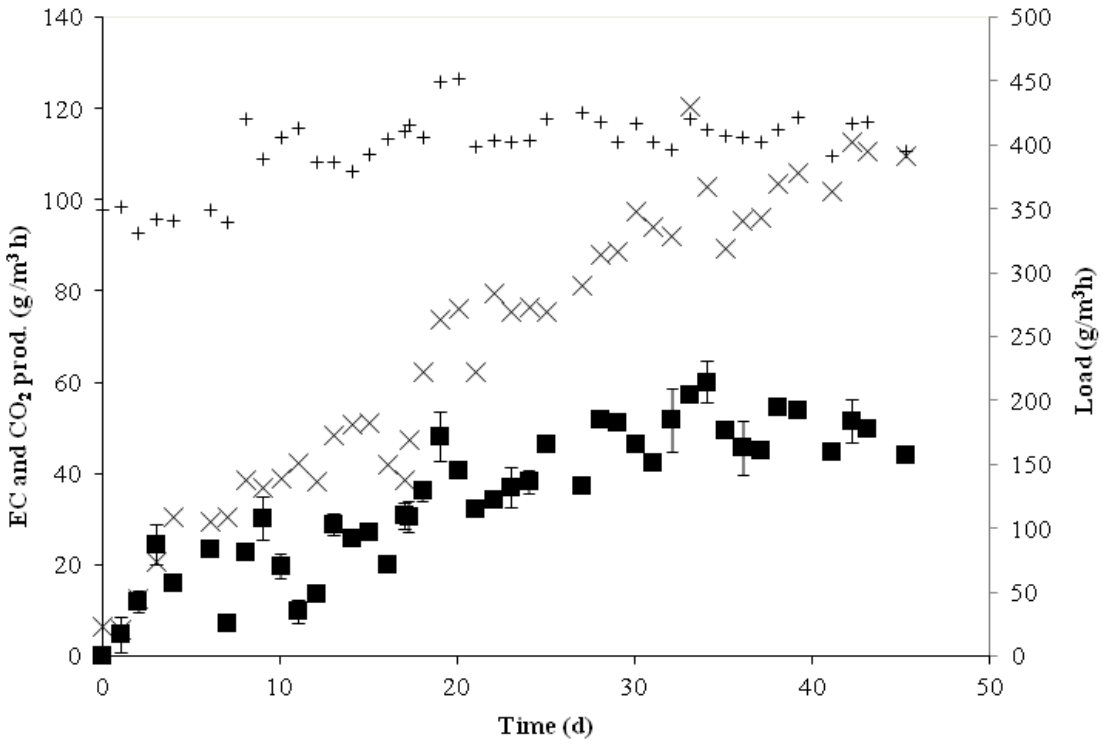

Figure 11 Time course of the BTF loading rate (+), EC (■) and CO_2 (X) production in the TPPB biotrickling filter.

TOC concentration remained constant at 36 ± 4.6 mg/L until day 20, gradually increasing afterwards to ~ 60 mg/L and further increasing and stabilizing to 76 ± 3.2 mg/L by day 34 (Figure 12). The first increase in TOC between days 21 and 25 correlated with a simultaneous EC increase, while the second increase recorded between days 33 and 35 corresponded to the peak in the EC value of 60.1 $g/m^3/h$ by day 34 and was correlated to a visible biomass increase. The later stabilization of TOC concentration at 76 ± 3.2 mg/L indicated a move towards a state steady microbial status. TN values, originated from the NO^-_3 present in the irrigation MSM, ranged between 80-170 mg/L (Figure 12). The two drops recorded at days 19 and 34 correlated with a higher microbial activity (thus a higher EC), which resulted in an increased consumption of nitrogen. The consumption of nitrogen slowed down as growth of new biomass mass also slowed down towards the end

of the experimentation period. This resulted in the accumulation of the unused nitrogen in the liquid phase, although inhibitory effects were not noticed in EC performance.

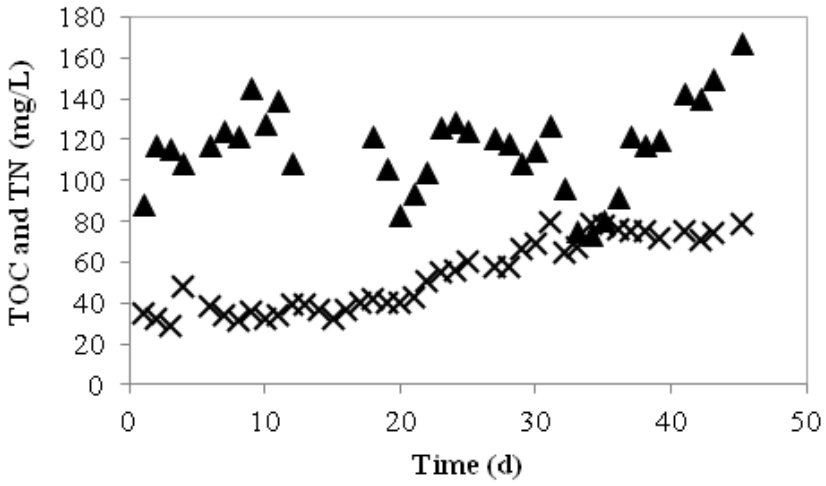

Figure 12 Time course TOC (×) and TN (▲) of the TPPBTF leachate

The initial objective of increasing CH_4 mass transfer rates and simultaneously achieving a high mineralization rate to avoid bed clogging was successfully fulfilled, although clogging of the packed bed might have occurred with a longer reactor run time. A BTF with the same configuration and operating parameters devoid of a NAP was previously operated in our laboratory, and a comparative improvement of approximately 110% in the EC value was observed in the TPPBTF. This experiment thus confirmed the positive effect of a NAP addition, which increased the CH_4 concentration gradient available for mass transport a result of an increased pollutant affinity. Unfortunately, a clear estimation on the level of hydrophobicity of the *Methylosinus Sporium* used in the BTF could not be obtained. However, visual observations clearly showed the capability of the microorganisms to migrate from one liquid phase to the other, according to the prevailing concentration gradients. This is supported by the research work on microbial chemotaxis (movement between phases) developed by Emerson et al. (1994).

5 Conclusions and Future Work

5.1 *Graphium* Sp.

Although *Graphium* sp. was not capable of utilizing CH_4 as the sole carbon and energy source, the results here obtained suggest the potential CH_4 degradation through methanol co-metabolism, which deserves further investigation, In this contex, the additions of small amounts of methanol to the MSM used to irrigate a *Graphium*-based biofilter might induce stable and high CH_4 removal efficiencies.

5.2 Fungal Biofilter

The low performance of the fungal BF demonstrated the difficulties of treating a stable compound such as CH_4 and the importance of considering the effects of several operating parameters to improve this removal. However, as fungal CH_4 treatment still remains a novel endeavor and since fungal biofilters have been shown previously to be capable of successful treatment of persistent pollutants (PPs) and VOCs, research under different operating conditions such as higher EBRT should be considered. Furthermore, the successful fungal CH_4 treatment is backed up by the positive results of co-metabolic degradation by *Graphium* sp. with methanol.

Possible full scale applications of fungal biofilters could be considered in landfill biocovers, coal mine ventilation air treatment and other environments where sub optimal growth conditions are encountered. The exploration of fungal biofiltration of CH_4 should focus on the following issues: isolation and characterization of fungal strains capable of CH_4 degradation and establishing the enzymatic pathway of CH_4 utilization by fungi.

5.3 Two-Phase Partitioning Biotrickling Filter

The maximum peak EC of 60.1 ± 4.6 g/m^3/h and the maximum stable EC of 49.5 ± 6.0 g/m^3/h exceeded previously reported CH_4 ECs in biotrickling filters. Despite the NAP addition improved mass transfer, more studies focusing on the evaluation of reactor configuration, such as the optimum NAP/MSM ratio, are still necessary in order to achieve higher CH_4 removal performance.

The possibility of replacing conventional BTFs by TPPBTFs has been proved to be feasible, although further studies on cost-effectiveness are still necessary. Future TPPBTF research should focus on practical applications where the benefits of a NAP can be fully utilized, such as remediation of polluted air, treatment of stored xenobiotics and contaminated water or soil.

Ever increasing data on biological treatment methods and their broad research will certainly provide us with sufficient, environmentally friendly, tools for emissions control.

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