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ENVIRONMENTAL BIOTECHNOLOGY

Assessing the influence of the carbon oxidation-reduction state on organic pollutant biodegradation in algal–bacterial photobioreactors

Melanie Bahr · Alfons J. M. Stams · Francisco De la Rosa · Pedro A. García-Encina · Raul Muñoz

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Abstract The influence of the carbon oxidation–reduction state (CORS) of organic pollutants on their biodegradation in enclosed algal–bacterial photobioreactors was evaluated using a consortium of enriched wild-type methanotrophic bacteria and microalgae. Methane, methanol and glucose (with CORS −4, −2 and 0, respectively) were chosen as model organic pollutants. In the absence of external oxygen supply, microalgal photosynthesis was not capable of supporting a significant methane and methanol biodegradation due to their high oxygen demands per carbon unit, while glucose was fully oxidized by photosynthetic oxygenation. When bicarbonate was added, removal efficiencies of 37±4% (20 days), 65±4% (11 days) and 100% (2 days) were recorded for CH_4, CH_3OH and $C_6H_{12}O_6$, respectively due to the additional oxygen generated from photosynthetic bicarbonate assimilation. The use of NO_3^- instead of NH_4^+ as nitrogen source (N oxidation–reduction state of +5 vs. −3) resulted in an increase in CH_4 degradation from 0 to 33±3% in the absence of bicarbonate and from 37±4% to 100% in the presence of bicarbonate, likely due to a decrease in the stoichiometric oxygen requirements and the higher photosynthetic oxygen production. Hypothetically, the CORS of the substrates might affect the CORS of the microalgal biomass composition (higher lipid content). However, the total lipid content of the algal–bacterial biomass was 19±7% in the absence and 16±2% in the presence of bicarbonate.

Keywords Carbon oxidation–reduction state · Microalgal–bacterial symbiosis · Nitrogen source · Photosynthetic oxygenation · Pollutant degradation

M. Bahr · A. J. M. Stams · P. A. García-Encina · R. Muñoz (✉)
Department of Chemical Engineering and Environmental Technology, University of Valladolid,
Dr. Mergelina s/n,
47011 Valladolid, Spain
e-mail: mutora@iq.uva.es

A. J. M. Stams
Laboratory of Microbiology, Wageningen University,
Dreijenplein 10,
6703 HD Wageningen, The Netherlands

F. De la Rosa
Department of Analytical Chemistry, University of Valladolid,
Dr. Mergelina s/n,
47011 Valladolid, Spain

Introduction

In wastewater treatment, aerobic conditions are often applied for biodegradation of organic pollutants. Traditionally, oxygen is introduced by air diffusion or surface aeration. These measures result in increased operating costs and cause a risk of volatilization of hazardous and odorous organic pollutants (Bell et al., 1993). Microalgal photosynthesis can provide an alternative to overcome the limitations of conventional mechanical aeration (Oswald, 1988).

Microalgal–bacterial systems were originally applied for the treatment of domestic wastewater in high-rate algae ponds (HRAPs) (Mara and Pearson, 1986; Oswald, 1988), and their application has been further extended to the biodegradation of toxic industrial pollutants (Borde et al., 2003; Muñoz and Guieysse, 2006). The implementation of a microalgal–bacterial consortium for the biodegradation of organic matter is based on the production of oxygen by microalgal photosynthesis. This oxygen is then used by heterotrophs as electron acceptor to degrade organic pollutants. In this symbiotic community, the carbon dioxide released by heterotrophic bacteria during pollutant mineralization is used by microalgae as carbon source during

microalgal photosynthesis (Oswald, 1988). Despite the significant breakthroughs in photobioreactor engineering, there is a lack of knowledge regarding the mechanisms underlying pollutant biodegradation in algal–bacterial systems (Janssen et al. 2003, Muñoz and Guieysse, 2006).

The carbon oxidation–reduction state (CORS) of pollutants is a key parameter determining the amount of oxygen needed in the overall biodegradation process, which is directly correlated to the chemical oxygen demand to total organic carbon ratio (COD/TOC). In addition, microalgal biomass composition, which is known to be highly sensitive to environmental growth conditions, could even change as a result of the different oxygenation needs imposed by the CORS of the pollutant. Thus, the more reduced the pollutant is, the higher the oxygen requirements are, which could promote the synthesis of a more reduced microalgal biomass. In this context, a more reduced biomass would result in a higher lipid content (Bordel et al., 2009). Finally, the nature of the nitrogen present in the wastewater has to be taken into consideration as well, since the stoichiometric oxygen required for pollutant biodegradation could significantly differ when either NH_4^+ or NO_3^-/NO_2^- are used as nitrogen source (Bordel et al., 2009).

Here, the influence of the CORS of pollutants on their biodegradation by an algal–bacterial community was investigated. Methane, methanol and glucose were selected as model organic pollutants based on their different CORS (−4, −2, and 0, respectively) and their biodegradability by the same bacterial consortium. The influence of the nitrogen source (ammonium or nitrate) on methane biodegradation was also determined in an independent set of experiments. Finally, the impact of the addition of inorganic carbon in enclosed photosynthetically oxygenated CH_4 biodegradation tests on the total lipid content of the biomass produced was assessed.

Materials and methods

Culture conditions and microorganisms

The methanotrophic bacterial consortium used in this fundamental study was enriched from the surface of an outdoors stabilization pond treating a CH_4-saturated effluent of an anaerobic digester treating swine manure in Almazán (Spain). Likewise, the mixed microalgal consortium was obtained both from a 500-L outdoors HRAP treating diluted swine manure (de Godos et al., 2009) and the upper surface of the river Duero in Valladolid (Spain), respectively. Despite the identification of both bacterial and microalgal species was not conducted, this fact does not invalidate the validity of the fundamental outcomes of this study. For microalgae and methanotrophs enrichment, a mineral medium as described by Whittenbury et al. (1970) was prepared with 10 mM ammonium sulfate or 10 mM potassium nitrate as nitrogen source. The trace element solution used was the same as described below. Methanotrophs were enriched in two series of 120-mL vials containing 50 mL of filter-sterilized medium (prepared with ammonium or nitrate) closed with butyl septa and aluminum caps. Air was used as the headspace atmosphere and 20 mL of pure methane was injected as carbon and energy source. The pH of both media was 7. Similarly, microalgae were enriched in two series of 1.25-L bottles with 250 mL of medium with either NH_4^+ or NO_3^- as nitrogen source and sodium bicarbonate (10 mM) as carbon source. The pH of the medium was adjusted to 7. Culture enrichment took place at room temperature under continuous illumination (4,500 lx) for microalgae and in the dark for methanotrophs. Finally, the corresponding microalgae and methanotrophs cultures were combined in fresh medium for subcultivation.

The mineral salt medium used for microalgal and bacterial growth (AMSM) during the biodegradation studies had the following composition (grams per liter): 2.44 Na_2HPO_4, 1.52 KH_2PO_4, 1.00 $(NH_4)_2SO_4$, 0.20 $MgSO_4 \cdot 7H_2O$, 0.05 $CaCl_2 \cdot 2H_2O$ and 10 mL/L of trace element solution containing (grams per liter): 0.5 EDTA, 0.2 $FeSO_4 \cdot 7H_2O$, 0.01 $ZnSO_4 \cdot 7H_2O$, 0.003 $MnCl_2 \cdot 4H_2O$, 0.03 H_3BO_3, 0.02 $CoCl_2 \cdot 6H_2O$, 0.001 $CuCl_2 \cdot 2H_2O$, 0.002 $NiCl_2 \cdot 6H_2O$, 0.003 $Na_2MoO_4 \cdot 2H_2O$. The final pH value of the medium was 7.0. In the experiments using mineral salt medium supplemented with NO_3^- (from now on referred to as NMSM) instead of medium supplemented with NH_4^+ (from now on referred to as AMSM) as nitrogen source, $(NH_4)_2SO_4$ was replaced by KNO_3 (1.53 g/L; equal N content). This mineral salt media allowed sterilization via autoclaving without salt precipitation. Fresh microalgal–bacterial consortium, sub-cultivated under test conditions in either AMSM or NMSM, was used as inoculum.

Chemicals

All chemicals and reagents with a purity of 99% were purchased from PANREAC (Barcelona, Spain). Methane and helium were purchased from Abello Linde (Barcelona, Spain). Prior to its use, methane was stored overnight in sealed glass flasks containing a sodium hydroxide solution (2 M) to eliminate eventual impurities of carbon dioxide.

Influence of the carbon oxidation–reduction state on pollutant degradation

All experiments were carried out batchwise in 1,250-mL glass flasks containing 400 mL of sterile AMSM. The bottles were flushed with helium, and closed with butyl septa and plastic caps to ensure that the O_2 needed for pollutant oxidation was only provided by microalgal

photosynthesis. Three series of experiments were conducted with organic pollutants (0.1 g of carbon) exhibiting different CORS (methane −4, methanol −2, glucose 0). In each series of experiments, pollutant biodegradation was carried out in the presence and absence of bicarbonate (5 mM). Following pollutant addition, the systems were inoculated with 10 mL of a fresh microalgal–bacterial inoculum pre-grown in AMSM. A series of control tests containing AMSM, 10 mL of algal–bacterial inoculum and bicarbonate (5 mM) was carried out to assess microalgae viability. Likewise, another series of control tests, supplied only with AMSM and 10 ml of inoculum, was conducted to assess the endogenous metabolism of the algal–bacterial consortium. These control tests were carried out in order to confirm that microalgae were active (and therefore capable of oxygenating the process) and that the consortium did not grow in the presence of external carbon source.

All tests were carried out at 30 °C (temperature controlled by a thermostatic water bath) under magnetic agitation (300 rpm) and continuous illumination at 5,500 lx (TL-D Philips, France, 36 W). Liquid samples of 1 mL were withdrawn periodically to monitor optical density (OD_{550}) and methanol or glucose concentrations. Gas samples of 100 μL were also taken from the flask's headspace to record CO_2, O_2, N_2 and CH_4 concentrations. All tests were carried out in duplicate and aliquots of culture broth were microscopically examined.

Influence of the nitrogen source on methane biodegradation

Methane biodegradation tests in AMSM and NMSM (inoculated with their corresponding inoculum) were conducted in the absence and presence of bicarbonate as described above. This experiment was carried out to determine the influence of the nitrogen source on the degradation in the worst-case scenario (the most reduced pollutant). In addition, aqueous samples were taken to monitor NO_3^- concentration in the liquid phase since this compound can act as an electron acceptor during pollutant oxidation. The corresponding control tests were also carried out as described above.

Influence of inorganic carbon supplementation on total biomass lipid content

Methane biodegradation tests were conducted in the absence and presence of sodium bicarbonate (5 mM) in eight bottles of 2,000 mL containing 1,000 mL of AMSM under a 70/30 *v*/*v* He/CH_4 atmosphere in order to obtain enough biomass for the lipid analysis. The systems were inoculated with 10 mL of the corresponding fresh microalgal–bacterial consortium and incubated as described above.

Analytical procedures

CO_2, O_2, N_2 and CH_4 concentrations in the gas phase were measured using a gas chromatograph-TCD (Varian CP-2800) equipped with a Molesieve 5A (15 m×0.53 mm×15 μm) and a CP-PoraBand Q (25 m×0.53 mm×10 μm) capillary column. The temperatures of injector, detector and oven were maintained at 150, 175 and 40 °C, respectively. Helium was used as the carrier gas (13.7 mL/min). External standards prepared in volumetric bulbs (Sigma Aldrich, USA) were used for CO_2, O_2, N_2 and CH_4 quantification.

The determination of methanol in the liquid phase was done by GC-FID (Hewlett Packard HP 6890 Series) using a SupelcoWax (15 m×0.25 mm×0.25 μm) column. Oven, injector and detector temperatures were maintained at 140, 250 and 200 °C, respectively. Helium was used as the carrier gas at 2 mL/min, while H_2 and air were fixed at 30 and 300 mL/min. N_2 was used as make up gas at 28 mL/min. External standards enabled the quantitative determination of methanol.

Glucose analysis was done by HPLC using a Waters 515 HPLC pump (Waters, Milford, USA) coupled with an Aminex HPX-87 C (0.3 m×7.8 mm) column (BioRad, USA) at 85 °C and an IR detector (Waters 410, USA) at 35 °C. Samples were eluted isocratically using ultrapure water as a mobile phase at 0.6 mL/min.

The determination of nitrate was done by HPLC-IC using an Waters 515 HPLC pump (Waters, Milford, USA) coupled with an ion conductivity detector (Waters 432, Milford, USA) using an IC-Pak Anion Guard-Pak column (Waters, Milford, USA) and an IC-Pak Anion HC (150 mm×4.6 mm) column (Waters, Milford, USA).

The optical density of the culture was measured at 550 nm with a HITACHI U2000 UV/visible spectrophotometer (Hitachi Ltd., Tokyo, Japan). Algal–bacterial floc formation hindered the accurate correlation between OD_{550} measurements and biomass concentrations. In addition, sampling volume limitations did not allow the monitoring of algal–bacterial concentration by dry-weight measurements.

The total lipid content was determined using a modified version of the method proposed by Bligh and Dyer (1959). The algal–bacterial biomass was centrifuged at 10,000 rpm for 10 min and the remaining pellet dried at 105 °C for 24 h. The dry biomass was then grinded to disrupt the cell walls, mixed with chloroform–methanol (1:2 *v*/*v*) in a proportion of 10 mL per gram dry biomass and the mixture agitated for 1 h at 80 rpm in order to extract the total lipid content. The lipid–solvent solution was separated from the biomass residue by filtration (0.45 μm Whatman filters) and then transferred to a pre-dried, pre-weighed round bottom flask. Finally, the solvent was evaporated using a rotary evaporator at 50 °C prior to total lipid measurement. Fatty acid methyl esters preparation

and analysis by GC-FID was carried out according to De la Rosa et al. (1998).

Microscopic pictures of the microalgal–bacterial community were taken with a light microscope (Leica DM 4000B, Germany) using the bright field transmitted light method. There was no control of the species present in the symbiotic consortium due to the short duration and batch mode of the experimentation.

Results

Influence of the CORS on pollutant degradation

No significant removal of methane and optical density increase were observed in the absence of bicarbonate, (Fig. 1a, b). Only a slight increase in optical density concomitant with a decrease in oxygen concentration was recorded from day 4 onwards (Fig. 1c). Similarly, carbon dioxide concentration was negligible during the course of the test (Fig. 1d). On the other hand, the microalgal–bacterial system was able to degrade 37±4% of the initial methane by day 20 in the presence of bicarbonate (Fig. 1a). The optical density of the culture rapidly increased within the first 2 days and coincided with an increase in oxygen concentration (up to 127±19 $g m^{-3}$) and a sharp decrease in CO_2 from 20±2 $g m^{-3}$ to almost complete depletion (Fig. 1b, c, d). From day 2 onwards, oxygen concentration gradually decreased along with an increase in culture absorbance and CH_4 degradation.

Optical density in the microalgae viability tests rapidly increased during the first 2 days of the experiment, remained constant afterwards, and was always correlated with the time course of O_2 and CO_2 concentration (Fig. 1b, c, d). Thus, O_2 concentration rose concurrently with the increase in OD_{550} and the depletion of CO_2 within the first 48 h of cultivation. No change in OD_{550}, CO_2 and O_2 concentrations were recorded in the tests conducted to assess the endogenous metabolism of the inoculum (Fig. 1b, c, d). The outcomes of these results applied also to the tests assessing CH_3OH and $C_6H_{12}O_6$ biodegradation

No major decrease in methanol concentration was recorded in the absence of bicarbonate, which also correlated with a negligible increase in culture absorbance (Fig. 2a, b). Similarly, the concentrations of carbon dioxide and oxygen did not show any significant variation over the course of methanol degradation (Fig. 2c, d). In the presence of bicarbonate, a methanol removal of 65±4% was recorded by day 11. Pollutant biodegradation and bicarbonate assimilation resulted in a steady increase in the OD_{550} of the culture and in the complete depletion of both CO_2 and O_2 in the headspace by day 4 (Fig. 2b, c, d). The oxygen concentration rapidly increased up to a maximum value of 63±2 $g m^{-3}$ within the first 2 days of the experiment (where methanol biodegradation did not occur to a significant extent) concomitantly with a decrease in carbon dioxide (Fig. 2d).

When glucose was used as model pollutant, its complete degradation occurred within the first 2 days of the experiment regardless of the absence or the presence of

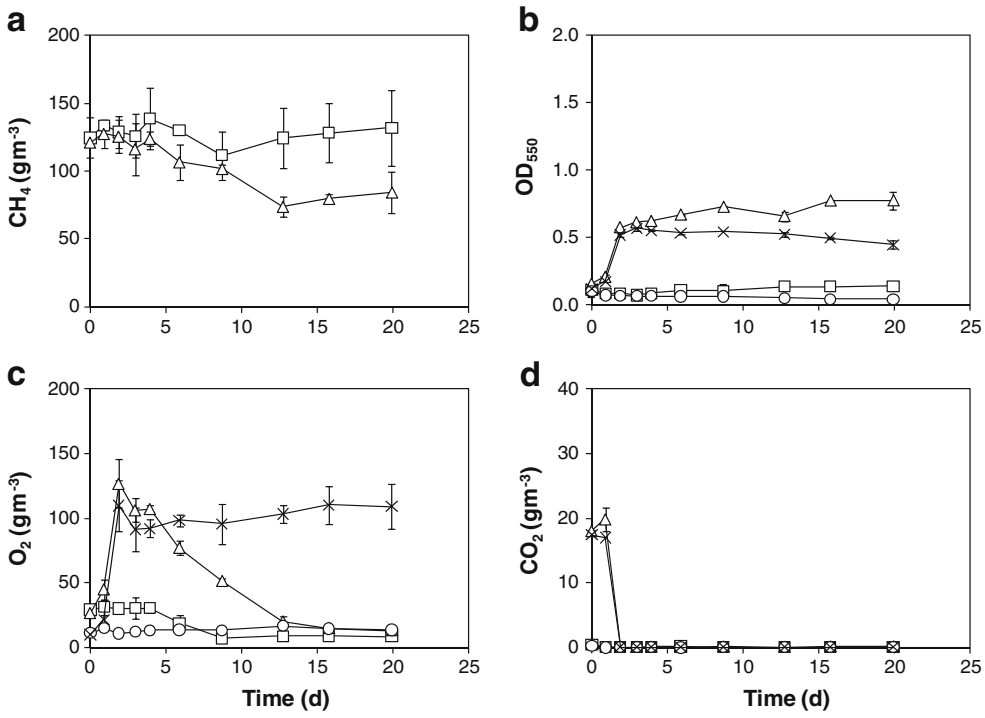

Fig. 1 Time course of methane (**a**), biomass (**b**), oxygen (**c**) and carbon dioxide (**d**) concentrations during methane biodegradation under photosynthetic oxygenation in the absence (*white squares*) and presence (*white triangles*) of bicarbonate (5 mM) in tests supplied with AMSM. Control tests to assess microalgae viability (*multiplication sign*) and the endogenous metabolism (*white circles*) were also conducted

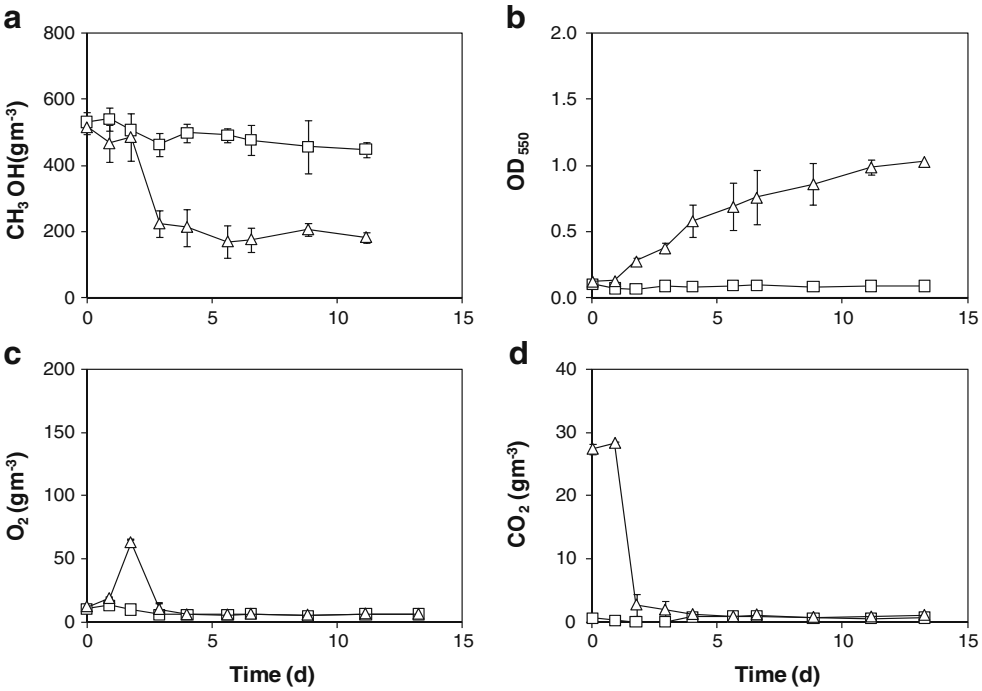

Fig. 2 Time course of methanol (**a**), biomass (**b**), oxygen (**c**) and carbon dioxide (**d**) concentrations during methanol biodegradation under photosynthetic oxygenation in the absence (*white squares*) and presence (*white triangles*) of bicarbonate (5 mM) in tests supplied with AMSM

bicarbonate, and an increase in OD_{550} was measured (Fig. 3a, b). In the absence of bicarbonate, the carbon dioxide concentration gradually increased up to 13±3 gm^{-3} on day 1.8 and was completely depleted by day 2.7 (Fig. 3d). While glucose was present and being degraded, the oxygen concentration remained very low (8±2 gm^{-3}) and increased up to 33±4 gm^{-3} on day 2.2 of experimentation. The presence of bicarbonate in the cultivation medium resulted in higher final O_2 concentrations and slightly lower OD_{550} values (Fig. 3b, c). The carbon dioxide concentration gradually increased in the early stage of the biodegradation process from 20±1 gm^{-3} up to 37±1 gm^{-3} on day 2 and was finally consumed in 3 days. Similarly, the O_2 concentration remained very low (12±3 gm^{-3}) while glucose was

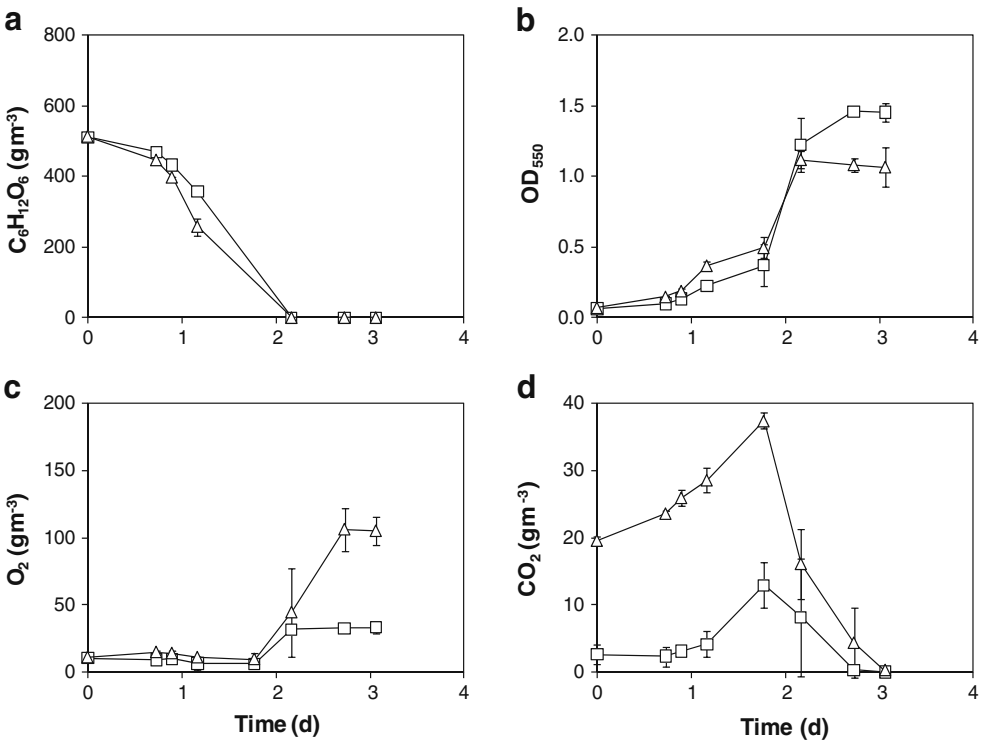

Fig. 3 Time course of glucose (**a**), biomass (**b**), oxygen (**c**) and carbon dioxide (**d**) concentrations during glucose biodegradation under photosynthetic oxygenation in the absence (*white squares*) and presence (*white triangles*) of bicarbonate (5 mM) in tests supplied with AMSM

present, and increased from day 2 onwards, reaching values of 106±16 gm^{-3} on day 2.7 (Fig. 3c).

No noteworthy variations in N_2 headspace concentrations were recorded regardless of the organic pollutant applied.

Influence of the nitrogen source on methane biodegradation

When nitrate was used as nitrogen source, methane removal rates of 33±3% were recorded in the absence of bicarbonate. This correlated with an increase in the optical density on day 11 (Fig. 4a, b). Carbon dioxide concentration remained very low during the course of the test, while oxygen concentration gradually declined from day 7 onwards, following a previous slight increase up to 32±9 gm^{-3} (Fig. 4c, d). In the presence of bicarbonate, complete methane depletion occurred in 18 days. The culture optical density rapidly increased within the first 3 days of incubation concomitantly with an increase in O_2 concentration up to 174±25 gm^{-3} and a sharp decrease in CO_2 from 27±2 gm^{-3} to almost complete depletion (Fig. 4b, c, d). From day 3 onwards, oxygen concentration gradually decreased along with an increase in optical density and a steady decrease in methane concentration.

Culture absorbance in the microalgae viability test increased rapidly during the first 2 days of the experiment and remained constant afterwards (Fig. 4b). Likewise, the oxygen concentration rose concurrently with a depletion of CO_2 within the first 3 days of cultivation (Fig. 4c, d). No significant variations in OD_{550}, CO_2 and O_2 concentrations were recorded in the tests conducted to assess microbial endogenous metabolism under cultivation with NO_3^- (Fig. 4b,c,d).

Influence of inorganic carbon supplementation on total biomass lipid content

The total lipid content (*w*/*w*) of the CH_4 degrading algal–bacterial biomass cultivated in the presence of additional bicarbonate was 16±2%, while a value of 19±7% was obtained in the absence of inorganic carbon. The growth of the algal–bacterial biomass in both test series was sufficient to neglect the effect of the lipid content of the inoculum. The GC-FID chromatograms obtained after methylation of the fatty acids present in the extracted lipids showed a comparable peak profile, with the six major peaks appearing at the same retention times in both tests (Fig. 5).

Discussion

Influence of the CORS on pollutant degradation

The relationship between microalgae and bacteria during photosynthetically oxygenated biodegradation processes is complex due to the mutual interactions between the two microbial populations. Oxygen is produced by the algae and is further used by the bacteria as electron acceptor to

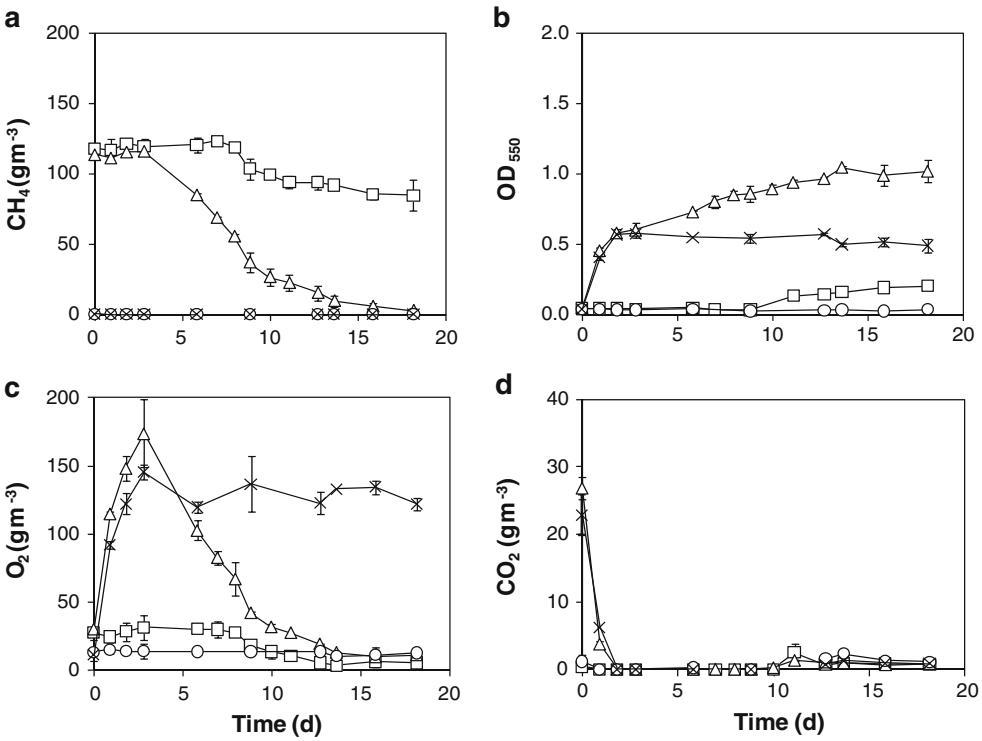

Fig. 4 Time course of methane (**a**), biomass (**b**), oxygen (**c**) and carbon dioxide (**d**) concentrations during methane biodegradation under photosynthetic oxygenation in the absence (*white squares*) and presence (*white triangles*) of bicarbonate (5 mM) in tests supplied with NMSM. Control tests to assess microalgae viability (*multiplication sign*) and the endogenous metabolism (*white circles*) were also conducted

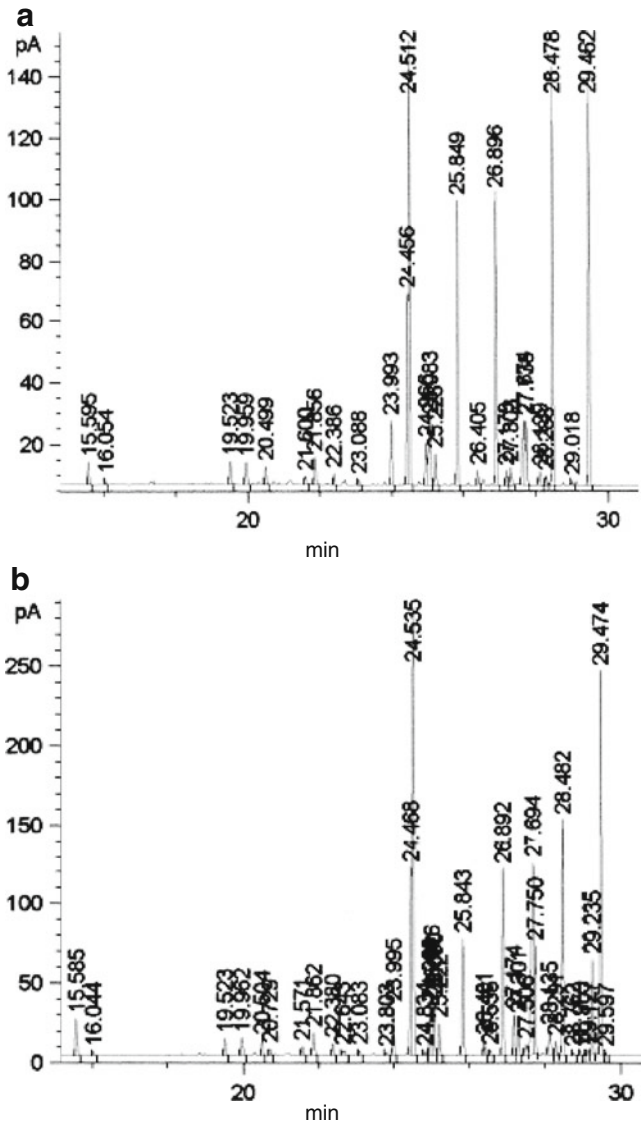

Fig. 5 Fatty acid methyl ester analysis by GC-FID from the CH_4 degrading microalgal bacterial biomass cultivated in the presence (**a**) and absence (**b**) of bicarbonate

degrade organic carbon and form bicarbonate, which is used by the algae for photosynthesis. In our particular study, microalgae and bacteria tend to form flocs during CH_4 biodegradation regardless of the nitrogen source, which suggests a more efficient symbiotic interaction (Fig. 6).

The effect of the CORS of the pollutant, corresponding to the COD/TOC ratio in wastewater, was herein studied. In the absence of bicarbonate, complete pollutant biodegradation was only observed for glucose (CORS=0), while no significant degradation was recorded for methane (CORS=−4) and methanol (CORS=−2). The addition of bicarbonate ($HCO_3^- \leftrightarrow H_2CO_3 \leftrightarrow H_2O+CO_2$), an oxygen precursor in the presence of light and microalgae, to the cultivation medium resulted in pollutant removals of 37±4,65±4 and 100% for CH_4, CH_3OH and $C_6H_{12}O_6$, respectively (Fig. 1a, 2a, 3a). The fact that the same algal–bacterial consortium was used in all biodegradation tests, together with the ability of the bacterial community to fully oxidize the target pollutant under mechanical aeration (empirically proven), confirmed the key role of the CORS in algal–bacterial-based pollutant biodegradation. A stoichiometric analysis was carried out to estimate the oxygen demand for complete degradation of each pollutant. It was assumed that 2/3 of the carbon present in each pollutant was used by the bacteria for energy formation (semi-reaction a) and 1/3 for biomass build-up (semi-reaction b) (Bowman and Sayler, 1994; Rocha-Rios et al. 2011; Tchobanoglous et al. 2003). Under this assumption, the overall equations 1, 2 and 3 yield oxygen demands of 1.65, 1.15 and 0.683 mol O_2per mol C for CH_4,CH_3OH and $C_6H_{12}O_6$, respectively.

$$10\,CH_4 + 20\,O_2 \rightarrow 10\,CO_2 + 20\,H_2O \quad (1a)$$

$$5\,CH_4 + NH_4^+ + 4.75\,O_2 \rightarrow C_5H_8O_2N + H^+ + 20\,H_2O \quad (1b)$$

$$10\,CH_3OH + 15\,O_2 \rightarrow 10\,CO_2 + 20\,H_2O \quad (2a)$$

$$5\,CH_3OH + NH_4^+ + 2.25\,O_2 \rightarrow C_5H_8O_2N + H^+ + 7.5\,H_2O \quad (2b)$$

$$10/6\,C_6H_{12}O_6 + 10\,O_2 \rightarrow 10\,CO_2 + 20\,H_2O \quad (3a)$$

$$5/6\,C_6H_{12}O_6 + NH_4^+ + 0.25\,O_2 \rightarrow C_5H_8O_2N + H^+ + 7.5\,H_2O \quad (3b)$$

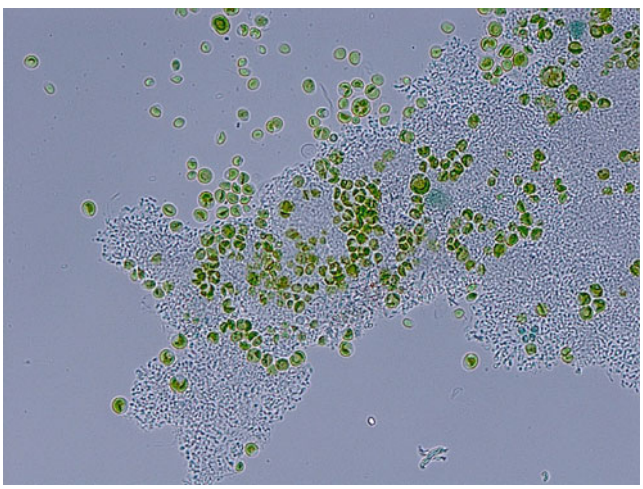

Fig. 6 Microscopic picture of the CH_4 degrading microalgal–bacterial biomass cultivated with NH_4^+ as N-source

Oxygen formation by microalgae is always linked to biomass formation, as described below. The amount of oxygen formed by consumption of the CO_2 produced by the heterotrophs was sufficient for degradation of glucose, but insufficient to degrade the more reduced substrates methane and methanol. Therefore, this research empirically confirmed that a lower CORS results in higher oxygen requirements per mole C for complete pollutant biodegradation due to an increased O_2 demand for bacteria biomass build-up. This confirms the experimental data obtained. In addition, our experimental findings were in agreement with the predictions of the algal–bacterial mechanistic model for pollutant biodegradation proposed by Bordel et al. (2009) that showed the need of external O_2 or CO_2 supply for complete biodegradation in case of reduced pollutants.

When bicarbonate was supplemented to the cultivation medium in the CH_4 and CH_3OH biodegradation tests, the process was initially characterized by a high photosynthetic activity as shown by the rapid O_2 increase concomitant with CO_2 depletion (Fig. 1, 2). In both tests, O_2 concentration decreased from the second day of experimentation concomitant with a decrease in pollutant concentration, which suggests that in the presence of bicarbonate, the biodegradation process was limited by bacterial rather than by microalgal activity during the initial stages. This high microalgal activity was confirmed by the rapid microalgal growth in the viability tests (Fig. 1). Conversely, during glucose biodegradation, O_2 concentrations remained close to zero during the initial stages of the biodegradation process despite the presence of bicarbonate, which indicates that microalgal rather than bacterial activity limited in this particular case. The analysis of pollutant biodegradation rates (and therefore O_2 consumption rates) suggests that bacterial activity increases with increasing CORS (Fig. 1, 2, 3). This hypothesis was further supported by the decreasing maximum O_2 concentration recorded at increasing CORS. However, glucose consumption occurred by non-methanotrophic population of the bacterial inoculum (a non-pure methanotrophic culture was here used) and methanotrophic bacteria are not capable of degrading glucose (Hanson and Hanson, 1996; Whittenbury et al., 1970). In addition, it cannot be excluded that the microalgal heterotrophic metabolism could have contributed to the high O_2 consumption recorded in the glucose biodegradation tests, as some microalgae are capable of using easily biodegradable substrates such as glucose or acetate as carbon and energy source (Lee, 2001).

In the absence of bicarbonate, the biodegradation process was always limited by microalgal photosynthetic activity, regardless of the pollutant tested, as shown by the low oxygen concentration present in the headspace (Fig. 1, 2, 3). In this context, Muñoz et al. (2003) reported low O_2 headspace concentrations and CO_2 accumulation during the mineralization of easily biodegradable organic substrates such as salycilate (CORS=0), followed by a rapid increase in O_2 concentration after pollutant depletion. Furthermore, the absence of significant methane and methanol degradation confirmed that microalgal activity is not capable of supporting the complete biodegradation of organic pollutants with very low CORS in the absence of bicarbonate. The results obtained by Tamer et al. (2006) confirmed this hypothesis. These authors recorded an increase in the continuous degradation of phenol (CORS=−0.67) by a *Chlorella vulgaris* and *Alcaligenes* sp. consortium from 28% to 100% when bicarbonate was supplemented to the artificial wastewater to enhance microalgal growth and thus photosynthetical oxygenation. In the light of these results, the removal of dissolved CH_4 (a strong greenhouse gas) in anaerobic effluents would be feasible in algal–bacterial photobioreactors as these effluents present high inorganic carbon concentrations.

Influence of the nitrogen source on methane biodegradation

The use of nitrate as N-source in the cultivation medium supported a CH_4 removal of 33±3% without bicarbonate supplementation, which compares positively with the absence of CH_4 degradation when NH_4^+ was the N-source. This better biodegradation performance in the presence of NO_3^- was also reflected in higher rates of CH_4 removal and O_2 consumption (Fig. 4). Similarly, while NH_4^+ supported CH_4 removals of 37±4% in the presence of bicarbonate, complete CH_4 elimination was achieved in 18 days with nitrate. The possibility of using nitrate as an electron acceptor for CH_4 anoxic oxidation as shown by Thalasso et al. (1997) was ruled out by the fact that no further methane degradation occurred after oxygen depletion but in excess of nitrate (870 mg/L). The analysis of equations 1 (CH_4 degradation using NH_4^+ as N-source) and 4 (methane biodegradation using NO_3^- as N-source) reveals lower bacterial oxygen requirements in the presence of nitrate (1.52 O_2/CH_4 vs. 1.65 O_2/CH_4) due to the difference in the nitrogen oxidation-reduction state (NH_4^+=−3, NO_3^-=+5).

$$10\,CH_4 + 20\,O_2 \rightarrow 10\,CO_2 + 20\,H_2O \quad (4a)$$

$$5\,CH_4 + NO_3^- + H^+ + 2.75\,O_2 \rightarrow C_5H_8O_2N + 6.5\,H_2O \quad (4b)$$

Furthermore, the oxygen produced from microalgae photosynthesis using nitrate as N-source is higher than the one using NH_4^+ (1.45 O_2 per mol CO_2 assimilated compared to 1.05 O_2/CO_2 for ammonium) (equations 5a,b).

$$10\,CO_2 + 2\,NH_4^+ + 5\,H_2O \rightarrow 2\,C_5H_8O_2N + 2\,H^+ + 10.5\,O_2 \quad (5a)$$

$$10\,CO_2 + 2\,NO_3^- + 2\,H^+ + 7\,H_2O \rightarrow 2\,C_5H_8O_2N + 14.5\,O_2 \quad (5b)$$

This explains that the use of nitrate as N-source resulted in a higher methane removal due to lower oxygen needs of the bacteria and higher oxygen production from microalgae photosynthesis.

In the presence of bicarbonate, methane biodegradation was characterized by a high microalgal activity in the early stages of the process (confirmed by the microalgal viability tests) as recorded in the tests conducted with ammonium as N-source (Fig. 1, 4). Bacterial activity limited pollutant biodegradation as suggested by the longer initial lag-phase and the gradual methane depletion in the excess of oxygen (Fig. 4a, c). However, microalgal activity limited CH_4 biodegradation in the absence of bicarbonate despite the lower oxygen demand mediated by the use of NO_3^- as N-source.

Influence of inorganic carbon supplementation on total biomass lipid content

The lipid content (19±7%) recorded in the algal–bacterial biomass cultivated in the absence of bicarbonate was comparable to that of the biomass grown in its presence (16± 2%) but the final algal–bacterial biomass productivity was negligible under the former cultivation conditions (Fig. 1b). These results were in agreement with those presented by Park and Craggs (2009) operating two 8 m^3-HRAPs (retention time of 4 days) treating domestic wastewater in New Zealand with and without CO_2 addition. The addition of CO_2 to the HRAP resulted in an improved biomass production (30%) but also in comparable average lipid content (19.8% vs. 20.9% without CO_2 supply). Hence, one of the initial hypotheses underlying this study (an enhanced lipid accumulation resulting from the higher oxygen demand during the degradation of pollutant with low CORS) could not be confirmed here. Apparently, in our tests, the CORS of the biomass was not affected by oxygen availability or bicarbonate availability.

In summary, the experimental findings herein presented limited the applicability of microalgal–bacterial systems in wastewater treatment to organic pollutants with moderate to high CORS. In this context, the biodegradation of very reduced pollutants such as methanol or methane in enclosed algal–bacterial photobioreactors would require the addition of external oxygen or inorganic carbon. The CORS also had an impact on the rate of pollutant biodegradation, being higher at increasing CORS. The use of nitrate as N-source instead of ammonium substantially improved the biodegradation of methane by lowering the oxygen demand and increasing the microalgal oxygen productivity. Finally, the potential synthesis of a more reduced microalgal biomass (higher lipid content) as a result of the higher oxygen requirements of pollutants with low CORS was ruled out by the results obtained during CH_4 biodegradation with and without bicarbonate supplementation.

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