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Effect of mineral nutrients on the kinetics of methane utilization by methanotrophs

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Abstract

The effect of different mineral nutrients on the kinetics of methane biodegradation by a mixed culture of methanotrophic bacteria was studied. The substrate factors examined were ammonia, iron, copper, manganese, phosphate, and sulphide. The presence of iron in the growth medium had a strong effect on the yield coefficient. Yield coefficients up to 0.49 mg protein per mg methane were observed when iron was added at concentrations of 0.10–5.0 mg/l. Iron addition also increased the maximum methane utilization rate. The same effect was observed after addition of ammonium to a medium where nitrate was the only nitrogen source. The observed Monod constant for methane utilization increased with increasing concentration of ammonia. This shows that ammonia is a weak competitive inhibitor as observed by other researchers. Relatively high levels of both ammonia (70 mg/l) and copper (300 µg/l) inhibited the methane degradation, probably due to the toxic effect of copper-amine complexes.

Introduction

The interest in methane-oxidizing bacteria, methanotrophs, has increased considerably in recent years since several studies have shown aerobic biodegradation of chlorinated aliphatics by these bacteria (Wilson & Wilson 1985; Henson et al. 1988; Henson et al. 1989; Oldenhuis et al. 1989; Tsien et al. 1989; Henry & Grbic-Galic 1990; Alvarez-Cohen & McCarty 1991). Methane-oxidizing bacteria occur widely in soil and groundwater containing both methane and oxygen, and therefore aerobic biodegradation has a potential for remediation of soil and groundwater contaminated with chlorinated aliphatics, e.g. trichloroethylene (TCE). The biodegradation of both methane and chlorinated aliphatics by methanotrophs is influenced by a range of factors, such as the substrate concentration and active biomass concentration, the availability of copper, nutrients, etc. However, the present knowledge is very limited. As pointed out by Henry and Grbic-Galic (1990): 'Researchers should consider manipulating mineral nutrient concentrations in order to enhance TCE oxidation rates, growth rates and growth yields'.

Copper seems to have an important influence on the activity of methanotrophs. During copper limitation higher degradation rates for chlorinated aliphatics (Oldenhuis et al. 1989; Tsien et al. 1989) and lower degradation rates for methane (Jørgensen & Degn 1987) were observed compared to situations with copper excess. Whittenbury et al. (1970), Ferenci et al. (1975) and recently Carlsen et al. (1991) have shown that ammonia inhibits the growth of

methanotrophs, probably due to competitive inhibition. Chlorinated aliphatics also inhibit the methane utilization competitively (Broholm et al. 1992). Henry and Grbic-Galic (1990) studied the growth of two *Methylomonas* sp. on mineral media which were modifications of the medium of Fogel et al. (1986) and Whittenbury et al. (1970). They observed that the lack of EDTA in the Whittenbury medium caused a strong reduction in the degradation rate of methane and TCE and a reduction in the growth yield of the bacteria. The reason was not identified. They also observed that for the same culture the methane utilization rate in the Fogel-medium was half the utilization rate as compared to the Whittenbury medium. Again, the reason was not identified, but the authors concluded that factors related to the mineral medium formulation other than copper availability could also have affected the methanotrophs. The discussion presented by Henry and Grbic-Galic (1990) illustrates the complexities of identifying the effect of nutrient components.

The purpose of this study was to examine the effect of the presence of the following nutrient factors: ammonia, iron, copper, manganese, phosphate and sulphide on the activity of a mixed culture of methanotrophs. The kinetics of methane utilization was characterized by the four kinetic constants: the maximum methane utilization rate (k_{max}), the methane Monod constant (K_s), the growth yield coefficient (Y), and the decay constant (b). The effects of the substrate factors were estimated based on statistical experimental design.

Materials and methods

Experimental system

The experiments were carried out in batch systems of 13.8 ml test tubes. They were equipped with screw caps containing a rubber membrane which enabled frequent air sampling from the test tubes. Each test tube had a total liquid volume of 5 ml made up of inoculum and a combination of the nutrient factors copper, ammonia, iron, manganese, sulphide, and phosphate added at different concentration levels (Table 1). The initial biomass concentration in experiment 1–4 was about 2.25 mg protein per liter and in experiment 5 about 8.0 mg protein per liter. Methane and pure oxygen were added with a syringe into the headspace of atmospheric air at concentrations of 2.6% (Vol/Vol) and 5.2% (Vol/Vol). The amount of oxygen added was sufficient to avoid oxygen limitation. The test tubes were incubated in the light at about 20° C. The test tubes were placed on a shaking table (IKA Werk, HS 500, 220 hub/min) in order to maintain equilibrium between the gas phase and liquid phase. Equilibrium was assumed due to the strong shaking action and the slow methane consumption (Fig. 1–3) which was a result of the small biomass concentration. The concentration of methane was monitored by taking air samples (10 µl) with a syringe directly from each test tube at different time steps during the experiments. The protein concentration was measured before and after the experiments.

In experiments 1–4 the basal nutrient solution A contained tap water with the following minerals per liter: 0.08 mg Fe, 3.30 mg K. 21.0 mg Mg, 0.015 mg NH_4-N., 30.0 mg SO_4, 89.0 mg Ca, 348.0 mg HCO_3, 32.0 mg Na, and 10.0 µg Mn. It was supplemented with 70.0 mg NO_3-N ($Mg(NO_3)_2 \cdot 7H_2O$). In experiment 3 and 4 phosphate-buffer was added: 78.0 mg P as $NaH_2PO_4 \cdot H_2O$ and 96.0 mg P as Na_2HPO_4. In experiment 5 the basal nutrient solution B contained the following minerals per liter of destilled water: 1.0 mg Fe ($FeSO_4 \cdot 7H_2O$), 5.0 mg K (K_2SO_4), 5.0 mg Mg ($Mg(NO_3)_2 \cdot 7H_2O$), 6.0 mg NO_3-N ($Mg(NO_3)_2 \cdot 7H_2O$), 30.7 mg SO_4 ($FeSO_4 \cdot 7H_2O$), 10.0 mg Ca ($CaSO_4 \cdot H_2O$), 60.0 mg HCO_3 ($NaHCO_3$), 23.0 mg Na ($NaHCO_3$), 27.0 µg Mn ($MnSO_4 \cdot H_2O$), 30.0 µg Co ($CoNO_3 \cdot 6H_2O$), 6.0 µg B ($Na_2B_4O_2 \cdot 10H_2O$), 32.0 µg Zn ($Zn(NO_3)_2 \cdot 6H_2O$), 48.0 µg Mo ($Na_2MoO_4 \cdot 2H_2O$), 15.0 µg Ni ($NiSO_4 \cdot 7H_2O$), 13.0 µg I (KI) and 1.0 mg EDTA. A phosphate-buffer identical to experiment 3 and 4 was added. The pH of the solutions were 7.

Chemicals

The methane was of 99.95% purity from A/S Dansk

Ilt & Brintfabrik, Denmark. The salts were of analytical grade quality.

Microorganisms

The inoculum used in the experiments was a mixed culture of bacteria grown on methane as the sole carbon source. The inoculum for the mixed culture consisted of biomass taken from the aeration system at a waterworks (Nykøbing Sjaelland waterworks, Denmark) treating methane-containing groundwater (1–2 mg/l) for public water supply. The biomass was mixed with nutrient solution B in a glass bottle, and methane and oxygen were added. Weekly, new methane and oxygen were added to the bottle. After about one month the mixed culture was used as inoculum for the experiments. The experiments were carried out under non-sterile conditions.

Levels of added mineral nutrients

The kinetic constants of methane utilization were estimated at different added levels of ammonia, iron, copper, manganese, phosphate and sulphide. The following salts were used: NH_4Cl, $FeSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $MnCl_2 \cdot 4H_2O$, $Na_2PO_4 \cdot 2H_2O$ and Na_2S. The results were obtained from five separate experiments, carried out at similar conditions. A summary of added substrate components and concentration levels is shown in Table 1. The levels of iron, manganese, sulphide and phosphate were fixed at concentrations realistic for Danish groundwaters. The concentrations of copper and ammonia were chosen according to levels which appear to affect the growth of the methane oxidizing bacteria (O'Neill & Wilkinson 1977; Leak & Dalton 1986; Prior & Dalton 1985; Oldenhuis et al. 1989).

Analytical procedures

Methane was analyzed on a Shimadzu GC-9A gas chromatograph (GC) equipped with a flame ionization detector connected with a Chromatopac C-R3H integrator. The GC column was an 18 m DB-5-column with an inner diameter of 0.53 mm. The GC was operated isothermally at a column temperature of 40° C and a detector temperature of 250° C. The carrier gas was nitrogen.

Protein measurements were performed according to Bradford (1976) on liquid samples to which 3 M trichloroacetic acid solution was added to extract the protein before storage at 5° C.

Experimental design

The five experiments were designed as 2^f and 3^f factorial experiments according to the plans: 2^3, 2^3, $^1/_2{*}2^5$, 3^2 and 3^3. The 2^f and 3^f factorials consider f factors at two and three levels as shown in Table 1 (Hicks 1982). The estimated kinetic constants were the dependent variables, the added substrate factors were the independent variables. The experimental design was based on the assumption that only main-factor and two-factor interactions might be significant. In addition to each of the experimental treatments, two additional batches were used as controls to correct for evaporation losses.

Table 1. Added levels of substrate factors in the five factorial experiments.

Exp.	Substrate factors	No. of lev.	NH_4-N (mg/l)	Fe (mg/l)	H_2S (mg/l)	Cu (µg/l)	Mn (mg/l)	PO_4 (mg/l)
1	NH_4, Cu	3	0/14/28	0	0	0/200/400	0	6
2	NH_4, CU, PO_4	3	0/14/28	0	0	0/200/400	0	6/36/66
3	NH_4, Fe, Cu	2	0/70	0/5	0	0/300	0	*)
4	NH_4, Fe, H_2S, Cu	2	0/70	0/5	0.1/0.5	300	0	*)
5	NH_4, Fe, H_2S, Cu, Mn	2	0/28	0/2	0/2	0/30	0/0.5	*)

* Approximately 530 mg/l PO_4-P was added as phosphate buffer.

Table 2. Significance of substrate factors.

Substrate factors	b (d^{-1})	Y (mg protein/mg CH_4)	k_{max} (mg CH_4/mg protein/d)	K_s (mg CH_4/l)
NH_4	–	+	++	++
Cu	–	–	+	–
Fe	–	++	++	–
H_2S	–	–	–	–
Mn	–	–	–	–
PO_4	–	–	+	–

++ significant effect in all experiments.
+ significant effect in experiment 1 and 2, not 3–5.
– no significant effect.

Mathematical model for methane degradation

The utilization rate of methane in the batches was described by the Monod equation adapted for two-phase (air and liquid) systems (Broholm et al. 1990):

$$-dS/dt = V_L/(V_L + F \cdot V_A) \cdot (X \cdot k_{max} \cdot S/(S + K_s)) \quad (1)$$

where S is the methane concentration (mg methane/liter), X is the biomass concentration (mg protein/liter), k_{max} is the maximum methane utilization rate (mg methane/ (mg protein · d)), K_s is the methane Monod constant (mg methane/liter), V_L and V_A are the volumes of liquid and air phases (liters), and F is the partition coefficient for methane between air and liquid (dimensionless). The growth rate of bacteria was described by the conventional equation (2) taking bacterial decay into account:

$$dX/dt = Y \cdot k_{max} \cdot S/(S + K_s) \cdot X - b \cdot X \quad (2)$$

where Y is the yield coefficient (mg protein/mg methane) and b is the decay constand (d^{-1}). The model is based on the assumption that the growth of bacteria is limited only by methane and not by oxygen or nutrients. It appears from eq. 1 and 2 that the kinetics of methane oxidation is characterized by four constants, k_{max}, K_s, Y, and b.

Equations 1 and 2 were solved numerically and a program for simulating the nonlinear equations was used to estimate the kinetic constants for each batch experiment, 68 in total. The best fit between model values, the experimentally determined methane concentrations and the final biomass concentration were found by combining visual comparison between fitted and measured curves and minimization of the sample variance (δ^2) between measured and fitted data.

Statistical analysis of the four estimated kinetic constants in relation to the substrate factors was performed by the statistical package SAS, Version 6, for personal computers, using the procedure GLM (SAS 1985). In all cases the significance of a variable was evaluated based on the observed P-value, i.e. the probability of accepting a hypothesis wich is wrong. The criterion for significance was set to $P \leq 5\%$.

Table 3. The lowest and highest estimated values of the affected kinetic constants and the average of the non affected constants.

Exp.	X_o (mg/l)	Medium	Y (mg protein/mg CH_4)	k_{max} (mg CH_4/mg protein/d)	K_s (mg CH_4/l)	b (d^{-1})
1	4.2	A[a]	0.15	2.88–8.64	0.05–0.11	0.192
2	4.4	A[a]	0.20	2.4–7.68	0.05–0.19	0.192
3	4.6	A	0.19–0.39	0.62–4.32	0.05–0.2	0.072
4	4.9	A	0.19–0.49	0.48–3.84	0.06–0.18	0.096
5	16.2	B	0.25–0.45	0.86–1.54	0.06–0.11	0.048

[a] no phosphate-buffer was added.

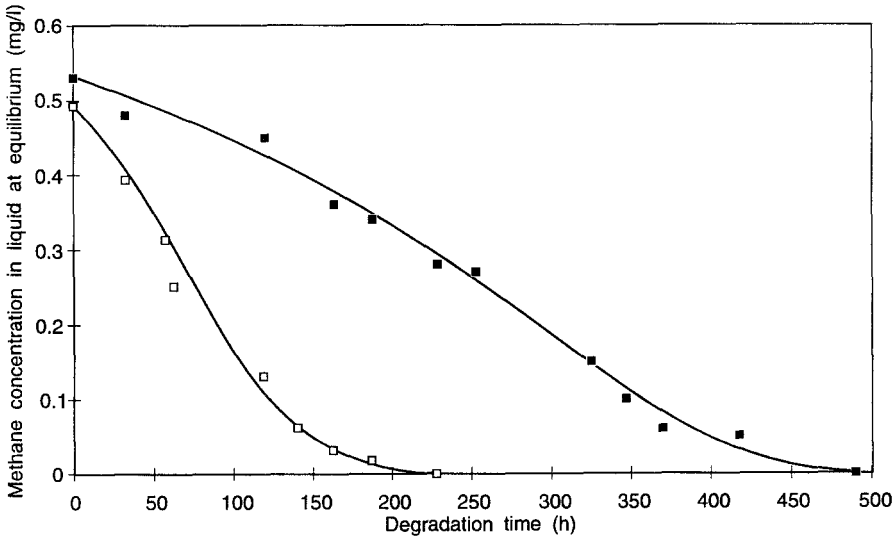

Fig. 1. Influence of added iron on the methane degradation (exp. no. 3). All other factors except iron were added at a low level. □ iron added at a high level (5.0 mg/l). ■ no iron was added.

Results

Significance of substrate factors

The effect of added substrate factors according to Table 1 was examined. The significant factors are summarized in Table 2. In particular the addition of ammonia and iron had an effect, as described in the following sections. In all cases of significance the effects were positive, i.e. the kinetic constants increased when the concentration of the substrate factors increased. A summary of the estimated kinetic constants is given in Table 3. The highest and lowest values of the affected kinetic constants together with the average values of the other constants are shown in the table. The initial biomass concentration of the experiments and the nutrient solution used are also shown.

The effect of iron

An increase of the maximum methane utilization rate, k_{max}, was observed when iron was added at a high level compared to degradation experiments at low levels of iron (Fig. 1). The plot is based on data from experiment 3 but the effect is characteristic of all five factorial experiments (Table 3). A strong increase of the estimated yield coefficient, Y, from about 0.20 mg protein per mg methane to 0.45 mg protein per mg methane was obtained when the amount of iron was changed from a low to a high level (Table 3, Exp. 3–5).

The effect of ammonia

A significant increase of the maximum methane utilization rate, k_{max}, was also observed in experiments where ammonia was added at high levels. This effect is shown in Fig. 2. The plot is based on data from experiment 3 but the effect was characteristic of all five factorial experiments. Another general effect of ammonia was a significant increase of the methane Monod constant in all five experiments when the amount of ammonia was changed from a low to a high level (Tables 2 and 3).

Effect of copper, manganese, phosphate and sulphide

An increase of the copper concentration from a low to a high level increased the maximum methane utilization rate in experiment 1 and 2 but not in experiments 3–5 (Tables 2 and 3). The addition of phos-

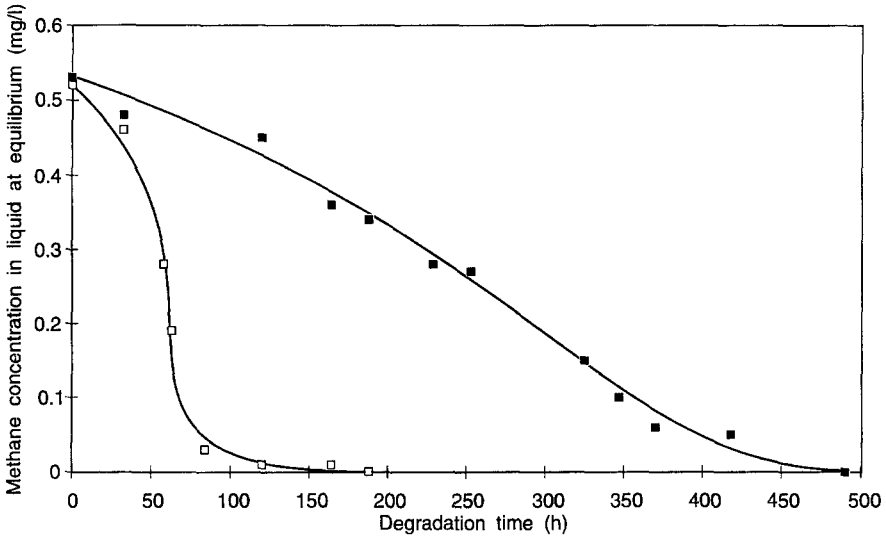

Fig 2. Influence of added ammonia on the methane degradation (exp. no. 3). All other substrate factors except ammonia were added at a low level. □ ammonia added at a high level (70 mg/l). ■ no ammonia was added.

phate also increased the methane removal rate in experiment 1 and 2. In experiments 3–5 the phosphate concentration was kept constant.

The other substrate factors sulphide and manganese did not effect the kinetic parameters.

Inhibition of methane degradation

Relatively high levels of ammonia (70 mg/l) and copper (300 μg/l) inhibited the methane degradation. The inhibition was not total since complete degradation of methane was obtained. However, the degradation time was much longer compared to an experiment where ammonia was the only factor added at a high level. Interestingly, when the three factors copper, ammonia, and iron were added in combination at high levels the degradation proceeded in the same way as when ammonia was the only factor added at a high level. Iron addition obviously neutralized the copper/ammonia inhibition.

Discussion and conclusions

An increase in the methane utilization rate and the growth yield was observed when the iron concentration was increased (Table 1 and 2). The enzyme responsible for the transformation of methane to methanol, methane monooxygenase (MMO) contains iron (Fox et al. 1989). An iron concentration corresponding to low levels may cause limitation of the MMO-synthesis resulting in a slow methane degradation compared to degradation of methane at high levels of iron.

A high effectiveness of carbon uptake when ammonia replaced nitrate as a nitrogen source (Table 2) can be explained by a low energy consumption when ammonium is assimilated compared to nitrate. However, at relatively high concentration ammonia acted as a weak competitive inhibitor as suggested by Carlsen et al. (1991). Competitive inhibition occurs when two substrates are converted by the same enzyme resulting in competition between the two substrates (Stainer et al. 1989).

The intracellular location of MMO (soluble or particulate) is dependent on the availability of copper in the growth medium. Experiments carried out by Tsien et al. (1989) showed that the methanotroph *Methylosinus trichosporium* OB3b expressed soluble MMO in a medium containing 0.25 μM or less of copper sulphate. At higher copper concentrations the particulate MMO was expressed. A shift from soluble to particulate MMO gave rise to a higher growth rate and a lower Monod constant (Jørgensen & Degn 1987). Therefore, an increase of the me-

thane uptake rate was expected in this work when the copper concentration was changed from a low to a high level (Table 1). This effect was observed in experiments 1 and 2, but not in experiments 3–5 (Tables 2 and 3). The high concentration of phosphate buffer in experiments 3–5 may have given rise to a significant reduction in the copper activity caused by precipitation of copper phosphate salts ($Cu_3(PO_4)_2$, etc.) and/or formation of copper complexes. This may explain the missing copper effect. Henry and Grbic-Galic (1990) also did not observe an effect of copper (zero and 1.6 μM Cu) on the growth of two *Methylomonas* species as long as EDTA was present. Absence of EDTA resulted in a low methane oxidation rate and a low growth yield.

Experiments carried out by Sato et al. (1988) showed an increased toxicity of copper to the nitrifying bacteria *Nitrosomonas europaea* with increasing ammonia concentrations. This phenomenon was explained by the formation of copper-amine complexes. The observed inhibition of the methane degradation at high copper and ammonia concentrations in this work may also be explained by a formation of copper-amine complexes. Surprisingly, the addition of iron neutralized the inhibition by the suspected copper-amine complexes. Perhaps due to the formation of iron-amine complexes.

In order to compare the growth yields and the Monod constants found in this study with literature values Table 4 was prepared. The growth yields of methanotrophs from this work was recalculated in terms of mg cells/mg methane converted by using a conversing factor of 0.5 mg protein per mg cells (Stainer et al. 1989). The estimated maximum yield coefficient of 0.98 mg cells per mg methane is much higher than the yields reported in the literature by several authors (Tabled 4) but is close to the maximum theoretical value of about 1.0 mg cells per mg methane (Arvin 1991). It is also close to the yield value of 1.1 mg cells/mg methane found by Whittenbury et al. (1970) for methanotrophs which were non-capsulate, non-slime-forming, with no detecta-

Table 4. Estimated values of the yield coefficient and the methane Monod constant of methanotrophs together with values from the literature.

Strain	Nitrogen source (mg/l)	Cu (μg/l)	Fe (mg/l)	Y (mg cells/ mg CH_4)	K_s (mg CH_4/l)	Reference
Methylosinus trichosporium	NO_3-N/140 NH_4-N/0.0	398	0.8	0.38	0.032	Jørgensen and Degn 1983
Strain OU-4-1	NO_3-N/140 NH_4-N/0,0	398	0.8	0.63	0.0128	Jørgensen and Degn 1983
Pseudomonas methanica	NO_3-N/0.0 NH_4-N/280	nm	nm	nm	0.24	Ferenci et al. 1975
Methylomonas	NO_3-N/140 NH_4-N/0.0	0/102	0.7	0.61–0.73	nm	Henry and Grbic-Galic 1990
Methylomonas	NO_3-N/165 NH_4-N/0.0	3.8	1.0	0.65	nm	Henry and Grbic-Galic 1990
Methylococcus NCIB11083	NO_3-N/0.0 NH_4-N/195	32	1.9	0.75–0.78	nm	Linton and Vokes 1978
Mixed culture	NO_3-N/5 NH_4-N/0.0	32	0.5	1.0	nm	Arvin 1991
Methylococcus capsulatus	NO_3-N/140 NH_4-N/42	635	2.8	nm	1.46	Carlsen et al. 1991
Mixed culture	NO_3-N/70 NH_4-N/70	300	5.0	0.78[a]	0.2[a]	This work
Mixed culture	NO_3-N/70 NH_4-N/0.0	300	0.1	0.43[a]	0.05[a]	This work

[a] Estimated values from experiment 3.
nm: not measured.

ble lipid inclusions. The Monod constants observed in the different studies are very different. This is probably due to the use of different types of microorganisms and growth substrates.

There are few studies dealing with the rate of methane utilization. Henry and Grbic-Galic (1990) found removal rates in the same range as found in this work in experiments 3–5 (21° C). Carlsen et al. (1991) found very high utilization rates compared to this study and Henry and Grbic-Galic (1990) but their experiments were done at 45° C. After temperature correction, assuming a two-fold increase of the rate per 10° C, the rates are similar to the rates found in this work in experiment 1 and 2.

Generally, this study has revealed that the kinetic characteristics of methanotrophs may be significantly influenced by different mineral nutrients. In particular, the iron and ammonia concentrations were important substrate components.

References

Alvarez-Cohen L & McCarty P (1991) A cometabolic biotransformation model for halogenated aliphatic compounds exhibiting product toxicity. Environ. Sci. Technol. 25: 1381–1387

Arvin E (1991) Biodegradation kinetics of chlorinated aliphatics with methane oxidizing bacteria in an aerobic fixed biofilm reactor. Wat. Res. 25: 873–881

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem 72: 248–254

Broholm K, Christensen TH & Jensen BK (1992) Modelling TCE degradation by a mixed culture of methane-oxidizing bacteria. Wat. Res. 26: 1177–1185

Broholm K, Jensen BK, Christensen TH & Olsen L (1990) Toxicity of 1,1,1-trichloroethane and trichloroethene on a mixed culture of methane oxidizing bacteria. Appl. Environ. Microbiol. 56: 2488–2493

Carlsen, HN, Jørgensen L & Degn H (1991) Inhibition by ammonia of methane utilization in *Methylococcus capsulatus* (Bath). Appl. Microbiol. Biotechnol. 35: 124–127

Ferenci, T, Strøm T & Quayle JR (1975) Oxidation of carbon monoxide and methane by *Pseudomonas methanica.* J. Gen. Microbiol. 91: 79–91

Fogel MM, Taddero AR & Fogel S (1986) Biodegradation of chlorinated ethenes by a methane-utilizing mixed culture. Appl. Environ. Microbiol. 51: 720–724

Fox GB, Froland AW, Dege EJ & Lipscomb DJ (1989) Purification and properties of a three-component system with high specific activity from a type II methanotroph. J. Biol. Chem. 264: 10023–10033

Henry S & Grbic-Galic D (1990) Effect of mineral media on trichloroethylene oxidation by aquifer methanotrophs. Microb. Ecology. 20: 151–169

Henson JM, Yates MV & Cochran JW (1989) Metabolism of chlorinated methanes, ethanes, and ethylenes by a mixed bacterial culture growing on methane. J. Ind. Microbiol. 4: 29–36

Henson JM, Yates MV, Cochran JW & Shackleford DL (1988) Microbial removal of halogenated methanes, ethanes, and ethylenes in an aerobic soil exposed to methane. FEMS Microbiol. Ecol. 53: 193–201

Hicks CR (1982) Fundamental concepts in the design of experiments, 3th edn, Holt, Reinhart & Winson, New York

Jørgensen L & Degn H (1983) Mass spectrometric measurements of methane and oxygen utilization by methanotrophic bacteria. Microbiol. Lett. 20: 331–335

—(1987) Growth rate and methane affinity of a turbidostatic and oxystatic continuous culture of *Methylococcus capsulatus* (Bath). Biotechnol. Lett. 9: 71–76

Leak DJ & Dalton H (1986) Growth yields of methanotrophs. 1. Effect of copper on the energetics of methane oxidation. Appl. Microbiol. Biotechnol. 23: 470–476

Linton JD & Vokes J (1978) Growth of methane utilising bacterium *Methylococcus* NCIB 11083 in mineral salts medium with methanol as the sole source of carbon. Microbiol. Lett. 4: 125–128

Oldenhuis R, Vink RLJM, Janssen DB & Witholt B (1989) Degradation of chlorinated aliphatic hydrocarbons by *Methylosinus trichosporium* OB3b expressing soluble methane monooxygenase. Appl. Environ. Microbiol. 55: 2819–2826

O'Neill GJ & Wilkinson FJ (1977) Oxidation of ammonia by methane-oxidizing bacterium and the effects of ammonia on methane oxidation. J. Gen. Microbiol. 100: 407–412

Prior SD & Dalton H (1985) The effect of copper ions on membrane content and methane mono-oxygenase activity in methanol-grown cells of *Methylococcus capsulatus* (Bath). J. Gen. Microbiol. 131: 155–163

SAS (1985) Version 6, release 6,02, SAS Institute, Inc., Cary, N.C.

Sato C, Leung SW & Schnoor JL (1988) Toxic response of *Nitrosomonas europaca* to copper in inorganic medium and wastewater. Wat. Res. 22: 1117–1127

Stainer, RY, Ingraham JL, Wheelis ML & Paisto PR (1989) General Microbiology, 5th edn, MacMillan Education Ltd, London

Tsien HC, Brusseau GA, Hanson RS & Wackett LP (1989) Biodegradation of trichlorothylene by *Methylosinus trichosporium* OB3b. Appl. Environ. Microbiol. 55: 3155–3161

Whittenbury R, Phillips KC & Wilkinson JF (1970) Enrichment, isolation, and some properties of methane-utilizing bacteria. J. Gen. Microbiol. 25: 225–233

Wilson JT & Wilson BH (1985) Biotransformation of trichloroethylene in soil. Appl. Environ. Microbiol. 49: 242–243