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Methane oxidising bacteria as environmental indicators

Svend Jørgen Binnerup, Mette Marianne Svenning, Anne Grethe Hestnes, Ingvild Wartiainen

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Nordic Council of Ministers
Store Strandstræde 18
DK-1255 Copenhagen K
Phone (+45) 3396 0200
Fax (+45) 3396 0202

Nordic Council
Store Strandstræde 18
DK-1255 Copenhagen K
Phone (+45) 3396 0400
Fax (+45) 3311 1870

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Nordic Co-operation in Agriculture and Forestry

Agriculture and forestry in the Nordic countries are based on similar natural pre-requisites, and often face common challenges. This has resulted in a long-established tradition of Nordic co-operation in agriculture and forestry. Within the framework of the Plan of Action 1996-2000, the Nordic Council of Ministers (ministers of agriculture and forestry) has given priority to co-operation on quality agricultural production emphasizing environmental aspects, the management of genetic resources, the development of regions depending on agriculture and forestry and sustainable forestry.

Nordic co-operation

Nordic co-operation, one of the oldest and most wide-ranging regional partnerships in the world, involves Denmark, Finland, Iceland, Norway, Sweden, the Faroe Islands, Greenland and Åland. Co-operation reinforces the sense of Nordic community while respecting national differences and similarities, makes it possible to uphold Nordic interests in the world at large and promotes positive relations between neighbouring peoples.

Co-operation was formalised in 1952 when *the Nordic Council* was set up as a forum for parliamentarians and governments. The Helsinki Treaty of 1962 has formed the framework for Nordic partnership ever since. The *Nordic Council of Ministers* was set up in 1971 as the formal forum for co-operation between the governments of the Nordic countries and the political leadership of the autonomous areas, i.e. the Faroe Islands, Greenland and Åland.

Contents

I and type II methanotrophs, it is known that type II methanotrophs dominate in environments of high methane production. Increased temperatures followed by elevated methane production can be reflected as changes in the methanotroph community. The community structure and quantification of methanotrophs can be promising methanotroph-related parameters to include as useful indicators of environmental changes as a result of e.g. global warming. However to evaluate this, further experimental microcosms studies and *in situ* measurements should be done.

The project has developed new techniques and established a solid collection of methanotrophs from Nordic soils. This allows high throughput screening of soil samples with respect to presence of type I and type II methanotrophs and it will be possible to include this indicator in already established or in upcoming soil monitoring programmes.

notroferne) generelt havde en større evne til vækst ved lave temperaturer end isolater tilhørende den anden hovedgruppe (Type II metanotroferne).

Nedbrydningspotentialet for de miljøfremmede stoffer diklorofluorometan (HCFC-21) og klorodifluoromethan (HCFC-22) blev undersøgt i et sammenlignende studie af metanoxiderende bakterier isoleret fra en dansk losseplads og en dansk strandeng. Der blev fundet en dominans af type II metanoxiderende bakterier i lossepladsen mens strandengen også havde et stort antal tilhørende type I hovedgruppen. Undersøgelsen kunne ikke demonstrere nedbrydning af gassen HCFC-22 (klorodifluorometan), hvorimod op til halvdelen af den tilsatte mængde HCFC-21 (diklorofluorometan) blev nedbrudt i renkultur studier. Der kunne ikke påvises nogen sammenhæng imellem nedbrydningspotentialet og isolaternes slægtskabsforhold eller metanmonooxygenasernes karakteristika med hensyn til substrat-specificitet.

Som en miljøindikator er der i projektet ikke blevet identificeret en simpel entydig sammenhæng imellem klimavariation eller miljøbelastning og sammensætningen af de metanotrofe samfund. Med hensyn til en potentiel anvendelse af metanotrofer som en indikator for klima forandringer vil vi vurdere at der er behov for mere viden som kan afdække samspillet imellem frekvensen af type I og type II metanotrofer i specielt arktiske og subarktiske tundra jorde og ændringer i temperaturforhold. Det vurderer vi ud fra den viden vi har om dels forskelle i temperatur optimum for de to hovedgrupper (type I og type II) og dels fra en viden om at den ene gruppe (type II) dominerer i organisk rige miljøer med stor metan-produktion. Et scenario med en temperaturstigning, og en deraf følgende forøgelse i tilgængeligt organisk materiale, samt øget metanproduktion vil samlet forventes at medføre en øget tilstedeværelse af type II metanotrofer, hvorimod det omvendte vil forventes ved et temperaturfald. Der eksisterer i dag ikke de systematiske beskrivelser af type I og type II metanotrofernes udbredelse i bestemte miljøer over tid som vil være en forudsætning for at kunne udvikle og validere en brugbar miljøindikator på dette område. Men med de nye teknikker som bl.a. er udviklet og anvendt i projektet til beskrivelse af de metanotrofe samfund, vil det dog være inden for rækkevidde at etablere dette f.eks. i tilknytning til et overvågningsprogram.

- Policy relevance
- Analytical soundness
- Measurability

However, interpretation of microbial indicator data beyond the information they offer to fulfil these criteria is not without problems. Kapusta (1999) argues that the microbial parameters of use do not represent the relevant assessment endpoints (defined as the valued resources to be considered, e.g. endangered species) but are rather "secondary or even tertiary endpoints tied to one of the primary endpoint categories". Another commentary highlights the difficulties in linking impact on measurable microbial activities to ecological impact (Sheppard 1999). According to Sheppard (op. cit.) the linkage fails due to problems such as: 1) compensation processes – the ecological process is continued by organisms or enzymes other than those measured; 2) use of non-specific assays; 3) difficulties in obtaining reproducible results due to the complex responses of the microbes during sampling and handling; and 4) ambiguous benefits/detriments – it is not clear weather a change should be interpreted as beneficial, detrimental or is ecologically neutral.

Certain features of methanotrophs and the ability of these bacteria to oxidise methane make them a potential microbial indicator, at least from a theoretical point of view. The unique capacity of methanotrophs to remove the greenhouse gas methane, and their ability to remove a number of halogenated C-1 or C-2 compounds of environmental concern, link them to one endpoint of clear political concern, namely global climate change and its consequences. Further, the narrow link between the primary ecological function of methanotrophs, i.e. removal of methane, and their relatively limited evolutionary versatility reduces the problem of compensation processes. Methanotrophs are therefore superior for studying the link between the diversity of a specific population and the ecological process they perform. Using molecular tools, it is possible to address changes in diversity or abundance of methanotrophs and relate these to how the specific process (removal of methane in the environment) proceeds. This is advantageous in the selection of microbes or processes as indicators of environmental disturbance.

However, other characteristics complicate the interpretation of data. The adverse effect of various halocarbons that on the one hand are cometabolised by the methanotrophs but on the other hand inhibit the oxidation of methane, is an example of complexity affecting the use of methanotrophs as indicators of pollution. Further, interpretation of possible changes in the diversity of methanotrophs seems complicated in the context of human activities that are considered harmful to the environment. The question of whether the observed change is a sign of a beneficial, detrimental or an ecologically neutral development is a difficult one, partly due to the lack of suitable baseline values or a frame of reference.

Methanotrophs are divided into two main groups: type I and type II, based on different physiological characteristics. The two groups are only distantly related. Type I methanotrophs belong to the gamma subgroup of the proteobacteria, and include the species *Methylomonas*, *Methylococcus*, *Methylocaldum*, *Methylosphaera*, *Methylomicrobium, Methylosarcina* and *Methylobacter*. They have intracytoplasmic membranes arranged in bundles of vesicular discs (Fig.1, right), 16-carbon phospholipid fatty acids, and assimilate formaldehyde by the ribulose monophosphate (RuMP) pathway (Fig. 2).

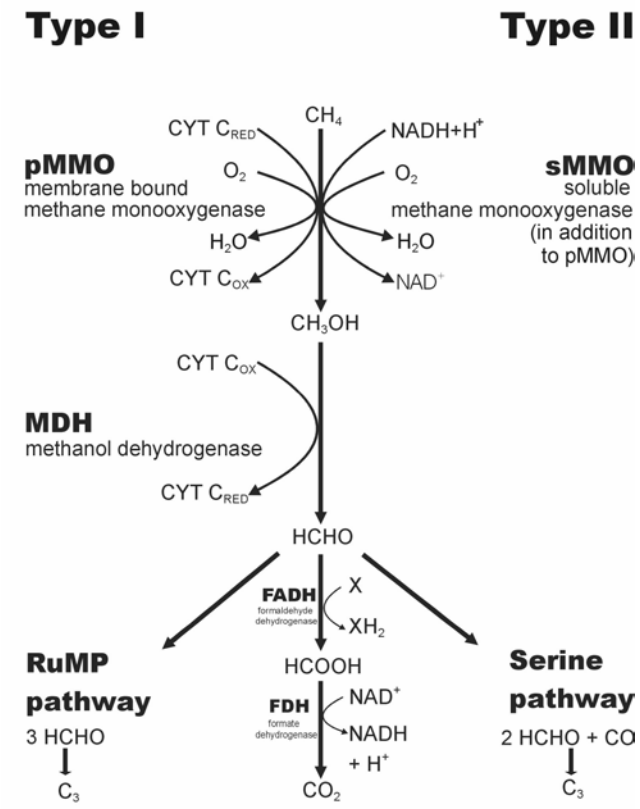

Fig. 2: Pathways for the oxidation of methane (CH4) and assimilation of formaldehyde (HCHO) for type I and type II methanotrophs (modified from Hanson & Hanson 1996). The enzymes involved are MMO (methane monooxygenase), MDH (methanol dehydrogenase), FADH (formaldehyde dehydrogenase) and FDH (formate dehydrogenase).

The type II methanotrophs belong to the alpha subunit of the proteobacteria and include the species *Methylocystis, Methylocella, Methylocapsa* and *Methylosinus*. They assimilate formaldehyde by the serine pathway, have 18-carbon phospholipid fatty acids and paired intracellular membranes aligned to the periphery of the cell (Fig. 1, left). The membrane arrangements of the type II genera *Methylocella* and *Methylocapsa* are,

however, different from other common described methanotrophs. For further reading on the species relations of methanotrophs, see Hanson et al. (1991). The characteristics of methanotrophs with respect to their phylogeny, membrane arrangements and phospholipid fatty acids are important components in the identification of methanotroph isolates obtained from the environment, and also for direct monitoring of methanotroph communities.

Methanotrophs contain the key enzyme methane monooxygenase (MMO). This enzyme oxidises methane to methanol. The reaction uses energy (reducing power) to break the O-O bound in dioxygen (O_2). One oxygen atom is incorporated into methanol (CH_3OH), while the other is used to form water (H_2O). The energy required for the oxidation of methane to methanol and for bacterial growth is derived from further oxidation of methanol, via formaldehyde (HCHO) and formate (HCOOH) to carbon dioxide (CO_2) (Fig. 2). The methane monooxygenase (MMO) enzyme exists in two different forms: a soluble form (sMMO) and a membrane bound form (pMMO). Most methanotrophs contain pMMO but some species also have sMMO, which is expressed by low copper concentration during growth in environments (Hanson & Hanson 1996).

The MMO enzyme has been the subject of extensive biochemical and molecular research. The particulate methane monooxygenase (pMMO) is integrated into the inner membrane of the bacterial cell wall and is a copper protein (Zahn & DiSpirito 1996). The other methane monooxygenase is present within the cellular fluid (cytoplasma) and is therefore called the soluble methane monooxygenase (sMMO). This enzyme differs biochemically from the pMMO by having a di-ion centre at the active site for methane oxidation (Rosenzweig et al., 1993). Further, it is inducible, meaning that it is only expressed when, for example, copper deficiency prevents expression of pMMO. Methanotrophs expressing pMMO have higher growth yield, as pMMO is more specific and has a higher affinity for methane than sMMO (Hanson & Hanson, 1996). Therefore, methanotrophs expressing sMMO can better utilise habitats with low concentrations of copper.

The most important difference between the two enzymes in an environmental perspective is that the function of the sMMO enzyme is less specific than the pMMO enzyme. The enzyme also has an affinity for other methane-like C-1 and even C-2 compounds, many of which are solvents of environmental concern. Assessment of methanotrophs as environmental indicators necessitates a thorough understanding of the regulation and function of the two enzymes in relation to the affinity for methane and other C-1 compounds of environmental concern. Since the different methanotrophs express either pMMO or sMMO, the bacteria are also expected to have different capacities for removal of methane and other harmful compounds from the environment. A thorough understanding of environmental effects on the structure of the methanotroph com-

munity and its metabolic competence is a prerequisite for the successful use of methanotroph-related parameters as environmental indicators.

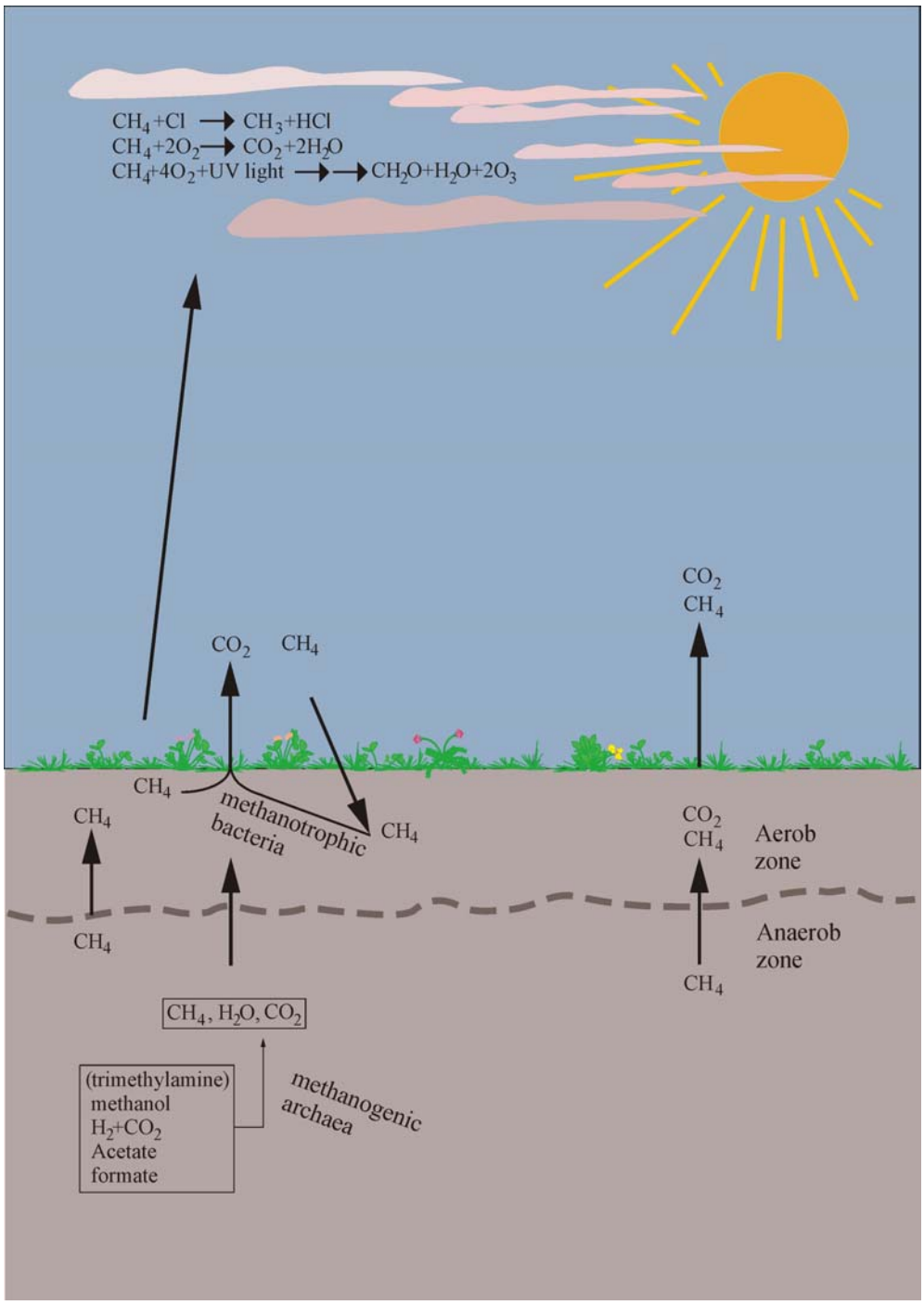

Fig. 3. A schematic diagram of methane (CH_4) flow in the terrestrial carbon cycle, and methane oxidation in the atmosphere.

favours high methane oxidation activity by the methanotroph communities present in the aerobic surface soil layers..(2) If global warming results in a more humid climate, there may be a significant increase in methane emission from Arctic environments. In this scenario, increased soil temperatures enhance biological activity and accelerate the decompo-

sition of organic matter, resulting in an expansion of the anaerobic soil layers, where methane production takes place. Methane production depends both on the growth conditions and the substrate availability of methanogenes. In this case, therefore, global warming will have a direct positive effect on methane production by influencing the growth and activity of methanogenic bacteria, and an indirect positive effect by increasing the substrate availability of these bacteria. Since methane is also a significant greenhouse gas, methanogenesis provides a positive feedback on climatic warming, and methane released from Arctic tundra may in this case be an accelerating factor.

The most important biological sink for methane is aerobic oxidation to carbon dioxide by the methane oxidising bacteria (methanotrophs). This group of organisms can be characterised as important biological "methane munchers" that dominate the drained soil layers above the waterlogged anaerobic zones and facilitate methane emission from deeper layers to the atmosphere (Conrad, 1996). The efficiency with which methane is removed during this shift of the aerobic soil layer toward the atmosphere depends on a number of factors. Short-term climatic factors such as precipitation and temperature will have a dramatic effect on the profusion of aerobic vs. anaerobic zones. High temperatures and high precipitation result in a widening of anaerobic zones at the expense of aerobic zones, which in turn results in more methane being produced and less removed, and thus more methane escapes to the atmosphere. Another short-term climatic factor influencing the degradation or movement of methane is atmospheric pressure change. This facilitates either the penetration of oxygen into the system or the release of methane to the atmosphere.

One of the major challenges in developing methanotroph-related measures into indicators of climate change is the need to differentiate between short-term climatic variability and the long-term effects of global warming. The need to reveal changes in the presence, structure and activity of methanotrophs over years, decades or even centuries, due to slight but permanent changes in climate, is heavily biased by short-term seasonal variability that often has dramatic effects on microbial populations in the soil. Tools are needed to enable descriptions of the methanotroph communities that on the one hand virtually eliminate the short-term fluctuations and on the other hand are sensitive enough to reveal the long-term changes.

Degradation of halogenated compounds

Another important aspect of methanotrophs is their capacity to degrade a number of halogenated C-1 compounds (halomethanes) and C-2 compounds (haloethenes and haloethanes). The class of halocarbons reported to be degradable by methanotrophs includes various chlorinated, fluori-

nated and brominated hydrocarbons (Table 1). These compounds have been or are still used in numerous applications such as pesticides (fumigants), solvents (e.g. in degreasing agents and dry cleaning agents), refrigerating aggregates and foaming agents. Such compounds have ozone-depleting or global warming potentials and are toxic to animals and humans. Due to the widespread use and high chemical stability of many of these compounds, some can be found in groundwater aquifers while others accumulate in the stratosphere. The methane monooxygenases of the methanotrophs responsible for oxidation of methane to methanol are also able to oxidise a variety of halogenated C-1 and C-2 compounds. The degradation of these compounds is co-metabolic, meaning that the methanotrophs do not gain energy from the reactions. Consequently, rates of conversion are very low when compared, for example, to the oxidation of methane.

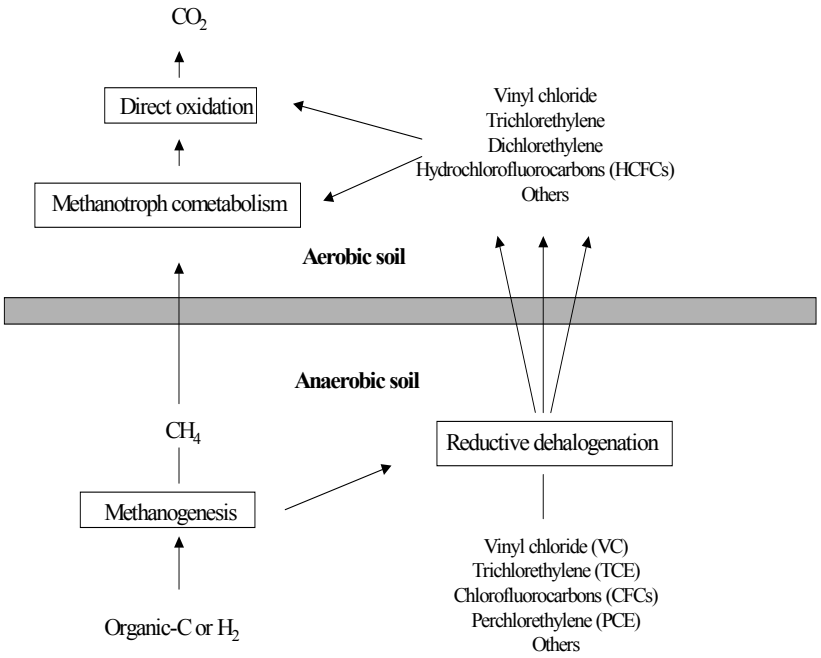

Fig. 4. Pathways of anaerobic and aerobic dehalogenation.

Biological dehalogenation (the removal of chlorine, bromine, or fluorine atoms from the compounds) proceeds in both anaerobic and aerobic environments (Fig. 4). However, fully halogenated compounds such as CFC gases or perchloroethylene will only be degraded by anaerobic reductive dehalogenation. In the aerobic soil layers, halocarbons with one or more hydrogen atoms (e.g. HCFC gases, TCE (trichloroethylene) and other

compounds in Table 1) can be degraded either by the co-metabolic process of the methanotrophs or by direct oxidation in an energy-yielding process performed by other aerobic bacteria (Lee et al., 1998). The degradation of many halocarbons very likely involves the activity of different micro-organisms that can live in close association to each other. In other cases compounds that are partly dehalogenated in anaerobic environments diffuse to aerobic soil layers, where they finally become fully dehalogenated and thus detoxified.

The presence and activity of methanotrophs as indicators of a potential for degradation of some halocarbons in the aerobic environment is very significant, but the exact functions of methanotrophs in the degradation process are still uncertain. They may participate directly in co-metabolic degradation of harmful compounds, or their metabolism of methane may provide organic carbon that is used by other micro-organisms participating in the dehalogenation process. It is very likely that methanotrophs perform both of these functions.

Table 1: Compounds reported to be transformed by- or in association with the activity of methanotrophs.

Compound name	Compound formula	Organism/community	Reference
Dichlorofluoromethane (HCFC-21)	$CHCl_2F$	*Methylosinus trichosporium* OB3b numerous landfill and wetland soil metha-notrph isolates agricultural soil landfill soil	Oremland et al., 1996 This study DeFlaun et al., 1992 Scheutz & Kjeldsen, 2003
Chlorodifluoromethane (HCFC-22)	$CHClF_2$	numerous landfill and wetland soil metha-notrph isolates. landfill soil. Methanotrophic mixed culture.	This study Scheutz & Kjeldsen, 2003 Chang & Criddle, 1995
1,1-dichloro-1-fluoromethane (HCFC-141b) 1,1,2-trichloro-2-fluoroethane (HCFC-131) 1,1,2-trifluoroethane (HCFC-143)	CH_3CFCl_2 $C_2H_2FCl_3$ $C_2H_3F_3$	*Methylosinus trichosporium* OB3b	DeFlaun et al., 1992
1-chloro-1,1-difluoroethane (HCFC-142b) 1,1-dichloro-2,2,2-trifluoroethane (HCFC-123) 1,2,2,2-tetrafluoro-ethane (HFC-134)	CH_3CF_2Cl $C_2F_3HCl_2$ CHF_2CHF_2	Methanotrophic mixed culture	Chang & Criddle, 1995
Dichloromethane (DCM)	CH_2Cl_2	*Methylosinus trichosporium* OB3b, *Methylomicrobium album* BG8 *Methylococcus capsulatus* Methanotroph culture (CAC1). Methanotro-phic mixed culture	Han et al., 1999 Oremland et al., 1994 Chang & Alvarez-Cohen 1996
Chloroform	$CHCl_3$	*Methylosinus trichosporium* OB3b *Methylococcus capsulatus* Methanotroph culture CAC1 Mixed chemostate culture mixed batch culture pure CAC1 methanotroph culture	Oremland et al., 1994 Chang & Alvarez-Cohen, 1996 Alvarez-Cohen & McCarty, 1991
Methyl Bromide	CH3Br	Methylococcus capsulatus	Oremland et al., 1994
Dibromomethane	CH2Br2	Methylomicrobium album BG8	Han et al., 1999

Trichloroethylene (TCE)	C2HCl3	Methylosinus trichosporium OB3b Methylosporovibrio methanica 81Z Methylocystis parvus OBBP? Methylococcus capsulatus Bath Methylomicrobium album BG8 Methylomonas sp. MN Methylomonas sp. A45 Methanotroph cultures (CACx). Methano-trophic mixed culture	Lontoh & Semrau, 1998 van Hylckama Vlieg et al., 1996 Oldenhuis et al., 1991 Chang & Alvarez-Cohen, 1996 Chu and Alvarez-Cohen, 1998 Chu and Alvarez-Choen, 2000 Alvarez-Cohen & McCarty, 1991 Brusseau et al., 1990 DiSpirito et al., 1992 Han et al., 1999
Vinyl chloride	C2H3Cl	Methylosinus trichosporium OB3b Methylomicrobium album BG8 Methylococcus capsulatus Methanotroph culture CAC1 Mixed chemostate culture mixed batch culture	Han et al., 1999 Oremland et al., 1994 Chang & Alvarez-Cohen, 1996
1,1-dichloroethylene trans-dichloroethylene cis-dichloroethylene	C2H2Cl2	Methylosinus trichosporium OB3b Methylomicrobium album BG8 Methylococcus capsulatus Methanotroph culture CAC1 Mixed chemostate culture mixed batch culture	Han et al., 1999 Oremland et al., 1994 van Hylckama Vlieg et al., 1996 Chang & Alvarez-Cohen, 1996
1,2 Dichloroethane	$C_2H_4Cl_2$	Methylosinus trichosporium OB3b Methylococcus capsulatus Methanotroph culture CAC1 Mixed chemostate culture mixed batch culture pure CAC1 methanotroph culture;	Oremland et al., 1994 Chang & Alvarez-Cohen, 1996
Chloromethane, 1,1,1-trichloroethane Chloroethane Ethane Ethene	CH_3Cl $C_2H_3Cl_3$ C_2H_5Cl C_2H_6 C_2H_4	Methylosinus trichosporium OB3b Methanotroph culture CAC1 Mixed chemostate culture mixed batch culture pure CAC1 methanotroph culture	Chang & Alvarez-Cohen, 1996
Chloroacetonitrile	$ClCH_2CN$	*M. trichosporium* OB3b	Castro et al., 1996

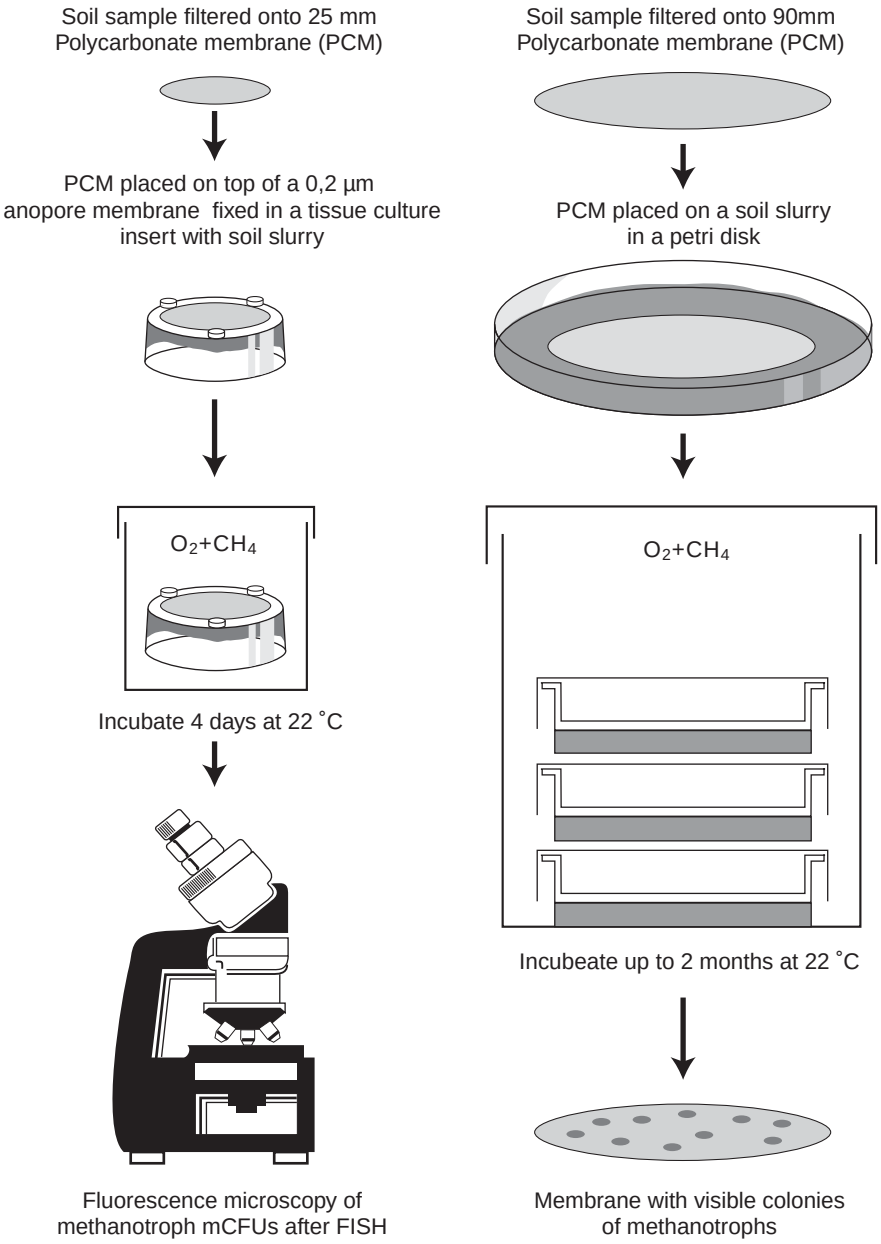

Fig. 5. Outline of the SSMS approach in the present study. Left: incubation and preparation of samples for microscopy. Right: incubation and preparation for quantification and isolation from visible methanotroph colonies.

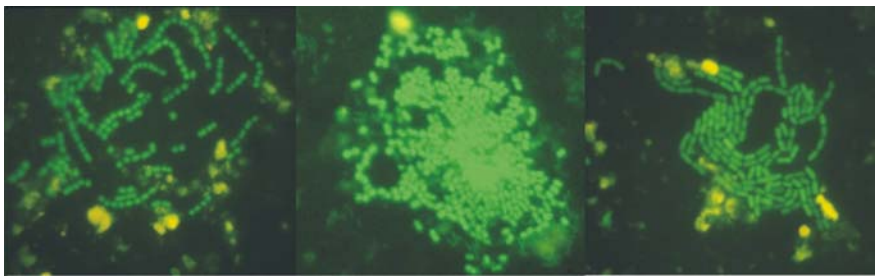

Fig. 6. Three day old microcolonies of type I methanotrophic bacteria hybridised with specific fluorescent DNA probes (630x magnification).

Traditional quantification and isolation of methanotrophs from environmental samples are biased by the growth of several other non-methanotrophic bacteria on the growth substrates, and these approaches have other serious drawbacks that typically underestimate the importance of methanotrophs in the environment. The highly selective growth of methanotrophs in our SSMS approach is apparently due to the non-sterile soil slurry used as the medium. Microbes in this slurry (which is void of methanotrophs) remove other organic carbon compounds produced by the methanotrophs growing on the filter. The slurry thus acts as an organic carbon sink that continuously prevents proliferation of other non-methanotrophic bacteria on the membrane filter.

The unique ability of the SSMS approach to isolate many different methanotrophic bacteria and study their physiological characteristics and species affiliations will offer new insights into the presence and function of methanotrophs in the environment. Most of the current knowledge about the physiology of methane oxidation and co-oxidation of other environmentally harmful compounds is based on a few comprehensively studied strains. It remains unclear how representative those few strains are for native methanotroph communities in different environments.

The development of methanotroph measures into environmental indicators requires more knowledge about the structure of methanotroph communities and about the physiological versatility of individuals that dominate the environment. Application of the SSMS approach should offer a better understanding of these features.

In our study, we obtained 22 different methanotroph isolates by the SSMS approach. These have been characterised (Svenning et al., 2003) and subsequently studied with respect to temperature optima and co-metabolism of the two environmentally harmful hydrochlorofluorocarbons HCFC-21 and HCFC-22. Results from these experiments are presented in the following two sections.

Further genetic analyses of the methanotrophs showed that the strains from the landfill soil at Skellingsted belonged to the same type II genus: *Methylosinus*. The strains from the wetland soil included type I and type II methanotrophs and could be grouped into the two genera *Methylobacter* and *Methylosinus*.

Direct community studies by DGGE

Molecular methods represent important tools for studying microbial communities *in situ*. These methods allow us to compare and follow microbial communities at different sites and over time, e.g. through seasons and years. The analyses are based on genetic material extracted from soil samples. From this total pool of genetic material we can select the groups of interest for further analyses.

The method applied in this project is called DGGE: Denaturing Gradient Gel Electrophoresis. It is a rapid molecular method for profiling complex microbial populations (Fig. 8). The band patterns shown in Fig. 8 indicate differences and similarities between samples, and illustrate the complexity of the community. To identify more specifically which types of methanotrophs are represented in the fingerprint patterns, the fragments have to be removed from the gel and further analysed for sequence composition.

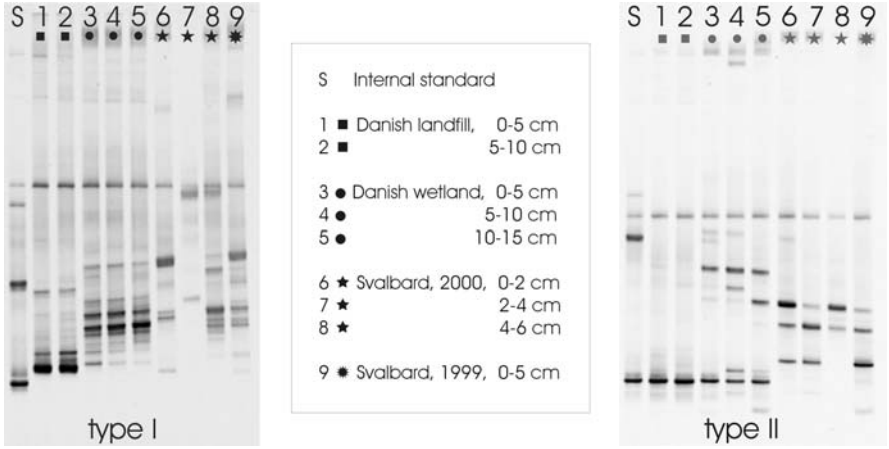

Fig. 8. DGGE community fingerprints of methanotrophic populations in soil from Denmark at 55°N and from Svalbard at 78°N. Left: type I methanotrophic profiles. Right: type II methanotrophic profiles.

DGGE analysis of soil samples from different Nordic high organic soils gave distinct community profiles from each sampling site (Fig. 8). Type I and type II methanotrophs were detected in both soil types (landfill and wetland). In general the wetland soils had more complex methanotroph

structures than the landfill soils. The profiles from the Danish wetland communities and those from northern high latitude wetlands have similar complexity. Within the sites, the profiles revealed small differences with respect to sampling depth. Samples from successive years gave similar profiles, indicating a stable methanotrophic community in each of the chosen sites, and thus allowed us to follow the composition of the methanotrophs over time.

Using the DGGE method, we were able to follow the stability and structure of the methanotroph communities both within and between seasons and for considerably longer periods. The method allowed us to describe methanotroph communities that were found to be stable between repeated samplings over years. This indicates that the method disregards temporal seasonal variation. The robustness of this direct molecular analysis of the methanotroph communities suggests that the method can possibly be developed into a useful indicator of long-term effects of global warming on the methanotroph communities, provided that more comprehensive studies prove the method to be sensitive to more permanent changes in the community structures.

Temperature responses in methanotrophic bacteria from different latitudes

Methanotrophic bacteria from a latitude gradient represented by soils collected in Ny-Ålesund (Svalbard, 78°N), Tromsø (Northern Norway, 69°N) and Seeland (Denmark, 55°N) were studied for temperature responses in the range 0-40°C. The results showed that psycrophilic bacteria with an optimal growth temperature below 20°C were not obtained from any of the sites. Growth studies gave optimal growth rates in the mesophilic range 20-30°C for all the studied isolates. Interestingly, the optimal temperatures and the growth curves measured in a controlled temperature gradient (Fig. 9) did not differ between localities. However, two characteristic growth profiles were observed, differentiating between the type I and type II isolates. Type II methanotrophs had a typical mesophilic growth curve, with little or no growth below 10°C, rising rapidly to the optimal temperature. A much broader temperature curve was described for the type I methanotrophs, with recorded growth nearly down to freezing point.

Our studies did not show a correlation between latitude origin and growth pattern of the methanotrophs within the temperature range 0-40°C. The northern methanotrophs have a mesophilic growth pattern.

However, the temperature gradient studies showed a different growth profile for type I methanotrophs than for type II: type I methanotrophs have a more psychotropic growth, allowing them to oxidize methane at lower temperatures than type II. The presence of type I methanotrophs in

high organic soil in sub-Arctic and Arctic climates may therefore be significant for the potential of methane removal in these soils. At present, methane production at high latitudes is restricted by low temperature, but this situation may change.

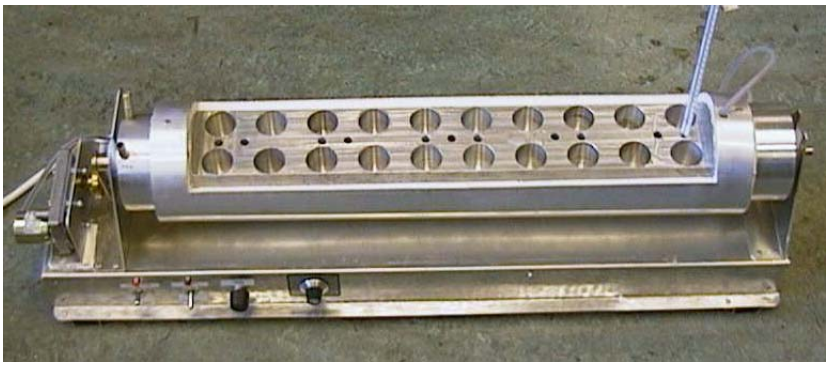

Fig. 9. Photograph of the temperature gradient equipment with two rows of holes for 27ml serum bottles and a series of smaller holes along the centre for thermometers. The left end attaches to a cooling water bath and the right end to a thermostat-regulated heater to induce a temperature gradient along the cylinder.

grade HCFC-21 and HCFC-22 gases. Landfill and wetland methanotrophs from the SSMS incubation, described in chapter 4, were selected and studied. The wetland isolates near Roskilde fjord was chosen as a reference, representing a non-polluted site. In addition to environmental origin, the presence and expression of the sMMO enzyme (considered important for successful dehalogenation) and species affiliation were considered (different species may have different degradation capacities). Finally, two comprehensively studied strains of methanotrophs (*Methylosinus trichosporium* OB3b and *Methylomicrobium album* BG8) were included as representatives of known strains with a capacity for co-metabolism of different halogenated methanes (see Table 1). It was hypothesised that the methanotroph community at this site somehow mirrors the flux of methane in high concentrations and the flux of chlorofluoromethanes (HCFC gases) through the surface soil layers.

The HCFC degradation potential was studied in sealed 35ml flasks containing 4ml bacterial culture adjusted to the same density (approximating the number of methanotrophs found *in situ* at the landfill site) and 100 µg/l HCFC-21 or HCFC-22 gas (concentrations equivalent to those locally found *in situ* at the landfill site). The disappearance of HCFC gases was measured after 22 hours by gas chromatography.

Copper is assumed to have a key function in the expression of the sMMO enzyme, which is also important in the degradation of HCFC gases. The biodegradation assay involving cultures of our methanotrophs was therefore performed with and without copper. This should reveal any copper dependent efficacy in the enzymatic dehalogenation process. Finally, the assay was performed with a co-substrate (formate) to ensure that the cultures were able to generate sufficient reducing power to maintain optimal activity of the MMO enzyme during co-metabolism (refer to chapter 2 for more details of methanotroph metabolism).

The results showed variable capacities for removal of HCFC-21 among different isolates of the tested methanotrophs (Table 2), but no significant capacity for degradation of HCFC-22 (data not presented). In no case was more than 25% of the added HCFC-21 degraded during incubation. Two of the strains did not show any significant degradation. Further, we were not able to demonstrate a clear correlation between the presence of and capacity to express the soluble MMO gene and successful degradation of HCFC-21.

Table 2. Capacities of different methanotrophs to degrade HCFC-21. The methanotrophs were isolated from a landfill site, a wetland area and obtained from bacterial culture deposits. The percentage of HCFC-21 (100ug/l) removed during 20-hour incubation of cultures grown with or without copper is indicated. Cultures grown without copper should express the soluble methane monooxygenase (sMMO), which is considered important for co-metabolic dehalogenation.

Isolate	Closest relative species	Origin	Type	*mmoX* genotype	sMMO phenotype	HCFC-21 (%)	
						Plus Cu	Minus Cu
BG8	*Methylomicrobium album*	strain collection	I	no	no	21,1	19,6
MSB3	*Methylobacter*	wetland soil	I	no	-[a)]	20,2	16,1
MSB8	*Methylobacter*	wetland soil	I	yes	-[a)]	25,1	16,3
MSB5	nd	wetland soil	I	no	yes	15,8	12,9
MSB7	nd	wetland soil	I	no	no	17,1	15,9
OB3b	*Methylosinus trichosporium*	strain collection	II	yes	yes	-[b)]	-[b)]
MSB6	*Methylosinus trichosporium*	wetland soil	II	yes	yes	10,4	21,8
MSB13	*Methylosinus trichosporium*	landfill soil	II	yes	-[a)]	23,5	10,6
MSB18	*Methylosinus trichosporium*	landfill soil	II	yes	yes	13,5	15,0
MSB12	*Methylosinus trichosporium*	landfill soil	II	no	yes	-[b)]	-[b)]
MSB14	*Methylosinus trichosporium*	landfill soil	II	no	-[a)]	15,3	13,3
MSB16	*Methylosinus* sp	landfill soil	II	yes	yes	15,9	9,2
MSB1	*Methylosinus sporium*	wetland soil	II	yes	yes	12,4	11,9
MSB15	*Methylosinus sporium*	landfill soil	II	yes	yes	7,2	13,8
MSB22	*Methylosinus sporium*	landfill soil	II	no	-[a)]	14,5	8,8

[a)] sMMO phenotype could not determined.
[b)] HCFC-gas concentrations were not significant different from controls.

No systematic differences in degradation were seen between the Cu-present and Cu-deficient assays, and no correlation was revealed between presence or absence of sMMO and the HCFC-21 degradation potential. These observations raise new questions about the specific role of the soluble versus the particulate methane monooxygenase enzyme in the co-metabolism of halogenated hydrocarbons such as HCFC-21.

It was clear from our HCFC-21 degradation experiments that complete degradation never occurred. Even when observed over time, one of our most efficient strains (MSB13) never demonstrated more than 25 % removal (Fig. 11), indicating inhibition of the organisms either by irreversible HCFC-21 binding to the MMO or by accumulation of produced toxic intermediates that prevented further degradation.

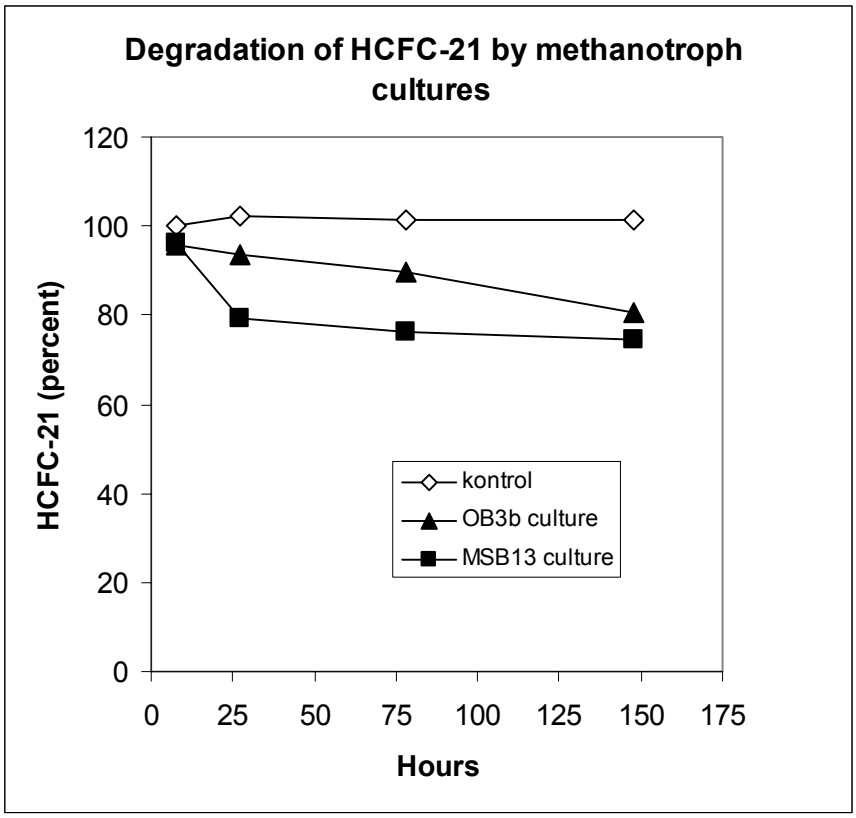

Fig. 11. Degradation of 100 µg/l HCFC-21 by *Methylosinus trichosporium* OB3b, a comprehensively studied strain of methanotrophs in the literature, and the methanotroph MSB13 from Skellingsted landfill isolated in this project.

The restricted degradation of HCFC-21 by very dense populations of pure methanotroph cultures contrasts with the frequent high concentrations of HCFC-21 (and HCFC-22) observed in the aerobic landfill topsoils. One hypothesis is that the high activity of methanotrophs in locations of methane production supports growth of other micro-organisms, some of which are able to dehalogenate the halocarbons by direct oxidation (see Fig. 4). Another explanation could be that the methanotrophs *de facto* participate

in degradation of the HCFC-21, but do so in consort with other micro-organisms that are able to remove toxic intermediates.

New SSMS incubations of Skellingsted landfill soils were performed, this time with either methane (CH_4) or HCFC-21 as the only substrates or with methane (CH_4) and HCFC-21 in combination. The purpose of the incubations was to quantify, isolate and characterise the HCFC-21 degrading bacteria that dominate the landfill soil.

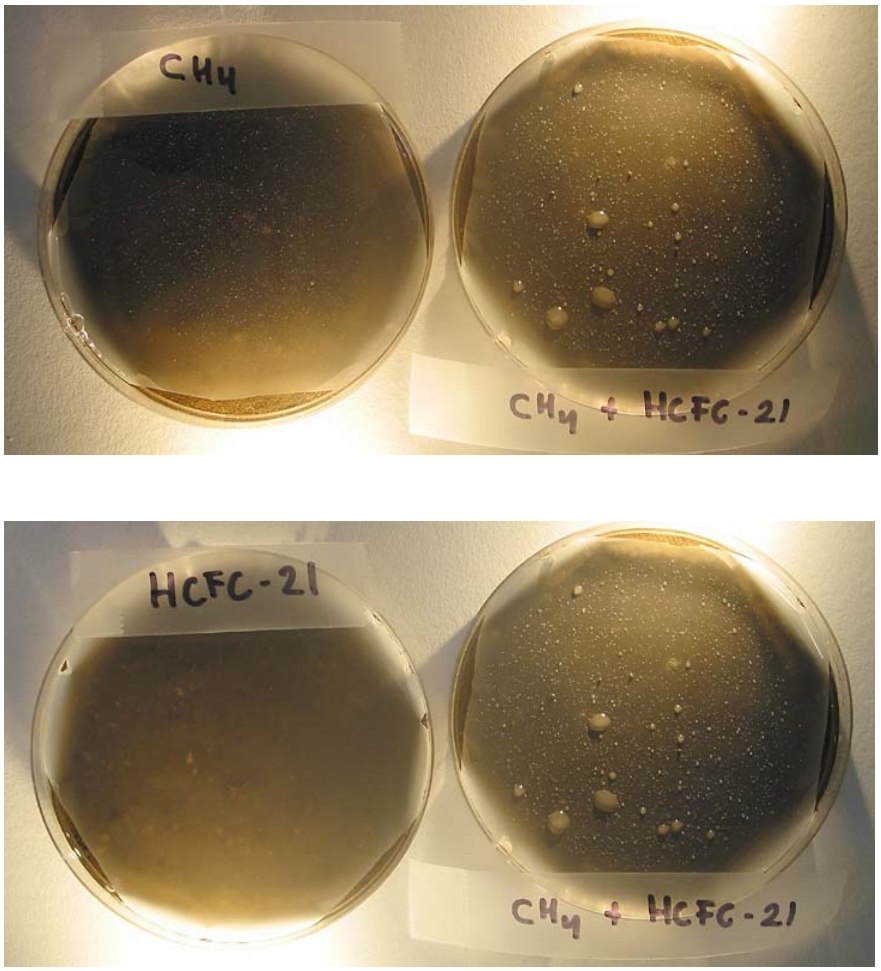

Fig. 12. Appearance of colonies on membranes after 1 month of SSMS incubation in atmospheres of methane (upper left), HCFC-21 (lower left) and HCFC-21 + methane (upper and lower right).

No growth was observed in the dishes containing only HCFC-21, while colonies were clearly visible in the dishes containing HCFC-21 + methane (Fig. 12). This indicates that the methanotrophs *do* play a key role in the degradation of HCFC-21. Quite surprisingly, colony development was more pronounced after the combined HCFC-21 + methane incubation than after incubation in a methane atmosphere only. The methanotrophs living in consort with other (unknown) bacteria thus seem to pro-

liferate more successfully when both methane and HCFC-21 is available during incubation. Although the methanotrophs appear to be the key organisms in HCFC-21 removal in this landfill soil, further investigation is required to establish the exact role of the methanotrophs in this context.

probes specific to the two methanotroph categories are available. The FISH method can be used as a true *in situ* method or be combined with the SSMS cultivation assay described in chapter 4.

The molecular approach (DGGE), employed to describe the diversity of methanotrophs within type I and type II, was found to be very robust and nearly identical diversity profiles were demonstrated over years (see chapter 5). From an indicator perspective this appears to be promising. However, the sensitivity of the approach towards more permanent environmental or climatic changes still needs to be demonstrated. Additionally, a basic understanding of the relationship between molecular diversity and environmental change needs to be developed.

Finally we investigated the ability of a variety of methanotrophs isolated from Danish landfill and wetland locations to degrade two hydrochlorofluorocarbons (HCFC-21 and HCFC-22) of environmental concern. However, we deduced no simple correlation between the HCFC degradation capacity and the type of methanotrophs exposed to the compound. This is contradictory to other studies which show that the type II methanotrophs expressing the soluble variant of methane monooxygenase (sMMO) are primary responsible for the co-metabolic dehalogenation. Even so, our data suggests that the presence of methanotrophs in the environment also indicates a potential for biodegradation of different hydrogenated halocarbons. More knowledge about the role of methanotrophs in this biodegradation process is required before it becomes practicable to use the presence of methanotrophs as either an indicator of environmental pollutants or as an indicator of an environment with a high bioremediation capacity.

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