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"Environental Assessment of Methane Oxidizers Nitrite Driven Bacteria"

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V˚B˚ of the ACADEMIC SUPERVISOR for the presentation and defense of the present memory:

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All the information presented in this research was obtained and analyzed in this institute and all data are owned by NIOO-KNAW.

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"Those who dream by day are cognizant of many things which escape those who dream only by night."
Edgar Allan Poe

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1. Resumen

El grupo de bacterias anaeróbicas N-DAMO llevan a cabo el proceso de oxidación del metano a través de la desnitrificación. Las bacterias N-DAMO fueron descubiertas en la década pasada y muy poco es conocido sobre su distribución ambiental y su contribución a la oxidación del metano (CH_4). Debido a que el metano es un problema para el medio ambiente por su contribución al calentamiento global, es muy importante investigar sobre metodologías que puedan ayudar a reducir los niveles de metano en la naturaleza.

El propósito de este proyecto es establecer técnicas moleculares y su aplicación en investigaciones de ecología microbiológica. En este proyecto el caso de estudio considerado es la distribución de metanotrofos anaerobios pertenecientes al grupo NC10 en muestras ambientales (lagos europeos, campos abandonados, áreas urbanas y áreas con animales domésticos).

La razón de escoger el uso de técnicas moleculares en esta investigación es debido a la dificultad de encontrar bacterias N-DAMO en el medio ambiente, debido a su distribución en pequeñas poblaciones y por su compleja tarea de cultivo o aislamiento. Además, el uso de métodos moleculares permite trabajar y obtener resultados rápidamente.

Una vez establecidas las técnicas moleculares utilizadas para la investigación (extracción de ADN y cadena de reacción de la Polymerasa (PCR)), éstas fueron optimizadas para detectar bacterias N-DAMO en las muestras a analizar.

Nuestros resultados muestran la presencia de bacterias N-DAMO en ecosistemas acuáticos. Conociendo que estos hábitats son los mayores contribuidores de las emisiones de metano, la presencia de bacterias N-DAMO en estos ecosistemas abren una puerta a la investigación sobre la reducción de metano en estos ambientes.

2. Summary

The nitrite-dependent anaerobic methane oxidation (N-DAMO) bacteria has been discovered in the last decade and there is little known about its environmental distribution and contribution to the oxidation of methane (CH_4). Because CH_4 is of environmental concern due to its contribution to global warming, it has become very important to look for ways to reduce it.

The purpose of this thesis is the acquisition of established molecular tools and their application in microbial ecology investigations. The microbial ecology case study chosen regarded the distribution of anaerobe methanotrophs belonging to the NC10 phylum in environmental samples (European lakes, neglected fields, urban areas and farming areas).

The reason for using molecular tools in this research is due to the difficulty of finding N-DAMO bacteria in the environment, due to their small populations and because it is arduous to cultivate or isolate them. Furthermore, with molecular tools it is possible to work and get results quickly.

Already established molecular techniques (DNA extractions and Polymerase Chain Reaction (PCR)) were optimized to detect n-damo bacteria in these samples.

Our results show the presence of n-damo bacteria in freshwater ecosystems. Knowing that these habitats are the major contributors of methane emissions, the presence of n-DAMO in these ecosystems opens a new way to investigate the reductions of methane in freshwater environments.

3. Introduction

3.1 Molecular techniques in Ecology

Molecular ecology is defined as "the application of molecular techniques to answer ecological questions" (Beebee & Rowe 2004).

Since traditional ecological approaches are based on direct observations of organisms, they frequently do not detect underlying variations in organisms that do not influence physical appearances. The study distribution of an organism in the environment can be relatively simple to assess when organism can be directly observed. But when the organism is a bacteria for instance, it is impossible to study their population or distribution in the environment, it is in this case when molecular tools are chosen.

The aim of this research is to connect Environmental Sciences with molecular techniques where the uses of molecular tools are useful and sometimes necessary for ecological studies.

Over the last few decades, the field of microbial ecology has seen tremendous progress, and a wide variety of molecular techniques have been developed for describing and characterizing the phylogenetic and functional diversity of microorganisms. These techniques have been classified broadly into major categories depending on their capability of revealing the microbial diversity structure and function (Rastogi et al., 2011):

1. *Partial community analysis methods*

These strategies generally include polymerase chain reaction (PCR)-based methods where total DNA/RNA extracted from an environmental sample is used as a template for the characterization of microorganisms.

2. *Whole community analyses methods*

Whole-genome molecular techniques offer a more comprehensive view of genetic diversity compared to PCR-based molecular approaches that target only a single or a few genes. These

techniques attempt to analyze all the genetic information present in total DNA extracted from an environmental sample or pure culture.

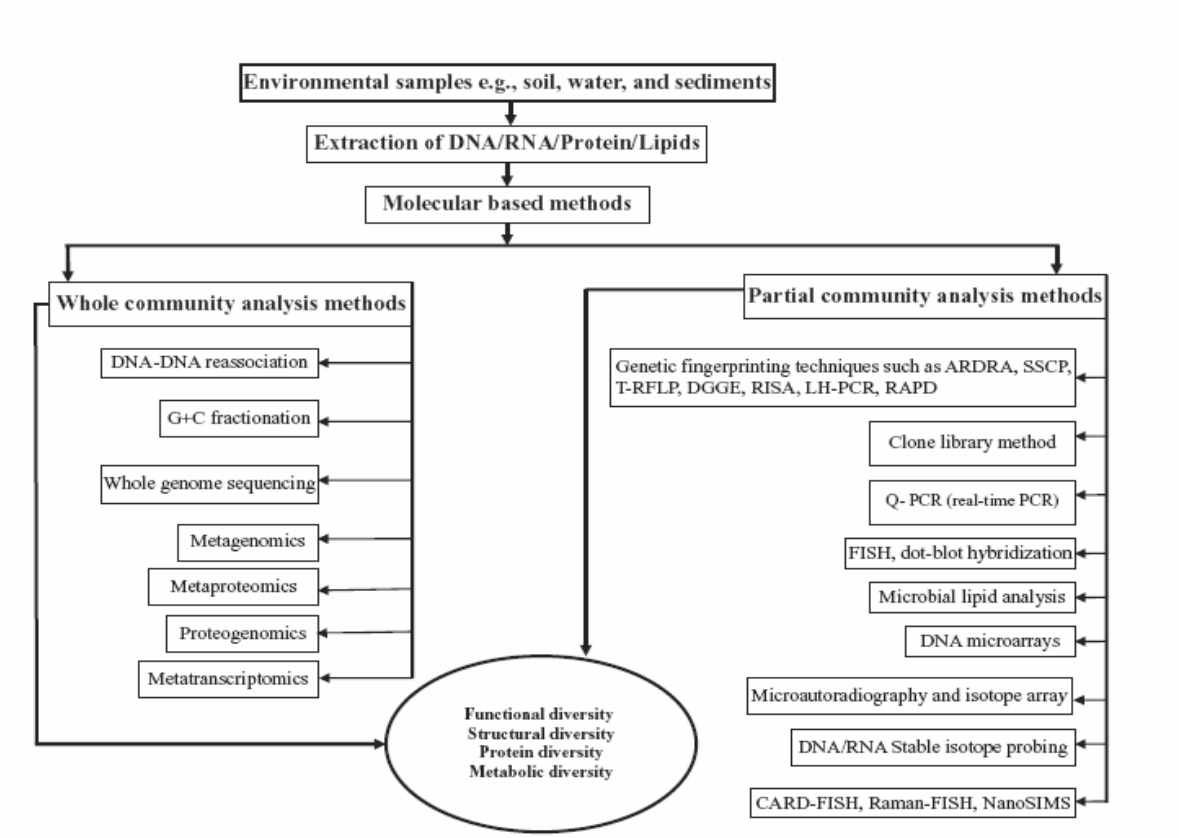


Figure 1. Molecular toolbox to characterize the structural and functional diversity of microorganisms in the environment (Rastogi et al., 2001)

Figure 1 shows all the molecular methods available in microbial ecology, where depending on the investigation is possible to chose one or different methods to carry out the investigation.

In this study, we focus on molecular techniques such as partial community analysis methods, where the use of DNA extraction and PCR techniques are useful in order to find anaerobe methane oxidizers in natural habitats. The decision to analyze the results with both techniques was because with DNA extraction we can obtain all the information we need from the environment (organisms, population…) and with the PCR it is possible to obtain specific information from the DNA extracted from the environment. The combination of both

techniques brings us all the information we need to know about the environmental distribution of methane oxidizers nitrite driven bacteria.

In restricted areas in anaerobic conditions, this type of bacteria lives; which makes it very difficult to find them because of the small amount of bacteria present. Thus, only with molecular techniques is possible to study the natural distribution of these bacteria.

3.2 Methane in the environment

Methane (CH_4) is mainly produced microbiologically in the final stage of anaerobic degradation of organic matter (Whalen, 2005). The current global budget of atmospheric methane is 500-600 Tg year^{-1}[1] (Conrad, 2009). Around 25% of all CH_4 emitted into the atmosphere is associated with mining and combustion of fossil fuel or with the burning of biomass (Conrad, 2009). But the emissions due to microbial processes are the most important. In fact, they can be accounted for 69% of the atmospheric CH_4 (Borrel et al., 2011).

Methanogenic bacteria are responsible for producing biogenic methane (Zeikus, 1977). They are a strictly anaerobic metabolic group that belongs to the Archaea. The anaerobic methabolism of CH_4 is carried out by using a narrow range of substrate (H_2/CO_2, formate, acetate, methanol, methylated-compound, CO, ethanol and secondary alcohol) which are generally the final products of organic matter degradation (Borrel et al., 2011).

Three main metabolic pathways for methane production have been identified: *Hydrogenotrophic methanogenesis*, *Acetoclastic methanogenesis* and *Methylotrophic methanogenesis* by Hedderich and Whitman (2006).

The importance of CH_4 in the atmosphere is due to its effect on global warming. Even though the amount of CH_4 concentration in the atmosphere is less than the one of carbon

[1] Tg = 1012 g

dioxide (CO_2), 1,7 ppmv/360 ppmv respectively (Van der Putte, 1995), it is the second largest contributor to the global warming of the planet after CO_2 (Hanson et al., 1996). Despite a short residence time in the atmosphere (about 10 years), the CH_4 ability to absorb infrared radiation makes it 20 to 30 times more efficient than CO_2 as a greenhouse gas (Blake & Rowland, 1988; Rodhe, 1990). CH_4 is mainly eliminated in the troposphere through oxidation by OH radicals. Furthermore, methane is also eliminated in soils by microbial oxidation, where they are responsible for 6% of all methane consumption (Le Mer et al., 2001)

Two biological pathways are involved in methane oxidation:

3.3 Aerobic methane oxidation

The aerobic methane oxidation pathway contains four sequential oxidation steps with methanol, formaldehyde and formate as metabolic intermediates (Borrel et al., 2011). In this pathway, oxygen is needed to oxidize methane in order to produce carbon dioxide.

All known aerobic methanotrophs are assigned to the domain of the Bacteria and constitute a polyphyletic group in which four families are distinguished (Borrel et al., 2011) and they are affiliated with:

- ɣ- Proteobacteria (Methylococcaceae, Methylocystaceae)
- α- Proteobacteria (Beijerinckiaceae)
- Verrumcombia (Methylacidiphilaceae)

3.4 Anaerobic methane oxidation (AMO)

Anaerobic methane oxidation is globally important because the methane is oxidized in environments without the presence of oxygen and needs an it external reduction component to obtain the oxygen necessary to oxidize the methane in the environment.

Three different pathways of anaerobic methane oxidation are described (Borrel et al., 2011):

1. AMO coupled to sulfate reduction: $CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O$ (ΔG^o = -14 KJ mol^{-1} CH_4)
2. AMO coupled to iron and manganese reduction

- AMO coupled to iron: $CH4 + 8Fe(OH)3 + 15H^+ \rightarrow HCO_3^- + 8Fe_2^+ + 21H2O$ (ΔG^o = -572 KJ mol^{-1} CH_4)
- AMO coupled to manganese: $CH_4 + 4MnO_2 + 7H^+ \rightarrow HCO_3^- + 4Mn^{2+} + 5H_2O$ (ΔG^o = -790 KJ mol^{-1} CH_4)

3. AMO coupled to denitrification: $3CH_4 + 8NO_2^- + 8H^+ \rightarrow 3CO_2 + 4N_2 + 10H_2O$ (ΔG° = -987 kJ/mol CH_4)

So far only ine type of N-DAMO bacteria, the NC10 group, has been able to consume methane and reduce nitrogen (Ettwig et al., 2010). Experimental evidence for the methane oxidation coupled with denitrification was first obtained from environmental samples originating from freshwater contaminated aquifer, these samples contained high concentrations of nitrate (until 16 mg of N per liter) (Smith et al., 1991). Raghoebarsing et al. (2006) demonstrated the presence of N-DAMO in enrichment cultures obtained from sediments of two freshwater ecosystems with high nitrate concentrations. This enrichment culture was dominated by a new bacterial division called NC10 (Ettwig et al., 2008). Using molecular tools, complete genome analysis of the enrichment was carried out and named *Candidatus Methylomirabilis oxifera,* which was shown to be affiliated with NC10.

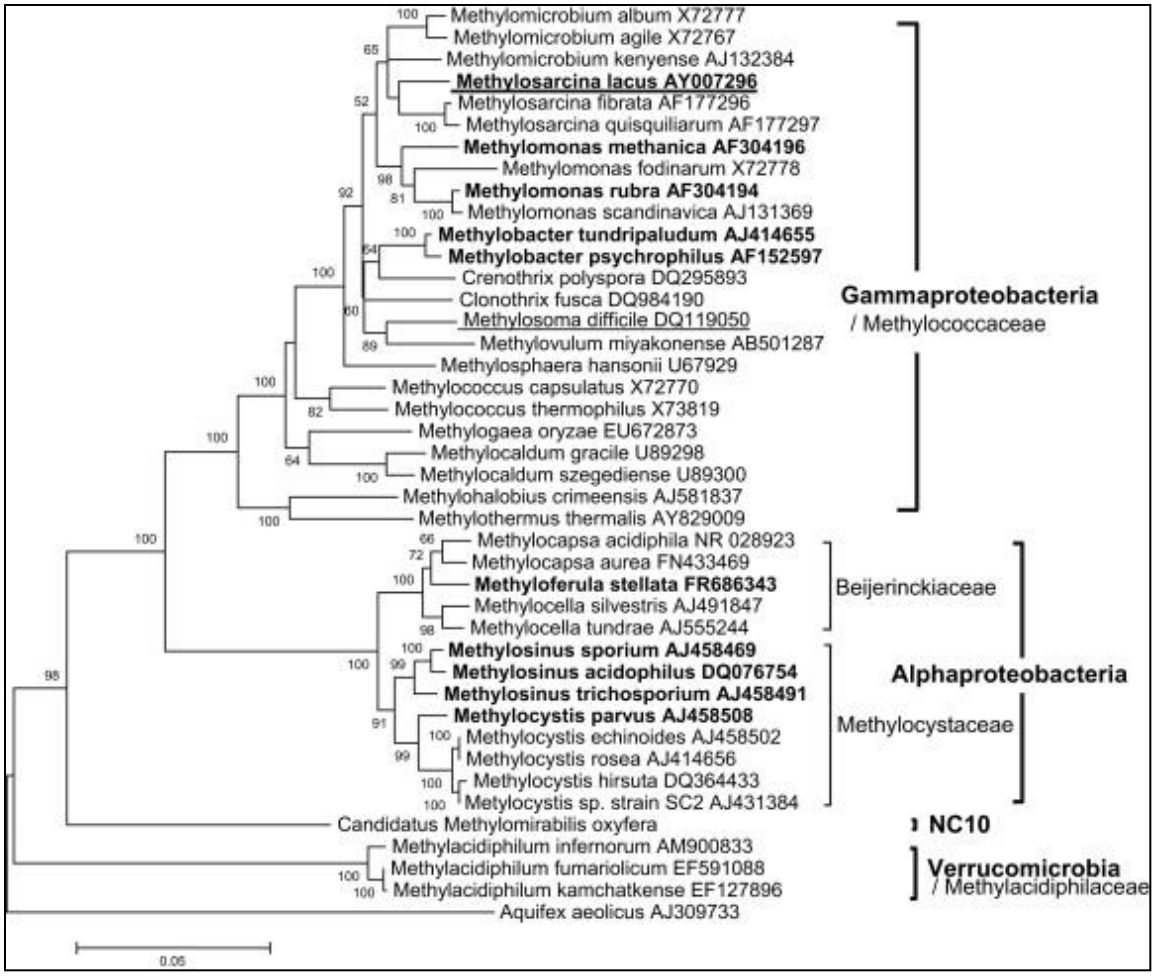


Figure 2. Phylogenetic tree illustrating relationships between the four families of aerobic methanotrophs and anaerobic methanotroph "*Candidatus Methylomirabilis Oxyfera*"(division NC10), all belonging to the bacterial domain (Borrel et al., 2011)

The pathway carried out by *Candidatus Methylomirabilis oxyfera*, is the process where methane is oxidized in anaerobic conditions, this bacteria can reduce nitrate (NO_3^-) and nitrite (NO_2^-) through nitric oxide (NO) to nitrous oxide (NO_2) and/or dinitrogen gas (N_2) in this way obtaining oxygen (Ettwig et al., 2010). The intracellularly produced oxygen is then used for the oxidation of methane by the classical aerobic methane oxidation pathway involving methane mono-oxygenase (Wu et al., 2011).

Figure 3 shows three significant pathways, where (a) is the canonical pathways of denitrification, (b) is aerobic methane oxidation and (c) is the proposed pathway of methane oxidation with nitrite of *Methylomirabilis oxifera* (Ettwig et al., 2010)

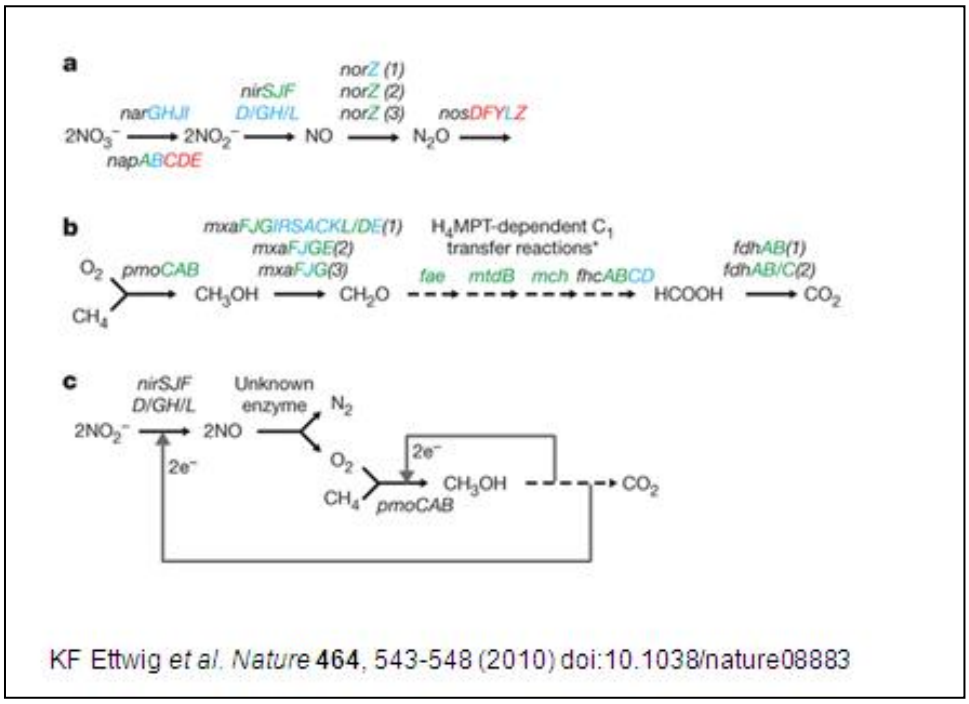


Figure 3. Significant pathways, a) canonical pathway of denitrification; b)aerobic methane oxidation; c)pathway of *Methylmirabilis oxyfera*.

Where narGHJI, nitrate reductase; *napABCDE*, periplasmic nitrate reductase; *nirSJFD/GH/L*, nitrite reductase; *norZ*, nitric oxide reductase; *nosDFYLZ*, nitrous oxide reductase; *pmoCAB*, particulate methane monooxygenase; *mxaFJGIRSACKL/DE*, methanol dehydrogenase; *fae*, formaldehyde-activating enzyme; *mtdB*, methylene-tetrahydromethanopterin (H_4MPT) dehydrogenase; *mch*, methenyl-H_4MPT cyclohydrolase; *fhcABCD*, formyltransferase/hydrolase; *fdhABC*, formate dehydrogenase. Genes in red are absent from the genome, those in blue are present in the genome and the genes in green are present in both the proteome and the genome. Asterisk, H_4MPT-dependent reactions involve the intermediates methylene-H_4MPT, methenyl-H_4MPT and formyl-H_4MPT.

In summary, the process of anaerobic methane oxidation coupled with denitrification refers to the following reaction of methane with nitrite (or nitrate) as oxidant (Wu et al., 2011):

$$3CH_4 + 8NO_2^- + 8H^+ \rightarrow 3CO_2 + 4N_2 + 10H_2O \qquad (\Delta G^\circ = -987 \text{ kJ/mol of methane})$$

This reaction is highly exergonic and yields free energy values comparable to those with oxygen (O_2) as electron acceptor. Aerobic methanotrophs overcome this high activation energy by using O_2 as a highly reactive co-substrate for the initial attack in a reaction catalyzed by methane monooxygenase (MMO)(Wu et al., 2011). It appears that *Candidatus Methylomirabilis oxyfera* is capable of producing O_2 via a new intra-aerobic pathway, which involves the dismutation of NO (nitric oxide) into O_2 and N_2 (nitrogen) (Etwig et al., 2011).

The ability of methanotrophs to oxidize methane is conferred by a methane monooxygenase (MMO) enzyme that converts methane to methanol. There are two types of MMO enzyme: a cytoplasmatic version (sMMO) and a membrane-bound version (pMMO). All methanotrophs including N-DAMO bacteria belonging to the NC10 phylum such as *Candidatus Methylomirabilis oxyfera* have pMMO enzyme in common. Each enzyme is controlled by a specific functional gene; in the pMMO enzyme is the pmoA functional gene which actives the pMMO enzyme to convert methane to methanol. (Dumond et al., 2005).

The alpha subunit of PMMO, encoded by the pmoA gene, is highly conserved and often used as a functional marker for analyzing methanotrophs in the environment. As the pmoA gene phylogeny is largely comparable to the 16S rRNA gene phylogeny (Holmes et al.1995), pmoA primers[2] provide a useful tool for obtaining simultaneous functional and taxonomic inventories of methanotrophs in the environments (Luesken et al., 2011).

[2] Primer: A primer is a strand of nucleic acid that serves as a starting point for DNA synthesis. They are required for DNA replication because the enzymes that catalyze this process, DNA polymerases, can only add new nucleotides

3.5 Study case

Since their discovery, methanotrophs were found in a variety of ecosystems, including soils, wetlands, freshwater and marine habitats.

In this study we analyze the habitat of N-DAMO bacteria belonging to NC10 phylum with molecular tools, in order to show more information about their environmental distribution. According to the bacteria, that seems to have a preference with fresh water sediments, we analyzed a large number of soil sediments from European lakes but did not discard the probability of finding these bacteria in other environmental areas in anaerobic conditions, where concentrations of methane and nitrates are present, due to the preferential habitat characteristics of these bacteria.

Wetlands and lakes soils are regarded as an important source of atmospheric methane. The contribution of these ecosystems to the global methane emission has been estimated at 55% (IPCC, 1994). According to previous studies, where it is known that the N-DAMO bacteria were found in freshwater sediments (Raghoebarsing et al., 2006), we monitored different lakes from Europe to obtain more information about environmental habitats where this type of bacteria can be found. For our research we studied lakes in the European countries of The Netherlands, Denmark, Switzerland, Finland and Sweden.

According to Le Mer & Rowe (2001), an environment is a methane source when the balance between production by methanogenic bacteria and consumption by methanotrophic bacteria is positive, leading to methane emission. When the balance is negative, the environment is a methane sink. Thus, in non-flooded upland soils, such as crop fields, forest or grasslands are regarded as the only biological sink of atmospheric methane and are responsible for 6% of the global methane consumption. For this reason, we decided to analyze neglected fields where methane is present because of the degradation of organic matter and because at one time the fields were fertilized and cultivated. However, over the years they were neglected and it is interesting to know if there is any relationship between the stage of succession of the fields and

the presence or absence of methanotrophic bacteria coupled with denitrification. This was our goal when we analyzed soils from different stages of succession; neglected fields between 15 and 23 years without cropping and neglected fields between 23 and 29 years without cropping. The soil was sampled from The Hoge Veluwe-National Park (The Netherlands).

Furthermore, soil samples from ditches and pools in Wageningen (The Netherlands) were taken. These samples were taken because these two ecosystems seemed to be anaerobic.

Figure 4. Samples of soil in Wageningen (The Netherlands)

Compost soils in anaerobic conditions were taken as well.

Thus, with these soil samples, we analyze the possible distribution of N-DAMO bacteria belonging to NC10 in the environment using molecular tools. In order to find this group of bacteria, functional gen pmoA related to NC10 was used for the identification of these bacteria in the environment. To effectuate this identification, DNA extractions and Polymerase Chain reaction (PCR) were carried out.

Thus, using molecular tools such as DNA extraction and PCR techniques in Environmental ecology, we expect to find the N-DAMO bacteria belonging to NC10 phylum such as *Candidatus Methylomirabilis oxyfera* in natural habitats.

4. Materials and methods

4.1 Soil Samples

Between 0.1 and 0.3 g wet weight (ww) per sample was used for DNA extraction. For molecular analyses of N-DAMO bacteria under field conditions, samples were stored at -20˚C. The soil samples used in this research are the following:

Soil Sample	Study Area	Soil Sample	Study Area
Mos.Veld	Neglected field	**Lak.NIOO**	Urban Area
De.Mos	Neglected field	**DLC**	Urban Area
Dit.NIOOS	Urban Area	**DLNC**	Urban Area
Dit.NIOOD	Urban Area	**BioF**	Farming Area
Dig	Farming Area	**Hacro**	Farming Area
Recro	Farming Area		

Soil Sample	Study Area	Soil Sample	Study Area
BUR	European lake	LAU	European lake
ERS	European lake	**LIL**	European lake
FP	European lake	**LOV**	European lake
GA	European lake	**MEK**	European lake
GLI	European lake	**MRH**	European lake
GRI	European lake	**NIM**	European lake
HAS	European lake	**NUS**	European lake
HAR	European lake	**ROT**	European lake
HIN	European lake	**SCW**	European lake
HUT	European lake	**SEE**	European lake
ILR	European lake	**SGL**	European lake
JYV	European lake	**SKO**	European lake
KIS	European lake	**ST**	European lake
LA	European lake	**STV**	European lake

Table 1. List of the samples used in this study.

4.2 DNA extraction

DNA extraction solution prepared:

-120 mM of sodium phosphate buffer ($NaPo_4$) (pH8) with 6,02g Na_2HPO_4 + 2 H_2O (12.87mM); 0,33g NaH_2PO_4 + 2 H_2O (7,12mM) and 300 ml of MiliQ water.

- TNS (Total nucleic acid solution) with 0,29g NaCl(100mM); 5g SDS (10% SDS) (w/v) and 50ml Tris-HCL pH 8 (500 mM).

- PEG with 150g polyesthylene glycol 6000 and 46,7g NaCl in 500 ml of MiliQ water.

Then these dilutions were autoclaved during 20 minuts at 117ºC.

The method used to extract DNA from the samples of soil was the following: 0,1-0,3 g (fresh weight without roots) portions of soil were placed in a sterile eppendorf tube filled with 0,5 g zirconium beads, 750 µL of sodium phosphate buffer (120mM; pH8), and 250 µL of TNS solution, were added to the soil, which was resuspended by vortexing. The cells were lysed by bead beating cell disrupter (mini bead beater, Biospec products) for 45 s at a setting of 6,5 m s^{-1}, afterwards it was centrifuged at maximum speed and 4˚C during 10 minutes in centrifuge 5804R, 700 µL of supernatant was collected, and the soil bead mixture was extracted a second time, with 750 µL of sodium phosphate buffer (120mM; pH8), and 250 µL of TNS solution, doing again the bead beating and centrifuge treatment.

Afterwards, it was extracted with 700 µL of Phenol/Chloroform/Isoamylalcohol (25:24:1) pH 8, which was vortexing and spined for 5 minutes, in order to remove the proteins in the DNA. Then 500 µL supernatant was collected and placed in a new 2 ml eppendorf tube. Again it was extracted with 500 µL of Chloroform/Isoamylalcohol (24:1), which was vortexing and spined for 5 minutes as well, in order to eliminate rest of the phenol, 250 µL of supernatant was collected in a new 2 ml eppendorf.

Then we added 1 ml of Polyethylene glycol **(**PEG) and it was precipitated by spinning 30 minutes at 4˚C in centrifuge 5804R, the liquid was removed and 500µL ice-cold 70% EtOH was added to wash the pellet. Later it was spinning down for 30 minutes at the maximum speed (14000 rpm) at 4˚C again. Finally the EtOH was removed carefully by pipetting and the DNA was drying 20 minutes in concentrator 5302 eppendorf machine. The pellet was eluted in 40 µL with PCR water.

- Absorbance spectrometry

Figure5.NanoDrop spectrophotometer

The amount of DNA was measured by absorbance spectrometry using NanoDrop apparatus.

NanoDrop is a biological machine that quantifies and assesses purity of samples, such as proteins and nucleic acids with less than 2µL of the sample.

For molecular analyses, DNA extractions were stored at -20˚C.

4.3 Polymerase Chain Reaction amplification (PCR)

Until 1980, the determination of microbial community structure and the identification of microorganisms in environmental samples depended on culture-based studies. But nowadays, two major discoveries have revolutionized microbial ecology and have permitted culture-independent characterization of the microbial community: the recognition that the phylogenetic relationship between microorganisms can be inferred from molecular sequences and the ability to selectively amplify minute amounts of nucleic acids extracted from environmental samples by the polymerase chain reaction.

Polymerase chain reaction (PCR) is a molecular technique which allows amplifications of a specific region of the DNA. A PCR reaction consists of a mixture containing at least: PCR buffer, to get the optimal pH for the PCR reaction, magnesium ions that are required as a cofactor by most NTP-binding proteins, free desoxynucleoside triphosphate (dNTPs: dATP, dTTP, dCTP, dGTP) that are responsible for building the blocks of DNA, oligonucleotide primers, the role of the primes is indeed to allow the polymerase to start with the amplification, but the most important is that the primers give the PCR specifity and lastly thermostable DNA polymerase are responsible for building each single strand of the DNA, and finally a template DNA.

Typically, PCR consist in a series of 20-40 repeated temperature changes, called cycles, with each cycle commonly consisting of 2-3 discrete temperature steps. The steps of the PCR are the following:

1. *Initialization step*: This step consists of heating the reaction to a temperature of 94–96 °C which is held for 1–9 minutes. It is only required for DNA polymerases that require heat activation by Hot Start PCR.

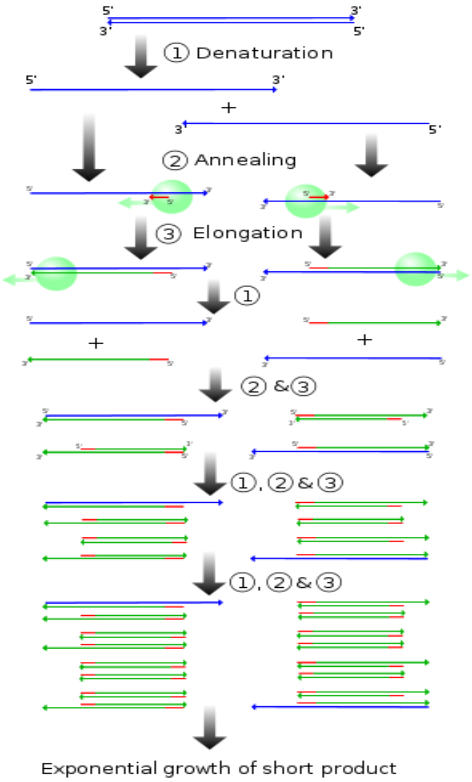


Figure 6.PCR cycles

2. *Denaturation step*: This step is the first regular cycling event and consists of heating the reaction to 94–98 °C for 20–30 seconds. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.

3. *Annealing step*: The reaction temperature is lowered to 50–65 °C for 20–40 seconds allowing annealing of the primers to the single-stranded DNA

template. Typically the annealing temperature is about 3-5 degrees Celsius below the melting temperature of the primers used. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA formation.

4. *Extension/elongation step*: The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75–80 °C, and commonly a temperature of 72 °C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. As a rule-of-thumb, at its optimum temperature, the DNA polymerase will polymerize a thousand bases per minute. Under optimum conditions, i.e., if there are no limitations due to limiting substrates or reagents, at each extension step, the amount of DNA target is doubled, leading to exponential (geometric) amplification of the specific DNA fragment.
5. *Final elongation*: This single step is occasionally performed at a temperature of 70–74 °C for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

6. *Final hold*: This step at 4–15 °C for an indefinite time may be employed for short-term storage of the reaction. (Polymerase chain reaction (2012, November 17). In *Wikipedia, TheFreeEncyclopedia*.from http://en.wikipedia.org/w/index.php?title=Polymerase_chain_reaction&oldid=533536291)

There are different types of PCR, but in this research we decided to use Hot Start PCR. Hot Start PCR is a technique that reduces non-specific amplification during the initial set up stages of the PCR. It may be performed manually by heating the reaction components to the denaturation temperature (e.g., 94°C) before adding the polymerase, afterwards the temperature decreases until 80°C, at which time the polymerase is added.

Hence, in our study the amplifications in the thermal block were carried out in the following order, starting with a preheat of 94°C , afterwards the thermal cycle profile consisted of initial denaturation for 5 min at 94˚C, 5 min at 80˚C in order to add the enzyme (Taq polymerase), followed by 35 cycles consisting of 1 min at 94˚C, 1 min at 56,5˚C (annealing temperature) and 1,5 min at 72˚C (elongation) and 10 min at 72˚C for the last cycle.

To carry out the Hot Start PCR it is important to choose the correct functional primer set to amplify the specific strand of the DNA. Thus, the set of primers used was cmo186 and cmo568. These pmoA primers were designed by Luesken et al. (2011) (table 3), because it was not possible to amplify pmoA genes in N-DAMO bacteria NC10 phylum with the most commonly used forward primer A189 and the reverse primer A682 was used for the rest of the methanotrophs. Thus, with these new designed primers, it is possible to analyze pmoA sequences closely related to NC10 phylum such as *Candidatus Methylomirabilis oxyfera* (at list 85.5% nucleotide identity and 92% protein identity) in different environments (Luesken et al., 2011).

Name	A189b	Cmo182	Cmo568	Cmo682
New pmoA primers for "ca.M.oxyfera"	GGNGACTGGGACTTYTGG	TCACGTTGACGCCGATCC	GATGGGGATGGAGTATGTGC	TCGTTCTTYGCCGGRTTT

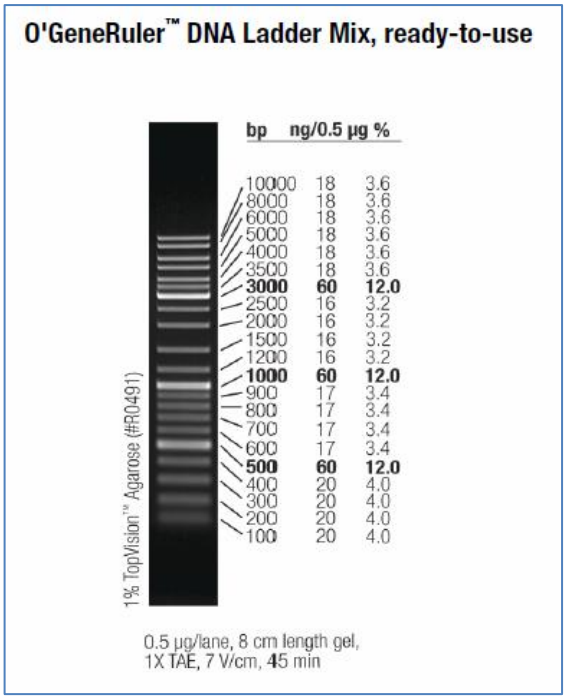
Table 3. Aligment using MEGA4 of newly developed primers for the alpha subunit of particulate methane monooxygenase (pmoA) of "*Candidatus Methylomirabilis oxyfera*"(Luesken et al., 2010).

4.4 Agarose Gel Electrophoresis (AGE)

Once the DNA is extracted or the PCR is carried out, it is always important to analyze the products with AGE. In this way it is possible to appreciate the amount of DNA or PCR product in a gel, and to know if there is the presence or absence of N-DAMO bacteria belonging to NC10 phylum in our case.

With the DNA extractions and the PCR products the protocol of the AGE was the same.

DNA samples were diluted with the 6X Orange DNA loading Dye, 1 µL of the dye solution with 5 µL of the DNA samples.



These samples were analyzed by electrophoresis on 1% agarose gel (1 g agarose/100 ml 0,5M TBE), 1 µL O'GeneRuler DNA ladder mix was used for calibration, at 130 V, for 45 minutes, stained with ethidium bromide for 20 minutes. The gels were photographed with image processing software: Proxima Q-4.

Figure 6. DNA Ladder Mix

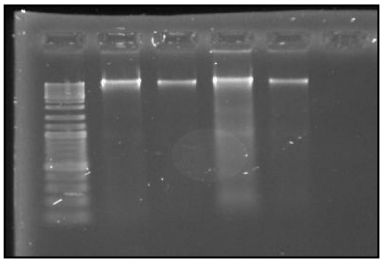
With the AGE we get information regarding the length and molecular weight of our samples of DNA or PCR products.

5. Results

5.1 DNA extraction

In relation to the samples of soil, the soils sediments from European lakes were provided with the DNA already extracted and for this reason it was not necessary to extract the DNA. Because these DNA samples were provide from an external company, their origin and characteristics are confidential. We are allowed to say if we did or did not detect the presence of N-DAMO bacteria in these lakes but we are not allowed to explain the characteristics of the lakes. On the other hand, soil samples from neglected areas, urbanized areas and farming area were sampled in field conditions and DNA extraction protocol with phenol/chloroform was carried out.

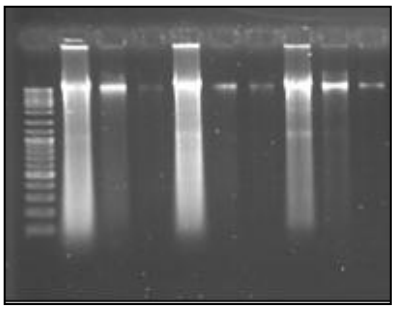
To examine the yield and quality (size/shearing) of DNA, agarose gel electrophoresis (AGE) were used. The following pictures shows two examples of AGE:



DNA analysis by A.G.E in order:

Marker/De Mos1/ De Mos 2/ Lak Nioo1/ Lak.Nioo 2

Figure 7. DNA analysis by AGE



DNA analysis by A.G.E in order:

Marker/De Mos1(diluted 10 and 100 times)/ De Mos 2(diluted 10 and 100 times)/ Lak Nioo1 (diluted 10 and 100 times)

Figure 8. DNA analysis by AGE

Pictures 8 and 9 show the DNA extracted from some soil samples. The first vertical line is the marker, which provides the information about molecular weight. A clean extraction without apparent contamination can be seen. For more information about concentration and contamination in the DNA, a NanoDrop analyses were carried out.

5.2 DNA concentration

With absorbance measurements by the Nanodrop apparatus it is possible to know the concentration of DNA in ng/µL and how purified the DNA samples are. It is possible to analyze two different ratios with the absorbance measurement:

- *260/280*

The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as "pure" for DNA; a ratio of ~2.0 is generally accepted as "pure" for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.

- *260/230*

This ratio is used as a secondary measure of nucleic acid purity. The 260/230 values for "pure" nucleic acid are often higher than the respective 260/280 values. Expected 260/230 values are commonly in the range of 2.0-2.2. If the ratio is appreciably lower than expected, it may indicate the presence of contaminants which absorb at 230 nm (NanoDrop, 2007).

Sample	Ng/uL	A260	260/280	260/230	Sample	Ng/uL	A260	260/280	260/230
Mos.veld	248.51	4.97	1.67	1.21	**DLNC2**	55.14	1.10	1.53	1.75
De Mos 1	23.05	0.64	1.65	1.72	**Biof1**	80.75	1.61	1.56	1.08
De Mos 2	54.88	1.09	1.51	1.78	**Biof2**	46.04	0.92	1.62	0.93
Dit.NIOOS	275.65	5.51	1.74	1.38	**Dig1**	135.33	2.70	1.68	0.94
Dit.NIOOD	341.99	6.84	1.75	1.36	**Dig2**	107.5	2.15	1.56	0.71
Lak.NIOO1	83.38	1.66	1.54	1.79	**Hacro1**	116.13	2.32	1.45	0.62
Lak.NIOO2	70.38	1.40	1.52	1.81	**Hacro2**	99.99	2.00	1.51	0.81
DLC1	33.12	0.66	1.58	1.58	**Recro1**	124.45	2.48	1.55	0.83
DLC2	41.39	0.82	1.57	1.76	**Recro2**	90.27	1.80	1.56	0.84
DLNC1	54.22	1.08	1.72	1.74					

Table 4. DNA measurements of the different stations sampled.

In the table 4 it is possible to see the DNA concentration using nanoDrop machine of the soil samples from neglected fields, urbanized area and farming area, the DNA was extracted by phenol/chloroform protocol. Thus, it is possible to see the high concentration of DNA from the soil samples such as 248,51ng/uL in the Mos.Veld sample. Regarding the 260/280 ratio the average from all the results is 1,60 which means that its accepted as pure DNA. On the other hand, the ratio 260/230 shows presence of a contaminant which absorbs at 230nm.

In these graphs made with Microsoft excel is possible to appreciate the relation between 260/280 and 260/230 ratios:

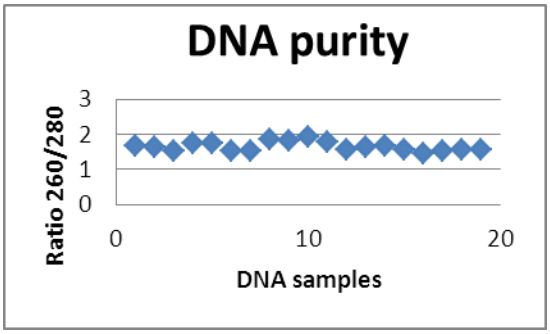


Figure 9-. DNA purity

This graph shows a 260/280 ratio between 1,5 and 2, in this case it is possible to conclude that the DNA are phenol or protein contamination free, because the ratio is around 1,8 that means purity.

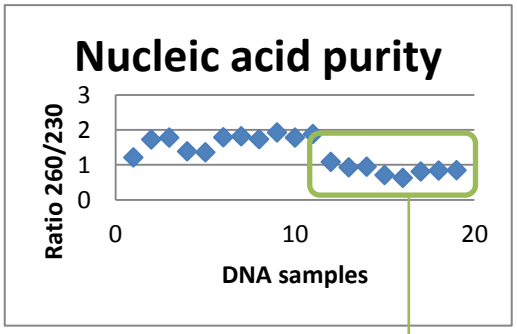


Figure 10. Nucleic acid purity

DNA samples from farming area

On the other hand, regarding to other types of contamination which absorbs at 230nm, we have a bad relation between the DNA samples from the farming area with values around 0,6 and 1, which means contamination in the samples, possibly because of the difficult to extract the DNA in this type of soil. (There was a lot of roots and wood pieces).

DNA measurements from the European lake's sediments:

In this case, the soil sediment samples from European lakes were available with the DNA already extracted by an external company. The DNA extraction was carried out by a DNA extraction kit and the information about concentration and ratios provided from the company is as follows:

Sample	260/280	260/230	Sample	260/280	260/230	Sample	260/280	260/230
BUR-S	2.4	0.11	**ILR-I**	1.30	0.32	**NIM-S**	1.83	0.37
BUR-I	2	0.68	**ILR-D**	1.06	0.14	**NIM-I**	1.84	1.03
BUR-D	1.98	1.07	**JYV-S**	1.95	0.32	**NIM-D**	1.90	1.20
ERS-S	1.49	0.35	**JYV-I**	1.72	0.53	**NUS-S**	2.40	0.08
ERS-I	1.61	0.52	**JYV-D**	1.50	0.40	**NUS-I**	1.88	0.29
ERS-D	1.82	0.22	**KIS-S**	1.52	0.19	**NUS-D**	1.98	0.57
GLI-S	1.4	0.19	**KIS-I**	1.05	0.09	**ROT-I**	1.81	0.18
GLI-I	1.68	0.55	**KIS-D**	1.29	0.13	**ROT-D**	20.08	0.58
GLI-D	1.75	0.50	**LA 2**	1.66	0.24	**SCW-S**	2.00	0.28
GRI-S	1.47	0.32	**LA 3**	1.58	0.40	**SCW-I**	2.38	0.11
GRI-I	1.35	0.32	**LA 1**	1.24	0.25	**SEE-S**	1.43	0.12
GRI-D	1.38	0.25	**LAU-S**	3.90	0.11	**SEE-I**	1.93	0.85
HAS-S	2.13	0.31	**LAU-I**	2.14	0.14	**SEE-D**	2.32	0.17
HAS-I	1.82	0.31	**LAU-D**	1.96	0.14	**SGL-S**	1.69	0.59
HAS-D	2.11	0.67	**LIL-S**	1.87	0.14	**SGL-I**	1.66	0.61
HAR-S	1.72	0.58	**LIL-I**	1.26	0.12	**SGL-D**	1.42	0.20
HAR-I	1.65	0.63	**LIL-D**	1.29	0.23	**SKO-S**	1.54	0.48
HAR-D	1.50	0.26	**LOV-S**	1.87	0.39	**SKO-I**	2.09	0.06
HIN-S	3.34	0.90	**LOV-I**	1.83	0.64	**SKO-D**	1.43	0.24
HIN-I	3.33	0.31	**LOV-D**	1.82	0.51	**ST 1.3**	1.71	0.23
HIN-D	2.05	0.85	**MEK-S**	1.73	0.57	**ST 1.9**	1.76	0.69
HUT-S	1.66	0.42	**MEK-D**	1.74	0.11	**ST 2.2**	1.77	0.77
HUT-I	2.63	0.22	**MRH-S**	1.55	0.51	**STV-S**	1.29	0.26
HUT-D	1.84	0.70	**MRH-I**	1.49	0.19	**STV-I**	1.45	0.31
ILR-S	1.02	0.07	**MRH-D**	1.16	0.22	**STV-D**	1.51	0.46

Table 5. DNA measurements from european lakes.

Where the first letters identify the lake's situation and the last letters -S,-I,-D means:

- S: soil sample which was collected on the surface of the lake.
- I: soil sample which was collected on the intermediate area of the lake.
- D: soil sample which was collected from the deeper area of the lake.

In these graphics made with Microsoft excel it is possible to appreciate the relationship between the 260/280 and 260/230 ratios:

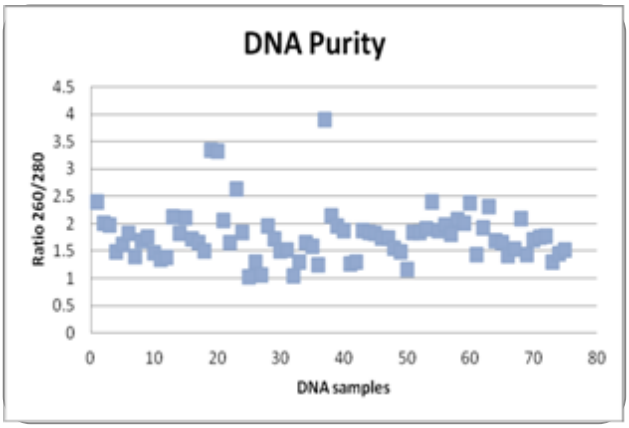


Figure 11. DNA purity from European lakes

This graph shows a 260/280 ratio between 1 and 4. In this case it is possible to conclude that the DNA extractions were dirty and contaminated, but because the origin and protocol used with these extraction are unknown, it was not possible to extract the DNA another time.

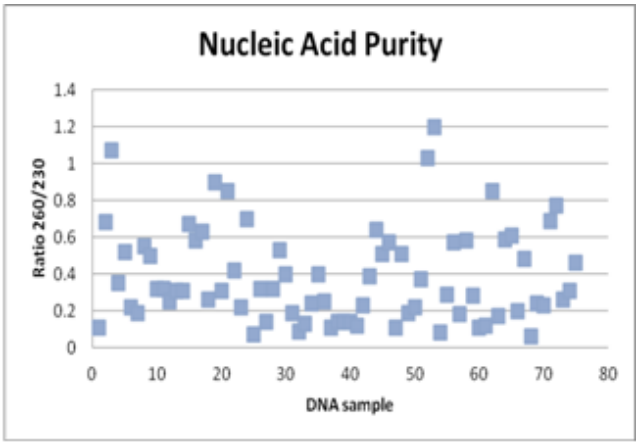


Figure 12. Nucleic acid purity from European lakes

As with the previous graph, the values obtained from the 260/230 ratio show a high contamination in the DNA samples, because the ratio shows low ratio (less than 1). These types of data with contamination make it really difficult to obtain available results, this creates more difficulties to obtain good results, but not impossible.

5.3 Analyses of PCR variables

Once we had all the DNA extractions and knew their concentrations; we did concentration dilutions ten times, because to carry out the PCR techniques the concentration of DNA must be between 0,1 and 3 ng/uL in order to obtain good results.

Different variables of concentration of PCR components were tried. Thus, it was possible to know the best combination of concentration components to obtain better results.

Variable		Result
Primer	A189b cmo682	-
	cmo 186 cmo 568	+
dNTP	2.5 mM	+
	5 mM	+
	0.2mM	+
	0.4mM	-
	0.6mM	+
	0.8mM	+
	1mM	-
	1.2mM	-
	1.4mM	-
	1.6mM	-
	1.8mM	-
	2mM	-
$MgCl_2$	1.5mM	+
	2mM	-
	2.5mM	-

Table 6. PCR variables where (+) means result and (-) means no result.

Different sets of primers were tested; A189b/cmo682 and cmo186/cmo568. The set of primers A189b and cmo682 only worked with the positive control of pmoA but in every DNA sample we obtained results, however with the set of primers cmo186 and cmo568 we got amplification in the positive control and in some DNA samples. With the concentration of the

primers we got results with different concentrations such as 2.5mM and 5mM, but in the end we decided to use the low concentration of primers in order to save PCR compounds.

With the concentration of dNTPs, we even got positive results with different concentrations we decided to use the lower one (0.2mM) because of the good results and also to save the PCR compounds.

According to the $MgCl_2$ only with the concentration of 1.5mM did we obtain positive results.

Hence, the final components with their respective concentrations to carry out all the PCR during our investigation were the following:

Concentration of components PCR-stock	**PCR concentration**	**Final PCR reaction Final volume 25 µL**
10 mM PCR buffer- $MgCl_2$	1 mM PCR buffer –$MgCl_2$	2,50 µL
50 mM $MgCl_2$	1,5 mM $MgCl_2$	0,75 µL
10 mM dNTP stock	0,2 mM dNTP	0,50 µL
	Primer cmo186	2,25 µL
	Primer cmo568	2,25 µL
	Taq DNA polymerase 5U/ µL	0,12 µL Taq + 4,87 PCR H_2O
	PCR H_2O	8,25 µL
	DNA template	3,00 µL

Table 7. PCR components

Another variable that we checked was the annealing temperature in the Hot Start PCR; even with all the temperatures checked we got positive results with the positive control of pmoA, only with the annealing temperature of 56.5˚C did we get positive results in most of our DNA samples. For this reason, we used this annealing temperature to analyze all our samples.

Variable	Temperature (˚C)	Results
Annealing Temperature	50,5	only positive control
	52,5	only positive control
	54,5	positive control and DNA sample 54.1
	56,5	all results
	58,5	positive control and DNA sample Nus-S
	60,5	only positive control

Table 8. Annealing Temperature variables

5.4 Analyses of Hot Start PCR (T=56,5˚C)

Hot Start PCR is a modified Polymerase chain reaction (PCR) which avoids non-specific amplification of DNA by inactivating the Taq polymerase at lower temperature (80˚C). With this technique it is possible to amplify a specific region of the DNA using specific primers. In this study case set primers cmo186/cmo568 were used, which were designed for detection of N-DAMO bacteria belonging to C10 phylum such as *Candidatus Methylomirabilis oxyfera*.

To interpret the results we need to understand three things;

1. The positive control has to appear in the Agarose Gel Electrophoresis (AGE), positive control means that we add to the PCR mix; DNA from pmoA purification, thus if the PCR works correctly which means that the primers amplified the specific strain of DNA from the sample, visible results in the positive control have to be seen.

2. Our possible results have to appear with the same length as the positive control, because the specific primers amplifies a concrete region of the DNA (the same as the positive control) and if in some samples we have N-DAMO bacteria belonging to NC10 phylum, we have to obtain results on it.
3. Negative control should not appear in the AGE. Negative control means that we do not add DNA in the PCR mix, for this reason we do not expect any result.

Here are some positives results we obtained with Hot Start-PCR:

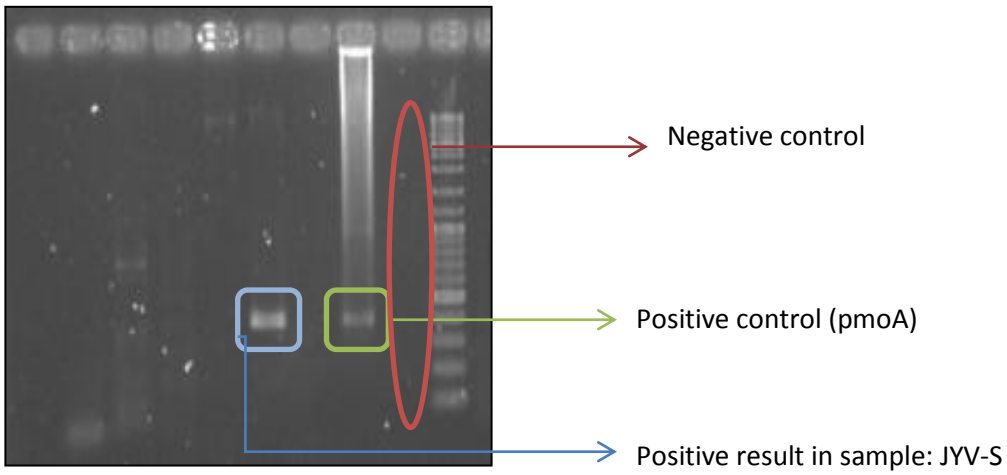


Figure 13. Positive results on JYV-S

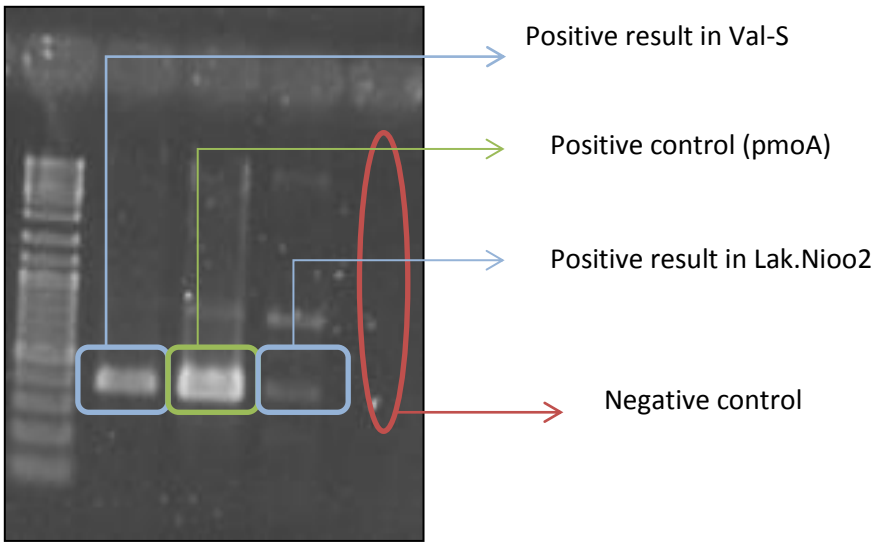


Figure 14. Positive results on Val-s and Lak.Nioo2

5.5 Positive results

This table summarizes the positive results from all the DNA samples analyzed. This means that in these samples of soil the N-DAMO bacteria belonging to the NC10 phylum are present.

Soil Sample	Study Area
BUR-D	European Lake
BUR-I	European Lake
GLI.I	European Lake
HIN-D	European Lake
HUT-D	European Lake
HUT-I	European Lake
JYV-S	European Lake
LAK.NIOO2	Urban Area
NUS-I	European Lake
NUS-S	European Lake
SEE-D	European Lake
SEE-I	European Lake

Table 9. Results with presence of N-DAMO bacteria belonging to NC10

Consequently, the positives results obtained during the investigation were from European lakes and from a lake in an urban area, confirming that the investigation of this group of bacteria from Smith et al. (1991) and Raghoebarsing et al. (2006), where the bacteria was found and cultivated in freshwater sediments in anaerobic conditions.

With this research, we can provide more information about which natural environments it is possible to find N-DAMO bacteria belonging to the NC10 phylum.

6. Discussion

As mentioned previously, the objective of this research was the application of molecular tools in the area of environmental research for screening N-DAMO bacteria in environmental samples. Using molecular tools we got accurate and quick results.

Pertaining to the DNA extraction, classical methods such as DNA extraction with phenol/chloroform was chosen. Thus, the extraction was slower than using extraction kits; we obtained clean extraction with good results according DNA purity or nucleic acids purity (figures 9 and 10). Using this method, it was possible to understand each step of the extraction, starting by the assemblage of soil samples and finishing by the removal of proteins by phenol, or the rest of proteins by chloroform and the precipitation of plasmids by PEG.

On the other hand, DNA extractions from European lakes sediment was already extracted when the samples arrived at NIOO-KNAW (research center where the investigation was carried out). Although in this case, the DNA extraction kit was used (DNA extraction with kit it is very quick), the DNA purity results with this technique was not as satisfactory as expected (figures 11 and 12).

Another molecular tool used in this research was Polymerase Chain Reaction (PCR). With this technique it was possible to amplify a specific region of the DNA. Hot Start PCR was chosen to screen all our DNA samples. This specific PCR allowed amplifying the DNA, while reducing non-specific amplification during the initial set up stages. This technique has the inconvenience of adding the polymerase manually during the first heating of the reaction components which may cause some contamination in the samples, because of the manipulation during this step. However, with accurate precision it is possible to obtain good results without contamination and without non-specific amplification.

To carry out the Hot Start PCR different concentrations of all the PCR components were tested, in order to find the best combination of concentration and to improve the PCR results. After some testing, table 7 shows the final PCR components and their concentrations. Testing

different concentrations of PCR components, made it possible to understand the small range where the PCR can works properly, otherwise small changes in the components may give negative results.

Temperature gradient were used in order to show which annealing temperature worked best. Thus we optimized the Hot Start PCR with 56,5˚C temperature annealing from which we obtained all our results.

Using these molecular tools and then analyzing the results with Agarose Gel Electrophoresis (AGE) we obtained the results of this research.

Showing positive results in amplifications of pmoA in some lakes from Europe and according to studies from Smith et al. (1991) and Ragohebarsing et al. (2006), it is possible to hypothesize that anaerobic freshwater sediments are a possible habitat where the N-DAMO bacteria can be present if the concentration of methane and nitrates are at the convenient levels.

However, the non-presence of N-DAMO bacteria in some freshwater sediment or non-flooded ecosystems does not mean that the bacteria cannot grow in these ecosystems. It would be very interesting to make more molecular analyses in order to demonstrate the presence or absence of N-DAMO bacteria in these kind of ecosystems. Furthermore molecular analyses may have some restrictions. This is the reason it is not possible to conclude in this research if in non-flooded ecosystems it is possible to find N-DAMO bacteria.

It would be interesting to go deeper with this research in order to continue analyzing the positive results that we obtained. Analyzing specifics layers from fresh water sediments could have shown us more information about in which random places these bacteria can grow and provide more details about the characteristics of the area where the samples of soil were taken, but as the data of this research is confidential (the data are provided by research center NIOO-KNAW) it is impossible to explain in which concentrations of nitrates and in which specific places were the bacteria found.

For this reason, we consider the following step in the habitat study research of N-DAMO bacteria belonging to NC10 should include this future investigations:

-Analyze the relationship of nitrate/nitrite amount and methane amount with bacteria presence.

- Monitor the pH in freshwater sediments and if the increase or decrease of the pH affect to the bacteria presence.

- Analyze the bacteria presence during different seasonal periods.

- Monitor anthropogenic lakes or natural lakes in order to know if the presence of human activity affects the bacteria.

7. Conclusions

The aim of this research was to investigate in which natural habitats it is possible to find methane oxidizers nitrite driven bacteria using molecular tools.

The innovative use of these techniques in the area of environmental sciences provides a new option for the investigations of microorganisms which are exceedingly difficult to find in the environment or when they are only present within the ecosystem in small amounts. Thus, it is possible to go ahead with environmental ecology investigations, whereas before it was unthinkable.

Nowadays where interdisciplinary science is appreciated, the combination of environmental ecology knowledge and molecular tools is a strong skill. In this research our goal was to test how it is possible to integrate two disciplines in order to obtain better results.

Hence, using molecular tools in the study and assessment of N-DAMO bacteria belonging to NC10 phylum in environmental areas; we obtained positive results of bacteria presence only in freshwater ecosystems. Thus, according to the results of this research, freshwater areas with anaerobic conditions and with methane/nitrate presence are favorable for the growing of N-DAMO bacteria.

However, more molecular analyses is necessary to conclude if solely in freshwater ecosystems it is possible to find N-DAMO bacteria or nevertheless it is possible to find in non-flooded areas as well.

Additionally, more studies are necessary to better understand the favorable conditions of N-DAMO bacteria in the environment. For example, studies using samples with different concentrations of methane and nitrates will be interesting to appreciate in which range of these variables the bacteria can live. Also studies during different seasonal periods of the year could be interesting in order to know the ranges of temperature and oxygen in the water.

With all this information, other important question is; can N-DAMO bacteria be cultured for the purposes of bioremediation? And is it possible their use to control the climate change? Thus, studies of nutrient requirements and environmental conditions are required.

Hopefully, with the integration of two disciplines such as Environmental Ecology and Microbial Ecology we can glean more information about the natural habitat of N-DAMO bacteria. Thus, possible studies regarding these bacteria and their use in bioremediation can be the solution to high methane/nitrate presence in wetlands and lakes.

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