Master's thesis

The spatial and temporal variation of nitrogen fixation in aquatic environments

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ABSTRACT

Nitrogen fixation is an essential process in the nitrogen cycle where atmospheric N_2 is made available for organisms to build nitrogenous compounds. Nitrogen fixation in aquatic environments has traditionally been considered a minor source of new nitrogen, but the recently discovered abundance of diazotrophic (nitrogen fixing) organisms in various aquatic habitats has changed this view. The main focus of aquatic nitrogen fixation research has been in the marine environment although lacustrine nitrogen fixation is also known to occur. Because of the limited recent information about lacustrine nitrogen fixation, the purpose of this master thesis is to investigate diazotrophy in different types of Finnish lake environments. The results indicate that, in addition to cyanobacterial nitrogen fixation in surface waters, diazotrophy can also be found from the anoxic and dimly lit regions of water columns and that nitrogen fixation in all these areas should be accounted for when nitrogen budgets are constructed. Another purpose of this master thesis is to investigate the nutrient dynamics that trigger nitrogen fixation and how they are reflected in the stable isotopic ratios of particulate organic matter (POM). Based on results, decreased nitrate and nitrite concentrations and low nitrate and nitrite to phosphate ratios correlate with low POM $\delta^{15}N$ values. However the correlation was not observed at all sites.

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TIIVISTELMÄ

Typen sidonta on yksi tärkeimpiä biokemiallisia prosesseita, jossa ilmakehän typpikaasu pelkistetään muotoon, jota organismit voivat käyttää typpiyhdisteiden rakentamiseen. Perinteisesti typen sidonnan on oletettu olevan vesistöissä marginaalista, mutta viimeaikaiset tutkimustulokset ovat osoittaneet, että typen sidonta ja typensitojaorganismit ovat oletettua yleisempiä. Koska valtaosa typensidontatutkimuksista on tehty merialueilla, tämän opinnäytetyön tarkoitus on tutkia typen sidontaa erityyppisissä suomalaisissa järvissä. Tutkimuksen tulokset osoittavat, että typen sidontaa tapahtuu pinnassa olevien syanobakteerikukintojen lisäksi myös pimeissä ja hapettomissa olosuhteissa. Tyypillisesti typen sidontaa eri vesikerroksissa ei ole sisällytetty typpibudjetteihin, mutta tulosten perusteella se olisi tärkeää joissakin järvissä. Lisäksi opinnäytetyössä tarkastellaan ravinteiden merkitystä typen sidonnalle ja sitä, kuinka ravinteiden konsentraatiot heijastuvat orgaanisen partikulaarisen typen isotooppisuhteisiin (δ^{15}N-arvoon). Tulokset osoittavat, että epäorgaanisen nitraatti/nitriittitypen konsentraatio sekä nitraatti/nitriitintypen ja fosfaattifosforin suhde korreloivat partikulaaristen orgaanisen typen δ^{15}N-arvojen kanssa. Korrelaatiota ei kuitenkaan pystytty osoittamaan kaikilla tutkimusalueilla.

Contents

1. INTRODUCTION

Nitrogen fixation is an essential biochemical process that sustains life (Postgate 1978, Capone *et al.* 2006). In the process atmospheric dinitrogen (N_2) is reduced to ammonia (NH_3) which is a form of nitrogen that can utilized by living organisms to build nitrogenous compounds (Capone *et al.* 2006). The capability for biological nitrogen fixation is limited to bacteria and archae that possess the gene operon *nif*, which encodes for the nitrogenase enzyme. This enzyme is the catalyst that enables the extremely energy expensive process of nitrogen fixation (Postgate, 1978).

Filamentous and colonial cyanobacteria are efficient diazotrophs (nitrogen fixers) in aquatic environments and cyanobacterial blooms are a common phenomenon in the surface layers of eutrophic lakes. Because cyanobacteria require high phosphorus concentrations, oligotrophic and mesotrophic lake ecosystems generally lack cyanobacterial blooms and nitrogen fixation has been considered as a negligible source of nitrogen in these types of lakes (Howarth *et al.* 1988a). However, research is indicating that nitrogen fixation can also occur below the photic zone and in the sediments which have not been traditionally included into nitrogen budgets. The methodological difficulties have hindered research in these areas but the development of stable isotope mass spectrometry and molecular biology has broadened the possibilities to investigate them (Howarth *et al.* 1988a, Capone 1993, MacGregor *et al.* 2001, Zehr *et al.* 2001). Research by Wasmund *et al.* (2001), Zehr *et al.* (2001) and Montoya *et al.* (2004) in oceanic areas has revealed that a significant portion of the nitrogen fixation occurring in these areas is mediated by small bacterioplankton that can also inhabit the dimly lit and anoxic regions of the water column. The diversity analyses of these species have revealed them to be unicellular cyanobacteria and anaerobic bacteria. It is not confirmed that a similar phenomenon exists in the lake environments, but this view has gained support because Breznik & Harper (1969) detected small nitrogenase activity in the anoxic and aphotic conditions of dystrophic Lake Mize in Florida. Similarly, in oligotrophic Lake Michigan amplified *nif* sequences and low natural abundance of ^{15}N indicated that nitrogen fixation might be occurring below the photic zone although the actual rate was not measured (MacGregor *et al.* 2001).

The rate of nitrogen fixation is controlled by various environmental parameters such as oxygen concentration, nutrient concentrations and light availability (Howarth *et al.* 1988b). The oxygen concentration is an important regulatory factor because nitrogenase enzyme is easily destroyed by oxygen. This has lead to an evolution of various protective mechanisms such as the heterocysts in the filamentous cyanobacteria and alternation of nitrogenase production with photosynthesis in unicellular diazotrophic bacteria (Postgate 1978, Wasmund *et al.* 2001). Non-photosynthetic diazotrophs are less vulnerable to oxygen because they can inhabit the anoxic region of the water column and their energy is provided by other mechanisms than oxygen evolving photosynthesis (Montoya *et al.* 2004). Besides oxygen concentration, nutrients are important controlling factors for nitrogen fixation. The overall limiting nutrients for primary production in the aquatic environments are generally considered as nitrogen and phosphorus, and the Redfield ratio, where by the molar ratio of nitrogen to phosphorus equals 16, has become a tenet of aquatic biology. Any deviations from this ratio are considered as a sign of nutrient limitation (Howarth *et al.* 1988b, Arrigo 2005). The capability to fix nitrogen is often considered an advantage in environments where nitrogen limitation is a common phenomenon, thus the genetic potential for nitrogen fixation has been detected in various organisms inhabiting aquatic environments (Zani *et al.* 2000, Arrigo 2005)

Although nitrogen fixation was known to be a common phenomenon for a long time, the wide scale measurements of nitrogenase activity did not begin until the 1960s when the acetylene reduction technique was developed. This is based on the fact that besides atmospheric dinitrogen, nitrogenase can reduce other triply bound molecules such as acetylene, azide and cyanide. Because the concentrations of acetylene and its reduced form ethylene are relatively simple to measure using the flame ionization detector (FID) of the gas chromatograph (GC), the acetylene reduction technique became a common method to detect nitrogenase activity. The acetylene reduction technique is a simple, inexpensive and precise method, but since it is an indirect measurement, the ethylene production rate has to be converted into the nitrogen fixation rate and the conversion factor is known to vary (Capone 1993, Montoya *et al.* 1996, Moisander *et al.* 1997). Acetylene is also known to starve and inhibit the metabolic functions of some diazotrophs, so the suitability of the method to various organisms is questionable (Oremland & Taylor 1975, Capone 1993).

The development of stable isotope mass spectrometry has generated good alternatives to the acetylene reduction technique and stable isotope analyses have become a common method in nutrient and food web studies (Peterson & Fry 1987). Stable isotope analyses measure certain isotopic forms of elements that are found in low concentrations in nature and the stable isotopic ratio of an element (δ-value) reflects the source of nutrients and the processes that have occurred during the nutrient utilization (Peterson & Fry 1987). Stable isotope analyses can be used in two ways in nitrogen fixation measurements. In the ^{15}N-tracer method, the diazotrophic organisms are given the heavy isotope nitrogen gas, $^{15}N_2$, and the amount incorporated into tissues as particulate organic nitrogen (PON) is measured by using the isotopic ratio mass spectrometer (IRMS). This is a direct measurement of nitrogen fixation and is currently utilized in modern research of nitrogen dynamics (Montoya *et al.* 1996). The measurement of the natural abundance of ^{15}N is a non-manipulative method to investigate nitrogen fixation. The method is based on the fact that low $\delta^{15}N$-values reflect nitrogen fixation because diazotrophs are utilizing atmospheric dinitrogen, which $\delta^{15}N$-value by definition equals zero, and the fractionation during nitrogen fixation is negligible. It should be noted though, that using solely this method is not recommended because various factors, such as effluents, can affect the $\delta^{15}N$-values (Peterson & Fry 1987, Robinson 2001).

The genetic potential for diazotrophy has also received increased attention and the methods in molecular biology have enabled these analyses. Currently the presence and the expression of the nitrogenase encoding *nifH* gene are generally analyzed by using the quantative polymerase chain reaction (Q-PCR) and the reverse transcriptase polymerase chain reaction (RT-PCR) (Burns *et al.* 2002, Short & Zehr 2005).

In the light of the recently discovered common occurrence of diazotrophy in the marine environment, the purpose of this master's thesis is to investigate whether diazotrophy is as common a feature in the lacustrine environment. The spatial variation of nitrogen fixation was investigated in detail, and dark and anoxic waters received special attention because the nitrogen fixation has not, to the author's knowledge, been previously investigated in these types of lakes in Finland. Another goal of this thesis was to investigate how well the $\delta^{15}N$ values of particulate organic matter (POM) correlates with nutrient concentrations and whether decreased $\delta^{15}N$ values can be used as an indicator of nitrogen limitation. Prior to the actual measurements lots of emphasis was put on establishing and comparing the methods.

2. BACKGROUND

2.1. Nitrogen cycle

Nitrogen, among other essential recycling elements such as carbon and sulfur, is a key element sustaining life (Capone *et al.* 2006). In the nitrogen cycle (Figure 1) atmospheric dinitrogen (N_2) becomes bioavailable when it is reduced into ammonia (NH_3) in a process called nitrogen fixation. Nitrogen fixation can be either biotic or abiotic. The biotic nitrogen fixation is mediated by certain limited bacteria that posses the gene operon *nif* which encodes for the nitrogenase enzyme. The abiotic nitrogen fixation can occur for example during lightning events and fertilizer production (Capone *et al.* 2006, Arrigo 2005, Postgate 1978). The ammonia produced in nitrogen fixation can be further on oxidized into nitrite (NO^-_2) or nitrate (NO^-_3) by nitrification which is mediated by specific bacteria *Nitrosomonas* and *Nitrobacter* (Postgate 1978). Ammonia, nitrite and nitrate are assimilated by various organisms, such as plants and phytoplankton, to build amino acids and proteins which can be consumed by animals to build complex protein compounds (Postgate 1978). Nitrogenous compounds are converted back to ammonia by ammonification that occurs during decay, autolysis and putrefaction of biological material. Another ammonia producing pathway is the dissimilatory nitrate reduction, where nitrate is reduced to ammonia in a highly reducing environment such as the hypolimnion (Arrigo 2005). The losses of nitrogen from the nitrogen pool to the atmosphere can either occur by denitrification or anammox. In denitrification, two nitrate molecules are reduced to dinitrogen whereas in anammox one nitrite and one ammonium molecule are reduced to dinitrogen. Anammox did not become familiar to scientists until the mid-1990s when bioreactors were developed to remove ammonium from waste water (Wetzel 2001, Arrigo 2005). Overall, the nitrogen cycle is complex and includes various processes that are mediated biologically.

There has long been a heated debate about the imbalance of the nitrogen cycle. Based on budgets, there is less nitrogen coming in to the nitrogen pool than is lost by denitrification and anammox. The recently discovered abundance of diazotrophic organisms in the oceans is filling the shortfall, but it still seems that budgets are imbalanced and all components are not included correctly to the calculations (Arrigo 2005). There have been several attempts to quantify the components of the nitrogen cycle but this is very complicated because nitrogen exists in variable redox states and many of the conversion processes are mediated biologically (Vitousek 1994). In an estimate prepared by Soderlund & Rosswall (1982) nitrogen fixation by terrestrial ecosystems would yield approximately 100 Tg and lightning 10 Tg of nitrogen or less per year globally. Biological nitrogen fixation in the oceans estimated by Carpenter & Capone (1983) would yield 5-20 Tg. However this figure is probably an underestimate because present studies indicate that nitrogen fixation in the oceans is more important that previously understood (Montoya *et al.* 2004). Currently anthropogenic nitrogen fixation, for example during fertilizer production, is yielding more fixed nitrogen than natural biotic and abiotic pathways. Industrial nitrogen fixation produces more than 80 Tg of nitrogen per year and 25 Tg are released by internal combustion engines as nitrogen oxides. Additionally 30 Tg of nitrogen is fixed in legume crops. Humans also affect the global nitrogen cycle by releasing long term storage pools by burning biomass and draining wetlands (Vitousek 1994). In the removal processes, denitrification has been estimated to remove 175-450 Tg of fixed nitrogen per year. The role of anammox in the nitrogen budgets is not well known, but research is indicating that this process has the potential to remove significant amounts of fixed nitrogen (Arrigo 2005). Based on the higher estimates

of denitrification rates, it appears that the nitrogen removal processes are more efficient than the natural nitrogen input processes.

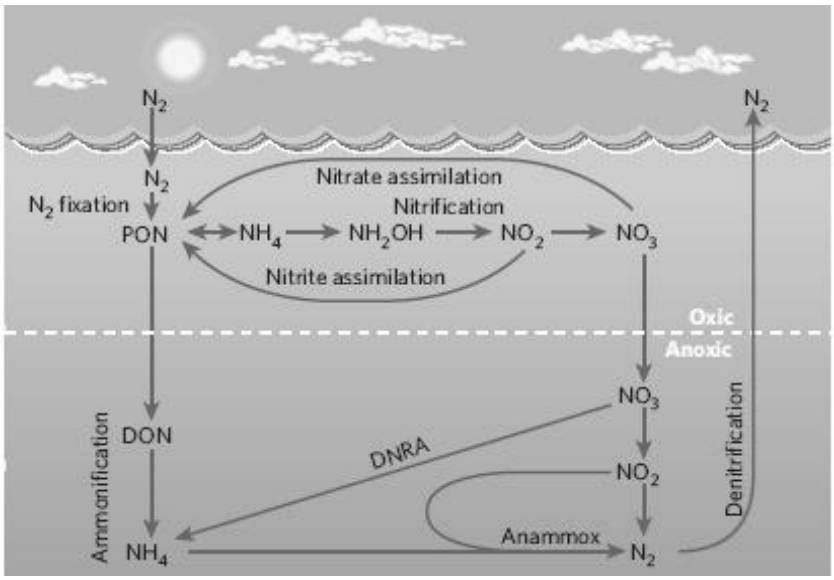

Figure 1. Marine N cycle. PON = particulate organic nitrogen, including phytoplankton; DON = dissolved organic nitrogen, DNRA = dissimilatory nitrate reduction to ammonium (Arrigo 2005).

2.2. Structure of the nitrogenase enzyme

2.2.1 Physical structure of the nitrogenase enzyme

Nitrogen fixation is mediated by the nitrogenase enzyme which presence is limited to certain archae and bacteria (Capone 1993). The presence of the nitrogenase enzyme was detected long before the enzyme was isolated in 1960 by a group of researchers in the Central Research Laboratories of Dupont de Nemours. The isolation took a long time and was extremely difficult because of the oxygen sensitivity of the enzyme (Postgate 1978). The isolation revealed that the nitrogenase enzyme consists of two distinct proteins, dinitrogenase and dinitrogenase reductase. The larger protein, dinitrogenase, consists of four sub-units that can be divided into two types, and the small protein, dinitrogenase reductase, consists of two sub-units that are similar with each other. Both proteins contain iron and sulfur and dinitrogenase also molybdenum. The molybdenum and iron in dinitrogenase are contributed by the cofactor known as FeMoCo and the actual reduction of dinitrogen involves participation of this iron-molybdenum center (Postgate 1978, Brock *et al.* 1994). During molybdenum limitation so called alternative-nitrogenases are formed. Alternative nitrogenases do not contain molybdenum but instead vanadium and iron or iron only. The alternative nitrogenases are presumed to be a back-up mechanism in molybdenum limiting conditions (Postgate 1978, Howarth *et al.* 1988b).

2.2.2 Genetic structure of the nitrogenase enzyme

The biological reduction of dinitrogen to ammonia is an extremely complex process that is genetically controlled by the *nif* regulon which encodes for the nitrogenase enzyme. The genetic information of the *nif* regulon has been determined mainly by sequencing the nitrogen fixing organism *Klebsiella pneumoniae*. The regulon contains over 30 genes including 24206 base pairs that encode for nitrogenases structural units, FeMoCo, and regulatory genes (Table 1). What is peculiar about the *nif* regulon is that some of the *nif*

genes overlap and it has several binding sites in different operons for the main regulatory *nif A* protein (Brock *et al.* 1994).

Table1. The identified subunits and their function in the *nif* regulon. Additionally n*if X* and *Z* has been identified and most likely serve as structural genes (Brock *et al.*1994, Johnston *et al.* 2005)

***nif D*:** the α subunit of the dinitrogenase
***nif K*:** the β subunit of the dinitrogenase
***nif H*:** the dinitrogenase reductase protein dimmer
***nif N, E, B, V* and *Q*:** FeMoCo
***nif A*:** positive regulatory protein, activates transcription of other *nif* genes.
***nif L*:** negative regulatory protein, represses transcription of other *nif* genes.
***nif F*:** flavodoxin, physiological reductant of the Fe protein.
***nif J*:** Pyruvate-flavodoxin-oxidoreductase
***nif M*:** activation of the Fe protein
***nif S, U and Y*:** Processing of FeMo protein
***nif W and Z*:** required for full activity of FeMo protein

2.3. Methods to measure nitrogen fixation

There are four methods that are commonly used to investigate nitrogen fixation. All of the methods have their pros and cons which should be considered carefully when research projects are planned and when results are interpreted.

2.3.1 Acetylene reduction method

The nitrogenase enzyme has a very high reducing power when it reduces dinitrogen to ammonia as follows:

$$N_2 + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2$$

The sequence of electron transfer in nitrogenase is: electron donor → dinitrogenase reductase → dinitrogenase → N_2 → $2NH_3$. The electrons are transferred to dinitrogenase reductase from ferroxin or flavodoxin, which are low-potential iron-sulfur proteins. Electron transfer is a very energy expensive because 4 to 5 ATPs are hydrolyzed for every $2e^-$ transferred (Brock *et al.* 1994). Although six electrons are needed to reduce N_2 to $2NH_3$, careful measurements have indicated that in fact eight electrons are consumed because of two electrons are lost as H_2 for each mole of N_2 reduced (Capone 1993).

The acetylene reduction method is based on the fact that the nitrogenase enzyme is unspecific. Besides N_2, nitrogenase can reduce other triple bonded compounds such as acetylene, azide, and cyanide. Reduction of acetylene by nitrogenase produces ethylene and the production can be easily measured by using gas chromatograph's (GC) flame ionization detector (FID). The acetylene reduction technique is based on the reduction reaction where:

$$C_2H_2 + 2H^+ + 2e^- \rightarrow C_2H_4$$ (Capone 1993)

Although acetylene reduction is very simple, precise and inexpensive, it is an indirect measurement of nitrogen fixation. Because the organism is reducing acetylene instead of dinitrogen, the acetylene reduction rate has to be converted into the dinitrogen reduction rate. The theoretical conversion ratio is 3 moles of acetylene reduced per 1 mole of dinitrogen reduced, thus the six moles of reductant required to reduce one mole of dinitrogen would instead reduce three moles of acetylene. The hydrogen evolution occurring during nitrogen fixation involves additional electron transfers hence it is

generally assumed that the conversion factor is 4:1 (Capone 1993). However, it should be noted that this conversion factor is known to vary, especially in natural environments (Moisander *et al.* 1996, Kayanne *et al.* 2005). Acetylene also interferes with the natural nitrogen metabolism and acetylene reduction (instead of nitrogen fixation) causes the organism to be nitrogen depleted and possibly to reduce more acetylene that it would naturally (Capone 1993). The suitability of the acetylene to some organisms is questionable because for example nitrogen fixing methanotrophic bacteria cannot tolerate acetylene since it interferes with methanogenesis and causes starvation or death to the organism (Oremland & Taylor 1975).

2.3.2 ^{15}N-tracer method

Although acetylene reduction is still probably the cheapest and easiest method to test for nitrogen fixation, the ^{15}N-tracer method is becoming more widely utilized because of the improvements in isotopic ratio mass spectrometers (IRMS). The method is based on the ^{15}N stable isotope that naturally appears in low concentrations. When the ^{15}N-tracer method is utilized, the nitrogen fixing organism is given $^{15}N_2$ gas and the amount of heavy nitrogen isotope incorporated into the particulate organic nitrogen (PON) is measured using a stable isotope mass spectrometer (Montoya *et al.* 1996).

The ^{15}N-tracer method is convenient because it is a direct measurement of nitrogen fixation and does not interfere with the organism's natural metabolism. With the improvement of IRMS it has become more precise and above all, more available than previously (Montoya *et al.* 1996). The downside of this method is that it only measures the net nitrogen fixation which can give false results when nitrogen fixation activity is vigorous and part of the fixed nitrogen is released from the nitrogen fixing organism (Ohlendiek *et al.* 1998, Mullholland *et al.* 2006).

2.3.3 ^{15}N natural abundance

Natural abundance of stable isotopes can be used as one method to investigate the dynamics of the nitrogen cycle. The method is based on the two natural stable isotopes of nitrogen existing in nature, ^{14}N and ^{15}N. Of the nitrogen atoms on earth 99.6337% are ^{14}N and 0.3663% are ^{15}N. To make the stable isotopic ratios more convenient to handle, the ratios are generally reported on a δ-scale where:

$$\delta^{15}N\ ‰ = 1000 \times [(R_{sample} - R_{Standard})/R_{sample}]$$

R_{sample} is the ^{15}N: ^{14}N ratio of the sample and, by definition, the standard for nitrogen is atmospheric N_2 when $R_{standard}$= 0.0036765 (Mariotti 1983, Robinson 2001).

The δ^{15}N-value reflects the sources of nitrogen and the processes that have occurred during utilization. In chemical reactions the lighter ^{14}N is preferred over ^{15}N because the chemical bonds of the lighter isotope are broken more easily than the bonds of the heavier isotope. This leads to fractionation in which the product contains more lighter isotope and the substrate is left enriched with the heavier isotope unless the substrate is consumed completely (Peterson & Fry 1987, Robinson 2001). During nitrogen fixation, the main source of nitrogen is atmospheric N_2, for which δ^{15}N value by definition equals 0‰. Because the fractionation during nitrogen fixation is negligible small, the δ^{15}N-value of the organism will become close to 0 ‰. Hence low δ^{15}N-values are an indicator of nitrogen fixation (Figure 2). This phenomenon has been demonstrated in various studies in lakes and marine environments (Montoya *et al.* 2002, Gu *et al.*2006, Patoine *et al.* 2006). It should be however noted, that using solely this method is not recommended since it does

not measure the actual volumetric rate of nitrogen fixation and the sources of nitrogen cannot be known precisely in all cases (Robinson 2001).

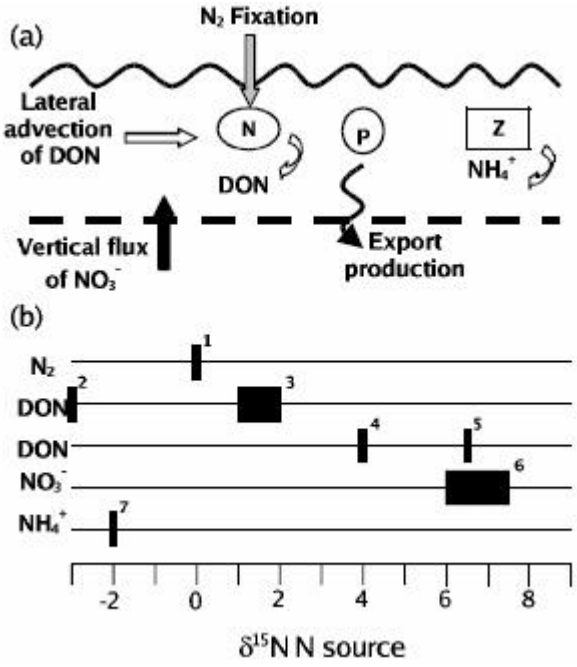

Figure 2. A schematic figure depicting (a) the likely nitrogen (N) sources to phytoplankton (P) in the surface ocean from nitrogen fixation, lateral advection of nitrate and dissolved organic nitrogen (DON), vertical transfer of nitrate (NO_3^-) from deep ocean and ammonium (NH^+_4) excreted by zooplankton (Z), and (b) the stable nitrogen isotopic values ($\delta^{15}N$) associated with the N_2 source. 1: atmospheric nitrogen (N_2). 2 and 3: DON from a N_2 fixing source. 4 and 5: DON from non-N_2 fixing source. 6: NO_3^- from deep ocean.7: NH^+_4. See Mahaffey *et al.* (2004) for further information.

3.3.4 Measurements by applying modern molecular biological methods

The development of molecular biology has enabled investigations of the genetic potential and the genetic expression of nitrogen fixation. This is generally done by investigating the *nifH* fragments with quantitative polymerase chain reaction (Q-PCR) and quantitative reverse transcriptase polymerase chain reaction (Q-RT-PCR). These methods measure the potential and the activity of the *nifH* but not the actual volumetric rate of nitrogen fixation (Short & Zehr 2005). Besides *nifH* activity measurements, lots of emphasis has been put on to quantify the diversity of the diazotrophic species. This is generally done by constructing phylogenetic trees from amplified 16S rDNA and comparing *nifH* sequences (Falcon *et al.* 2004).

2.4. Stable isotopic variation in the nitrogen cycle

The sources of nitrogen have distinct δ^{15}N-values (Figure 2) and there are several studies where they have been used to determine the nitrogen dynamics. In lacustrine environments, there is substantial variation in the isotopic signatures within taxa, between seasons and lakes. However, there are taxon-specific and seasonal patterns that show some consistency from year to year (Vuorio *et al.* 2006). In a Finnish lake study, the δ^{15}N-values among phytoplankton varied in between -2,1 ‰ to 12,8 ‰ and the values were highest among non-nitrogen fixers such as chrysophytes, dinophytes and diatoms, and lowest among nitrogen fixing species *Anabaena* spp., *Aphanizomenon* spp. and *Gloeotrichia*

echinulata. The amplitude of taxon-specific temporal changes was 2-6 ‰ and for the whole water sample 11 ‰ (Vuorio *et al.* 2006).

When the natural abundance of the ^{15}N in particulate organic nitrogen (PON) was investigated in Lake Michigan, the results indicated that the nitrogen fixation might be a significant source of new nitrogen although Lake Michigan is naturally oligotrophic and lacks cyanobacterial blooms. The low $\delta^{15}N$-values varying between 0.5-1.5 ‰ of particulate organic nitrogen (PON) were found from the depth of 10-15 meters where the lowest N: P ratio occurs. What makes these results important is that nitrogen fixation has not been previously looked for at these depths in lakes (MacGregor *et al.* 2001). In a more eutrophic lake system locating in the Northern Great Plains U.S.A, low $\delta^{15}N$-values of particulate organic matter (POM) were noticed during a cyanobacterial bloom and the low values radiated through to individual zooplankton taxa (Patoine *et al.* 2006).

For marine environments Montoya *et al.* (2002) and McCelland *et al.* (2003) have demonstrated that nitrogen fixation is a significant source of nitrogen in the tropical North Atlantic Ocean by using the natural ^{15}N-abundance. The low $\delta^{15}N$-values originating from nitrogen fixation were also reflected in the $\delta^{15}N$-values of suspended particles and zooplankton. Similarly Pantoja *et al.* (2002) indicated that this phenomenon exists also in the Mediterranean Sea. The $\delta^{15}N$-values of nitrate are significantly smaller in the eastern basin suggesting that the nitrate is derived from nitrogen fixation when in the western basin it is derived from the Atlantic Ocean.

2.5 Nitrogen fixation in lacustrine water columns

In lake ecosystems nitrogen dynamics are complex because of the seasonal vertical mixing of water and large impact from the drainage basin. Especially drainage basins that are dense with agricultural practices and sewage treatment facilities tend to lead to eutrophicated lakes and high nitrogen concentrations in the water. Although much improvement has happened in waste water treatment facilities and in agricultural practices for phosphorus reduction, nitrogen is still considered less harmful and the removal of nitrogen is not as efficient. Compared to the oceanic regions, lacustrine environments also have more potential sources of nitrogen such as precipitation directly to the lake, and inputs from the ground water and surface water drainages (Wetzel 2001).

The amount of recent information about the nitrogen fixation in lacustrine ecosystems is very limited. According to the data, nitrogen fixation rates vary according to the trophic status of the lake and the highest nitrogen fixation rates have been observed in eutrophic and hypereutrophic lakes (Table 2). Most of these types of lakes are characterized as shallow and high in phosphorus concentration. It should be noted that majority of the data has been gathered by assaying the acetylene reduction method so the results can be biased.

Table 2. The percent of total nitrogen input to the system contributed by nitrogen fixation in various types of lakes. AR notes for acetylene reduction method.

Lake:	**Trophic state**	**% of the annual nitrogen loading**	**Measuring method**	**Reference**
Lake Erken, Sweden	Eutrophic	82	AR	Howarth *et al.* (1988a)
Clear Lake, U.S.A	Eutrophic	43	AR	Horne & Goldman (1972)
Lake Võrtsjärv, Estonia	Eutrophic	2.34	AR	Tõnno & Nõges (2003)
Lake Verevi, Estonia	Hypereutrophic	Less than 1	AR	Tõnno *et al.* 2005
Lake Washington, U.S.A	Mesotrophic	0.29	AR	Howarth *et al.* (1988a)
Pyramid Lake, U.S.A	Oligotrophic (saline)	81	AR	Howarth *et al.* (1988a)
Lakes Superior, Huron and Michigan, U.S.A	Oligotrophic	0.02	AR	Howarth *et al.* (1988a)
Mono Lake, U.S.A	Oligotrophic (saline)	0	^{15}N-tracer	Steward *et al.* (2004)

Nitrogen fixation in oligotrophic lakes has been presumed to be minor (Howarth *et al.* 1988a). However recent evidence is suggesting that oligotrophic lacustrine environments are inhabited by various organisms that have the potential for nitrogen fixation although the contribution of these organisms is quite unknown. A study conducted in the hypersaline, meromictic Mono Lake in California revealed that there were anaerobic bacteria in all depths that possessed potential to diazotrophy. Although there was a large amount of potential for nitrogen fixation, nitrogen fixation was not detected when tested with the ^{15}N-tracer technique (Steward *et al.* 2004). In oligotrophic Lake Michigan, there was an indication that nitrogen fixation could be a source of new nitrogen in the deeper water layers. This notation was supported by low δ^{15}N-values in particulate organic matter (POM) and the presence of the *nifH* sequences. However the actual rate of nitrogen fixation was not measured (MacGregor *et al.* 2001). When the expression of *nifH* in the meso-oligotrophic Lake George in New York State was investigated, the results indicated that the lake supports a diverse group of diazotrophs that belong to various phylogenies such as unicellular and filamentous cyanobateria, α- and γ- proteobacteria and a previously undefined group of bacteria. The RT-PCR revealed that the species actively expressing the *nifH* were unicellular and filamentous cyanobacteria, α-proteobacteria and the novel bacterial cluster. Again, the actual nitrogen fixation rate was not measured (Zani *et al.* 2000). Based on these results it seems that the possession of the *nif* is an advantageous trait in environments such as Lake George where nitrogen limitation is a common phenomenon.

Dystrophic lakes also contain diazotrophs. In an early study Brezonik *et al.* (1969) detected nitrogen fixation in Lake Mize that is characterized as highly dystrophic and stratified. Additional research in these types of lakes has been hindered due to

methodological problems because generally diazotrophs inhabiting the anoxic regions of highly dystrophic lakes are very sensitive to oxygen and are often destroyed when the nitrogen fixation rate is measured by using the acetylene reduction technique (Bergstein *et al.* 1981).

2.6 Nitrogen fixation in sediments

Nitrogen fixation in the sediments by heterotrophic bacteria is a common phenomenon and the highest rates have been measured in organic rich sediments. For example, in Lake Sonachi in Kenya, nitrogen fixation by heterotrophic bacteria in the sediments was estimated to be 0.015 g N m^{-2} yr^{-1} (Howarth *et al.* 1988a). A study by MacKenzie (1984) revealed that in two eutrophic lakes, Lake Okaro and Lake Rotongaio in New Zealand, the sediments had acetylene reduction capacity, especially in the upper layers where ammonia concentrations were low. The maximum rates of acetylene reduction were 7 nM C_2H_4 g^{-1} h^{-1} (by dry weight of the sediment), and occurred during winter. Low nitrogen fixation rates were also detected in Chesapeake Bay (North Carolina) sediments. The rates were varying between 0.76 and 1.16 nM C_2H_4 g^{-1} h^{-1} (by dry weight of the sediment) (Burns *et al.* 2002). However it should be noted, again, that the results can be biased because they have been obtained by using acetylene reduction technique which can give biased results under certain circumstances (Oremland & Taylor 1975, Capone 1993).

The difficulty of applying conventional nitrogen fixation measurement methods in sediments has led to a wider use of modern methods based on molecular biology. The advantage of using these methods is that, besides measuring the RNA synthesis activity, they also give information about the diversity of diazotrophs. However, they do not measure the volumetric rate of nitrogen fixation. For Chesapeake Bay in North Carolina that is receiving high organic loads, the sediment DNA extractions revealed several unique sequences for organisms possessing the genetic potential for nitrogen fixation. The sequences obtained clustered closely with anaerobic organisms, such as sulfate reducing bacteria, and with each other, suggesting a low abundance of aerobic or facultative diazotrophs in the sediments. The diversity of diazotrophs from the high organic load area was compared to a site in the same region but with less organic load. In site where the organic load was low, the sequences obtained were highly divergent and clustered with deeply branching sequences from anaerobic environments, and included *nif* phylotypes reported from marine mats and from *Spartina* sp. rhizosphere. This study illustrates how the composition of diazotrophic community is highly dependent on the quality of the sediment (Burns *et al.* 2002).

2.7 Oceanic nitrogen fixation

The nitrogen dynamics in the open oceans are different from lakes and estuaries because of the negligible influence from the drainage basin and the lack of seasonal vertical mixing. The potential sources of nitrogen can generally be narrowed down to four: nitrogen fixation, lateral transport of dissolved organic nitrogen (DON), ammonia release by zooplankton and deep water nitrate from the thermocline (Figure 2). It has been well demonstrated that in the tropical North Atlantic Ocean and in the subtropical North Pacific Ocean, nitrogen fixing cyanobacteria *Trichodesmium* spp. are important sources of new nitrogen (Mullholland *et al.* 2006). *Trichodesmium* spp. are also important component supporting growth of other organisms in the oligotrophic ocean areas because they release ammonia, dissolved organic nitrogen and amino acids during blooms (Mullholland & Bernhardt 2005, Mullholland *et al.* 2006). A similar phenomenon has also been noticed in

the Baltic Sea, where cyanobacterial blooms are common. When the rate of nitrogen release was investigated during the growth phase, the filamentous cyanobacteria *Aphanizomenon* and *Nodularia* released 5-10% of their fixed nitrogen. This portion was further utilized by the non-diazotrophic picoplankton fraction. Cyanobacteria were also significant nitrogen contributors during senescence when the cellular contents were released to the overlying water (Ohlendiek *et al.* 1998)

Although many of the phytoplanktonic nitrogen fixers are bloom forming and easy to observe, Zehr *et al.* (2001) and Montoya *et al.* (2004) have discovered that small pico- and bacterioplankton size diazotrophs in various parts of the Pacific ocean create a significant fraction of the total nitrogen. These small organisms have been noted to belong to α- and γ- proteobacteria and to small unicellular cyanobacteria. A similar phenomenon has also been observed in the Baltic Sea (Wasmund *et al.* 2001). What is intriguing in these findings is that since these small organisms also inhabit the dimly lit and anoxic areas of the water column, they have been neglected from the early nutrient budgets. Preliminary results indicate that they might in fact have a more important role in nitrogen fixation than the large easily observed organisms (Montoya *et al.* 2004).

The diversity of small pico- and bacterioplankton diazotrophs has received a good deal of attention. In a study conducted by Falcón *et al*. (2004) small unicellular cyanobacteria, that are found in North Atlantic and subtropical North Pacific have a common ancestor in both locations based on the 16S rDNA sequences, but have diverged *nifH* sequences indicating adaptation to the surrounding environment. In station ALOHA locating in the North Pacific Ocean, the *nifH* phylotypes were amplified from various depths and the results indicated that in the surface cyanobacterial *nifH* phylotypes were similar to *Trichodesmium* spp., *Cyanothece* sp., and *Chlorosphaera watsonii*. Cluster III *nifH* phylotypes were found from the dimly lit areas and the sequences resembled strict anaerobes such as green sulfur bacteria (Church *et al*. 2005)

2.8. Factors controlling nitrogen fixation

2.8.1 Light and oxygen

Nitrogen fixation is a very oxygen sensitive process and diazotrophic species have developed various mechanisms to protect the nitrogenase enzyme (Postgate 1978). For example filamentous cyanobacteria physically separate the nitrogen fixing unit from the rest of the cell with heterocysts. Heterocysts are separate cell units with thick cell walls that exclude oxygen and protect the oxygen sensitive nitrogenase enzyme so that photosynthesis can be maintained during nitrogen fixation. This is very beneficial because photosynthesis supplies energy for nitrogen fixation. However not all phototrophic diazotrophs have heterocysts. Several unicellular phototropic diazotrophs have developed a cycle, where nitrogen fixation is separated temporarily so that the oxygen evolving photosynthesis occurs during the day and nitrogen fixation during respiration in low oxygen concentrations at night (Postgate 1978).

The temporal, spatial and functional variation in nitrogen fixation activity can be observed in various examples. Some photosynthetic diazotrophs have well developed light harvesting regulators that can adjust the photosynthetic rate accordingly. Cyanobacteria that are able to synthesize the red accessory pigment protein, phycoerythrin, can regulate their light intake in changing wavelengths by regulating their light harvesting accessory pigment production. This ability is called complementary chromatic adaptation (CCA). Postius *et al.* (2001) discovered that the ability to fix nitrogen and to perform CCA varies according to the habitat. Cyanobacteria isolated from the stone crusts in the supralittoral

zone were able to fix nitrogen but did not posses CCA. Cyanobacterial species isolated from periphyton of submerged macrophytes were capable of nitrogen fixation as well as to perform CCA. Pelagic cyanobacterial species were neither able to fix nitrogen nor to perform CCA although they were able to produce phycoerythrin.

More robust mechanisms often involve adjusting the nitrogenase activity with the light availability instead of regulating the light harvesting pigments. Depending on the environment and the species adaptations, light can either inhibit or stimulate nitrogen fixation rate. In Lake Valencia in Venezuela, heterocyst bearing cyanobacteria exhibited strong dial variation in their nitrogen fixation behavior. Increasing light intensities stimulated nitrogen fixation and cyanobacteria began migrating towards light. The cyanobacteria migrated to the surface during the highest light intensities and ascended when light availability decreased. The migration and nitrogen fixation pattern varied between species (Levine & Lewis 1984). When nitrogen fixation rates of heterocyst bearing *Anabaena cylindrical* was measured, it was observed that *Anabaena cylindrical* was fixing nitrogen only under lit conditions and there was no nitrogenase activity during the dark period (Millineaux *et al.* 1981). Similar results were obtained in a study, where cyanobacteria of the Tikehau Lagoon in French Polynesia sediment were investigated. The sediment cyanobacteria were showing a strong dial variation in the nitrogenase activity but in contrast to *Anabaena cylindrical,* small nitrogenase activity was detected also during night times although the night time rates were 1.7 to 7 times less than during day (Charpy-Roubaud *et al.* 2001). When two types of diazotrophic communities in Mono Lake in California were investigated, the results indicated that nitrogen fixation can in some cases be independent from the amount of light and that light can affect the nutrient intake. A diazotrophic community consisting of anaerobic bacteria in the flocculent surface layers of the sediment was fixing nitrogen under anaerobic conditions, was not stimulated by organic substrates or light and was inhibited by oxygen, ammonia and nitrate. Another diazotrophic community in Mono Lake, known as *Ctenocladus* balls consisting of eukaryotic algae and non-heterocystous anaerobic diazotrophic cyanobacteria, nitrogen fixation was usually, but not always, stimulated by light. When incubated under air, nitrogen fixation rate in light and in dark were equal. Nitrogen fixation rate of *Ctenocladus* balls in lit conditions was stimulated by 3-(3,4-dichlorophenyl)-1,1-dimethylurea and by propanil [N-(3,4-dichlorophenyl)] (Oremland 1990).

Microbial mats consisting of unicellular thermophilic cyanobacteria in Yellowstone National park, U.S.A were exhibiting opposite dial variation in nitrogenase activity. During the day when photosynthetic rate was high, nitrogen fixation rate was slow due to high oxygen concentration in the mat. When the mat turned anaerobic during the dark period, nitrogen fixation rate increased dramatically. The energy for nitrogen fixation was generated by fermentative processes in which glycolate photosynthesized during the day was converted to acetate at night. Fermentation is a very energy inefficient pathway to produce ATP, so hypothetically nitrogen fixation rate during the dark period is limited by the inefficiency of the fermentation (Steunou *et al.* 2006). The unicellular cyanobacteria in the subtropical North Pacific at the depth of 25 m were exhibiting similar behavior by having the highest nitrogenase synthesis rates at midnight, whereas at greater depths the nitrogenase synthesis rate was highest at noon (Zehr *et al.* 2001). Some cyanobacterial strains isolated from Antarctica also exhibited nitrogen fixation in complete darkness, although most strains of cyanobacteria were fixing nitrogen only in lit conditions (Pandey *et al.* 2004).

In most examples, the photosynthetic cyanobacteria are the sub-group of diazotrophs that exhibit strong dial variation in nitrogen fixation. However there are other non-

photosynthetic diazotrophs that are affected by light availability. A study with pure cultures of green sulfur bacterium, *Chlorobium phaeobacteroides,* indicated that the bacterium fixes nitrogen only when light is available and the activity increases with light intensity until a certain limit. When samples of *Chlorobium phaeobacteroides* from Lake Kinneret were tested for nitrogen fixation, there was no indication of activity, probably because this oxygen sensitive bacterium was exposed to air during sampling and the bacteria's nitrogenase enzyme was destroyed (Bergstein 1981).

2.8.2 Temperature

Diazatrophs are well adapted to different kinds of temperatures. Although the temperature optimum for most diazotrophs is well above 15°C, they are found even from the Antarctic, where temperatures are extremely low and the light availability is very restricted. Cyanobacterial strains isolated from Antarctica were able to perform their metabolic functions normally in temperatures as low as 5°C and their temperature optima was 10°C lower than for their counterparts in the tropics (Pandey *et al.* 2004). Results obtained from the Lake Bonney in Antarctica indicated similar results. In Lake Bonney, the nitrogenase activity was extremely low compared to tropical systems but it was detected in all sites. Molecular characterization demonstrated that this harsh environment has a diverse and periodically active diazotrophic community consisting of heterotrophic and autotrophic species (Olson *et al.* 1998)

2.8.3 N:P Ratio

It is a widely accepted assumption that the most important nutrients that determine primary production in aquatic ecosystems are nitrogen and phosphorus. The net primary production in lake and estuarine environments is typically considered to be phosphorus limited with periodic nitrogen limitation during midsummer (Howarth *et al*. 1988b, Wetzel 2001). Oligotrophic ocean areas instead tend to be nitrogen limited (Arrigo 2005). It has been proposed that nitrogen fixation by cyanobacteria is triggered by low N:P ratio and there are various examples where nitrogen limitation is observed during cyanobacterial blooms (Howarth *et al.* 1988b, Flett *et al.* 1980). This is plausible because nitrate and ammonia assimilation require less energy expenditure than nitrogen fixation. Especially nitrate is an efficient inhibitor of heterocyst formation although the reason is not clear (Howarth *et al.* 1988b). However, low N:P ratio does not always trigger nitrogen fixation. A study conducted in Shelburne Pond in Burlington Vermont, indicated that although the N:P ratio was low and the cyanobacteria made up 81-98% of the phytoplankton biomass, less than 2% of the nitrogen was acquired through nitrogen fixation. The possible reason for cyanobacterial dominance was that the cyanobacteria were superior competitors even without nitrogen fixation by creating dense mats that shaded other phytoplankton (Ferber *et al*. 2004). It should be noted though that besides nutrient concentrations, other factors such as turbulence, grazing by zooplankton and light intensity control cyanobacterial nitrogen fixation (Howarth *et al.* 1988b).

For decades Liebig's law of the minimum was the dominant theory stating that only one resource is limiting the plant, or in the case of aquatic environments phytoplankton, growth (Arrigo 2005). According to Falkowski (1997) this limiting nutrient in oceans for carbon fixation over a geological timescale has been nitrogen. This notation is also supported by results obtained by Mills *et al.* (2004) from the North Atlantic. However, the information gathered during the past couple of decades indicates that there is in fact a multiple resource limitation in the oligotrophic ocean areas (Arrigo 2005). Mills *et al* (2004) demonstrated that although the overall phytoplankton community in the North

Atlantic was limited by nitrogen, the nitrogen fixation in this area was limited by iron and phosphorus. These results illustrate the complexity of the nutrient dynamics.

It has been argued that there is so little phosphorus and iron available in the oligotrophic ocean regions, that it will not be enough to support a substantial amount of nitrogen fixing species. This dilemma is getting closer to be resolved because it appears that at least the common marine cyanobacteria *Trichodesmium* spp. has lower iron requirements than presumed earlier and they are quite superior in phosphorus utilization when compared to other phytoplankton (Arrigo 2005). Dyhrman *et al.* (2006) demonstrated that *Trichodesmium erythraeum* IMS 101 encode for proteins associated with high affinity transport and hydrolysis of phosphonate compounds by carbon-lyase pathway. This pathway allows the utilization of phophonates that have not previously been considered as a bioavailable source of phophorus in the oceans. Also, new potential sources of nutrients have been identified. It has been assumed that in oceanic regions most of the nitrogen and phosphorus in the epilimnion has updwelled from the thermocline. However, recent evidence is suggesting that in the Atlantic Ocean the lateral transports of dissolved organic nitrogen (DON) and dissolved organic phosphorus (DOP) can be significant sources of nutrients. Especially the lateral transport of semilabile DOP seems to be very important periodically (Mahaffey *et al.* 2004)

2.8.4 The Redfield ratio

In early part of the twentieth century Alfred Redfield noticed that the elemental composition of phytoplankton was very similar to the major dissolved nutrients in the deep ocean. Based on these observations, Redfield proposed that the nitrogen to phosphorus ratio of 16:1 is controlled by the requirements of the phytoplankton, which release nitrogen and phosphorus in this ratio when broken down during remineralization. This ratio has been confirmed several times and this stoichiometry of phytoplankton and seawater has become the widely accepted tenet of marine ecology (Arrigo 2005). Although the Redfield ratio holds well overall in the oceanic environment, the ratio is highly dynamic and varies spatially and temporarily. In a model proposed by Klausmeier *et al.* (2004) phytoplankton can be divided into three different classes based on their growth strategies: the survivalist, the bloomer and the generalist (Figure 3). What is intriguing in this model is that none of the classes exhibit the 16:1 ratio but overall the phytoplankton composition does.

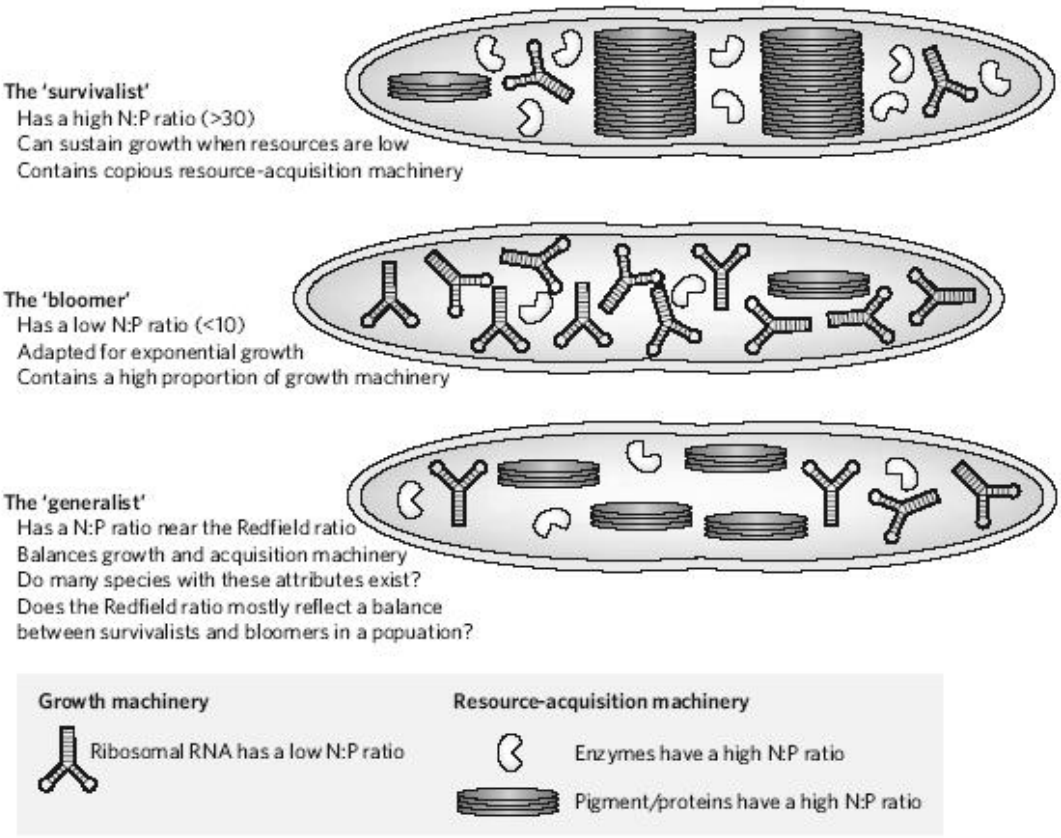

Figure 3. The three classes of phytoplankton proposed by Klausmeier *et al.* (2004), modified by Arrigo (2005).

2.8.5 Salinity

Salinity can have a significant effect on the nitrogen fixation rate. Between cyanobacterial species there is a physiological salinity tolerance range that affects nitrogen fixation. For example, freshwater species are least tolerant for salinity, when species from the Baltic Sea can tolerate a little salinity. The most salt tolerant species of cyanobateria are found from the open ocean areas. Salinity can be a controlling factor in example in estuarine environments when there is a gradient with increasing salinity towards the sea (Moisander *et al.* 2002). Because cyanobacteria in estuaries are generally intolerant to high salinities, the component of phytoplanktonic nitrogen fixation is often missing from those waters (Howarth & Marino 2006). Traditionally estuaries were viewed similar to lakes in terms of nutrient limitations and until 1980s there was a strong emphasis on phosphorus removal with little interest to limit nitrogen loadings. However recent evidence is suggesting that productivity of phytoplankton in estuaries is more determined by nitrogen than phosphorus loadings because salinity limits nitrogen fixation. This notation has lead to increasing interest to reduce nitrogen loadings and has been considered in the EU water framework directive (Howarth & Marino 2006).

2.9 Conclusios

The recently discovered abundance of diazotrophic organisms in aquatic environments has revolutionized the fundamental ideas about nitrogenase activity potential and the diversity of diazotrophic organisms. It appears that nitrogen fixation potential is a more common trait than previously presumed and diazotrophic species are found even

from the harshest environments. The environmental parameters control nitrogen fixation and cause nitrogenase activity to vary spatially and temporally. The modern methods based on stable isotopes and molecular biology have allowed more detailed investigations of these regulatory parameters and have brought more insight to variability of nitrogenase activity in between taxa and environments.

It has to be remembered that anthropogenic nitrogen fixation exceeds the natural nitrogen fixation in various areas (Vitousek 1994). One of the future challenges will be to determine how the anthropogenic nitrogen fixation will affect the nitrogen cycle. The increase in anthropogenic nitrogen fixation has been dramatic during the past few decades and it appears that it will still increase while the population on earth is multiplying. Also the global warming will most likely have an effect to the nitrogen cycle since most of the processes are biologically mediated. It may well be that nutrient budgets prepared right now will not hold for long because the rates of nitrogen redox processes will change with increasing temperatures.

3. MATERIAL AND METHODS

3.1 Preliminary preparations

The reliability of the gas chromatograph (FID, Perkin-Elmer Elite Alumina, at 80°C, H_2 as a carrier gas flowing 45 ml min^{-1}) used in the acetylene reduction measurements was tested by measuring the linear correlation between the peak areas and the ethylene concentrations. In the experiment, ethylene standards were prepared in 21.3 ml bottles in following concentrations: 0.005%, 0.01%, 0.05%, 0.1%, 0.5%, 1% and 2 %. From each sample bottle, three 100 µl samples of gas were withdrawn with a gas tight syringe needle and injected to the FID. The peak areas from each bottle were averaged and the linear correlation between ethylene concentrations and peak areas was determined.

When the enrichments in ^{15}N go well above the internal standard, the precision of the stable isotope mass spectrometer can be hindered (Tuula Sinisalo, pers. comm.). To test how the precision of the stable isotope mass spectrometer is interfered by high $\delta^{15}N$ values, samples from non-enriched moss ($\delta^{15}N$ =-3.6 ‰) and enriched moss ($\delta^{15}N$ = 132. 5 ‰) were prepared. In the first set of samples the total weight of each sample was 2 mg from where 0, 5, 10, 30, 50, 60, 80 and 100 % of the total weight was the enriched moss and the rest non-enriched moss. In the second set of the samples, the sample weight was 5 mg from where 0, 10, 30, 50 and 60 % was enriched moss and the rest non-enriched moss. Higher concentrations than 60 % were excluded from the 5 mg samples in order to protect the IRMS from contamination caused by the highly enriched samples. In both sets the samples were wrapped into tin cups (Elemental Microanalysis) and each concentration had three replicate samples. The details of the stable isotope mass spectrometer's settings are presented in section 3.2.

3.2. The acetylene reduction method and ^{15}N-tracer method

The experiment was executed by using water samples from Lake Muurikaisjärvi, locating in Leppälahti near Jyväskylä, which had a cyanobacterial bloom during the experiment. The water was concentrated by filtering through 60 µm plankton net prior to incubations the get enough biomass for analysis. The experiment followed the procedure described by Capone (1993) with some modifications. In the experiment, there was a total of six incubation bottles (glass septum bottles with grey butyl rubbers) of which three were darkened with aluminium foil so that no light was getting into the bottles. One additional

bottle was prepared with distilled water and acetylene to check the purity of the acetylene. The volume of the incubation bottles was 120 ml and in each bottle there was 50 ml of concentrated lake water with 10 ml of acetylene. Rest of the space was natural atmospheric air. After preparing the bottles, they were placed on a table shaker (100 rpm) under natural daylight outdoors. The weather of the incubation day was cloudy with temperature around 22°C. The production of ethylene was measured after 2, 4, 6 and 24 hours by injecting with a gas tight syringe needle 100 µl samples from each bottle to the gas chromatograph (FID, Perkin-Elmer Elite Alumina, at 80°C, H_2 as a carrier gas flowing 45 ml min^{-1}). The ethylene standards were prepared in 21.3 ml bottles in 0.01%, 0.1% and 1% concentrations and they were injected during every measuring period to check the linearity of the peak areas. The production of ethylene was calculated by the following formulas provided by Capone (1993):

$$\frac{(pkht, unk)}{(pkht, std)} * [std] * (GPV) * (SC) = nmolC_2H_4$$

where: (pkht,unk)= peak height response for C_2H_4 for 100 µl sample from an assay vessel; (pkht,std)= peak height response for 100 µl of an C_2H_4 standard; [std] = concentration of C_2H_4 standard used, expressed in nmol ml^{-1}, as determined by the ideal gas law corrected for room temperature and pressure; GPV = gas phase volume, the total volume of the head phase of the assay vessel in ml; and SC = solubility correction for C_2H_4 in aqueous phase.

Because C_2H_4 is relatively soluble in water, the ratio of the total volume (M) of C_2H_4 formed to the volume transferred to the gaseous phase (X) equals:

$$SC = M / X = 1 + (\alpha * A / B)$$

where: α = the Bunsen coefficient for C_2H_4 at the appropriate temperature and salinity; A = the volume of the aqueous phase; and B = the volume of the gas phase (or GPV)

The actual rate of nitrogenase activity is calculated as:

$$\frac{(nmolC_2H_4, t_n) - (nmolC_2H_4, t_0)}{t_n - t_0} = nmolC_2H_4$$

where: t_n is the time after n hours and t_0 is the time in the beginning of the experiment

The conversion factor between C_2H_2 produced and N_2 fixed was calibrated with the ^{15}N-tracer method. In the ^{15}N-tracer method, 0.610 L gas tight high density polyethene (HDPE) incubation bottles (Penli, Finland) were filled with concentrated lake water as full as possible without any air in the bottle. After careful filling and closing of the bottles, 2 ml of $^{15}N_2$-gas (98% purity, CK Gas Products LTD., Hampshire, UK, NML-361-1) was injected, and the bottles were placed on the same shaker table as the bottles with the acetylene. The purity of $^{15}N_2$-gas was known to be lower than 100% because there was some atmospheric air mixed into the bottle. Since atmospheric air contains 78.08 % nitrogen and 20.95% oxygen (Haavisto *et al.* 1996), the amount of atmospheric nitrogen in the bottle was determined by measuring the amount of oxygen in the bottle by gas chromatograph and using the ratio of atmospheric O_2/N_2 to determine atmospheric N_2 concentration. The atom percent of the $^{15}N_2$ in the bottle was calculated by the following formula:

$$Atom\%\ N_2 = \left(\frac{\left(mol\ \ {}^{15}N_2 * 98.00\%\right) + \left(mol\ \ N_{2\ atmosphere} * 0.366\%\right)}{mol\ \ total\ \ N} \right)$$

where: the atom % of $^{15}N_2$ was reported to be 98 % by the manufacturer, and the atom % of the atmospheric N_2 is 0.366 % (Montoya *et al.* 1996).

The ^{15}N enrichment of the POM was checked after 0, 2, 4, 6 and 24 hours by filtering 100 ml of water with a gentle vacuum through Whatman GF/F glass fibre filters (nominal pore size 0.7 μm). Each time interval had two replicate samples. After drying the filters overnight in 60°C, the surface of the dry filters was peeled of and wrapped into tin cups (Elemental Microanalysis). Later the isotopic ratio of the samples was analysed with Thermo Finnigan Delta Plus isotope ratio mass spectrometer (IRMS) where the combustion of samples was under ultra pure helium gas flowing 90 ml min^{-1}. The temperature in the oxidation furnace was 900°C, in the reduction furnace 640°C, and in the gas chromatograph 80°C. N_2-gas served as an internal reference and all isotopic values are reported relative to the atmospheric N_2 ($\delta^{15}N$-scale). The software used to calculate the $\delta^{15}N$-values was ISODAT 2.0. Potato leaves ($\delta^{15}N$ = 4.302, $\delta^{13}C$ = -26.261, N% = 4.21, C%=34.18) served as an internal standard which was calibrated with IAEA standards acetanilide and atropine prior to analyses. The $\delta^{15}N$-values and the N percentages were corrected for linearity and drift as described by Werner & Brand (2001).

The nitrogen fixation rate was calculated by using the following formulae presented by Montoya *et al.* (1996). The obtained $\delta^{15}N$ values were first converted to atom percentages:

$$A(atom\%) = 100 \times \left| \frac{\left(10^{-3}\delta^{15}N + 1\right)\left({}^{15}N/{}^{14}N\right)_{atmosphere}}{1 + \left(10^{-3}\delta^{15}N + 1\right)\left({}^{15}N/{}^{14}N\right)_{atmosphere}} \right|$$

where: $(^{15}N/^{14}N)_{atmosphere}$=0.003676.

Then the actual turnover of N through N_2 fixation was calculated by the following formula:

$$V\left(T^{-1}\right) = \left(\frac{1}{\Delta t}\right)\left(\frac{A_{PN_f} - A_{PN_0}}{A_{N_2} - A_{PN_0}}\right)$$

where: $V(T^{-1})$ represent the specific rate of uptake of N_2 particles in the experimental bottle and Δt the duration of the experiment. A_{PN0} is the ^{15}N enrichment of the particles in the beginning of the experiment and A_{PNf} in the end of the experiment. A_{N2} represents the enrichment of N_2 available for fixation.

Finally the volumetric rate N_2 fixation in mol of N_2 fixed per litre of water per hour was calculated:

$$\rho\left(M * L^{-3} * T^{-1}\right) = \frac{V}{2}\left[\overline{PN}\right]$$

where $\left[\overline{PN}\right]$ is the mean concentration of particulate nitrogen during the experiment.

3.3 Spatial variation of nitrogenase activity in the Lammi area

In the Lammi area lakes Pääjärvi, Ormajärvi, Valkea-Kotinen and Mekkojärvi (Appendix 1) were tested in summer 2006 for nitrogenase activity from three different

depths. The lakes were selected to represent different lake types so the limnological features between the lakes varied a lot (Table 3, Appendix 1, Appendix 2).

Table 3.The limnological features of the experimental lakes in Lammi.

Lake	**Pääjärvi**	**Ormajärvi**	**Valkea-Kotinen**	**Mekkojärvi**
Sampling date	July 25th	July 26th	July 24th	July 24th
Type	Dystrophic	Clearwater/eutrophic/ cyanobacterial blooms	Highly dystrophic	Highly dystrophic
Area	13.4 km^2	6.5 km^2	0.04 km^2	0.004 km^2
Max. temp.(°C)	19.2	20.5	21.7	18.3
Min. temp. (°C)	10	6.1	5.0	4.4
Max. O_2 %	92.2	111	105	73
Min. O_2 %	78.8	32.1	0	0

The sampling depths were the hypolimnion, the anoxic-oxic interphase in metalimnion and epilimnion (Appendix 1). Because Pääjärvi and Ormajärvi did not have an anoxic-oxic interphase, the second sampling depth in these lakes was in the middle of the water column. Every sampling depth had three replicate incubation bottles and three control samples, and in each lake there were additional incubation bottles in the surface where the filamentous cyanobacteria were excluded by filtering the water through 100 μm plankton net prior to the final filtration (noted as fraction). In the sampling procedure, the water was lifted from the incubation depth and poured into 0.610 L gas tight high density polyethene (HDPE) incubation bottles (Penli, Finland) until overflowing. After that, each bottle was enclosed with a gas tight cap and injected with 2 ml of $^{15}N_2$-gas (98% purity, CK Gas Products LTD., Hampshire, UK, NML-361-1, corrected for mixing of atmospheric air as in section 3.2), shaken for one minute to ensure the mixing of the gas, and lowered back for incubation. All bottles that were filled with water from the aphotic zone were darkened during the filling in order to protect the light sensitive diazotrophs. After 24 hour incubation in the lakes, the bottles were lifted up and brought to the laboratory in the Lammi Biological Station where the contents of the bottles were filtered on Whatman GF/F filters (nominal poresize 0.7 μm) with gentle vacuum. After filtration, the filters were dried in 60 °C overnight and approximately 2/3 of the surface of the dry filter papers was scraped off and placed into tin cups (Elemental Microanalyses) for stable isotope analysis. The stable isotope analysis was performed as described in the section 3.2. The samples from Mekkojärvi were sent to the stable isotope laboratory of Cornell University because the analysis was not satisfactory when analysed as in section 3.2 in Jyväskylä. In Cornell University the samples were analysed with Thermo Finnigan Delta Plus IRMS where the combustion of samples was in the presence of ultra pure helium gas with a flow of 120 ml min^{-1}. The oxidation furnace contained copper oxide and chromium oxide and the temperature was 1000°C. The temperature was 750°C in the reduction furnace and 80°C in the gas chromatograph. Methionine served as an internal standard and N_2-gas as an internal reference. The standard deviation of the $\delta^{15}N$-values of the methionine during the analysis was 0.1. All isotopic ratios are reported on a $\delta^{15}N$-scale and the $\delta^{15}N$-values were calculated by using the ISODAT 2.0. software. The actual nitrogen fixation rates were calculated by using the formulas by Montoya *et al.* (1996) presented in section 3.2.

3.4 Temporal variation in the $\delta^{15}N$ value of POM

The natural variation of $\delta^{15}N$ value of particulate organic matter (POM) was analyzed from four different sites of the Äänekoski water course (Appendix 4). The sampling sites were Poronselkä and Ristinselkä in Lake Päijänne, Lake Vatianjärvi and Rapid Kapeenkoski. Two replicate water samples were taken from the surface of the water column on every two weeks from the beginning of June until end of August. Once the samples were collected, they were brought to the laboratory where one litre of lake water from each site was filtered with a gentle vacuum through Whatman GF/F filters (nominal poresize 0.7 µm) unless the filter was clogged earlier. After filtration, the GF/F filters were dried in 60°C overnight and the dry surface of the filter paper was scraped off, ground to a fine powder and wrapped into tin cups (Elemental Microanalyses). The $\delta^{15}N$-value of the samples was analyzed by using the Thermo Finnigan Delta Plus IRMS in Jyväskylä with parameters described in section 3.2. Additionally during each sample collection time, total nitrogen, ammonia, nitrate & nitrite, total phosphorus and phosphate concentrations were analyzed in the Environmental Research Centre laboratory in Jyväskylä by using standard methods SFS-EN ISO 11905-1:1998, SFS3032:1976, SFS-EN ISO 13395:1996, A40 B Aquakem and A40 A Aquakem, respectively.

4. RESULTS

4.1 Preliminary preparations

The relationship between the peak areas and the ethylene concentrations was very linear (R^2= 0.998) when measured with the gas chromatograph. These results suggest that gas chromatograph reliably measures ethylene production (Figure 4).

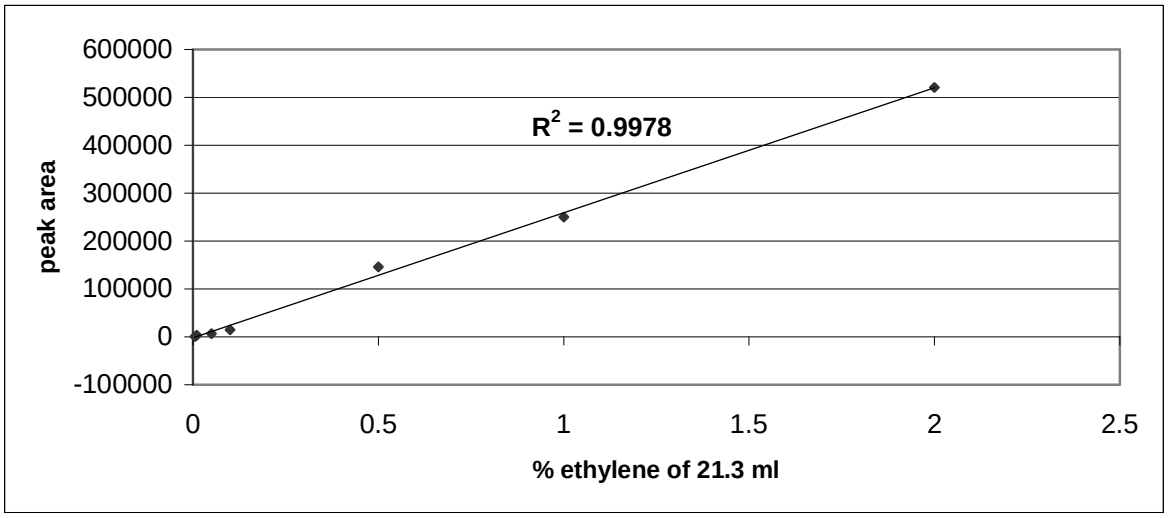

Figure 4. The correlation between the peak area and ethylene concentration

Increased $\delta^{15}N$ values seemed to have very little effect on the precision of the IRMS because the $\delta^{15}N$-value remained relatively linear even when the $\delta^{15}N$-value was as high as 133 ‰ (Figure 5). The weight of the samples did not affect the linearity of the $\delta^{15}N$-values because the R^2-value of the 5 mg samples was 0.9852 and for the 2 mg samples 0.9833.

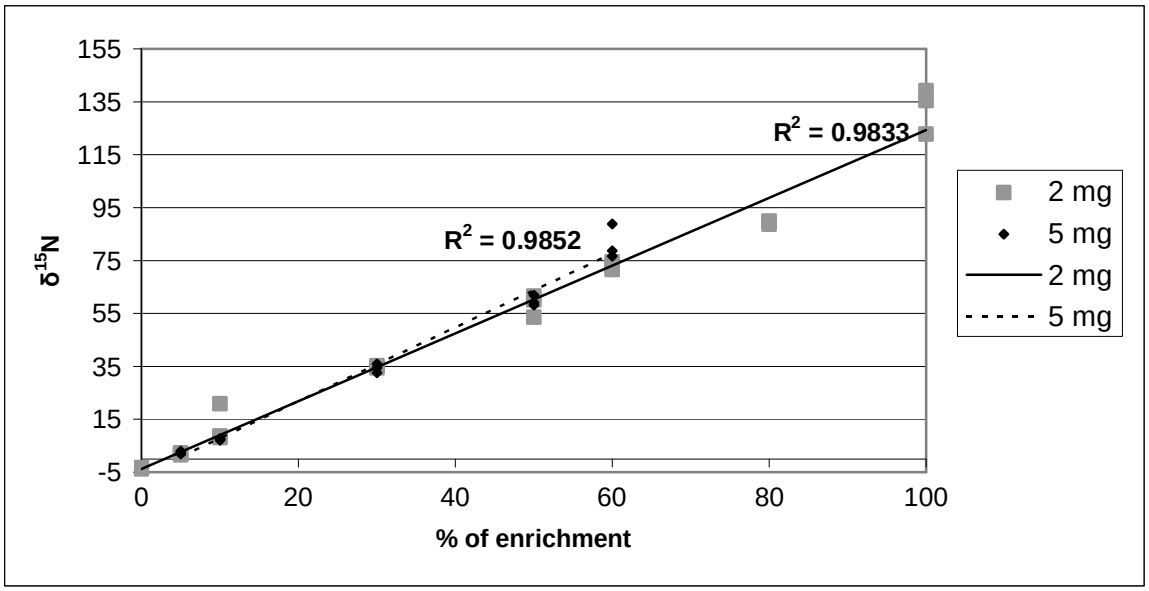

Figure 5. The increase of the $\delta^{15}N$-value when the concentration of artificially enriched moss increases in the sample.

4.2 The acetylene reduction method

The nitrogenase activity was very high in Lake Muurikaisjärvi. It has to be noted that these results are not quantative because of the concentrated water used in the experiment. Instead these results determine the conversion factor between the ethylene production and the nitrogen fixation and investigate how light availability affects the nitrogen fixation rate.

The ethylene production rate after two hours of incubation in the light bottles was 20.81 x 10^{-7} mol of C_2H_2 l^{-1} h^{-1} and the production remained linear throughout the experiment (Figure 6). The rate of nitrogen fixation was much slower in darkened bottles compared to light bottles and ethylene production nearly stopped after 6 hours (Figure 7). This suggests that nitrogenase activity is highly dependent on light availability in the Lake Muurikaisjärvi. The bottles that included distilled water with acetylene did not yield any ethylene peaks, so the acetylene did not have any ethylene contamination. The ethylene standards remained relatively linear during all measurement periods (R^2= 0.974-0.986) indicating the FID was measuring ethylene concentrations correctly.

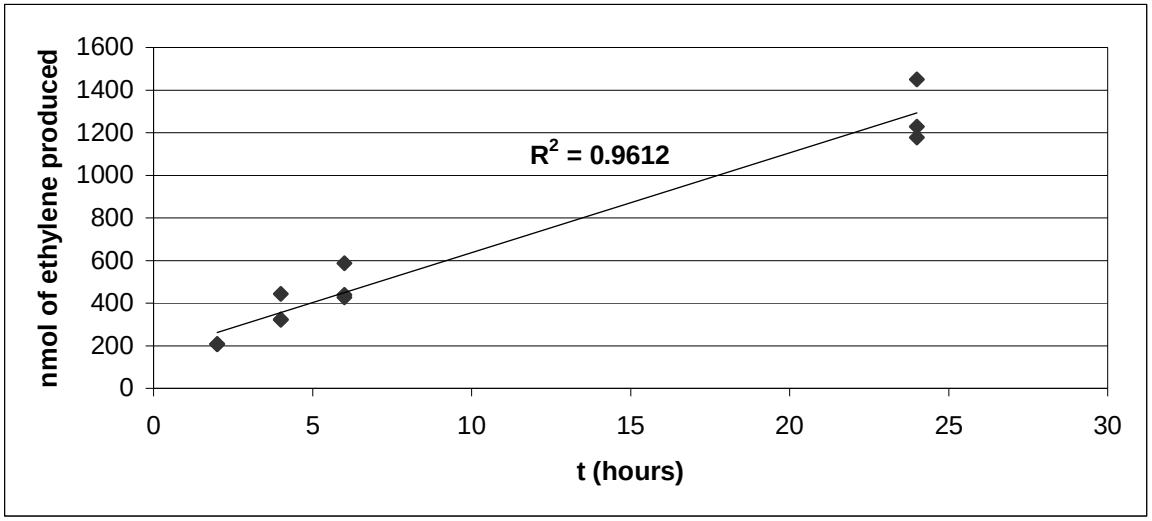

Figure 6. The production of ethylene in light bottles. The R^2 = 0.9612 which indicates that the production of ethylene (nitrogen fixation) is linear.

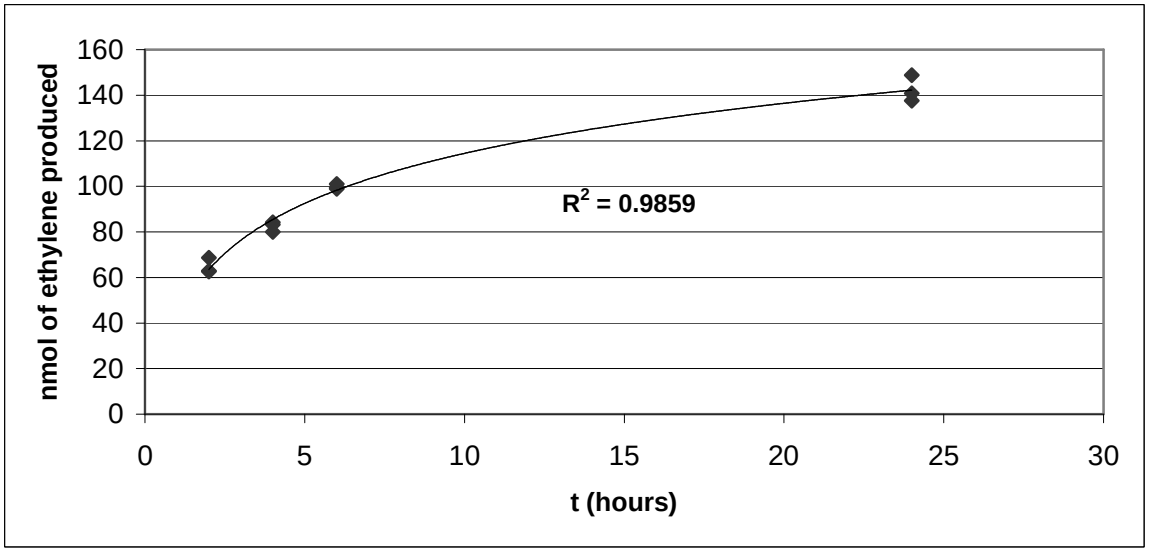

Figure 7. The production of ethylene in darkened bottles. The R^2 = 0.9859 when the trend line is logarithmic.

When nitrogen fixation in Muurikaisjärvi was measured by using the ^{15}N-tracer method, the volumetric rate of nitrogen fixation after two hours was 2.62 mol of N_2 $l^{-1}h^{-1}$ (Figure 8) and the ratio for moles of ethylene produced per 1 mole of N_2 gas fixed was 7.68. Unfortunately the nitrogen fixation was so vigorous that the enrichment in ^{15}N became too high to measure after 2 hours.

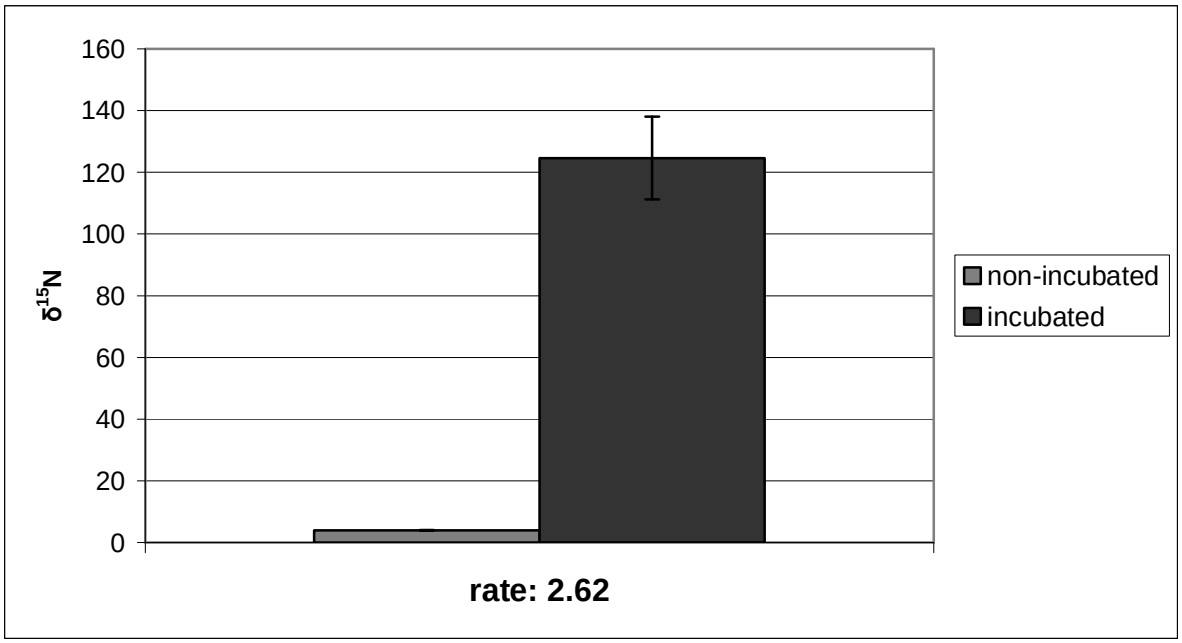

Figure 8. The $\delta^{15}N$ values of non-incubated and incubated concentrated water samples after two hours from Muurikaisjärvi. Error bars represent the standard error of the average and rate notes for the measured nitrogen fixation rate in 10^{-7} moles of N_2 $h^{-1}l^{-1}$.

4.3 Spatial variation in nitrogen fixation in the Lammi area

The stable isotope analysis of the POM from the Lammi area was equivocal. The samples from Pääjärvi and Valkea-Kotinen did not have enough biomass on the filters for reliable stable isotope analyses, so they were excluded from the data. There was some variation in $\delta^{15}N$-values between replicate samples in Mekkojärvi (Figure 9) but variation between replicates was especially apparent in the epilimnetic samples from Ormajärvi (Figure 10). The determinations whether the $\delta^{15}N$-values were significantly different before and after the incubation was determined by using the Whitney-Mann U-test because the values were not normally distributed (Kolmogorov-Smirnov statistic = 0.299 for non-incubated and 0.335 for incubated samples in Mekkojärvi and 0.324 for non-incubated and 0.291 for incubated in Ormajärvi, all values statistically significant $p< 0.05$). The sites in Mekkojärvi where the $\delta^{15}N$ values were significantly different before and after the incubation were the epilimnion (non-fractioned and fractioned samples) and the metalimnion. The rates of nitrogen fixation in Mekkojärvi were 3.60 x 10^{-11} moles of N_2 $h^{-1}l^{-1}$ in the non-fractioned epilimnion, 2.55 x 10^{-11} moles of N_2 $h^{-1}l^{-1}$ in the fractioned epilimnion, and 2.47 x 10^{-11} moles of N_2 $h^{-1}l^{-1}$ in the metalimnion (Figure 9). In Ormajärvi nitrogen fixation was detected in the epilimnion (fractioned and non-fractioned samples) as well as in the hypolimnion. The nitrogen fixation rates in Ormajärvi were in the non-fractioned epilimnion 3.80 x 10^{-9} moles of N_2 $h^{-1}l^{-1}$, in the fractioned epilimnion 1.80 x 10^{-9} moles of N_2 $h^{-1}l^{-1}$ and in the hypolimnion 0.07 x 10^{-9} mol of N_2 $h^{-1}l^{-1}$ (Figure 10).

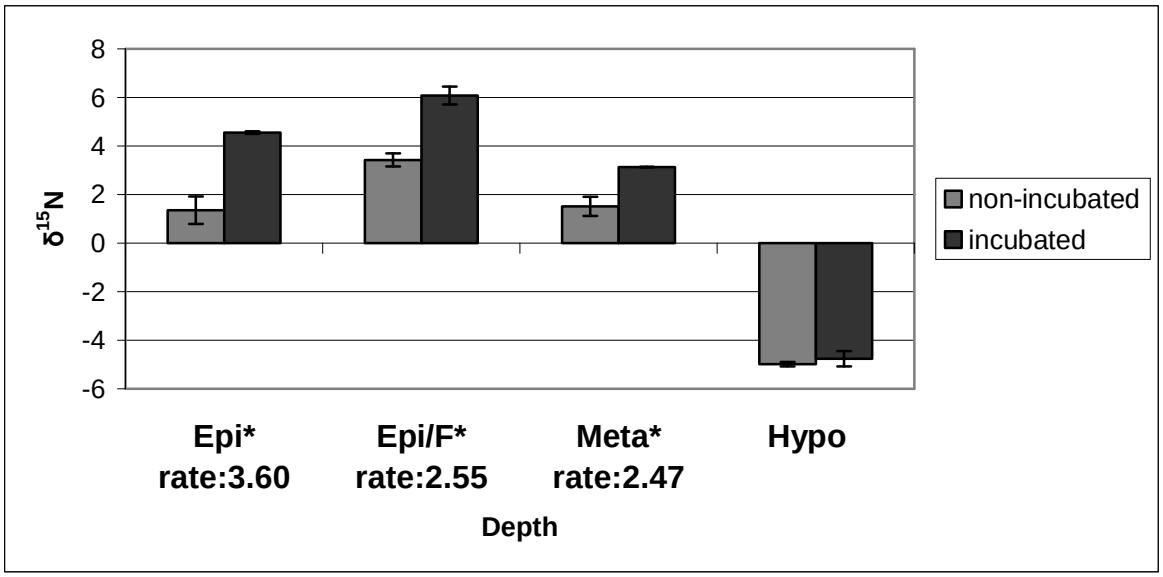

Figure 9. The $\delta^{15}N$ values of incubated and non-incubated samples from different depths in Lake Mekkojärvi. Epi=epilimnion. Epi/F=fractioned epilimnion, Meta=metalimnion and Hypo=hypolimnion. Error bars represent the standard error of the average and rate notes for the measured nitrogen fixation rate in 10^{-11} moles of N_2 $h^{-1}l^{-1}$. *The incubated and non-incubated samples are significantly different (Whitney-Mann p = 0.05).

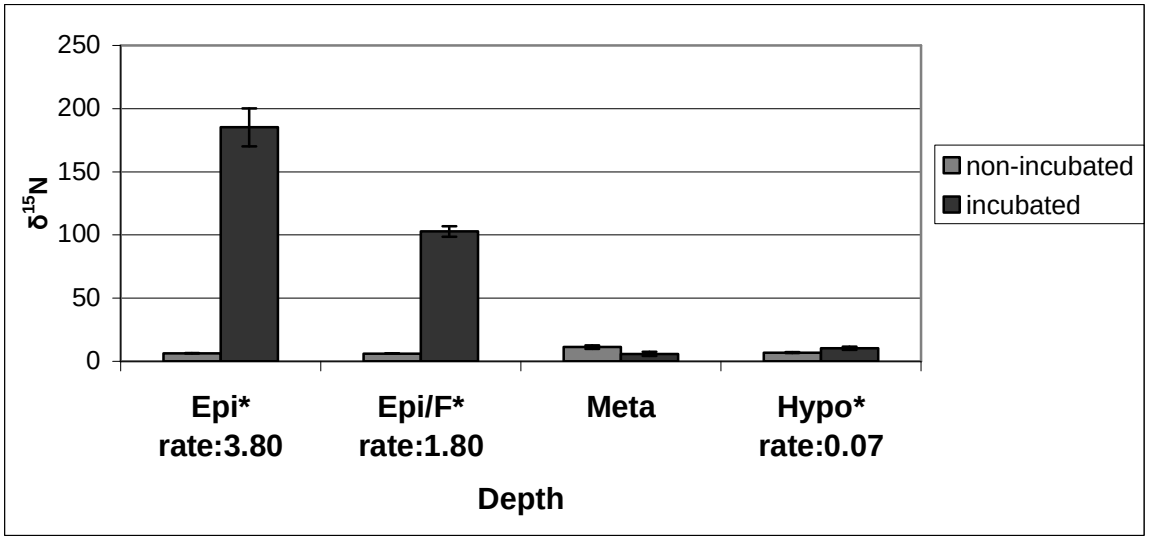

Figure 10. The $\delta^{15}N$ values of incubated and non-incubated samples from different depths in Lake Ormajärvi. Epi=epilimnion. Epi/F=fractioned epilimnion, Meta=metalimnion and Hypo=hypolimnion. Error bars represent standard error of the average and rate notes for the measured nitrogen fixation rate in 10^{-9} moles of N_2 $h^{-1}l^{-1}$. *The incubated and non-incubated samples are significantly different (Whitney-Mann p = 0.05).

4.4 Temporal variation in the $\delta^{15}N$ of the POM

The $\delta^{15}N$ values of particulate organic matter (POM) were decreasing towards the end of the July and the beginning of August. The decrease in $\delta^{15}N$-values is noticed especially in Poronselkä and Ristinselkä but was less notable in Vatia and Kapeenkoski (Figure 11). It has to be noted that the precision of the data is not statistically satisfactory because part of the samples was lost during the analysis and in some cases variation

between replicates is very high. From the available data the correlations between the δ^{15}N-values of POM and the nutrient concentrations were determined by using Spearman's rho 1-tail correlation analysis.

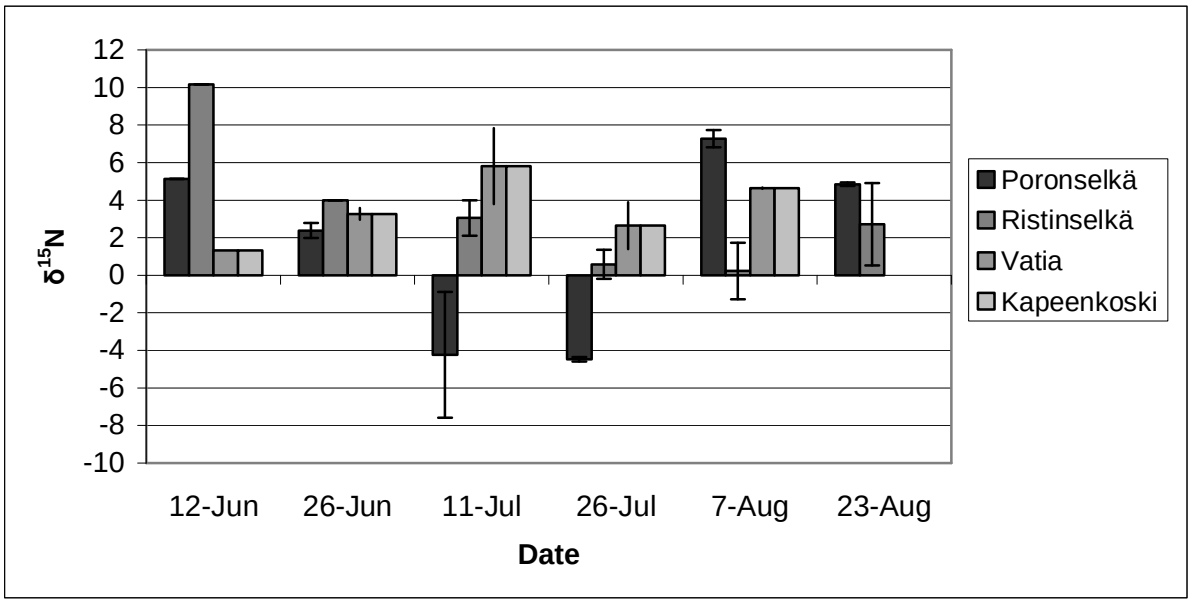

Figure 11. The variation of the natural δ^{15}N values of POM in four different locations of the Äänekoski water course. Error bars are indicating the standard error of the average when replicate samples were available. The data is missing from Vatia and Kapeenkoski on August 23rd.

The nitrate concentrations were decreasing at all sites towards end of the summer. Simultaneous decrease of nitrate and nitrite concentration and POM δ^{15}N-values was observed in Poronselkä and Ristinselkä (Figure 11, Figure 12). Nitrate and nitrite concentrations were reflected into POM δ^{15}N-values with a delay and there was a significant correlation between nitrate and nitrite concentration and POM δ^{15}N-values observed two weeks later in Ristinselkä ($r = 0.900$, $p = 0.019$) and nearly significant correlation in Poronselkä ($r = 0.700$, $p = 0.094$) (Figure 13). Similarly, a significant correlation existed when the data from Poronselkä and Ristinselkä was combined ($r = 0.614$, $p = 0.029$). In Ristinselkä, a significant correlation also existed between the nitrate and nitrite concentration and simultaneous POM δ^{15}N-values ($r = 0.771$, $p = 0.036$).

There was a decreasing trend in the nitrate and nitrite to phosphate ratio towards the mid-summer (Figure 14) and a significant correlation was observed between the POM δ^{15}N-values and simultaneous nitrate and nitrite to phosphate ratio in Poronselkä ($r = 0.771$, $p = 0.036$) (Figure 15). A significant correlation was also observed when the data from Poronselkä and Ristinselkä was combined ($r = 0.524$, $p = 0.040$). Although there was a decreasing trend in the total nitrogen to total phosphorus ratio, it did not correlate in any site with the POM δ^{15}N-values (Figure 16). None of the nutrient concentrations correlated with δ^{15}N-values of POM in Vatia or Kapeenkoski.

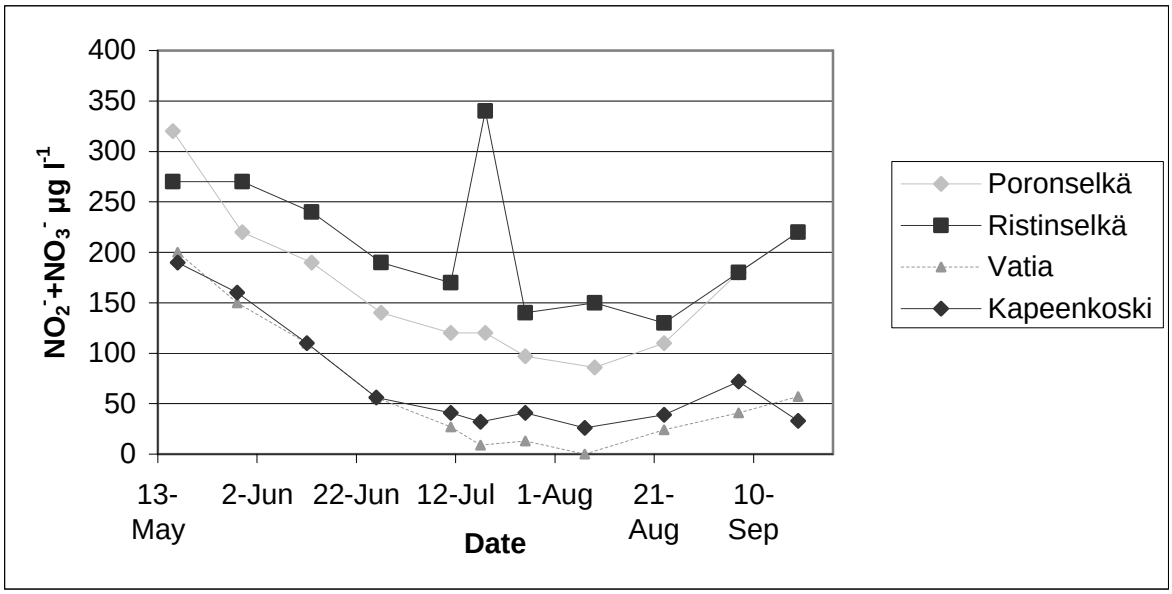

Figure 12. The concentration of NO_2^- + NO_3^- in four different locations in the Äänekoski water course.

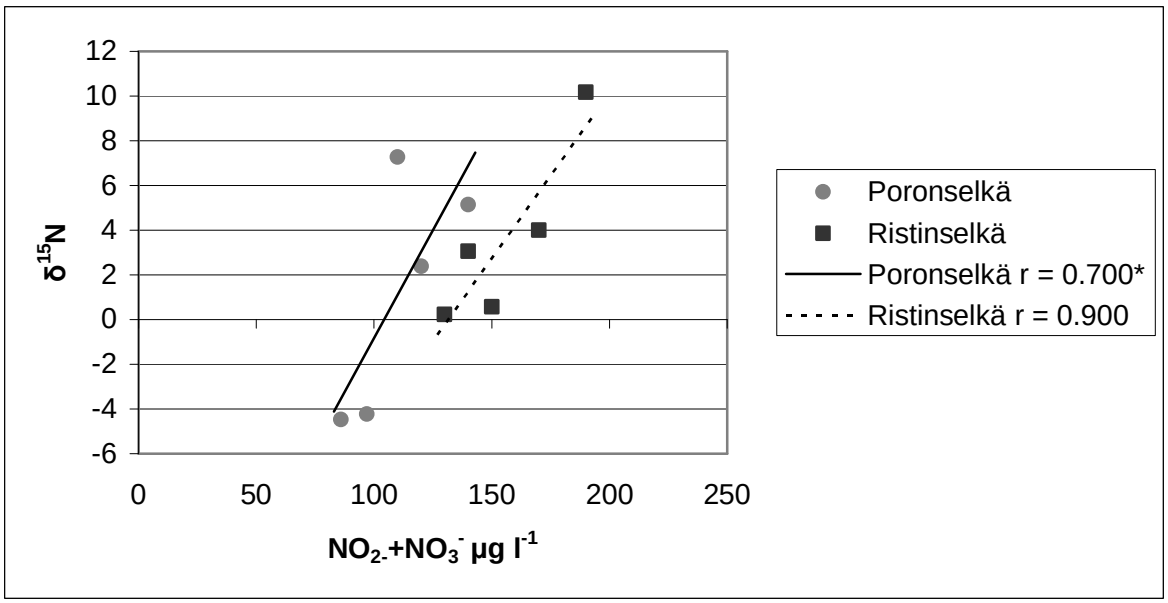

Figure 13. The NO_2^- and NO_3^- concentrations plotted against POM $\delta^{15}N$-values observed two weeks later. When the data from both sites was combined r = 0.614 (p = 0.029, significant at the 0.05 confidence level). *Value is not significant at the 0.05 confidence level (p = 0.094)

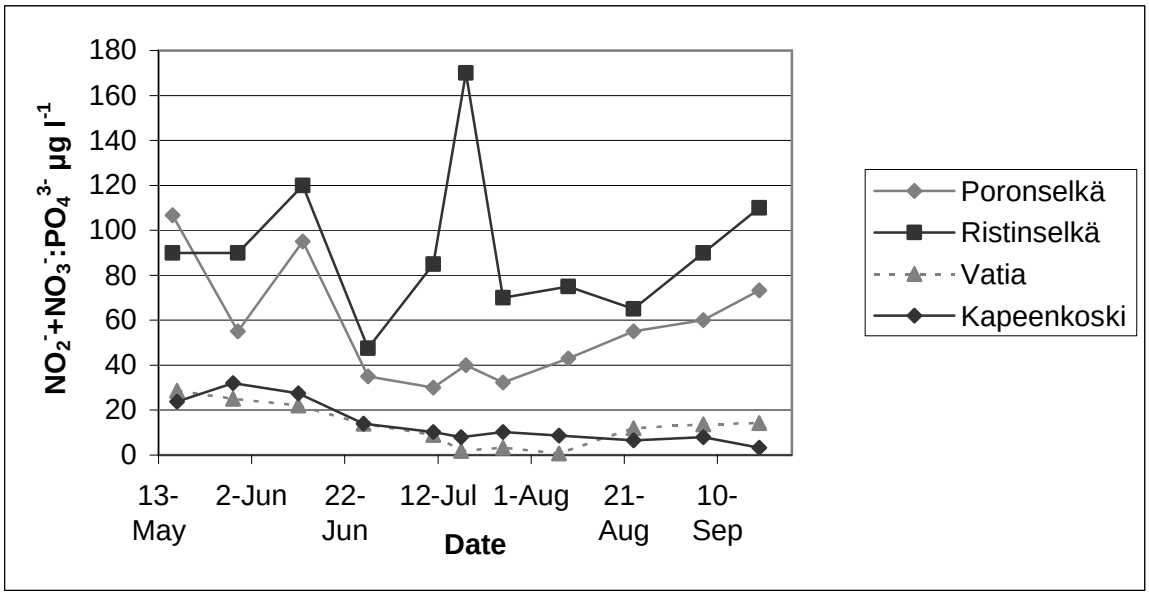

Figure 14. The NO_2^- + NO_3^- to PO_4 ratio by weight in the Äänekoski water course.

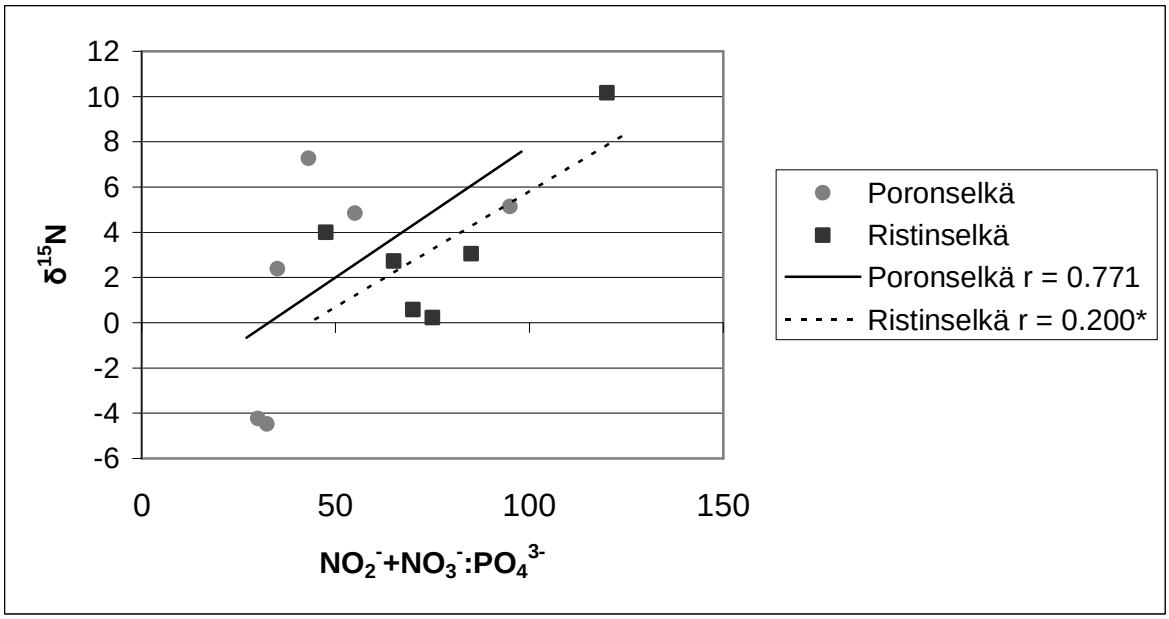

Figure 15. The nitrate & nitrite to phosphate ratio plotted against simultaneous POM $\delta^{15}N$-values. When the data was combined from both sites r = 0.524 (p = 0.040, significant at the 0.05 confidence level). *Value is not significant at the 0.05 confidence level (p = 0.352).

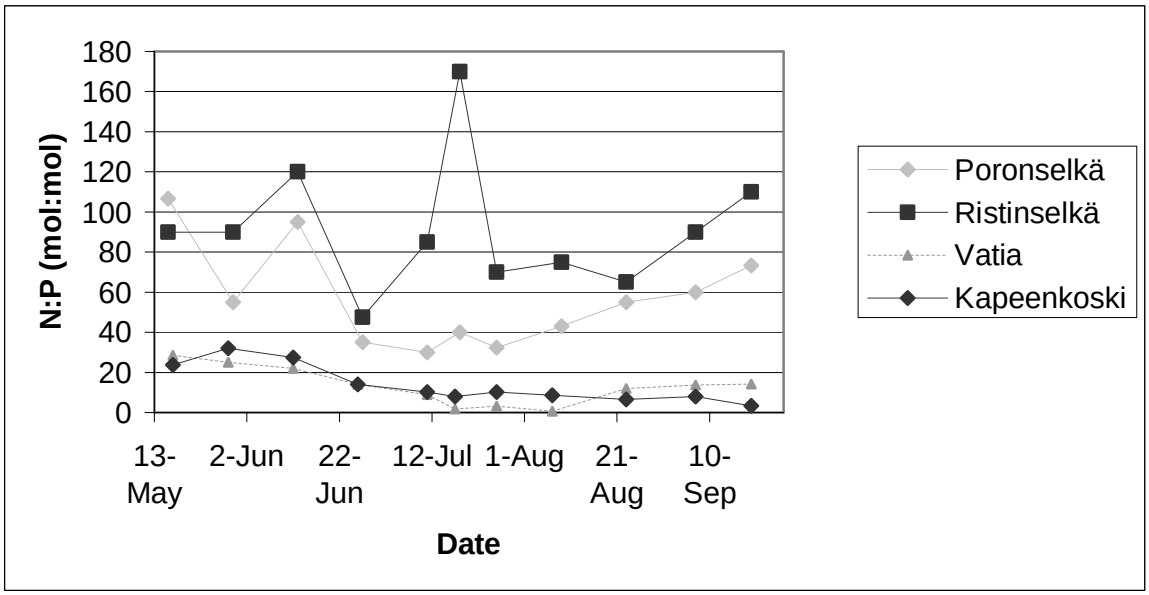

Figure 16. The molar total nitrogen to total phosphorus ratio.

5. DISCUSSION

5.1 Conversion factors for ethylene produced to dinitrogen fixed in the acetylene reduction technique

The comparison between the acetylene reduction and the ^{15}N-tracer techniques gave information about the applicability of these methods. It was unfortunate that the enrichment in ^{15}N became too high to analyze from the samples after two hours because it would have indicated how the conversion factor varies over time.

Table 4: The conversion ratio for C_2H_2 produced to N_2 fixed, n/a notes for data not available. *After two hours of incubation with two replicate samples.

	mol:mol	standard deviation
Mague *et al* (1974)		
Rhizosolenia spp.	9.29:1	5.34
Capone (1993)		
Theoretical	3:1	n/a
Corrected for hydrogen evolution	4:1	n/a
Montoya *et al*. (1996)		
Field samples	4.68:1	n/a
Moisander *et al* (1997)		
In pure cultures of cyanobacteria		
for *Nodularia spumigena*	3.5:1	1.9
for *Nodularia sphaerocarpa*	4.5:1	2.5
In field samples		
no concentration (light)	11.1:1	6.8
concentrated (light)	12.3:1	4.0
Mullholland *et al.* (2006)		
In field samples from gulf of Mexico	15.7-3.7:1	
Muurikaisjärvi, August 2006*	7.68:1	1.26

The conversion factor in Muurikaisjärvi settles in the mid-range when compared to results obtained by other researchers (Table 4). The standard deviation of conversion factor might have been higher if more replicate samples and time intervals would have been available. There are also some factors that might have influenced the results. Unlike the GC, the precision of the IRMS varied especially when δ^{15}N-values were high. The problems in the stable isotope analyses were mainly caused by incomplete burning of GF/F filters. The melted GF/F filters clogged the furnace and formed gas pockets that were bursting randomly and altering the δ-values. Also, high enrichments were problematic because there was no reliable standard to compare the results to. Although the IRMS gave reliable results with the moss samples to the enrichment levels of 133 ‰ (Figure 5), the δ^{15}N-values from Ormajärvi were at their highest around 200‰ and in Muurikaisjärvi around 140‰, and the variability between replicates was high (Figure 8, Figure 10). The large variation between replicate samples when δ^{15}N-values are high can also be caused by a "memory effect" where a high δ^{15}N-value of a sample carries-over to the next sample because of the natural degradation of the reduction furnace during the sample analyses (Kasson 2007).

The variation between the nitrogen fixation-acetylene reduction conversion factors is high (Table 4) and it is always necessary to calibrate nitrogen fixation rate with the ^{15}N-tracer technique. This can be difficult if part of the fixed nitrogen is secreted as organic nitrogen outside the cells, as was the case in the Gulf of Mexico (Mullholland *et al.* 2006). The advantage of using the acetylene reduction technique in these cases is that it is measuring the gross nitrogen fixation when the ^{15}N-tracer technique measures only the net nitrogen fixation. The downside of using the acetylene reduction technique is that it requires plenty of biomass since it is not as sensitive as the ^{15}N-tracer technique (Wasmund *et al.* 2001). This problem is often solved by concentrating water but in turn this can create problems because some of the small diazotrophs are excluded from the analyses and the analysis is not quantative. Also as mentioned earlier, the suitability of acetylene is questionable to some diazotrophs.

Nitrogen fixation in Lake Muurikaisjärvi stopped soon after darkening of the bottles (Figure 7). It can be suspected that nitrogen fixation in the epilimnion of Muurikaisjärvi is fuelled by photosynthesis. Thus, when the photosynthesis stops during darkness, nitrogenase production is exhausted. The reason why ethylene production continued in the dark bottles for a short period is probably because there is a lag period between the nitrogenase production and nitrogen fixation. Hence, dinitrogen, or in this case acetylene, reduction continues for a short period after photosynthesis stops until all nitrogenase has been used up. The linearity of ethylene production in the light bottles was not expected because there was a short dark period during the incubation. If the samples would have been taken more often, the line might not be as linear because of the darkness and decreased carbon availability in the bottles. It may well be that actually the line was increasing only during the first light period and levelled off when darkness began.

5.2 Nitrogen fixation in the dimly lit and anoxic waters

The development of the methods in stable isotope spectrometry and molecular biology has increased the research in aquatic nitrogen dynamics. Stable isotope analyses allow surveying areas that have not been previously investigated and provide precision for more accurate measurements. Molecular biology has enabled more precise diversity analyses of diazotrophs, and this has opened new views to investigate the potential for nitrogenase activity. Although majority of data is from marine environments, it can be

assumed that in the future the information in this area will increase dramatically also for the lacustrine environments.

The common occurrence of diazotrophy and nitrogenase activity in the dimly lit areas has been one of the most important discoveries during the past decade in marine nutrient dynamics (Wasmund *et al.* 2001, Zehr *et al.* 2001, Montoya *et al.* 2004). However, light independent nitrogen fixation was noticed in the anoxic regions of dystrophic lakes already in the late 1960s (Brezonik & Harper 1969), but this early discovery was largely ignored. This can possibly be because there have not been sensitive enough methods available to test for low rates of nitrogen fixation until now, when IRMS are more common and easier to use than previously. Knowing all this, it is not surprising that the results obtained from dystrophic Mekkojärvi indicate that nitrogen fixation was also occurring in this highly dystrophic lake, especially because there was very limited amounts of nitrogen present in the water (Figure 9, Appendix 1). It might be that nitrogen fixation is also occurring in the hypolimion but it did not show in this experiment because there was a little oxygen mixed in to the injected $^{15}N_2$-gas which might have destroyed the nitrogen fixation capacity of the hypolimnetic diazotrophs. The nitrogen fixation in Mekkojärvi is probably mediated by the diazotrophic green sulphur bacteria *Chlorobium* spp. because the lake has a dense population of this species (Marja Tiirola pers. comm). In contrast to Brezonik & Harper's (1969) discovery, in Mekkojärvi nitrogen fixation was also detected at the surface where oxygen saturation was 73%. The lack of cyanobacterial blooms and the fact that *Chlorobium* spp inhabit only the anoxic and aphotic regions of the water column leads to an interesting question: what are the organisms that fix nitrogen at the surface? One possible explanation could be the small, non-heterocystous and diazotrophic cyanobacteria that are known to be efficient nitrogen fixers in various aquatic environments (Zani *et al.* 2000, Wasmund *et al.* 2001, Zehr *et al.* 2001, Montoya *et al.* 2004). The diversity analysis of *nifH* gene from the surface could give more insight to this question and it is currently being analyzed by the research team. Also, it would be informative to know whether nitrogen fixation in Mekkojärvi is also occurring during the winter, because it seems to be light independent in the metalimnion. In order to investigate this, the analyses should be repeated when the lake is covered with ice.

5.3 Nitrogen fixation in eutrophic lakes

It was hypothesized that Ormajärvi should have a high nitrogen fixation rate at the surface because there was a cyanobacterial bloom during the experiment. This was indeed observed although there was no nitrogen limitation in any of the sampling depths (Figure 10, Appendix 1). Similar rates have been observed in various eutrophic aquatic systems in the Northern Hemisphere (Table 5).

Table 5. Maximum nitrogen fixation rates observed in various eutrophic aquatic systems. *The initial rate was reported as g of N m^{-2} yr^{-1}. The hourly rate was calculated assuming a 5 m deep zone of nitrogen fixation, 65 d nitrogen fixation period and 10h of nitrogen fixation activity per day.

Site:	**Maximum nitrogen fixation rate in mol of N_2 $l^{-1}h^{-1}$**	**Measuring method**	**Reference**
Baltic Sea	3.9×10^{-9}	^{15}N-tracer	Ohlendieck *et al.* 2000
Baltic Sea	6.5×10^{-9}	^{15}N-tracer	Wasmund *et al.* 2001
Lake Verivi/ Estonia	8.9×10^{-9}	Acetylene reduction	Tõnno *et al.* 2005
Lake Võrtsjärv/ Estonia	7.7×10^{-9}	Acetylene reduction	Tõnno & Nõges 2003
Lake Erie/ Canada	5.05×10^{-9} *	Acetylene reduction	Howarth *et al.* 1988a
Lake Ormajärvi July 26th.	3.80×10^{-9}	^{15}N-tracer	

Nitrogen fixation was also detected in the fractioned samples from which filamentous cyanobacteria were excluded. This can suggest two things; either the filamentous cyanobacteria release part of their fixed nitrogen outside their cells and this nitrogen is utilized by other small organisms (Ohlendieck *et al.* 2000, Mullholland *et al.* 2006) or there are small picoplankton cells that are fixing nitrogen (Zani *et al.* 2000, Wasmund *et al.* 2001, Zehr *et al.* 2001, Montoya *et al.* 2004). Nitrogen fixation was also detected in the hypolimnion close to the bottom. It should be noted that although the rate of nitrogen fixation was much smaller compared to the surface, it might be constant around the year since it has to be light independent. This may provide a significant portion of the total nitrogen and in order to confirm this, the analyses should be repeated during the winter. These results strengthen the idea that nitrogen fixation occurs also in the dimly lit areas and these areas should be included into nitrogen budgets since their contribution can be significant. The loss of samples from lakes Pääjärvi and Valkea-Kotinen was unfortunate because they would have given more insight to the variability of nitrogenase activity.

5.4 Temporal variation of the δ^{15}N-value in POM

In this part of the research project the problems with the stable isotope analyses hindered most the accuracy of the results. Several samples were lost during the analysis, and the data from the Äänekoski water course is incomplete. From the available data it was observed that the nitrate and nitrite concentrations and, nitrate and nitrite to phosphate ratio correlate with the POM δ^{15}N-values (Figure 13, Figure 15). This is reasonable because nitrogen limitation generally triggers nitrogen fixation, and hence should decrease POM δ^{15}N-values. There appears to be a small delay between decreased nitrate and nitrite concentrations and the decrease of POM δ^{15}N-values. Thus, it seems that it takes a short period before nitrogen fixation initiates when nitrate and nitrite concentrations decrease, or it takes a short period before nitrogen fixation is reflected to the POM δ^{15}N-values, or both. The correlation between nitrate and nitrite to phosphate ratios and simultaneous POM δ^{15}N-values is probably because the ratio reached its lowest values earlier than the nitrate and nitrite concentrations (Figure 12, Figure 14). This is because the concentration of

phosphorus did not fall as quickly as the nitrate and nitrite concentration. Interestingly the molar total nitrogen to total phosphorus ratio did not decrease dramatically in unison with the POM δ^{15}N-values. It was not expected because it is often considered to be one of the factors that trigger nitrogen fixation (Howarth *et al.* 1988b). This can be because in lakes, the organic phosphorus is more available than the organic nitrogen hence the total nitrogen to total phosphorus ratio does not reflect the availability of these nutrients (Howarth *et al.* 1988b). The critical total nitrogen to total phosphorus molar ratio for nitrogen limitation in lakes has been proposed to be 65:1 (Howarth *et al.* 1988b) and in Vatia and Kapeenkoski indeed this was observed (Figure 16) although it was not reflected into the δ^{15}N-values.

The reason why the δ^{15}N-values of POM did not decrease very much in Vatia and Kapeenkoski (Figure 11) might be because they are heavily influenced by the paper and pulp industry and the POM in these areas contains fibre originating from the mills. Hence, POM in this area does not reflect well the δ^{15}N-value of the biomass. These results indicate that using solely the natural δ^{15}N-values is not recommended in nitrogen fixation studies because the sources of nitrogen cannot be precisely known. However, the nitrate & nitrite to phosphate ratios in Vatia and Kapeenkoski were below 16 and the total nitrogen to total phsphorus ratios were below 65 suggesting nitrogen limitation. Based on the nitrogen limitation occurring in Vatia and Kapeenkoski it can be suspected that δ^{15}N-values of the POM could have been low if the wood fibres would have been separated from the POM.

5.5. Conclusions

There are various methods to test for nitrogenase activity. Based on these results it appears that the ^{15}N-tracer method measures nitrogenase activity relatively reliably and since it is a direct measurement of nitrogen fixation, it can be recommended in future nitrogen fixation studies. However getting the IRMS in Jyväskylä to run reliably with GF/F filters will still require some additional work so that the precision of the method can be increased. Relating the natural δ^{15}N-values to nitrogen fixation is questionable. It can work in some oceanic regions where the number of sources for nitrogen are limited, but in lakes there are various factors, such as the vertical mixing and effluents, that can have an effect to the δ ^{15}N-values. In order to detect nitrogen fixation in lakes it is more reliable to measure nitrogen fixation with the ^{15}N-tracer method than relating low δ^{15}N-values to nitrogen fixation. Also modern methods based on molecular biology can yield important information about nitrogen fixation and currently the research group is analyzing the diversity of *nifH* gene from Mekkojärvi and Ormajärvi in order to identify the organisms actively fixing nitrogen.

When nitrogen budgets for lakes are being prepared they often lack completely the nitrogen input resulting from biological nitrogen fixation. In some cases however it can provide significant amounts of nitrogen and can occur in various water layers, as was the case in Ormajärvi. Also understanding the nitrogen dynamics in different types of lakes is important. Based on the results from Mekkojärvi it appears that this extremely harsh environment supports diazotrophic species and nitrogen fixation might be an essential component that is supporting growth in this nitrogen depleted environment. For further analyses it would be interesting to see whether nitrogen fixation radiates throughout the food web and how significant a factor it actually is in the nitrogen cycle of Mekkojärvi.

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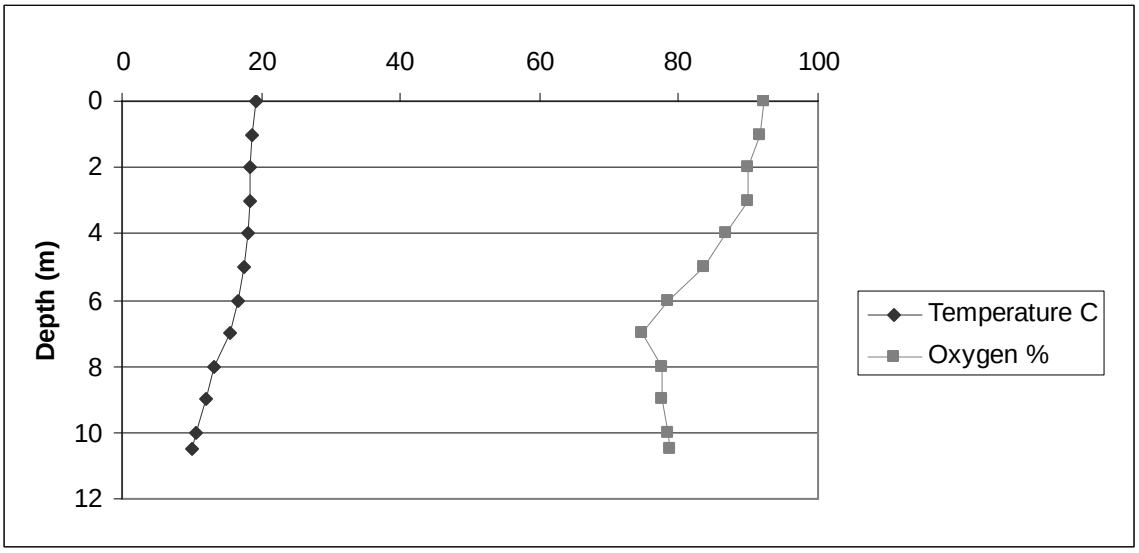
Appendix 1. The nutrient concentrations and temperature/oxygen gradients of the four experimental lakes in Lammi. The lowest sampling depth was in the deepest part of the lake except in Pääjärvi where the sampling depth was logistically more convenient. The NO_3^- & NO_2^-, PO_4^{3-} and NH_4^+ concentrations were analysed by using the QuikChem 8000 Flow Injection Analysis. *The value was interfered by the bacterial chlorophyll.

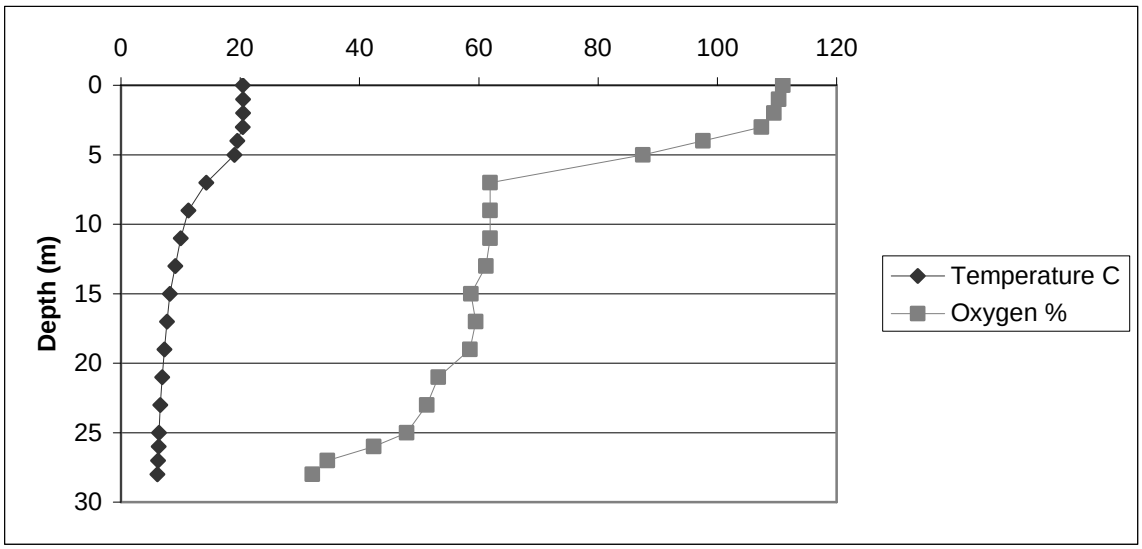
Pääjärvi:

	Epilimnion	**Metalimnion**	**Hypolimnion**	**Measurement standard**
Sampling depths (m)	0	7.5	11	
PO_4^{3-} ($\mu g\ l^{-1}$)	1	2	1	QuikChem method 10-115-01-1-B
NO_3^- & NO_2^- ($\mu g\ l^{-1}$)	875	1011	1012	QuikChem method 10-107-04-1-B
NH_4^+($\mu g\ l^{-1}$)	21	10	8	QuikChem method 31-107-06-1-A
Iron ($mg\ l^{-1}$)	0.10	0.10	0.11	Varian spectrometer AA 220 FS
Chlorophyll-a ($\mu g\ l^{-1}$)	4.2	0.5	0.9	SFS 5772



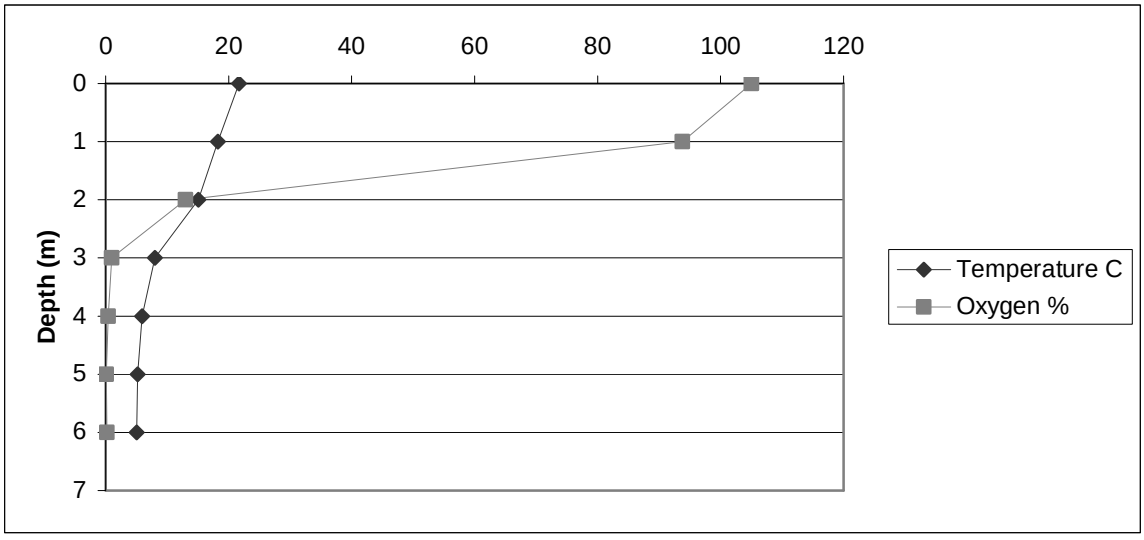
Ormajärvi:

	Epilimnion	**Metalimnion**	**Hypolimnion**	**Measurement standard**
Sampling depths	0	14.5	29	
PO_4^{3-} (µg l^{-1})	1	2	7	QuikChem method 10-115-01-1-B
NO_3^- & NO_2^- (µg l^{-1})	86	484	578	QuikChem method 10-107-04-1-B
NH_4^+(µg l^{-1})	9	3	99	QuikChem method 31-107-06-1-A
Iron (mg l^{-1})	0.08	0.12	0.18	Varian spectrometer AA 220 FS
Chlorophyll-a (µg l^{-1})	4.8	0.8	2.0	SFS 5772



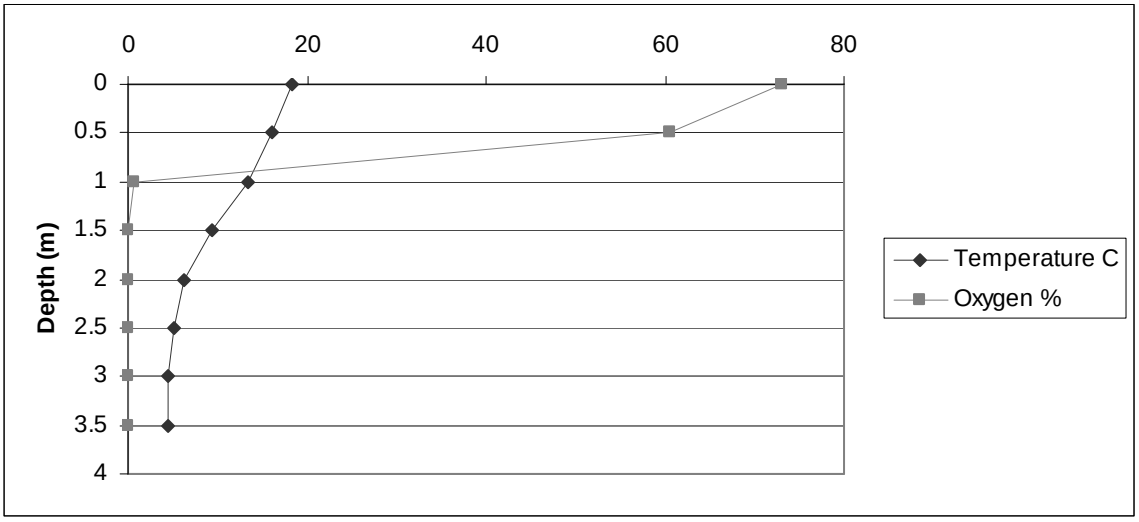
Valkea-Kotinen:

	Epilimnion	**Metalimnion**	**Hypolimnion**	**Measurement standard**
Sampling depths (m)	0	2.5	6	
PO_4^{3-} (μg l^{-1})	1	1	1	QuikChem method 10-115-01-1-B
NO_3^- & NO_2^- (μg l^{-1})	16	18	19	QuikChem method 10-107-04-1-B
NH_4^+ (μg l^{-1})	9	9	240	QuikChem method 31-107-06-1-A
Iron (mg l^{-1})	0.18	0.77	0.81	Varian spectrometer AA 220 FS
*Chlorophyll-a (μg l^{-1})	12.1	28.3	10.0	SFS 5772

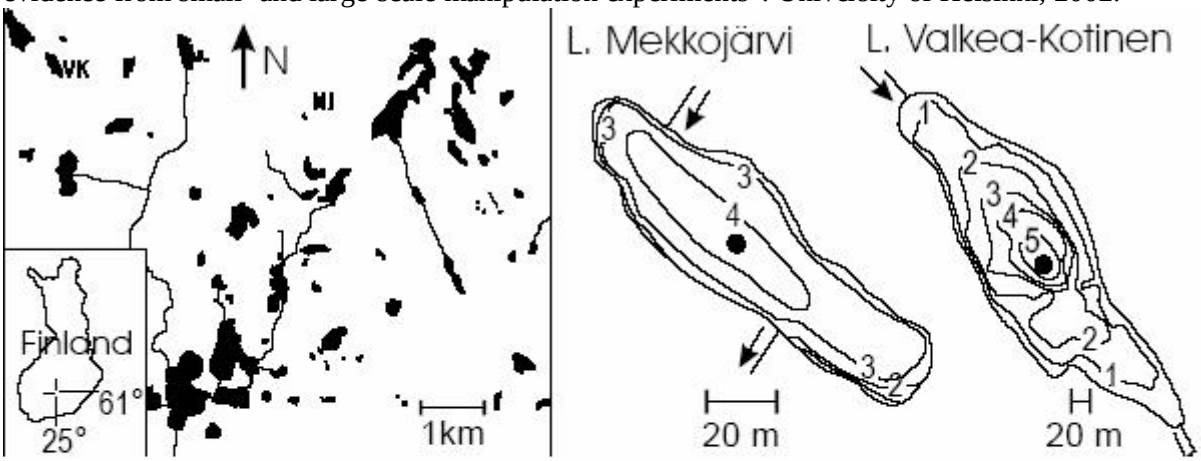


Mekkojärvi:

	Epilimnion	**Metalimnion**	**Hypolimnion**	**Measurement standard**
Sampling depths (m)	0	1	3.5	
PO_4^{3-} (μg l^{-1})	1	1	6	QuikChem method 10-115-01-1-B
NO_3^- & NO_2^- (μg l^{-1})	24	31	23	QuikChem method 10-107-04-1-B
NH_4^+ (μg l^{-1})	14	15	390	QuikChem method 31-107-06-1-A
Iron (mg l^{-1})	0.47	1.44	3.86	Varian spectrometer AA 220 FS
*Chlorophyll-a (μg l^{-1})	3.9	39.6	83.5	SFS 5772



Appendix 2. Mekkojärvi and Valkea-Kotinen. Map presented in Marko Järvinen's Academic Dissertation "Control of plankton and nutrient limitation in small boreal brown water lakes: evidence from small- and large-scale manipulation experiments". University of Helsinki, 2002.



Appendix 3. The Äänekoski water course. Map provided by Jyväskylän seudun puhdistamo