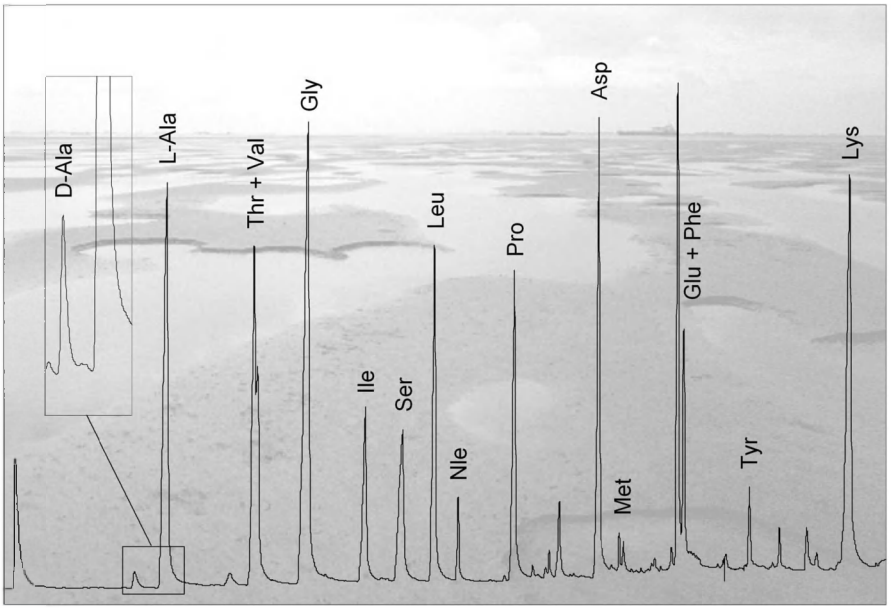
A new method for tracing flows of nitrogen and carbon through bacteria and algae in aquatic microbial communities

Analysis of ^{15}N- and ^{13}C-incorporation into D-alanine and other hydrolysable amino acids



Bart Veuger

2006

NIOO Thesis 47

GEOLOGICA ULTRAIECTINA
Mededeling van de Faculteit
Geowetenschappen
Universiteit Utrecht
No. 265

Dit proefschrift in een notendop

Dit proefschrift gaat over de ontwikkeling en toepassing van een nieuwe methode waarmee de opname van stikstof (N) en koolstof (C) door bacteriën en algen in water en sediment (de zeebodem) kan worden gemeten. De methode omvat analyse van de opname van de stabiele isotopen ^{15}N en ^{13}C (welke model staan voor de "gewone" stikstof en koolstof zoals die in de natuur voorkomen) in aminozuren. Omdat organismen voor een groot deel uit aminozuren bestaan geeft de totale opname van ^{15}N en ^{13}C in aminozuren een goede indicatie van de totale opname door alle (micro)organismen samen. Daarnaast komt één van de aminozuren (D-alanine) alleen voor in bacteriën waardoor de gemeten opname van ^{15}N en ^{13}C in D-alanine kan worden gebruikt om de opname door bacteriën te berekenen. De methode is succesvol gebleken en toepassing in verschillende studies (op locaties variërend van de Westerschelde tot de Australische oostkust) heeft interessante informatie opgeleverd over het opnemen en vasthouden van stikstof en koolstof door bacteriën en algen in sedimenten. De resultaten van dit fundamentele onderzoek dragen bij aan ons begrip van de stikstofkringloop in water en sediment. Deze kennis komt bijvoorbeeld van pas bij de aanpak van waterkwaliteitsproblemen.

This thesis in a nutshell

This PhD-thesis deals with the development and application of a new method for analysis of uptake of nitrogen and carbon by bacteria and algae in water and sediment (the sea bottom). The method concerns analysis of uptake of the stable isotopes ^{15}N and ^{13}C (representing "common" nitrogen and carbon as they occur in nature) in amino acids. Since amino acids are a major component of all organisms, the total uptake of ^{15}N and ^{13}C in amino acids provides a good indication of total uptake by all (micro)organisms together. In addition, one of the amino acids (D-alanine) is only present in bacteria which means that uptake of ^{15}N and ^{13}C into D-alanine can be used to calculate uptake by bacteria. The method proved to be successful and application in various studies (on locations ranging from the Dutch Scheldt Estuary to the Australian east coast) yielded interesting information on the uptake and retention of nitrogen and carbon by bacteria and algae in sediments. Results from this research add to our understanding of the nitrogen cycle in water and sediment. This knowledge can for example be applied in water quality improvement work.

Diese Doktorarbeit in Kürze

In dieser Doktorarbeit geht es um die Entwicklung und Anwendung einer neuen Methode. Mit dieser kann die Aufnahme von Stickstoff (N) und Kohlenstoff (C) durch Bakterien und Algen im Wasser und Sediment (Meeresboden) gemessen werden. Die Methode beinhaltet die Analyse der Aufnahme von stabilen Isotopen ^{15}N und ^{13}C (stellvertretend für den „normalen", in der Natur vorkommenden Stickstoff und Kohlenstoff) in Aminosäuren. Organismen bestehen größtenteils aus Aminosäuren. Deshalb kann man mit der Aufnahme von ^{15}N und ^{13}C in Aminosäuren die Aufnahme der Menge von ^{15}N und ^{13}C in den gesamten (Mikro-) Organismen berechnen. Zudem kommt eine der Aminosäuren (D-Alanine) nur in Bakterien vor, wodurch die gemessene Aufnahme von ^{15}N und ^{13}C in D-Alanine benutzt werden kann, um die Aufnahme durch Bakterien zu berechnen. Die Methode hat sich als erfolgreich erwiesen. Ihre Anwendung in verschiedenen Studien (an Orten von der Westerschelde bis zur australischen Ostküste) hat zu wesentlichen Erkenntnissen über die Aufnahme und Speicherung von Stickstoff und Kohlenstoff durch Bakterien und Algen im Wasser und Sediment geführt. Die Ergebnisse dieser Grundlagenforschung helfen, den Stickstoffkreislauf im Wasser und Sediment besser zu verstehen. Dieses Wissen kann man beispielsweise beim Verfahren zur Verbesserung von Wasserqualität einsetzen.

A new method for tracing flows of nitrogen and carbon through bacteria and algae in aquatic microbial communities: Analysis of ^{15}N- and ^{13}C-incorporation into D-alanine and other hydrolysable amino acids

Een nieuwe methode voor het traceren van stikstof- en koolstofstromen door bacteriën en algen in aquatische microbiële gemeenschappen: Analyse van ^{15}N- en ^{13}C-incorporatie in D-alanine en andere hydrolyseerbare aminozuren

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. W.H. Gispen, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 24 oktober 2006 des middags te 12.45 uur

door

Bart Veuger

geboren op 23 mei 1979 te Purmerend

Promotoren: Prof.dr. J.J. Middelburg
Prof.dr. C.H.R. Heip

Het in dit proefschrift gepresenteerde onderzoek is uitgevoerd binnen de werkgroep Ecosysteem Studies op het Centrum voor Estuariene en Mariene Ecologie van het Nederlands Instituut voor Ecologie (NIOO-KNAW) en werd financieel ondersteund door de EU (EUROTROPH) en NWO (PIONIER).

ISBN: 90-5744-129-2

CONTENTS

GENERAL INTRODUCTION

Although this thesis may seem to be somewhat different from the average PhD-thesis in the sense that it is based on a method, the presented applications of this method all concern flows of nitrogen and carbon through aquatic microbial communities, with special attention to bacteria. Here "flows" involve both the short term (hours to days) uptake and incorporation of nitrogen and carbon by the microorganisms and the fate of nitrogen and carbon from microbial biomass over longer periods over time (days to months). The primary focus of this thesis is on nitrogen and most of the presented studies concern work on intertidal, estuarine sediments. Below, I will first give a brief introduction on nitrogen cycling in aquatic systems, focusing on nitrogen uptake by bacteria and algae and the fate of microbial nitrogen. Thereafter, I will introduce the use of stable isotopes and biomarkers in ecological and biogeochemical studies and the Why? and How? of the method presented in this thesis.

The aquatic nitrogen cycle

The aquatic nitrogen cycle is a complex system consisting of several different forms of nitrogen with different oxidation states that are interconnected by numerous conversion reactions (Fig. I). The different components of the aquatic nitrogen cycle can be separated from each other in space and/or time. The nitrogen cycle plays an important role in aquatic systems since nitrogen is usually the element limiting microbial production in these systems. However, due to its complexity, various aspects of the nitrogen cycle are still poorly understood.

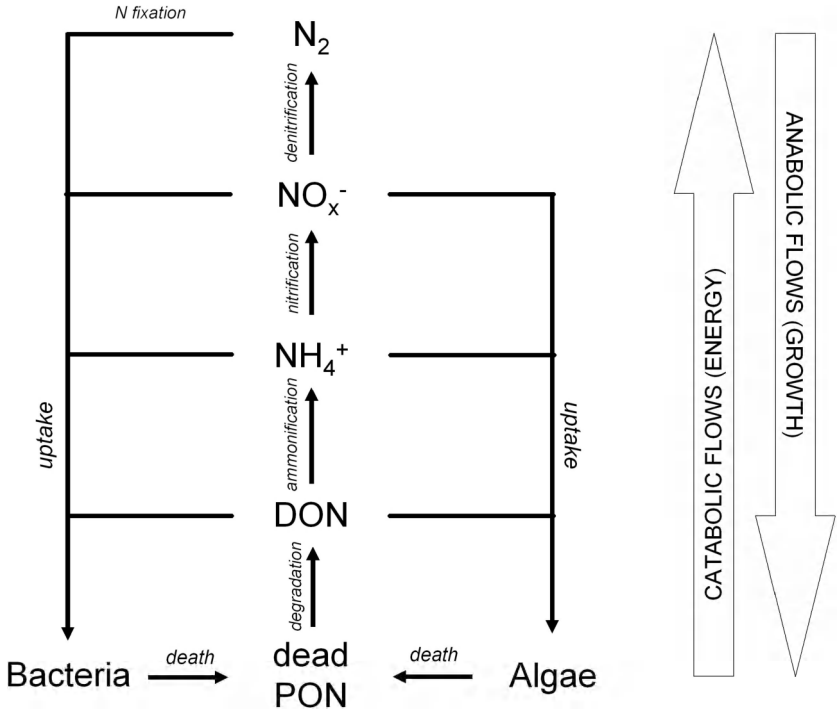


Figure I. General overview of nitrogen transformation processes within the nitrogen cycle, including nitrogen flows through bacteria and algae.

Bioavailable nitrogen is present in the environment as "free" nitrogen (atmospheric N_2) and "fixed" nitrogen. The latter can roughly be divided into *particulate* nitrogen (functionally defined as all nitrogenous material that can be captured by filtration) and *dissolved* nitrogen (all nitrogenous material that passes through a filter, typically GF/F). The dissolved fixed nitrogen pool can be subdivided into dissolved *inorganic* nitrogen (DIN) and dissolved *organic* nitrogen (DON). The DIN pool includes ammonium (NH_4^+), nitrate (NO_3^-) and nitrite (NO_2^-) of which the last two can be taken together as NO_x^-. The DON pool is a more complex pool of which only a small fraction (< 20%) has been chemically identified (Benner 2002, Bronk 2002). The identifiable fraction mainly consists of urea, dissolved free amino acids (DFAAs) and dissolved combined amino acids (DCAAs, mainly proteins and peptides), see Hansell and Carlson (2002) and Berman and Bronk (2003) for more information on DON.

The conversion reactions within the nitrogen cycle can have two "directions" (Fig. I). In catabolic (or "dissimilatory") reactions, nitrogenous substrates are used as a source of energy, these reactions are generally mediated by bacteria. In anabolic (or "assimilatory") reactions, nitrogenous substrates are used as a source of nitrogen for growth (i.e. production of biomass), which requires energy. The uptake and incorporation of various forms of fixed nitrogen by natural aquatic microbial communities is one of the main subjects in this thesis and is introduced in more detail below. More information on aquatic nitrogen cycling can be found in reviews by Herbert (1999), Capone (2000) and Zehr and Ward (2002).

Microbial nitrogen uptake

Natural aquatic microbial communities (mainly bacteria and algae) can use various forms of nitrogen as nitrogen sources for growth. Although N_2 is abundantly present in the atmosphere, its importance as a nitrogen source for aquatic microbial communities is limited, especially in systems with high availability of fixed nitrogen sources, because the use of nitrogen from N_2 for growth ("nitrogen fixation") requires a high energy investment and only a limited group of organisms (cyanobacteria and some heterotrophic bacteria) is capable of nitrogen fixation (Herbert 1999). Therefore, the main nitrogen sources for microbial communities in coastal waters and sediments are DIN and DON. Traditionally, studies on aquatic microbial nitrogen uptake focused on uptake of DIN by algae while bacteria were only considered as remineralizers of organic matter. However, the present view of microbial nitrogen uptake is considerably more complex since DON (especially amino acids and urea) has been found to be a potentially important nitrogen source as well and because it is now clear that bacteria are also important players in aquatic microbial nitrogen uptake (see e.g. Kirchman 2000, Bronk 2002, Zehr and Ward 2002, Berman and Bronk 2003). In addition to bacteria and algae, aquatic microbial communities can also include Archaea. However, at present their role in total microbial nitrogen uptake is unknown.

The pathways for uptake of dissolved nitrogen by algae and bacteria are schematized in figure II. The uptake process consists of two steps that are not necessarily coupled. The first step is the uptake of dissolved nitrogenous compounds from the surrounding water into the cells. Small molecules like NH_4^+, NO_x^- and urea can easily be taken up into cells while larger compounds like proteins and peptides first need to be broken down into individual amino acids that

can either be directly taken up into the cells or be broken down to NH_4^+ which is then taken up into the cells. The second step is incorporation of the nitrogen in new biomass. For this, intracellular NO_3^- and urea are first converted to NH_4^+ which is used to synthesize amino acids that form the building blocks for proteinaceous biomass components. Since amino acids can directly be incorporated into biomass after uptake (i.e. little or no conversion is required), these are a highly preferred nitrogen source. Conversely, the microbial preference for NO_x^- as a nitrogen source is low because of the relatively high energy investment required for its conversion to NH_4^+. Preferences for NH_4^+ and urea are generally in between those for amino acids and NO_x^-.

More information about microbial nitrogen uptake and its role within the aquatic nitrogen cycle can be found in reviews by Flynn and Butler (1986), Antia et al. (1991), Capone (2000), Kirchman (2000), Bronk (2002), Zehr and Ward (2002) and Berman and Bronk (2003).

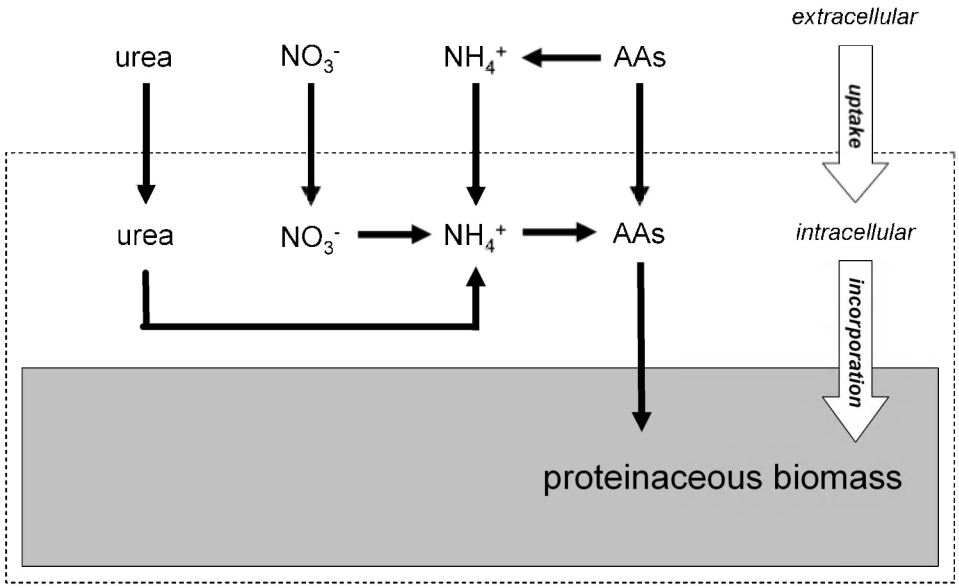


Figure II. Pathways for uptake and incorporation of nitrogen from DIN (NH_4^+ and NO_3^-) and DON (amino acids (AA) and urea).

Fate of microbial nitrogen

An interesting, yet poorly studied, aspect of the aquatic nitrogen cycle is the fate of microbial nitrogen (i.e., what happens with the nitrogen after it has been incorporated into microbial biomass?) which is coupled with the fate of other elements in microbial biomass such as carbon. Although nitrogen can be retained in microbial biomass during continued growth (by cell division), microbial biomass (especially that of bacteria) is often relatively constant over time, meaning that production of new biomass is balanced by loss of biomass due to cell death. Death of microorganisms can be due to various causes such as viral lysis, predation and deteriorating environmental conditions. After cell death, the leftovers (remnants) of microbial biomass are subject to degradation. During degradation, nitrogen is released back into the system as DON and/or DIN that can be used as nitrogen source for production of new microbial biomass or be lost from the system. Some microbial biomass components are more resistant to degradation ("refractory") than others and may accumulate in the system where they serve as a long term sink for microbial-derived nitrogen. Another potential fate of microbial nitrogen is transfer to higher trophic levels of the food web when microbial biomass is consumed by larger organisms.

Stable isotopes in ecological and biogeochemical studies

Elements such as carbon (C) and nitrogen (N) can occur in different forms called isotopes. Different isotopes of a single element have the same number of protons but a different number of neutrons. Some isotopes are radioisotopes, meaning that these isotopes are subject to radioactive decay. Radioisotopes have been used in ecological and biogeochemical studies (for example for measuring primary production using ^{14}C). However, work with radioisotopes comes with a potential health risk and can be restricted by law. Moreover, there is no convenient radioisotope of nitrogen which makes that the use of radioisotopes in studies on nitrogen cycling is restricted to work with the short-lived radioisotope ^{13}N (e.g., Zehr et al. 1988). Isotopes that are not subject to radioactive decay are stable isotopes. A single element can have two or more stable isotopes, of which one is by far the most abundant (e.g. ^{12}C and ^{14}N) while the other forms are rare (for example: 99.6% of all nitrogen in nature is ^{14}N and only 0.4% is ^{15}N). The abundance of these rare isotopes is measured by isotope ratio mass spectrometry (IRMS), a technique that uses a magnetic field to separate gas molecules (CO_2 in case of ^{13}C analysis and N_2 in case of ^{15}N analysis) of different molecular weights resulting from different

stable isotope compositions (for example: $^{15}N_2$ is heavier than $^{14}N_2$ and $^{15}N^{14}N$). The relative abundance of these molecules with different molecular weights can be used to determine the relative abundance of the rare (or "heavy") isotope. This abundance is typically expressed by the delta notation (δ) which is the ratio between the rare ("heavy") isotope and the common (or "light") isotope relative to that of a standard in permille (‰) units. More technical background information on IRMS can be found in a review by Brenna et al. (1997).

Table I. Carbon and nitrogen isotopes

		type of isotope	*natural abundance*
Carbon	^{10}C	radio (½ life 17 sec)	
	^{12}C	stable	98.9%
	^{13}C	stable	1.1%
	^{14}C	radio (½ life 5730 yr)	
Nitrogen	^{13}N	radio (½ life 10 min)	
	^{14}N	stable	99.6%
	^{15}N	stable	0.4%
	^{16}N	radio (½ life 7 sec)	

Stable isotopes can be used in ecological and biogeochemical studies in two different ways. The first way is to study the natural abundance of the rare, heavy isotope. Here, the relevant information lies in small differences in relative abundance of the heavy isotope between different compartments of a system. These differences are the results of isotopic fractionation, which involves changes in the natural abundance of the heavy isotope during reactions caused by small differences in mass-dependent reaction rates of heavy and light isotopes. The second way to use stable isotopes is by stable isotope labeling. In this approach, a substrate that is highly enriched in the heavy isotope is deliberately added to a system after which the abundance of the heavy isotope (the "label") is measured in various components of the system which allows to trace and quantify flows of the label trough these components. The material presented in this thesis all concerns stable isotope labeling work with the nitrogen isotope ^{15}N and the carbon isotope ^{13}C. More information and examples of the use of stable isotopes in ecology and biogeochemistry can be found in e.g. Lajtha and Michener (1994), Griffiths (1998) and Boschker and Middelburg (2002).

Biomarkers

Biomarkers are compounds that are unique to a specific group of organisms. Ideally, biomarker compounds are part of the organism's biomass and make up a relatively constant fraction of that biomass so that biomarker concentrations can be converted to total biomass of the group it represents. A variety of compounds have been used as biomarkers for various microbial groups (see Boschker and Middelburg 2002). Some of these compounds are rapidly degraded after death of the organism, which makes them specific for living biomass ("ecological biomarkers"), for example phospholipid-derived fatty acids (PLFAs). Other components are more resistant to degradation ("biogeochemical biomarkers"), for example D-amino acids from bacterial cell walls, which makes that these components can accumulate in a system, particularly in sediment where the abundance of these biomarkers can provide information on the contribution of material from the corresponding group of organisms to the total organic matter pool in the sediment. A third group of biomarkers are highly refractory molecules ("geological biomarkers"). The concentrations and characteristics of these "molecular fossils" in sediments can be used for paleoenvironmental reconstructions (e.g., Sinninghe Damsté et al. 2004).

A valuable application of biomarkers in ecological- and biogeochemical studies is analysis of their stable isotope composition, which requires compound-specific stable isotope analysis. This can be done at natural abundance level or in combination with stable isotope labeling where the latter provides the unique opportunity to directly link microbial identity (biomarker), biomass (biomarker concentration) and activity (label incorporation). Figure III shows a selection of bacterial biomarkers that have been used in combination with stable isotope analysis. More information on biomarkers and stable isotope analysis of biomarkers, including some examples of applications in microbial ecology, can be found in Boschker and Middelburg (2002) while technical aspects of compound-specific stable isotope analysis are presented in Brenna et al. (1997).

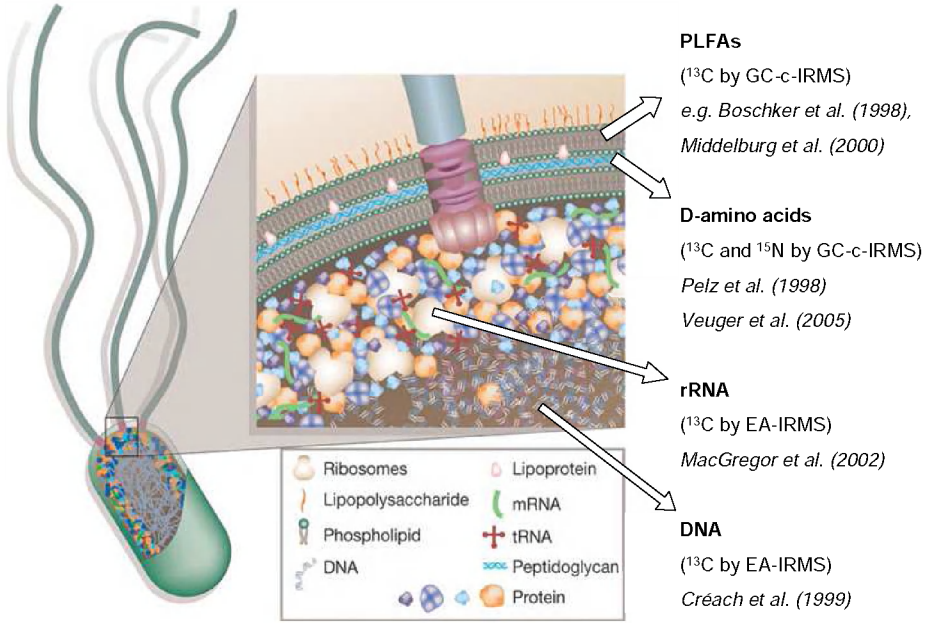


Figure III. A selection of bacterial biomarkers used in combination with stable isotope analysis.

The problem

Microbial uptake of nitrogen from various nitrogenous substrates is generally studied by analysis of uptake of ^{15}N from ^{15}N-labeled substrates. This approach has frequently been used in water column studies where ^{15}N enrichment of total particulate material in the water is considered to represent total microbial ^{15}N uptake. However, more detailed assessment of ^{15}N uptake by specific microbial groups (bacteria versus algae) has long been hampered by methodological problems. Until recently, two different approaches have been used to discriminate ^{15}N uptake by bacteria versus algae. The first approach is size fractionation, which concerns physical separation of relatively small bacteria from relatively large algae by filtration (e.g. Wheeler and Kirchman 1986) or flow cytometry (Lipschultz 1995). Although this approach may work in clear waters when a size difference between bacteria and algae is present, size fractionation is not suitable for studies in more turbid waters where bacteria and algae are closely associated with particulate matter. The second approach involves the use of inhibitors to eliminate activity of a specific microbial group, for example antibiotics to inhibit bacterial activity (e.g.

Wheeler and Kirchman 1986, Middelburg and Nieuwenhuize 2000b). However, the use of inhibitors is seriously limited by uncertainties concerning both the specificity and efficiency of inhibitors (Oremland and Capone 1989). Next to the problems in water column studies presented above, studies on microbial nitrogen uptake in *sediments* have been hampered by even more fundamental methodological problems because of the inability to distinguish between ^{15}N taken up by the microbial community and the remaining added ^{15}N-substrate and/or possible other ^{15}N-labeled extracellular compounds bound to the sediment. Moreover, another limitation of traditional ^{15}N-labeling work is that it does not allow clean assessment of immobilization of dissolved nitrogen into microbial biomass (the relevant process from a nitrogen cycling perspective as well as from a food web point of view) since total ^{15}N "uptake" also includes uptake of nitrogenous substrates into microbial cells, which is not necessarily directly coupled to incorporation into biomass (Fig. II). The method presented in this thesis is a new approach that has the potential to overcome the problems mentioned above and therefore may help to further clarify nitrogen flows through bacteria and algae in natural microbial communities.

The solution

The method presented in this thesis involves the use of compound-specific stable isotope analysis of biomarkers (see above). This approach has already been used at NIOO-CEME for some years in ^{13}C-labeling studies where analysis of ^{13}C incorporation into various group-specific PLFAs provided valuable information on ^{13}C flows through different microbial groups, including bacteria and algae (e.g. Boschker et al. 1998, Middelburg et al. 2000, Moodley et al. 2000). However, since PLFAs do not contain nitrogen, another type of compounds was required for ^{15}N-labeling studies. This thesis investigates the potential of ^{15}N analysis of hydrolysable amino acids (HAAs), including the bacterial biomarker D-alanine (D-Ala). HAAs are amino acids that can be released from proteinaceous biomass (proteins and protein-like material) by hydrolysis in hot acid. HAAs make up a large fraction (50-80%) of total microbial biomass (Simon and Azam 1989, Cowie and Hedges 1992), meaning that analysis of ^{15}N incorporation into total HAAs (THAAs) should provide a good indication of ^{15}N incorporation into total microbial biomass. Amino acids can occur as D- and L-stereoisomers (or "mirror images", see figure IV). Most HAAs are L-amino acids that are common biomass constituents of all organisms and therefore have very limited biomarker potential (Cowie and Hedges 1992).

Conversely, hydrolysable D-amino acids are rare and only occur in bacterial biomass, more specifically in peptidoglycan, a structural component of bacterial cell walls (Koch 1990, Madigan et al. 2000). Therefore, these D-amino acids can be used as bacterial biomarkers. Although different D-amino acids can be present in peptidoglycan, our method focuses on D-Ala because this is the only D-amino acid that is present in all bacteria and because it makes up a relatively stable fraction of total bacterial biomass. Moreover, D-Ala has been shown to be most suitable for stable isotope analysis by GC-c-IRMS (Pelz et al. 1998). The method can also be used to measure incorporation of the carbon isotope ^{13}C into D-Ala and other HAAs, which allows assessment of carbon flows through bacteria and algae. This is particularly interesting in direct combination with analysis of nitrogen flows. More information about the method is provided in chapter 2.

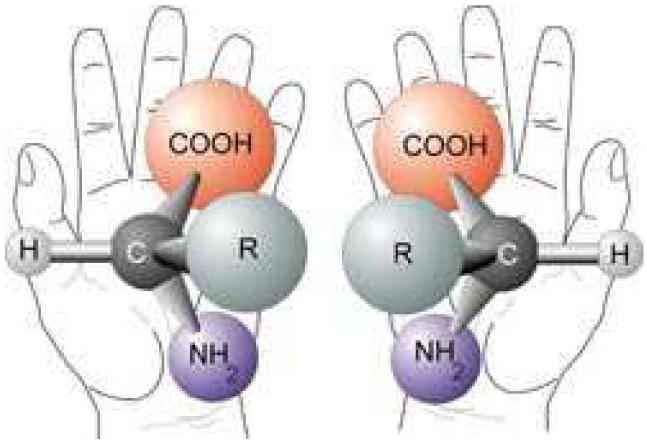


Figure IV. Amino acid D- and L-stereoisomers.
source: *http://web99.arc.nasa.gov/~astrochm/aachiral.html*

THESIS OUTLINE

Chapter 1 is a "classical" ^{15}N-labeling study in which total microbial uptake of ^{15}N from $^{15}NH_4^+$, $^{15}NO_3^-$,^{15}N-urea and a ^{15}N-amino acid mixture was measured in water samples from Randers Fjord (Denmark) to clarify the relative importance of these substrates (i.e. DIN versus DON) as nitrogen sources for the planktonic microbial community. In addition, uptake of a ^{15}N-labeled algal-derived DON mixture was measured in order to mimic uptake of total DON (including the large, chemically unidentified fraction of the DON pool). Moreover, an attempt was made to clarify the relative contributions of bacteria versus algae to total microbial ^{15}N uptake using traditional methods which yielded unsatisfactory results and illustrates the strong need for a new method.

Chapter 2 introduces the method that forms the basis of this thesis. It includes a detailed description and assessment of the analytical aspects of the method as well as results from a simple ^{15}N-labeling experiment with surface sediment from an intertidal mudflat in the Scheldt Estuary to illustrate the potential of the method. After publication of this chapter, the method has been applied in various ^{15}N- and ^{13}C-labeling studies, including those presented in chapters 3, 4 and 5, which served as a thorough validation and yielded valuable information concerning the interpretation of results obtained with the method. This information is presented in an ***update*** of the original manuscript.

Chapter 3 concerns a short-term (24 h) dual-labeling experiment in which surface sediment from two intertidal mudflats in the Scheldt Estuary was incubated with dual labeled (^{15}N and ^{13}C) urea and amino acids. Application of the method in this study allowed us to trace total ^{15}N- and ^{13}C incorporation by the benthic microbial community and to clarify the bacterial contribution to total microbial ^{15}N incorporation in the sediment that was characterized by a high abundance of benthic microalgae. Moreover, comparison of incorporation of ^{15}N versus ^{13}C yielded interesting information concerning the (un)coupled uptake and incorporation of nitrogen and carbon from urea and amino acids.

Chapter 4 presents a study on the uptake and retention of nitrogen by the microbial community in the sediment of the subtropical Brunswick Estuary (New South Wales, Australia). The main experiment involved in situ injection of $^{15}NH_4^+$ into the sediment of an intertidal mud bank after which the method was used to trace ^{15}N into HAAs, including D-Ala, over a 30-day sampling period. In addition, ^{15}N was also traced into fauna (hand picked). Additional experiments involved short-term incubations of sediment cores injected with different ^{15}N-labeled substrates and core incubation to measure fluxes of DIC, oxygen and nitrogenous substrates between the sediment and the overlying water. The unique combination of ^{15}N-labeling and specific assessment of ^{15}N incorporation into total microbial biomass (THAAs) and bacteria (D-Ala) allowed us to clarify the hypothesized strong retention of nitrogen within the sediment of the Brunswick Estuary and to identify the mechanisms and organisms responsible for this retention.

In ***Chapter 5***, the method is used to investigate the fate of different bacterial cell components in an intertidal sediment. This paper is part of a larger in situ ^{13}C-labeling study in which the bacterial biomass of an intertidal mudflat in the Scheldt Estuary was labeled with ^{13}C via injection of ^{13}C-glucose into the sediment. While two companion papers focus on the fate of total bacterial ^{13}C and the transfer of ^{13}C to fauna from an "ecological" point of view (van Oevelen et al. 2006a+b), this paper deals with the fate of specific compounds from the ^{13}C-labeled bacteria from a "biogeochemical" perspective. Analysis of the retention of ^{13}C in THAAs and D-Ala as well as that in bacteria-specific PLFAs during a 4.5-month period in situ allowed us to compare the fate of peptidoglycan (a bacterial cell wall component represented by D-Ala) relative to that of total bacterial proteinaceous biomass (THAAs) and cell membrane material (PLFAs). Using the PLFAs as a proxy for living bacteria, we were able to discriminate between retention of ^{13}C in living bacteria versus that in bacterial remnants ("leftovers") and hence to investigate the degradation versus accumulation of these remnants in the sediment.

C H A P T E R 1

Microbial uptake of dissolved organic and inorganic nitrogen in Randers Fjord

Bart Veuger, Jack J. Middelburg, Henricus T.S. Boschker, Joop Nieuwenhuize, Pieter van Rijswijk, Emma J. Rochelle-Newall and Nuria Navarro

Estuarine, Coastal and Shelf Science 61 (2004): 507-515

ABSTRACT

Uptake of ^{15}N labeled NH_4^+, NO_3^-, urea and dissolved free amino acids (DFAA) was measured in April and August 2001 at 6 stations along the salinity gradient of Randers Fjord (Denmark) in order to clarify the relative importance of dissolved organic and inorganic nitrogen (DON and DIN) as N sources for the estuarine planktonic microbial community. Although microbial N uptake was generally dominated by DIN (especially NH_4^+), DON (both urea and DFAA) also served as important N sources and occasionally dominated uptake. Uptake of ^{15}N labeled algal derived DON was measured in an attempt to mimic uptake of the complex ambient DON pool. Uptake rates for the algal derived DON in April were similar to those for NH_4^+ and even higher than the summed uptake of NH_4^+, NO_3^-, urea and DFAA in August, suggesting that DON other than urea and DFAA, likely dissolved combined amino acids (DCAA), also served as an important N source. In addition, the relative contribution of heterotrophic bacteria versus phytoplankton to total microbial N uptake was estimated from bacterial productivity and measured by inhibiting bacterial N uptake with antibiotics.

INTRODUCTION

The N cycle plays a central role in estuarine and marine systems. However, despite major research efforts, various aspects of the N cycle are still poorly understood. Elucidating these aspects will help to unravel the N cycle and to understand e.g. how the estuarine microbial community deals with eutrophication, a common problem in many estuarine and marine systems, including Randers Fjord. One of these aspects is the uptake of different nitrogenous substrates by the microbial community. Traditionally, research on N uptake focused on dissolved inorganic nitrogen (DIN), especially ammonium (NH_4^+) and nitrate (NO_3^-), while organic nitrogen was usually ignored as a potential N source. However, nowadays different studies showed that dissolved organic nitrogen (DON) can also serve as an important N source (e.g. Antia et al. 1991, Middelburg and Nieuwenhuize 2000b, Bronk 2002, Zehr and Ward 2002, Berman and Bronk 2003). Estuarine DON, which is typically defined as all organic N that passes through a filter (usually GF/F), can be derived from external sources (terrestrial and atmospheric inputs) and local sources (direct or indirect release by phytoplankton and bacteria) (Berman and Bronk 2003). The DON pool consists of a continuum of compounds, ranging from low molecular weight compounds like urea and amino acids on one side to high molecular weight compounds and aggregates of different compounds on the other (Zehr and Ward 2002). Up to now, only a small fraction of the estuarine and marine don pool can be chemically identified. This fraction includes urea, dissolved free amino acids (DFAAs), dissolved combined amino acids (DCAAs) and nucleic acids and typically accounts for less than 20% of the total DON pool (Benner 2002, Bronk 2002). Urea, DFAAs and DCAAs (proteins, peptides, etc.) have been shown to be potentially important N sources for the microbial community (Coffin 1989, Antia et al. 1991, Keil and Kirchman 1993, Middelboe et al. 1995, Middelburg and Nieuwenhuize 2000b). Studies on DON bioavailability showed that up to ~ 70% of the total DON pool can be bioavailable, suggesting that a considerable part of the unidentified fraction of the DON pool can also serve as a N source for the microbial community (Stepanauskas et al. 2002, Seitzinger 2002). However, studies like these typically involve incubations of several days and do not distinguish between uptake and regeneration. Therefore, the quantitative importance of DCAAs and the unidentified fraction of the DON pool as N sources at a shorter timescale (hours) remains largely unclear. In this study, we tried to clarify the relative importance of urea and DFAAs compared to

NH_4^+ and NO_3^- as N sources to the microbial community along the salinity gradient of Randers Fjord using ^{15}N-labeled substrates. In addition, we tried to mimic uptake of the total ambient DON pool by using a complex algal-derived $DO^{15}N$ mixture in order to get an indication of the importance of the total DON pool as a N source.
Another aspect of microbial N uptake is the relative importance of phytoplankton versus heterotrophic bacteria (the two key players in microbial N uptake). Traditionally, microbial N uptake has been attributed to phytoplankton while the role of heterotrophic bacteria in the N cycle was thought to be restricted to decomposition of organic matter and regeneration of nitrogen. Nowadays, bacteria have been shown also to be able to take up DIN and DON (e.g. Wheeler and Kirchman 1986, Kroer et al. 1994). However, the relative importance of bacterial N uptake in natural microbial communities is still largely unclear and results are characterized by considerable variation within and between studies (e.g. Kirchman 1994, Middelburg and Nieuwenhuize 2000b, Zehr and Ward 2002). In this study, the contribution of heterotrophic bacteria to total microbial N uptake was estimated from bacterial productivity data and measured by inhibiting bacterial N uptake with antibiotics.

MATERIALS AND METHODS

Randers Fjord

Randers Fjord is a small estuary in the north-east of Jutland (Denmark), with a length of 27 km, a catchment area of 3260 km^2 and an average depth of 1.7 m, except for the navigation channel which is dredged to a depth of ~ 8 m. The fjord is characterized by a negligible tide (0.2 - 0.3 m) and a strong stratification (Nielsen et al., 2001). Temperature during the field campaigns was 7 - 10 °C in April and 16 – 20 °C in August and salinity in the fjord ranged from 0 in the river Guden Å, the main fresh water input to the estuary, to 25 - 30 in the adjacent open sea (the Kattegat). Concentrations of suspended particulate matter (SPM) ranged from 1.5 to 43 mg l^{-1} in April and 0.6 to 12 mg l^{-1} in August.

Sampling

Fieldwork was performed during two campaigns in late April and late August 2001. During both campaigns, water was sampled at 6 stations along the salinity gradient of the fjord, including one station in the adjacent open sea. Stations were sampled at different days within a 10 day period. For each station, water was sampled from the surface and near-bottom layer, using a Niskin bottle.

Nutrients and pigments

Water samples were filtered through pre-weighed, pre-combusted Whatman GF/F filters, which were stored at - 20 °C and later analyzed for particulate nitrogen, using an elemental analyzer (Nieuwenhuize et al., 1994). GF/F filtered water was stored frozen and later analyzed for various nitrogenous compounds. Concentrations of NH_4^+, NO_3^-, NO_2^- and urea were measured by automated colorimetric techniques (Middelburg and Nieuwenhuize, 2000b). Total dissolved nitrogen (TDN) was measured as dissolved NO_3^- by spectrophotometry after oxidation in alkaline persulfate (120 °C for 30 min) (Grasshoff et al., 1999). DON concentrations were obtained by subtracting summed concentrations of NH_4^+, NO_3^- and NO_2^- from those for TDN. Amino acid concentrations were measured by HPLC (Fitznar et al., 1999). DCAA concentrations were determined by subtracting DFAA concentrations from total concentrations of dissolved amino acids (DFAA + DCAA) after hydrolysis in 6 M HCl at 110°C for 24 h. For analysis of chlorophyll a (Chl *a*), water was filtered through GF/F filters which were stored frozen until analysis by HPLC (Barranguet et al., 1997).

Bacterial numbers and productivity

Separate samples were taken for analysis of bacterial numbers (BN) and bacterial productivity (BP). BN were measured by a direct count method using DAPI-staining and epifluorescent microscopy (Porter and Feig, 1980). BP was determined by measuring uptake of ^{3}H-labeled leucine. Briefly, unfiltered 1.5 ml aliquots were incubated in the dark at ambient temperature after addition of 50 nM ^{3}H leucine. After 1 h incubation, samples were killed with 100% TCA and processed according to Smith and Azam (1992). The amount of radioactivity taken up in the TCA insoluble fraction was measured in a scintillation counter using Ultima Gold scintillation cocktail (Packard Instruments). BP in µM-C h^{-1} was calculated using a conversion factor of 1.5 ng C $pmol^{-1}$ Leu.

Preparation algal derived DON

Algal derived DON (ADDON) was obtained from a culture of *Skeletonema costatum* (a diatom) that had been grown on F2 medium containing 98% $Na^{15}NO_3$. After 3 weeks of incubation at 16 °C, cells were harvested by centrifugation and freeze-dried. Dried cells were resuspended in distilled water containing Devarda's alloy (to convert any remaining NO_3^- to NH_4^+) and MgO (to raise the pH) and shaken for 48 h to remove all inorganic nitrogen. Particulate material was removed by centrifugation and the remaining DO^{15}N solution was stored frozen until used. This procedure to remove NH_4^+ and NO_3^- has been tested extensively during our efforts to measure natural abundance δ^{15}N values for NH_4^+ and NO_3^- in three European tidal estuaries (Middelburg and Nieuwenhuize, 2001). In addition, DFAA and DCAA concentrations in the ADDON solution were measured by HPLC as described above (concentrations were 0.7 mM-N and 2.2 mM-N respectively).

Uptake measurements

Within ~ 2 h after sampling, water samples (100 - 200 ml) were spiked with one of the different ^{15}N substrates, yielding concentrations of 0.1 µM $^{15}NH_4^+$, 1 µM $^{15}NO_3^-$, 0.1 µM-N ^{15}N-Urea, 0.1 µM-N ^{15}N-DFAA (a mixture of 17 algal amino acids, Cambridge Isotope Laboratories, NLM-2161, see chapter 3 for composition) or 100 µl ADDO^{15}N solution per 100 ml. Samples were incubated for ~ 3 h in transparent 250 ml polystyrene culture bottles in a water bath at ambient temperatures. For each substrate, incubations were performed under ambient light conditions as well as in the dark and in the dark after pre-treatment with an antibiotic mixture (SIGMA P3664, containing penicillin-G, streptomycin and neomycin). The

antibiotics were added ~ 2 h before addition of the labeled N substrates at a final concentration of 10 mg l^{-1}.

Incubations were ended by filtration through precombusted Whatman GF/F filters. Filters were stored at - 20 °C and freeze-dried later. The atom percentage ^{15}N (at. % ^{15}N) of the material on the filters was measured by EA-IRMS (see Middelburg and Nieuwenhuize, 2000a for details). Specific uptake rates (V) were calculated by dividing the excess ^{15}N on the filter by the excess ^{15}N dissolved per hour incubation time. Uptake rates in nM-N h^{-1} (transport rate, ρ) were obtained by multiplying V (unit: h^{-1}) with the PON concentration at the start of the incubation (assuming PON concentrations did not change during incubation). Uptake rates were corrected for isotope dilution due to substrate regeneration according to Kanda et al. (1987). However, this correction was generally negligible. Inhibition by antibiotics was calculated by dividing uptake rate in the dark with antibiotics by uptake rate in the dark without antibiotics and expressed as %.

RESULTS

Microbial community

Bacterial numbers (BN, Fig. 1) were similar in April (2.1 - 4.9 x 10^6 ml^{-1}) and August (3.0 - 5.3 x 10^6 ml^{-1}) and showed a slight decrease with increasing salinity. Bacterial productivity (BP, Fig.1) in August was ~ 2 × higher than in April and showed more variation (0.06 - 0.75 µM-C h^{-1} versus 0.06 - 0.17µM-C h^{-1} in April). Concentrations of Chl *a* (indicator for total phytoplankton biomass) showed a clear peak in the freshwater in April (5 – 21 µg l^{-1}) while concentrations in the rest of the estuary were in the same range as the ones in August (0.5 - 5 µg l^{-1}) where concentrations showed a slight decrease with increasing salinity (Fig. 2).

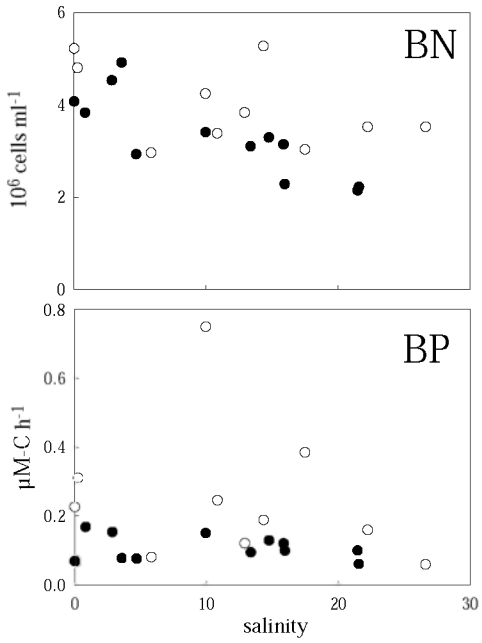


Figure 1
Bacterial numbers (BN) and productivity (BP) versus salinity in April (●) and August (○)

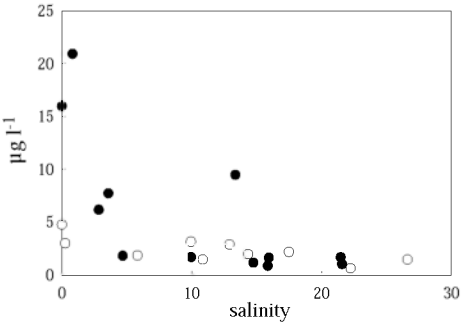


Figure 2
Concentrations of chlorophyll *a* in April (●) and August (○).

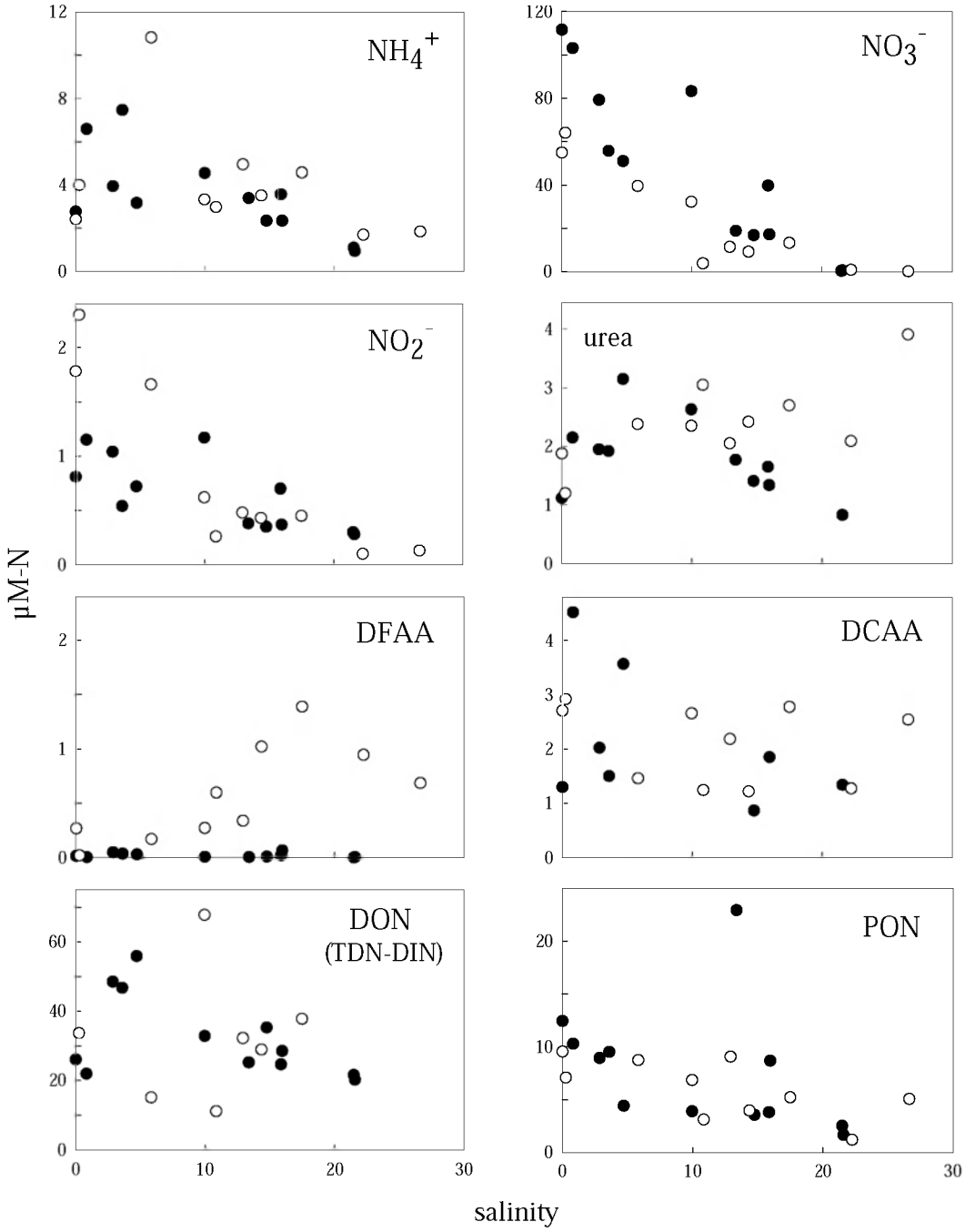


Figure 3. N substrate concentrations versus salinity in April (●) and August (○).

N substrate concentrations

Concentrations of the different N substrates (Fig. 3) did not show consistent differences between samples from the surface and bottom and are therefore not shown separately. Note that all concentrations are presented in µmoles *nitrogen* per liter. For most substrates, concentrations were similar during both campaigns, except for ~ 2 × higher NO_3^- concentrations in April and ~ 30 × higher DFAA concentrations in August. Out of the different N substrates, NO_3^- concentrations were high (0.4 - 110 µM in April and 0.2 - 65 µM in August), with highest concentrations in the fresh water and a strong decrease with increasing salinity. Concentrations of NH_4^+ (0.9 - 7.5 µM in April and 1.7 - 11 µM in August), urea (0.8 - 2.2 µM-N in April and 1.2 - 3.9 µM-N in August) and DCAA (0.9 – 4.5 µM-N in April and 1.2 – 2.9 µM-N in August) were in the same range while DFAA (0 - 0.65 µM-N in April and 0.02 - 1.4 µM-N in August) and NO_2^- (0.3 - 1.2 µM in April and 0.1 - 2.3 µM in August) were least abundant. Furthermore, particulate organic nitrogen (PON) concentrations ranged from 1.7 to 23 µM-N in April and 1.2 to 10 µM-N in August. DON (TDN - DIN) concentrations were 1 – 10 × higher than the PON pool during both campaigns and contributed 17 – 92 % and 22 – 69 % to the TDN pool in April and August respectively. Summed concentrations of urea, DFAA and DCAA accounted for 6 – 30 % (average 12 %) of the total DON pool in April and 8 – 44 % (average 20 %) in August.

Uptake rates

The uptake rates for the different substrates (Fig. 4) did not show consistent differences between surface and bottom samples and are therefore not shown separately. Out of the four substrates, NH_4^+ generally showed highest uptake rates (10 - 150 nM-N h^{-1}) with one exceptionally high value in August (280 nM-N h^{-1}). On average, uptake rates in August were slightly higher than those in April. Uptake rates for NO_3^- and urea were in the same range during both campaigns (1 - 50 nM-N h^{-1}) except for three high values for NO_3^- at low salinities in August and one value for urea in the open sea. DFAA were the only substrate that showed a clear difference between the two campaigns: uptake rates in April were very low throughout the entire estuary (2 - 12 nM-N h^{-1}) while values in August were much higher (4 - 80 nM-N h^{-1} in the fjord and up to 180 nM-N h^{-1} in the open sea).

The different substrates showed some consistent differences for uptake in the dark versus light (Fig. 4), except for NO_3^- that showed rather variable results with uptake

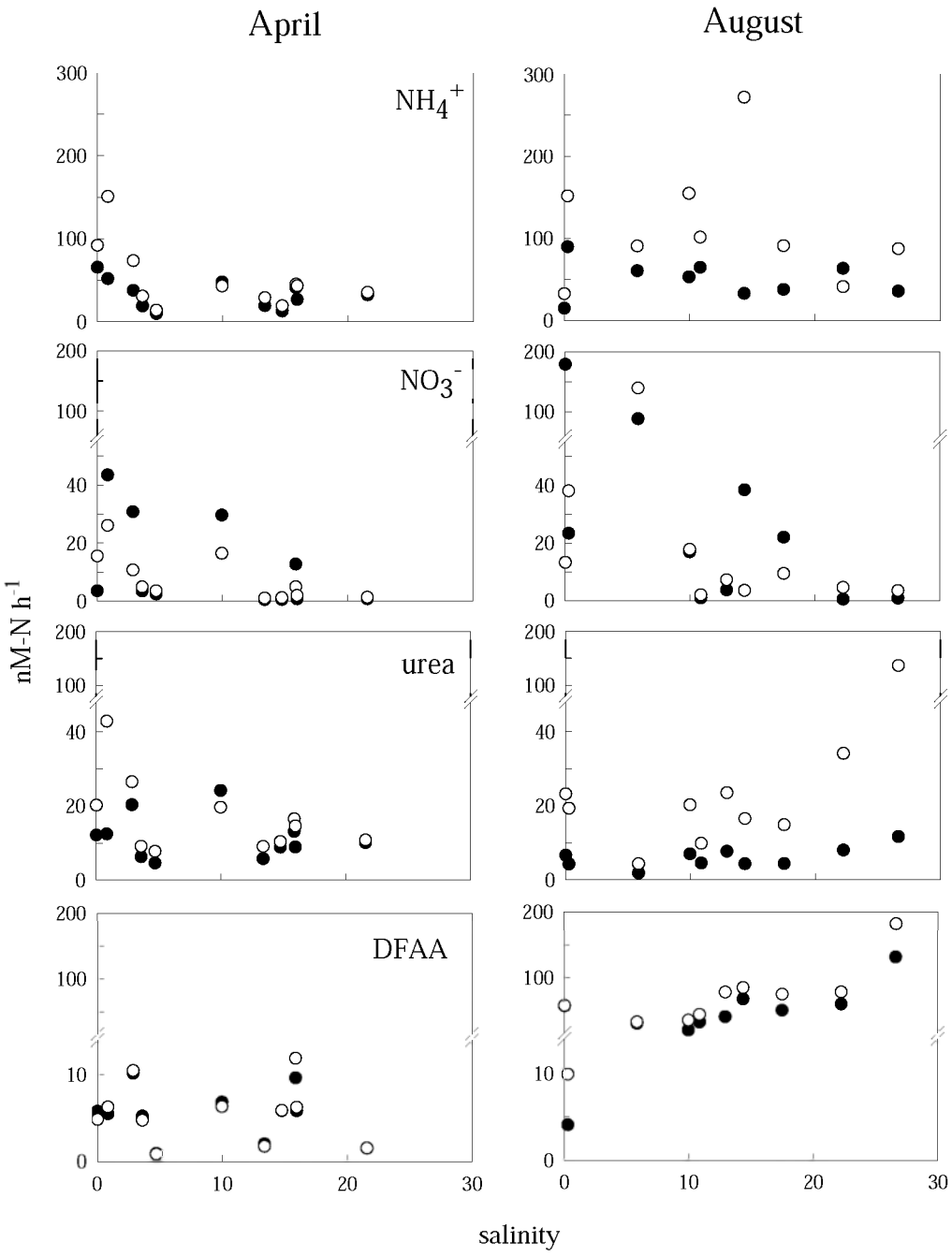


Figure 4. Uptake rates in the light (○) and in the dark (●) for NH_4^+, NO_3^-, urea and DFAA versus salinity in April and August. Notice the differences and breaks in scale.

in the dark sometimes exceeding uptake in the light. Out of the other substrates, urea showed highest differences (uptake in the dark on average being 73 ± 24 % of that in the light in April and 32 ± 8 % in August). Little difference occurred for DFAA (102 ± 12 % in April and 71 ± 19 in August) while NH_4^+ showed intermediate differences (71 ± 21 % in April and 60 ± 42 % in August).

The relative contributions of the four N substrates to their summed uptake (Fig. 5) showed a clear difference between the two cruises: Relative contributions in April were very similar all along the salinity gradient with NH_4^+ as the main contributor (50 - 70 %). In August, relative contributions clearly varied along the salinity gradient with a relatively high contribution (up to 90%) of NH_4^+ and NO_3^- at low salinities and a relatively high contribution (up to 80%) of DFAA and urea towards the open sea.

Uptake rates for the ADDON (Fig. 6) represent the uptake of added $DO^{15}N$ only (i.e. the ambient DON pool was not included in calculations). Uptake of the ADDON showed some clear differences between the two campaigns: uptake rates in April ranged from 10 to 60 nM-^{15}N h^{-1}, while values for August were an order of magnitude higher (100 - 500 nM-^{15}N h^{-1}) with similar values all along the salinity gradient of the Fjord and relatively low values in the open sea. Uptake rates in the dark were similar to those in the light (93 ± 22 % in April and 86 ± 11 % in August).

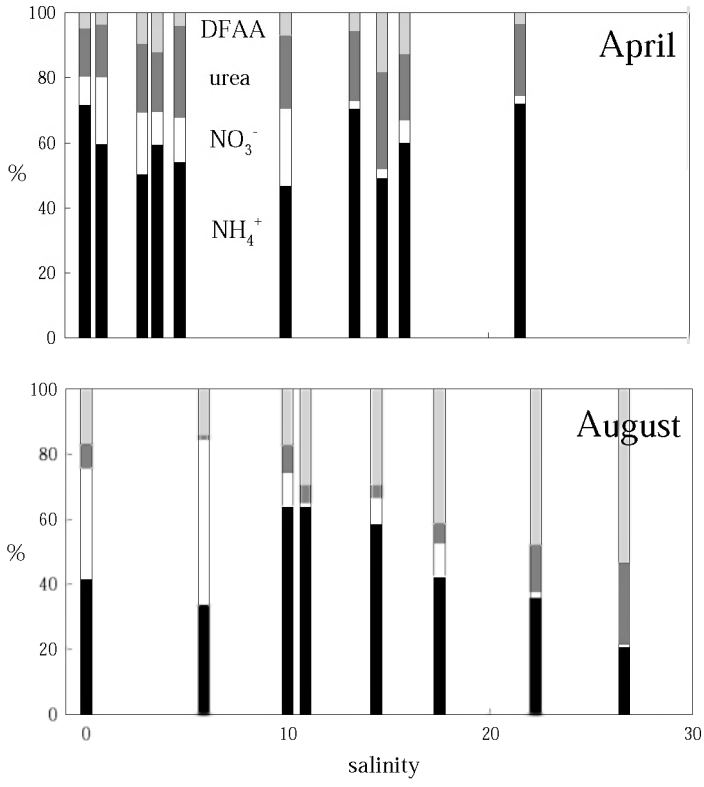


Figure 5

Relative contributions of the four N substrates to their summed uptake versus salinity in April and August

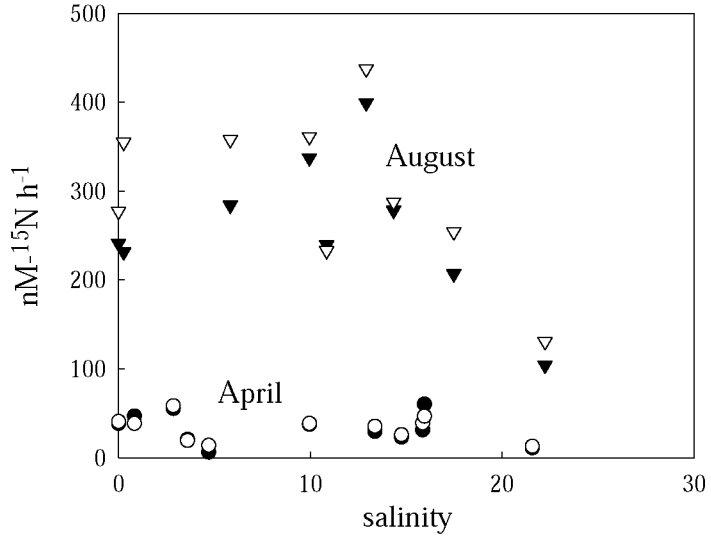


Figure 6

ADDON uptake rates versus salinity: ○: April in the light, ●: April in the dark, ▽: August in the light, ▼: August in the dark

Phytoplankton versus heterotrophic bacteria

Different approaches were used to estimate the relative importance of phytoplankton and heterotrophic bacteria to the uptake of the different N substrates (Table 1): For direct comparison of phytoplankton and bacterial abundance, biomasses of both groups were converted to µg-C l^{-1}. Bacterial biomass was calculated from bacterial numbers (BN, Fig 1) assuming an average bacterial C content of 20 fg per cell (Lee and Fuhrman, 1987). Phytoplankton biomass was calculated from Chl *a* concentrations (Fig. 2) assuming a C : Chl *a* ratio of 40. The abundance of phytoplankton and bacteria was generally similar in terms of biomass, except for in the fresh water in April where phytoplankton biomass was about one order of magnitude higher. In a similar way, bacterial productivity data (BP, Fig. 1) were used to calculate the bacterial N demand (the N uptake rate necessary to support bacterial growth), assuming a bacterial C : N ratio of 5 (Goldman & Dennett, 2000). The bacterial N demand is presented as absolute rates and as percentage of the summed uptake rates for NH_4^+, NO_3^-, urea and DFAA. Values are well within the range of measured uptake rates for the different N substrates. Results for the antibiotic treatments showed considerable variation (from < 0 to 100%), especially in August, and lacked consistent trends. Therefore, these results are only presented as average values for the whole estuary with their standard deviations to indicate the level of variation. Results for NO_3^- in August were not included in Table 1 since these were too inconsistent.

Table 1. Results concerning the relative contribution of heterotrophic bacteria to total microbial N uptake. Values are averages for the whole estuary (with indication of variation). See "Results" for details.

		April		August	
Bacterial biomass (µg-C l^{-1}) [a]		66	(40 - 100) [d]	80	(60 - 105) [d]
Phytoplankton biomass (µg-C l^{-1}) [b]		233	(35 - 840) [d]	93	(25 - 190) [d]
Bacterial N demand (nM-N h^{-1}) [a]		22	(12 - 34) [d]	50	(12 - 150) [d]
Bacterial N demand (% of summed uptake NH4+, NO3-, urea and DFAA)		35	(10 - 63) [d]	27	(3 - 66) [d]
Uptake inhibition by antibiotics (%)	NH_4^+	74	(± 17) [e]	54	(± 30) [e]
	NO_3^-	75	(± 43) [e]	[c]	
	urea	45	(± 27) [e]	57	(± 30) [e]
	DFAA	84	(± 17) [e]	34	(± 17) [e]
	ADDON	62	(± 19) [e]	4	(± 40) [e]

[a, b]: see Fig. 1 and 2 respectively for trends along salinity gradient
[c]: values too inconsistent
[d]: (minimum – maximum)
[e]: (± standard deviation)

DISCUSSION

Uptake of NH_4^+, NO_3^-, urea and DFAA

Concentrations and uptake rates for NH_4^+ and NO_3^- in Randers Fjord were within the range of values reported for six European turbid, tidal estuaries by Middelburg and Nieuwenhuize (2000a). Comparison of concentrations and uptake rates for urea and DFAA with those reported for other estuarine and marine systems (Bronk, 2002) shows that our results fit within the range of previously reported values, with concentrations and uptakes rates for DFAA in the open sea in August slightly exceeding highest reported values. The contribution of the summed concentrations of urea, DFAA and DCAA to the total DON pool (on average 12 % in April and 20 % in August) also agrees with previously reported values of identifiable DON contributing only ~ 20 % to the total DON pool (Benner, 2002; Bronk, 2002).

We are aware that, for some substrates, addition of a fixed amount of ^{15}N-labeled substrate caused differences in the ^{15}N enrichment of the dissolved substrate pool due to differences in ambient substrate concentrations along the salinity gradient. However, the ^{15}N enrichment of the dissolved substrate pool normally did not exceed the generally used upper limit of 10 % enrichment. Exceptions to this rule are the data for NO_3^- at salinities > 20 and DFAA at all stations in April and in the freshwater in August due to very low ambient concentrations. Enrichment > 10% is generally believed to increase uptake rates due to the increase in total substrate concentration. Since the points that clearly exceeded the 10% limit all showed very low uptake rates, it is unlikely that these high enrichments altered our results in respect to trends along the salinity gradient and differences between substrates and campaigns.

Although NO_3^- was the most abundant N substrate in Randers Fjord during both cruises, NO_3^- uptake rates were similar to those for the other N substrates. Apparently, the microbial affinity for NO_3^- was relatively low. On the contrary, DFAA uptake rates in August were in the same range as those for NH_4^+ and NO_3^- while DFAA was the least abundant N substrate, which implies that the microbial affinity for DFAA was high. Affinities for NH_4^+ and urea appeared to be similar and in between those for DFAA and NO_3^-. This order of preference for the different N substrates is consistent with results from culture studies as well as field measurements (e.g. McCarthy et al., 1977; Admiraal et al., 1987; Antia, 1991; Middelburg and Nieuwenhuize, 2000a,b; Bronk, 2002). The low microbial preference for NO_3^- is generally attributed to the relatively high energy investment

required for its reduction (Zehr & Ward, 2002). Although NO_2^- has also been shown to be a potentially important N substrate (McCarthy et al., 1977), in this study it probably was not since NO_2^- concentrations were much lower than those for NO_3^- and NH_4^+ while the microbial affinity for NO_2^- is similarly low as for NO_3^- (McCarthy et al., 1977). Microbial uptake of NO_3^- and NO_2^- seems to be important only when the availability of other N substrates is insufficient to meet the microbial N demand and / or when concentrations of NO_3^- / NO_2^- are very high (e.g. Middelburg and Nieuwenhuize, 2000b).

Figure 5 shows a clear seasonal difference in the relative importance of DIN versus DON as N sources to the microbial community along the salinity gradient of Randers Fjord. The high contribution of DON (urea and DFAA) in August (up to 80% in the open sea) is comparable with results by Middelburg and Nieuwenhuize (2000b) who found a similar high contribution for DFAA in the North Sea near the mouth of the Thames estuary. Given the high microbial preference for DFAA, it seems that the actual uptake of the different available N substrates was governed by a balance between the microbial affinity for the various substrates and their availability. Apparently, the abundance of DFAA was only sufficient to serve as the main N source at highest salinities in August. In the other cases, the microbial community had to turn to less favorable, but more abundant alternatives like NH_4^+ and even NO_3^- to support their N demand.

Uptake algal derived DON

The ADDON incubations (Fig. 6) were additional to those with the "standard", well-defined substrates in order to clarify the importance of the total ambient DON pool compared to individual substrates. Similar work with complex $DO^{15}N$ pools has been reported by Bronk and Glibert (1993) and Berg et al. (1997) who used $DO^{15}N$ extracted from a natural microbial community and an algal culture respectively. While these studies mainly focused on DFAA, our ADDON pool also contained a large DCAA fraction (~ 3 × higher than DFAA) and possible other (unidentified) compounds. Since the measured uptake rates concern uptake of the added $ADDO^{15}N$ only (i.e. the ambient DON pool was not included), these values are best considered as *potential* rates. The high uptake rates for the ADDON indicate that it was a favorable N substrate, which is not surprising as concentrations of the favorable DFAA in the ADDON pool were similar to those in the DFAA incubations. However, uptake rates for the total ADDON pool were actually about ~ 3 × higher than those for DFAA only, which indicates that other

compounds from the ADDON pool also served as an important N source. Out of these other compounds, DCAA concentrations were ~ 3 × higher than those for DFAA. Since DCAA have also been shown to be a favorable N substrate for the microbial community, especially bacteria (Coffin, 1989; Keil and Kirchman, 1993; Kroer et al., 1994; Middelboe et al., 1995), the majority of the ADDON uptake likely involved uptake of DCAA. Furthermore, DFAA and DCAA concentrations in the ADDON incubations were very similar to the ambient DFAA and DCAA concentrations in August. This suggests that the *potential* uptake rates for the ADDON provide a good indication of *in-situ* uptake rates for DFAA + DCAA in August (assuming a similar lability for ambient and added DCAA).

Phytoplankton versus heterotrophic bacteria

Although the biomass estimates for phytoplankton and bacteria do not necessarily reflect their contributions to N uptake, their comparable abundance does indicate that both groups required a similar level of N uptake to support their biomass production, except for in the freshwater in April where microbial biomass was dominated by phytoplankton. The latter was likely the result of a diatom bloom as suggested by high concentrations of the diatom biomarker pigments fucoxanthin and Chl *c* (data not shown). A more direct indication of the bacterial contribution to total microbial N uptake is provided by the bacterial N demand that was estimated from the measured BP. The fact that the estimated values were within the range of measured uptake rates for the different N substrates indicates that a considerable part of the total microbial N uptake was used to support bacterial biomass production. However, this approach is not precise because of uncertainties associated with the conversion factors used for calculation of BP and the bacterial N demand and the fact that the summed uptake rates for the tested N substrates do not represent total N uptake (as discussed above).

Interpretation of the results concerning the inhibition of bacterial N uptake by the antibiotics is limited by the large variation and the lack of consistent trends. Although average values confirm the large bacterial contribution to total microbial N uptake, as suggested by the other results, their quantitative interpretation is prohibited by the limited efficiency and specificity associated with antibiotics (Oremland and Capone, 1988).

The methods used in this study only provided an indication that both phytoplankton and heterotrophic bacteria were important in the uptake of the different N substrates. The only alternative method for measuring group specific N

uptake is size fractionation (Lipschultz 1995). However, the overlap in phytoplankton and bacterial cell size range and the close association of bacteria with larger particles (especially in turbid systems like Randers Fjord) are fundamental limitations to this method. In general, none of the existing methods are suitable for full quantification of the relative importance of heterotrophic bacteria versus phytoplankton, therefore a new method is required for further elucidation of this aspect of microbial N uptake. The use compound specific isotope analysis of biomarkers in combination with ^{15}N labeling is one, but promising approach (Boschker & Middelburg, 2002).

In conclusion, this study shows that DON (urea and DFAA) were important N substrates to the microbial community of Randers Fjord next to DIN (NH_4^+ and NO_3^-) and that other DON compounds were likely important N substrates as well. Therefore, studies on estuarine and marine microbial N uptake that do not consider urea, DFAA, DCAA and the unidentified fraction of the DON pool as potential N sources are likely to underestimate total microbial N uptake while overestimating the relative importance of substrates like NH_4^+ and NO_3^-. Finally, although both heterotrophic bacteria and phytoplankton have been identified as major contributors to total microbial N uptake, full quantification of their role is prohibited by a lack of adequate methodology.

Acknowledgements

We thank our EUROTROPH colleagues for the cooperation during the field work and Alex Parker, Dave Kirchman and an anonymous reviewer for evaluating this paper. This research was supported by the EU (EUROTROPH, EVK3-CT-2000-00040) and a PIONEER grant of the Netherlands organization for scientific research (NWO, 833.02.002).

C H A P T E R 2

Analysis of ^{15}N incorporation into D-alanine: a new method for tracing nitrogen uptake by bacteria

Bart Veuger, Jack J. Middelburg, Henricus T.S. Boschker and Marco Houtekamer

Limnology and Oceanography: Methods 3 (2005): 230-240.

ABSTRACT

The quantitative contribution of bacteria to the total microbial uptake of nitrogenous substrates is an aspect of the aquatic nitrogen cycle that is still largely unclear, mainly because existing methods are generally inadequate. We investigated the feasibility of measuring ^{15}N incorporation into bacterial D-amino acids by gas chromatography-combustion-isotope ratio mass spectrometry (GC-c-IRMS) and the potential of this method as a new tool for quantification of ^{15}N uptake by bacteria. The presented method allowed analysis of ^{15}N incorporation into various hydrolysable amino acids (HAAs), including the bacterial biomarker D-alanine (D-Ala), at trace levels. The potential of the method was tested in a ^{15}N-labeling experiment in which sediment slurries were incubated with $^{15}NH_4^+$ and a ^{15}N-labeled amino acid mixture. The ^{15}N from both substrates was rapidly incorporated into the HAAs, including D-Ala. ^{15}N incorporation into D-Ala was used to calculate total bacterial ^{15}N uptake while comparison of ^{15}N incorporation into D-Ala versus L-Ala provided a direct measure for the relative contributions of bacteria versus algae to the total microbial ^{15}N uptake (bacteria + algae). Subsequently, it was also possible to calculate ^{15}N uptake by the algae. Results for the test experiment showed that bacteria accounted for 38% of total $^{15}NH_4^+$ uptake and dominated uptake of the ^{15}N-amino acid mixture (90%). Analysis of ^{15}N incorporation into the other (non-biomarker) HAAs yielded useful additional information on the transformation of these HAAs during organic matter degradation. In conclusion, GC-c-IRMS analysis of D-Ala combined with ^{15}N labeling is a unique approach in aquatic sciences that provides a powerful new method for quantification of nitrogen flows through bacteria in natural microbial communities.

INTRODUCTION

The nitrogen cycle plays a central role in aquatic systems and although it has been studied extensively, various aspects are still poorly understood (Zehr and Ward 2002). One of these aspects is the uptake of nitrogenous substrates by bacteria. Although bacteria were traditionally considered as remineralizers of organic matter, it is now evident that they can also take up various forms of dissolved inorganic and organic N (e.g. Coffin 1989, Kirchman 1994, Kroer et al. 1994). The importance of N uptake by bacteria is especially interesting in comparison with that by algae (the other main group involved in microbial N uptake) as these two groups form different parts of the food web. Therefore, the relative contributions of bacteria versus algae can determine the subsequent fate of the N after uptake (Caraco and Cole 2002). However, the quantitative importance of bacterial N uptake in natural microbial communities is still largely unclear due to a lack of adequate methodology. The common technique for measuring microbial N uptake is to measure the uptake of nitrogenous substrates labeled with the stable isotope ^{15}N. Total microbial ^{15}N uptake can generally be measured relatively easy as ^{15}N enrichment of the bulk particulate material. However, specifically measuring ^{15}N uptake by bacteria or algae within the total microbial community is far more difficult. Up to now, two different methods have been used to distinguish N uptake by bacteria and algae. The first method is size fractionation, which involves physical isolation of microbial cells by filtration or flow cytometry with cell sorting (Lipschultz 1995). Although this method can work for oligotrophic waters, its use is limited by an overlap in bacterial and algal cell size range. Furthermore, size fractionation in turbid waters and sediment is prohibited by practical aspects like filter clogging and the close association of bacteria with larger particles. The second method involves the use of antibiotics to inhibit bacterial ^{15}N uptake. Although this method can sometimes provide interesting results (e.g. Wheeler and Kirchman 1986, Middelburg and Nieuwenhuize 2000), their interpretation is limited by uncertainties concerning the efficiency and specificity of inhibitors (Oremland and Capone 1988). In response to this lack of a suitable method for measuring ^{15}N uptake by bacteria in natural microbial communities, we developed a new approach that involves the use of biomarkers (compounds unique to a specific group of organisms). In combination with stable isotope labeling, biomarkers provide the possibility to directly link microbial identity (biomarker), biomass (biomarker

concentration) and activity (label incorporation into biomarker) (Boschker and Middelburg 2002).

Analysis of isotopic enrichment in biomarkers at trace levels requires compound-specific isotope ratio mass spectrometry (IRMS) (Brenna et al. 1997, Boschker and Middelburg 2002). Compound-specific analysis requires isolation of individual compounds prior to IRMS analysis, which can be done by liquid chromatography (LC) or gas chromatography (GC). Although interfaces for online LC-IRMS analysis are starting to become commercially available, these are so far only suitable for ^{13}C analysis. Therefore, the technique for online compound-specific ^{15}N analysis is GC coupled to IRMS via a combustion interface (GC-c-IRMS). The range of compounds suitable for GC-c-IRMS analysis is restricted to smaller molecules that are volatile or can be made volatile by derivatization. A group of biomarkers that meets these requirements are the phospholipid-derived fatty acids (PLFAs). The use of these membrane-associated compounds has yielded interesting results on bacterial and algal uptake of ^{13}C-labeled substrates (Boschker et al. 1998, Middelburg et al. 2000, Moodley et al. 2000). However, PLFAs are not suitable for ^{15}N studies as these compounds do not contain N. Therefore, we investigated the potential of D-amino acids (D-AAs) from peptidoglycan as alternative bacterial biomarkers. Peptidoglycan, a cell wall component unique to bacteria, consists of strands of sugar derivatives that are cross-linked by peptide bridges consisting of both the D- and L-enantiomers (or "stereoisomers") of alanine (D-Ala and L-Ala), D-glutamic acid (D-Glu) and either diaminopimelic acid (DAP) or L-lysine (L-Lys). In addition to this basic structure, over 100 variations of peptidoglycan have been reported (Madigan et al. 2000), including the presence of other D-AAs. Since bacteria are the only organisms to incorporate D-AAs into their biomass, D-AAs can be used as bacterial biomarkers (e.g., Ueda et al. 1989, Pelz et al. 1998, McCarthy et al. 1998, Grutters et al. 2002). Out of the different D-AAs, D-Ala is the most promising bacterial biomarker for our application since it is the only D-AA that is present in all bacteria (providing a good link with total bacterial biomass). Furthermore, D-Ala has been shown to be most suitable for GC-c-IRMS analysis (Pelz et al. 1998) and to be relative insensitive to racemization (the abiotic formation of D-AAs from their respective L-enantiomers) during acid hydrolysis (Erbe and Brückner 2000). Next to D-Ala, DAP has also been tested and used as a bacterial biomarker amino acid (Pelz et al. 1998, Tobias et al. 2003). Although DAP has the advantage of containing twice as much N as D-Ala, it seems to be less suitable for the current application as DAP is not present in all bacteria (Madigan et al. 2000) and because

GC-c-IRMS analysis of DAP in environmental samples proved to be more problematic than D-Ala (Pelz et al. 1998).

^{15}N incorporation into D-Ala was already measured in 1985 by Tunlid et al. in an *E.Coli* culture. However, they used GC-MS, which only allows analysis of very high levels of isotope enrichment (typically > 1 atom %). Hence, this technique is not suitable for environmental labeling studies that typically work at trace levels and therefore require GC-c-IRMS. Applications of GC-c-IRMS analysis of hydrolysable AAs (HAAs) in aquatic sciences and related fields of research usually involve studies on ^{13}C without chiral resolution (i.e. not distinguishing between D- and L-AAs) (e.g. Uhle et al. 1997, Fantle et al. 1999, Keil and Fogel 2001). Analysis of ^{13}C in D-AAs is restricted to Pelz et al. (1998) and Glaser and Amelung (2002). However, these studies all concern the *natural abundance* of ^{13}C, we are not aware of any relevant studies combining GC-c-IRMS analysis of D-AAs with ^{13}C *labeling*. Applications of GC-c-IRMS analysis of ^{15}N in HAAs are few: McClelland and Montoya (2002) and McClelland et al. (2003) measured natural abundance of ^{15}N in various HAAs from zooplankton and phytoplankton and Tobias et al. (2003) measured assimilation of ^{15}N from $^{15}NO_3^-$ into DAP. However, these studies used a non-chiral column, which does not allow analysis of D-AAs. Although the analytical aspects of ^{15}N analysis of D- and L-AAs by GC-c-IRMS were already presented by Macko et al. (1997), we are not aware of any applications in aquatic sciences or related fields of research. This seems to be due to the analytical difficulties associated with GC-c-IRMS analysis of ^{15}N compared to ^{13}C resulting from the relatively low N content of amino acids (C : N ratio of 2 to 9) and the additional reduction step required to convert NO and N_2O to N_2 prior to IRMS analysis (Brenna et al. 1997). To the best of our knowledge, we are the first to combine GC-c-IRMS analysis of D-AAs with ^{15}N labeling and to use this as a method to quantify ^{15}N incorporation by bacteria.

MATERIALS AND PROCEDURES

The protocol used for extraction of the HAAs from the sediment and subsequent purifcation of the samples is primarily based on work by Pelz et al. (1998) and Amelung and Zhang (2001) and similar to McClelland and Montoya (2002). All steps were individually validated and fine-tuned in order to maximize their efficiency and yield. The protocol for sample preparation and analyses is summarized in Fig. 1 and will be described in detail below.

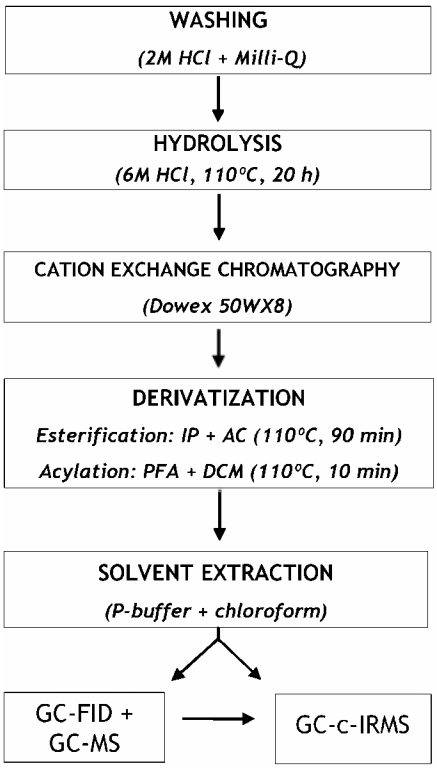


Figure 1. Schematic overview of the sample preparation protocol.

Washing

Samples (1 g) of ground, freeze-dried sediment were suspended in 6 ml 2M HCl in 10 ml glass test tubes to remove carbonates (Pelz et al. 1998) and to destruct microbial cells. After centrifugation (10 min at 600 g) pellets were washed with 10 ml Milli-Q water twice in order to further remove water-soluble compounds, including dissolved and / or adsorbed L-AAs.

Hydrolysis

HAAs were liberated from (microbial) cell walls, proteins, etc. by hydrolyzing the washed pellets in 4 ml 6M HCl at 110°C for 20 h in test tubes that were sealed tight with teflon-lined screw caps after replacing the headspace with N_2. After hydrolysis, samples were cooled to room temperature and spiked with L-Norleucine (L-Nle) that was used as an internal standard to determine the recovery

of the HAAs after further sample processing. Samples were centrifuged (10 min at 600 g) and supernatants (containing the HAAs) transferred to glass test tubes (25 ml). Pellets were washed by resuspension in 10 ml Milli-Q water followed by centrifugation (10 min at 600 g). After repeating the latter step, the resulting Milli-Q supernatants were pooled with the original 6M HCl supernatant, yielding a 24 ml 1M HCl solution that could directly be purified by cation exchange chromatography.

Cation exchange chromatography

Glass columns were filled with 5 ml of Dowex 50WX8 resin (50-100 mesh) between two pieces of quartz wool. Columns were prerinsed with 50 ml NH_4OH (2M), 50 ml HCl (2M) and 50 ml Milli-Q water. After adding the samples (HAAs in 1M HCl) to the columns, salts and organic contaminations were washed out with 50 ml Milli-Q water. Amino acids were eluted from the column by rinsing with 30 ml 2M NH_4OH which was subsequently evaporated to dryness in glass beakers (250 ml) on a heating plate (~ 60°C) under a warm air flow. Dried samples were resuspended in 2 ml acidified Milli-Q water (0.1 M HCl), transferred to glass screw cap vials (12 × 32 mm) and stored at -20°C until derivatization.

Derivatization

The protocol for derivatization (making the amino acids amenable to GC analysis) is primarily based on those of Macko et al. (1997) and Pelz et al. (1998). All steps were tested individually in order to maximize derivatization efficiency and AA recovery. Samples were evaporated in the 12 × 32 mm vials at 50°C under a gentle flow of N_2. The amino acids were esterified by adding 500 µl isopropanol (IP) that had been freshly acidified with acetylchloride (AC) in a 4:1 ratio, and heating for 90 min in a block heater at 110°C. Vials were sealed tight with teflon-lined screw caps in order to prevent loss of amino acids during heating. After the esterified samples had been cooled and evaporated at 50°C under a gentle flow of N_2, 200 µl dichloromethane (DCM) was added to the dried samples and subsequently evaporated in order to remove residual water and IP (Macko et al. 1997). The AA-IP esters were acylated by adding 150 µl DCM and 50 µl pentafluoropropionic anhydride (PFA) and heating for 10 min in a block heater at 110°C. After acylation, samples were cooled and further purified by solvent extraction.

Solvent extraction

The solvent extraction protocol was based on Ueda et al. (1989): 0.5 ml chloroform and 1 ml P-buffer (KH_2PO_4 + Na_2HPO_4 in Milli-Q water, pH 7) were added to the samples. Firm shaking caused the AA-PFA/IP esters to be transferred to the chloroform fraction while contamination ended up in the P-buffer. Separate testing of this step showed full recovery of the AA-PFA/IP esters in the chloroform fraction. The two solvents were separated by centrifugation (10 min at 600 g) after which the clear chloroform fraction was transferred to a crimp-cap GC vial, evaporated under a gentle stream of N_2, dissolved in ethyl acetate (25 - 200 µl) and stored capped at -20°C until analysis.

GC-FID analysis

Amino acid concentrations were measured by GC with Flame Ionization Detection (GC-FID) within 24 h after derivatization. A Carlo Erba Mega 2 series GC was equipped with a capillary Chirasil-L-Val column (Alltech, 50m, ID: 0.32 mm, film thickness: 0.2 µm), which is the standard column for separating D- and L-AAs (e.g. Macko et al, 1997; Pelz et al. 1998). Helium was used as the carrier gas at a pressure of 150 kPa and samples (0.5 - 1 µl) were injected in splitless mode (120 sec) at 200°C. The oven program started with 10 min at 80°C, then increased with 3°C min^{-1} to 190°C where it was held for 5 min. AA-PFA/IP esters eluting from the column were detected by FID, which yielded peak areas that were tested to be linearly correlated with amino acid concentrations over several orders of magnitude, including relevant concentrations for this study. Peak areas were converted to concentrations using separate conversion factors for all individual amino acids (obtained via analysis of standards) and corrected for losses during sample processing using the recovery of the internal standard.

GC-MS analysis

A selection of samples was measured by GC-MS using the same GC, but coupled to a quadrupole mass spectrometer (Thermo Finnigan Voyager). This technique uses electro ionization to split the compounds eluting from the GC column into characteristic mass fragments. The resulting mass spectra were used for direct identification of the amino acids and to check their peak purity.

GC-c-IRMS analysis

For stable isotope analysis, amino acids were separated on an HP 6890 GC using the same column and settings as for the GC-FID/MS analyses except for the temperature program which was modified to increase chromatographic performance and decrease analysis time. This modified temperature program started with 2 min at 60°C, followed by a 3°C min^{-1} increase to 120°C, then a 10°C min^{-1} increase to 190°C and finally 10 min at 190°C. The GC was coupled to a Thermo Delta Plus IRMS via a combustion interface (Thermo type III). Briefly, AA-PFA/IP esters eluting from the GC column were combusted to CO_2, H_2O, N_2, N_2O and NO in a combustion furnace at 940°C. Subsequently, N_2O and NO were reduced to N_2 in a reduction furnace at 600°C while H_2O and CO_2 were removed. The IRMS detected masses 28 ($^{14}N_2$) and 29 ($^{14}N/^{15}N$) and used the $^{15}N/^{14}N$ ratio (R) for the individual AA-PFA/IP esters to calculate their $\delta^{15}N$ (relative to standard N_2 reference gas pulses that were measured at the start and end of each run): $\delta^{15}N$ (‰) = $((R_{sample} / R_{standard}) - 1) \times 1000$. Unlike for ^{13}C analysis, the measured $\delta^{15}N$ values are the same as those for the underivatized amino acids since no extra N is added during derivatization and therefore do not require correction. ^{15}N enrichment resulting from incorporation of ^{15}N into the amino acids is reported as $\Delta\delta^{15}N$ ($\Delta\delta^{15}N_{sample} = \delta^{15}N_{sample} - \delta^{15}N_{control\ (unlabeled)}$). Furthermore, the atom percentage ^{15}N was calculated ($at\%^{15}N = (100 \times R_{standard} \times ((\delta^{15}N_{sample} / 1000) + 1)) / (1 + R_{standard} \times ((\delta^{15}N_{sample} / 1000) + 1))$), which was subsequently used to calculate the excess ^{15}N in the amino acids ($excess\ ^{15}N = (at\%^{15}N_{sample} - at\%^{15}N_{control}) \times AA\ concentration_{sample}$).

ASSESSMENT

Test experiment

A simple ^{15}N-labeling experiment was performed in order to test and validate the method. Briefly, surface sediment (upper 10 mm) was collected from an intertidal mudflat (Appelzak) in the Scheldt estuary (The Netherlands) in October 2003. In the laboratory, 20 ml samples of homogenized sediment were placed in glass beakers and diluted with 20 ml water from the same location. The resulting slurries were allowed to settle overnight. The next day, slurries were spiked with 0.4 µmol $^{15}NH_4^+$ or 0.4 µmol of a ^{15}N-labeled algal amino acid mixture (Cambridge Isotope Laboratories, NLM- 2161, see chapter 3 for composition). Resulting $^{15}NH_4^+$ and ^{15}N-DFAA concentrations in the slurries were 1.8 × and 6.5 × higher than the respective ambient NH_4^+ and DFAA concentrations. Due to this strong increase in total substrate concentrations, the resulting ^{15}N uptake cannot be extrapolated to ambient N uptake. Therefore, measured ^{15}N uptake should be considered as potential uptake (rates) for the two substrates. However, these increased concentrations did not affect the main focus of this test experiment, which was to determine the relative contributions of bacteria versus algae to the uptake of ^{15}N labeled substrates by a natural, mixed microbial sediment community. Samples were incubated at ambient temperature and light conditions for 6 h. Incubations were terminated by transferring the slurries to centrifuge tubes for centrifugation. Resulting pellets were frozen (-20°C), freeze-dried and stored frozen until analysis. In addition to the test experiment, we also analyzed material from various bacterial cultures (pure strains and mixed cultures, see Table 2) to determine the D-Ala content and D/L-Ala ratio of bacterial biomass.

Sample preparation

The protocol for washing, hydrolysis and cation exchange chromatography allowed processing of ~ 30 samples in 4 work days (not including derivatization and solvent extraction). Results for the sediment samples confirmed the need to wash the sediment with 2M HCl and Milli-Q water prior to hydrolysis. Washing not only eliminated carbonates that would otherwise interfere with hydrolysis, but also removed contamination (including a compound interfering with GC analysis of D-Ala) and dissolved and / or adsorbed free amino acids. This removal of free L-AAs decreased the abundance of the total L-AAs in the samples and thereby effectively

increased the relative abundance of the D-AAs (that are predominantly present in the HAA fraction and were therefore not removed by washing) which facilitated their analysis. A potential problem related to acid hydrolysis of organic material is racemization (the abiotic formation of D-AAs from their respective L-enantiomers). Although we did not measure racemization during hydrolysis, we are confident that it was negligible since we only detected peptidoglycan-associated D-Ala and D-Glu. In case of significant racemization, other D-AAs also should have been present in detectable amounts, especially D-Asp since Asp is considered to be relatively sensitive to racemization in 6M HCl (Erbe and Brückner 2000). In addition, similar studies also concluded that the effect of racemization is negligible (Tunlid and Odham 1983, Sonesson et al. 1988, Pelz et al. 1998). Washing the sediment material with Milli-Q water after hydrolysis proved to be an important step that was necessary to recover the significant amount of HAAs still present in the pellet after removal of the 6M HCl. As an additional advantage, dilution of the 6M HCl with the Milli-Q water used for washing resulted in a 1M HCl solution that could directly be purified by cation exchange chromatography. This eliminated the need for the time consuming additional step of evaporating the HCl solution and subsequent redilution in Milli-Q water as required for samples in 6M HCl. Protocols for cation exchange chromatography and derivatization are based on established protocols and will therefore not be discussed separately. Additional sample purification by solvent extraction was included because sediment samples still contained considerable amounts of impurities after derivatization. Solvent extraction proved to be simple, fast and very effective in removing these remaining impurities. We also tested solvent extraction as an alternative for cation exchange chromatography, but found that only the combination of both steps resulted in sufficient purification.

The HAA recovery after purification and derivatization (as determined by the recovery of the internal standard Nle) was 30 - 53% (average 43%). This apparently low recovery was the result of the extensive sample processing, where the overall recovery reflects the sum of smaller losses during the various individual steps. Incomplete recovery introduces the risk of isotopic fractionation (preferential loss of ^{14}N). The latter was tested by comparing the $\delta^{15}N$ of Nle that was derivatized directly (i.e. full recovery) with the $\delta^{15}N$ of Nle that had been added to the sediment samples after hydrolysis (same recovery as for the other amino acids). This yielded $\delta^{15}N$ values of 19.6 ± 1.8 ‰ and 23.1 ± 2.9 ‰ respectively, indicating a fractionation of + 3.5 ‰. Although this fractionation can be an issue for natural abundance studies, it is negligible for ^{15}N labeling studies where labeling clearly

exceeded natural abundance levels. Furthermore, this fractionation will not only occur in the labeled samples, but also in the control samples, which means that ^{15}N enrichment (labeled – control) is not influenced by the fractionation. Another potential risk of incomplete recovery is preferential loss of certain amino acids. However, comparison of samples with different recoveries showed no differences in the THAA pool composition (including the D/L-Ala ratio). Therefore, although not completely satisfactory, the overall recovery of 43% is acceptable for labeling studies like our test experiment.

GC-FID and GC-MS analyses

GC-FID and GC-MS analyses generally showed good chromatography (Fig. 2) with sharp and well separated peaks for most amino acids and little contamination, which confirms that sample purification was sufficient. Most of the 20 "common" L-AAs were detected in the sediment samples, except for His, Cys, Trp and Arg since these require special derivatization (Erbe 1999).

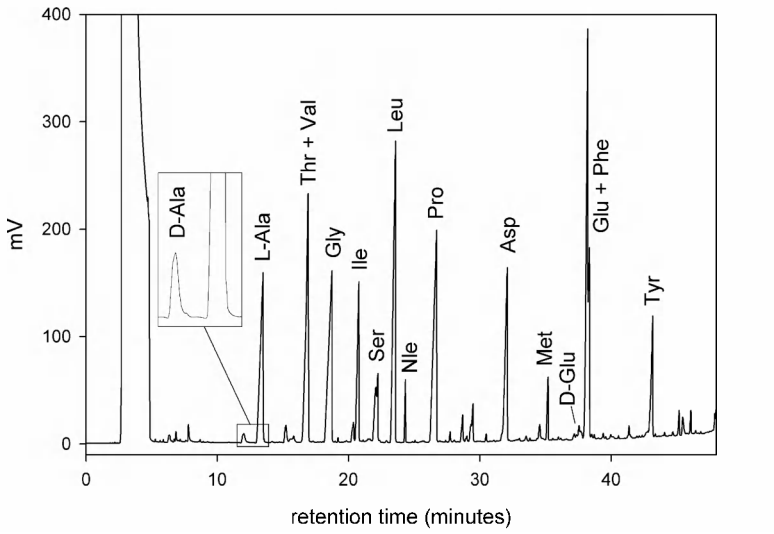


Figure 2. GC-FID chromatogram for a sediment sample from an intertidal mudflat in the Scheldt estuary. Insert shows magnification of D-Ala peak. Amino acids are all L-enantiomers, unless indicated otherwise. (Nle = internal standard).

During hydrolysis Gln and Asn are transformed to Glu and Asp respectively (Uhle et al. 1997, Erbe 1999), therefore, peaks for Glu and Asp represent the sum of Glu+Gln and Asp+Asn respectively. Peaks for Thr and Val as well as those for L-Glu and Phe were not always separated sufficiently and are therefore reported as one. The measured total HAA concentration (sum of individual amino acids) was 5.6 mg per gram dry sediment, which is similar to values reported for various other sediments (Dauwe and Middelburg 1998, Keil and Fogel 2001, Grutters et al. 2002). Out of the different D-AAs that could be used as bacterial biomarkers, only D-Ala and D-Glu were detected in the sediment samples (as identified by GC-MS). D-Ala was the first amino acid to elute from the GC column (retention time ~ 12 min), directly followed by L-Ala (~ 13 min), which resulted in optimal chromatographic performance (good peak separation and low column bleed). D-Glu eluted later (~ 37 min), resulting in poorer separation from L-Glu and higher column bleed. In addition, an unidentified compound co-eluted with D-Glu, which prohibited quantification of D-Glu. The average D-Ala concentrations in the sediment samples was 32 μg g^{-1}, which is somewhat higher than the range of 2 - 20 μg g^{-1} reported by Grutters et al. (2002). The D/L-Ala ratio (concentration D-Ala / concentration L-Ala × 100) in the test sediment was 5.2 ± 0.2 %, which is similar to values for marine sediment surface layers reported by Pedersen et al. (2001) and Grutters et al. (2002).

GC-c-IRMS analyses

GC-c-IRMS analyses of the amino acids showed lower peak separation than GC-FID/MS analyses due to peak broadening in the combustion interface. During earlier measurements (using the same temperature program as for the GC-FID/MS analyses) separation between D- and L-Ala was critical due to this reduced peak separation. However, our most recent measurements (with the adjusted temperature program and general optimization of GC-c-IRMS performance) showed improved peak separation with a clear and well separated peak for D-Ala (Fig. 3). Injected amounts of D-Ala were ~ 1 nmol (~ 0.1 μg), which resulted in co-injection of ~ 200 nmol (~ 25 μg) total HAAs. The feasibility of GC-c-IRMS analysis of ^{15}N in ~ 1 nmol D-Ala has already been reported by Macko et al. (1997). The injected amount of sample was < 1 % of the total sample derived from 1 g dry sediment. When assuming an injection volume of 1 μl and a minimum sample volume of 10 μl ethyl acetate, this means that the minimum amount of sample required for analysis is ~ 100 mg dry sediment.

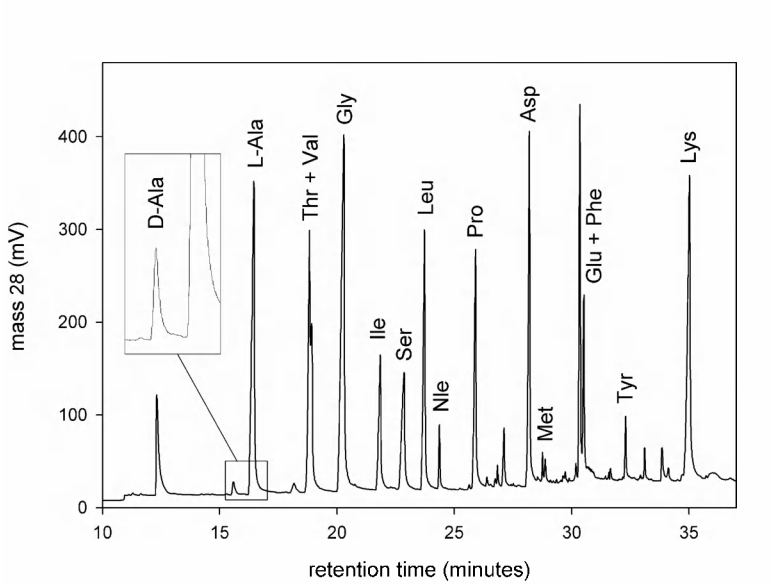


Figure 3. GC-c-IRMS chromatogram for a sediment sample from an intertidal mudflat in the Scheldt Estuary. Insert shows magnification of D-Ala peak. Amino acids are all L-enantiomers, unless indicated otherwise.

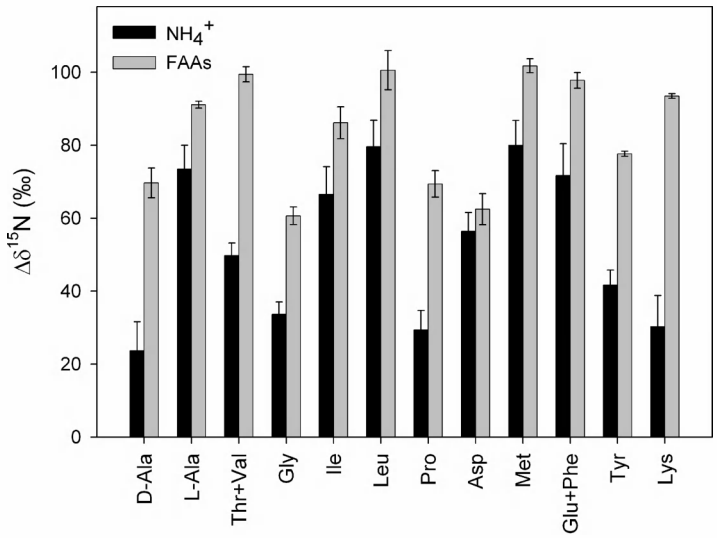


Figure 4. ^{15}N enrichment ($\Delta\delta^{15}N$) of individual HAAs after incubation with $^{15}NH_4^+$ (black bars) and the ^{15}N-labeled FAA mixture (grey bars). Amino acids are all L-enantiomers, unless indicated otherwise. Error bars indicate experimental variation (standard deviation for replicate incubations).

Table 1. Analytical variation for GC-c-IRMS analyses

	(n)	D-Ala stdev	other HAAs stdev
Replicate analysis (sample measured 2-3 times)			
Natural abundance ($\delta^{15}N$ ~ 15 ‰)	3	2.5 ‰	0.2 - 1.5 ‰ (avs 1.0 ‰)
Labeled ($\delta^{15}N$ 30 -150 ‰)	2	3.2 ‰	0.1 - 3.8 ‰ (avs 1.9 ‰)
Replicate sample preparation + analysis (identical samples processed separately)			
Labeled ($\delta^{15}N$ 30 -150 ‰)	2	6.3 ‰	1.3 - 7.5 ‰ (avs 2.8 ‰)

(stdev = standard deviation, avs = average).

^{15}N enrichment in D-Ala and the other HAAs

We were able to measure ^{15}N enrichment ($\Delta\delta^{15}N$) for D-Ala and various other HAAs with consistent $\delta^{15}N$ values well above natural abundance (Fig. 4) and little variation (Table 1). Standard deviations for the L-AAs in the unlabeled samples (Table 1) are similar to those reported for natural abundance $\delta^{15}N$ analyses by McClelland and Montoya (2002). The level of variation for D-Ala was somewhat higher (Table 1), but still very acceptable for ^{15}N labeling studies like our test experiment. We are confident that the measured ^{15}N enrichment of the amino acids in the samples incubated with the ^{15}N-FAAs actually represents incorporation of the ^{15}N into the HAAs without bias from the added ^{15}N-FAAs, because: 1. Washing the sediment with 2M HCl and Milli-Q water likely removed most of the adsorbed and dissolved amino acids. 2. Our results show clear incorporation of ^{15}N from $^{15}NH_4^+$ (without potential bias from the added ^{15}N-substrate). Since the microbial preference for FAAs is generally considered to be similar to or even higher than for NH_4^+ (Veuger et al. 2004), uptake of the ^{15}N-FAAs likely was similar to or even higher than for $^{15}NH_4^+$.

From ^{15}N in D-Ala to total bacterial ^{15}N uptake

The most straightforward way to interpret the results obtained with the method is to directly use $\Delta\delta^{15}N$ values for D-Ala as a semi-quantitative measure for bacterial ^{15}N incorporation. This can provide interesting results with respect to trends in bacterial uptake of a ^{15}N-labeled substrate over time. However, the potential of the method would be greatly enhanced if ^{15}N incorporation into D-Ala can be used to calculate total bacterial ^{15}N uptake. Calculation of total bacterial ^{15}N uptake requires a conversion factor based on the D-Ala content of bacterial biomass. The latter was

measured in five of our bacterial cultures (Table 2) which yielded an average value of 1.9 mg D-Ala per gram dry biomass. The measured values are similar to those measured by Sonesson et al. (1988) (Table 2). Assuming that dry bacterial biomass consists of ~ 12 % N (Maddigan et al. 2000), this yields a D-Ala content of 0.25 % (moles-N in D-Ala / moles-N in total bacterial biomass × 100) and a corresponding conversion factor of 400×. Application of this conversion factor to the measured excess ^{15}N in D-Ala yielded estimates of 12 and 36 nmol ^{15}N g^{-1} for total bacterial uptake of $^{15}NH_4^+$ and the ^{15}N-FAAs respectively (Table 3).

Bacterial contribution to total microbial ^{15}N uptake

Next to total bacterial ^{15}N uptake (as calculated above), the method can also be used to determine the relative contributions of bacteria versus algae to the total microbial uptake of the $^{15}NH_4^+$ and the ^{15}N-FAAs. One way to do this is by comparing the bacterial uptake as calculated above with the ^{15}N enrichment of the bulk sediment (measured by EA-IRMS, see Table 3) where the latter is considered to represent total microbial ^{15}N uptake. This approach yielded bacterial contributions of 36 % and 81 % to the total microbial uptake of the $^{15}NH_4^+$ and the ^{15}N-FAAs respectively. However, these estimates contain some major uncertainties because they are based on: 1) A relatively large conversion factor representing the average for the five bacterial cultures that showed quite variable D-Ala contents. 2) The assumption that the ^{15}N enrichment of the bulk sediment represents the total microbial ^{15}N uptake. It seems that this was true for our test experiment. However, the ^{15}N enrichment of the bulk sediment can also include part of the added ^{15}N substrates and/or ^{15}N labeled degradation and excretion products. When the latter occurs, total microbial ^{15}N uptake will be overestimated, resulting in an underestimation of the bacterial contribution.

Fortunately, the method also provides an alternative way to determine the bacterial contribution to total microbial ^{15}N uptake. This approach is based on the relative incorporation of ^{15}N into D-Ala versus L-Ala. Because L-Ala is a common amino acid that is abundant in biomass of all microorganisms (including bacteria) while D-Ala only occurs in bacterial biomass, comparison of ^{15}N incorporation into D- versus L-Ala provides a direct measure for ^{15}N incorporation by bacteria (D-Ala) versus that by the total microbial community (L-Ala). Since comparison of $\Delta\delta^{15}N$ values can be complicated by possible differences in isotope dilution by the (inactive) background pools of D- and L-Ala in the sediment, it is more practical to use excess ^{15}N values (Fig. 5).

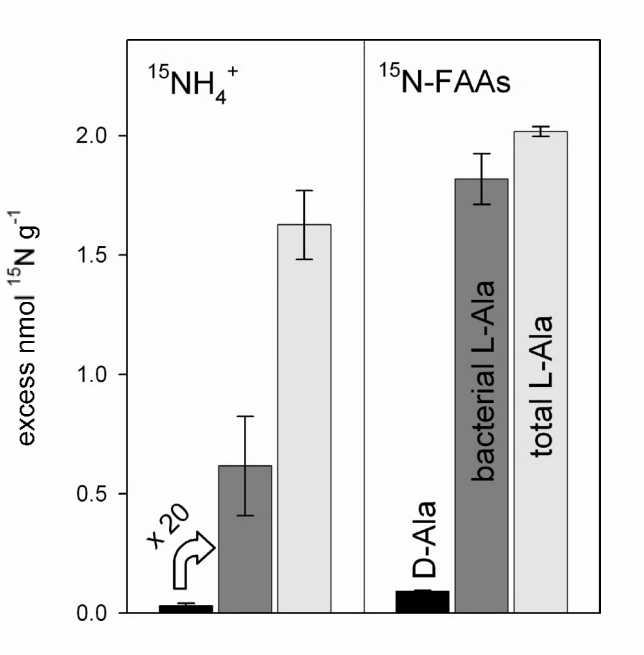


Figure 5. Measured excess ^{15}N in D-Ala (black bars) and L-Ala (light gray) and calculated values for bacterial L-Ala (dark gray) for incubations with $^{15}NH_4^+$ (left) and ^{15}N-FAAs (right). Values for bacterial L-Ala are calculated from the excess ^{15}N in D-Ala, assuming a bacterial D/L-Ala ratio of 5 %. Error bars indicate experimental variation (standard deviation for replicate incubations).

In figure 5, the measured excess ^{15}N in *total* L-Ala (representing ^{15}N incorporation by the total microbial community) is compared with the excess ^{15}N in *bacterial* L-Ala. The latter was calculated from the excess ^{15}N in D-Ala, assuming a bacterial D/L-Ala ration of 5 % (excess ^{15}N L-Ala $_{bacteria}$ = excess ^{15}N D-Ala × 20). Subsequently, excess ^{15}N in total and bacterial L-Ala were used to calculate the bacterial contribution to total microbial ^{15}N uptake (bacterial contribution (%) = [excess ^{15}N L-Ala $_{bacteria}$] / [excess ^{15}N L-Ala $_{total}$] × 100). This yielded bacterial contributions of 38% and 90% to total microbial uptake of the $^{15}NH_4^+$ and ^{15}N-FAAs respectively. These estimates are very similar to the estimates derived from the first approach (comparing total bacterial ^{15}N uptake with the ^{15}N enrichment of the bulk sediment). However, we prefer the second approach for ^{15}N uptake work in general as it is more direct and based on a relatively small and robust conversion factor (see Table 2 and text below).

Bacterial D/L-Ala ratios

The calculation of the relative contribution of bacteria to the total microbial ^{15}N uptake (Fig. 5) relies on the conversion factor of 20×, which was based on an average bacterial D/L-Ala ratio of 5%. This value was derived from our bacterial cultures and additional data from literature (Table 2). All of our mixed cultures and the pure G– strains showed a D/L-Ala ratio of ~ 5%, except for *Psychrobacter sp.* However, the fact that the D/L-Ala ratio of *Psychrobacter sp.* is very similar to those reported for four G+ strains by Sonesson et al. (1988) indicates that *Psychrobacter sp.* may have been a G+ bacterium that was identified falsely. The high D/L-Ala ratios for the G+ bacteria from Sonesson et al. (1988) are likely due to the fact that the peptidoglycan layer in G+ bacteria is much thicker than in G– bacteria (Madigan et al. 2000). Although the D/L-Ala ratios for the G+ bacteria are considerably higher than 5%, the bacterial communities in aquatic systems are generally thought to be dominated by G– bacteria. Therefore, it seems most likely that the average D/L-Ala ratio of the bacterial community in the sediment was ~ 5%, which was supported by the measured ratios in our mixed cultures. However, some uncertainty remains as these culture-based values only concern the culturable fraction of the total bacterial community in situ. Because of these potential problems related to the use of culture-based D/L-Ala ratios, we tested an alternative approach that allowed us to directly determine the D/L-Ala ratio of the active bacterial community in the sediment: Since the method presented in this paper also allows analysis of ^{13}C in D- and L-Ala (Pelz et al. 1998), it is possible to incubate the sediment with ^{13}C-glucose and to subsequently measure the incorporation of ^{13}C into D- and L-Ala. Glucose is used as a general C source by bacteria and by bacteria only (i.e. uptake by algae and other organisms is negligible). Therefore, the excess ^{13}C in D- versus L-Ala provides a direct measure for the D/L-Ala ratio of the active (^{13}C-incorporating) bacterial community in the sediment. This approach was applied to sediment from an intertidal mudflat in the Scheldt estuary in the months prior to our ^{15}N labeling experiment and yielded an average D/L-Ala ratio of 5.1 ± 0.5 %. These results further support the use of a D/L-Ala ratio of 5%. It seems that the ^{13}C-glucose approach provides a relatively simple and elegant tool to determine the D/L-Ala ratio for the active bacterial community in the same sediment, at the same time and under the same conditions as for the incubations with the ^{15}N labeled substrates.

Table 2. D-Ala contents and D/L-Ala ratios of bacterial cultures

	Gram (G+ or G–) [(a)]	D-Ala content (mg g^{-1})	D/L-Ala (%)
Halomonas pacifica (ATCC 27122)	G–	1.3	5.5
Marinobacter hydrocarbonoclasticus (ATCC 27132)	G–	0.9	5.2
Psychrobacter sp. (SW5H)	G–(?)	5.9	18
Pure culture I (isolated from Scheldt water)			5
Pure culture II (isolated from Scheldt water)			4.2
Mixed culture I (inoculated with sediment from Scheldt estuary)		1	4.2
Mixed culture II (inoculated with sediment from Scheldt estuary)		0.3	4
Mixed culture III (inoculated with water from Scheldt estuary)			4
Mixed culture IV (inoculated with water from Scheldt estuary)			5
Mixed culture V (inoculated with water from Scheldt estuary)			5.6
From Sonesson et al. (1988)			
Average for 4 Gram negative strains (± stdev)	G–	0.7 (± 0.1)	5.3 (± 1.8)
Average for 4 Gram positive strains (± stdev)	G+	1.9 (± 0.3)	19 (± 5)
From Pedersen et al. (2001)			
Bacterium AT	G–		6.9
Bacterium B	G–		5.1
Bacterium BJ	G+		5.9
Peptidoglycan (*S. aureus*)	G+		24.6

(a) G+ = Gram positive, G– = Gram negative,
(?) indicates doubt about identification as G– (based on D/L-Ala ratio)

Bacterial and algal ^{15}N uptake in the test experiment

So far, we calculated the total bacterial ^{15}N uptake (calculated from the excess ^{15}N in D-Ala) and the relative contribution of bacteria to total microbial uptake (comparing the excess ^{15}N in D- versus L-Ala). When these values are known, it is also possible to calculate the absolute ^{15}N uptake by the algae (assuming bacteria and algae were the only two groups involved in the uptake of the ^{15}N labeled substrates) (see Table 3 for results). Most likely, diatoms were the main contributor to this algal uptake as they were abundant in the sediment and have been shown to be able to take up both NH_4^+ and FAAs (Admiraal et al. 1987, Antia et al. 1991). The sum of the total uptake by the algae and bacteria was only slightly lower than the excess ^{15}N in the bulk sediment. The difference between these two values was most likely due to the presence of ^{15}N labeled substrates adsorbed to the sediment.

Table 3. Overview of excess ^{15}N (nmol ^{15}N g^{-1}) in different N pools for the incubations with $^{15}NH_4^+$ and the ^{15}N-FAAs.

	$^{15}NH_4^+$	^{15}N-FAAs
D-Ala (measured)	0.03	0.09
Bacteria	12 [(a)] (38%) [(b)]	36 [(a)] (90%) [(b)]
Algae	18 [(c)] (62%) [(b)]	4 [(c)] (10%) [(b)]
Total microbial community (bacteria + algae)	30 [(d)]	40 [(d)]
bulk sediment (measured by EA-IRMS)	34	45

(a): Calculated from excess ^{15}N in D-Ala (× 400)
(b): Relative contributions determined with D-Ala versus L-Ala approach
(c): Calculated by combining (a) and (b)
(d): Sum of (a) and (c)

Alternative use for non-biomarker amino acids

Although analysis of D-Ala was our primary focus, the method also allowed analysis of ^{15}N incorporation into other (L-) HAAs. Since the HAA pool composition of different groups of organisms is very similar (Cowie and Hedges 1992), these HAAs have limited biomarker potential and therefore cannot be used to measure group-specific ^{15}N uptake. However, the excess ^{15}N incorporated into the various HAAs directly reflects their relative contributions to the total HAA pool of the active (^{15}N incorporating) microbial community. This feature of the method can provide useful additional information for studies on organic matter degradation. In these studies, the composition of the HAA pool in the sediment is used as an indicator for the state of organic matter degradation. This is based on the principle that when fresh organic matter is degraded, some HAAs are preferentially degraded ("labile" HAAs) while others are less degradable and/or accumulate as degradation products ("refractory" HAAs). As a results, the labile HAAs decrease in relative abundance when degradation progresses while the refractory HAAs become more abundant. In general, the composition of the total HAA pool is measured at different sites, depths or time points representing different stages of organic matter degradation while cultures of various organisms are used as reference material for fresh organic matter (e.g., Cowie et al. 1992, Dauwe and Middelburg 1998, Pantoja and Lee 2003). The method presented in this paper offers the possibility to compare the composition of the "traditionally" measured total sediment HAA pool (fresh + degraded organic matter) with that of the active microbial community (i.e. fresh organic matter) in a single sediment sample. This is illustrated by the results from our test experiment (Fig. 6) where the relative composition of the total HAA pool in the sediment (calculated from HAA concentrations) is compared with the relative composition of the active (^{15}N incorporating) microbial community (calculated from the excess ^{15}N in the HAAs).

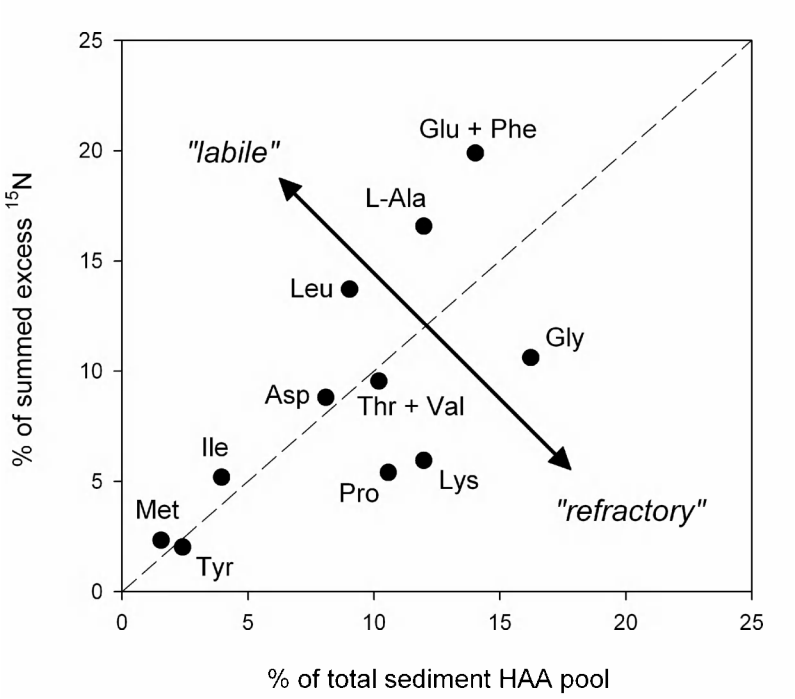


Figure 6. Relative abundance of individual amino acids in the total sediment HAA pool versus the ^{15}N-labeled HAA pool (excess ^{15}N) after labeling with $^{15}NH_4^+$.

When the relative abundance of an amino acid is the same in both pools, it will be on to the dashed 1:1 line in Figure 6. Gly, Lys and Pro are clearly below this line (i.e. relatively abundant in the total sediment HAA pool) while Glu+Phe, L-Ala, Leu and Ile are clearly above this line (i.e. relatively abundant in the microbial biomass = fresh organic matter). This indicates that the HAAs above the line are relatively labile amino acids (easily degraded) while the ones below the line are relatively refractory (accumulating in the total sediment HAA pool). These results are similar to those reported by e.g. Dauwe and Middelburg (1998) and Pantoja and Lee (2003). Although, these results are only a small example, it seems that analysis of stable isotope incorporation into the non-biomarker amino acids may provide a useful new tool for organic matter degradation studies. Applications to further exploit the potential of this tool may include the use of different ^{13}C- and /or ^{15}N-labeled substrates (labeling different fractions of the total microbial community) and / or tracing changes in the labeled HAA pool composition over time.

DISCUSSION

The presented method proved to be suitable for analysis of ^{15}N incorporation into D-Ala and other HAAs in the sediment. The excess ^{15}N in D-Ala could be used to determine the relative contributions of bacteria versus algae to total microbial ^{15}N uptake as well as to calculate total bacterial ^{15}N uptake (and indirectly algal ^{15}N uptake). Although sediment samples were used for the test experiment, there seem to be no fundamental problems prohibiting the use of the method for analysis of suspended matter in water samples.

These promising first results open the door for various applications in ^{15}N-labeling studies where the method can provide a powerful new tool for quantification of bacterial ^{15}N uptake, which will help to clarify the relative importance of bacteria versus algae in the uptake of various nitrogenous substrates. Furthermore, the method can also be used to measure bacterial uptake of ^{13}C labeled substrates. This creates the unique possibility to directly compare bacterial uptake of ^{15}N versus ^{13}C from dual-labeled organic substrates to clarify whether bacteria use these substrates as C and/or N sources. All together, these applications will ultimately help to increase our understanding of the role of bacteria in the cycling of N and C in aquatic food webs.

In addition, the method may provide a useful addition to existing methods for quantification of bacterial production based on incorporation of isotopically labeled amino acids (e.g., Kirchman et al. 1985, Smith and Azam 1992) since it eliminates the potential bias from additional amino acid incorporation by algae which is inherent to the existing methods.

Finally, the method can also be used as a new tool to study the dynamics of HAAs during organic matter degradation. Not only is it possible to determine the THAA pool composition of fresh organic matter (i.e. microorganisms incorporating ^{15}N and/or ^{13}C) produced in situ, but it can also be used to trace the fate of the labeled THAA pool (including D-Ala) over time. The fate of D-Ala during organic matter degradation is especially interesting since it provides an indication for the degradability of peptidoglycan, which is an outstanding topic in aquatic biogeochemistry (e.g., McCarthy et al. 1998, Jørgensen et al. 2003, Nagata et al. 2003).

UPDATE OF CHAPTER 2

(In revision for *Limnology and Oceanography: Methods* as update)

After publication of the original manuscript, the presented method has been applied in various ^{15}N- and ^{13}C-labeling studies to trace incorporation of ^{15}N- and/or ^{13}C by bacteria and incorporation into total (microbial) biomass. These applications included studies on various sediments (Chapters 3, 4 and 5, Cook et al. in prep., Gribsholt et al. in prep., Evrard et al. in prep.) and some water column SPM samples (Van den Meersche et al., unpublished). In addition, the method was used to analyze the D-Ala content of some algal- and cyanobacterial cultures (Table 4). These applications yielded valuable new information on the interpretation of ^{15}N and ^{13}C incorporation into D-Ala and other HAAs, which is presented in this update.

Calculation D/L ratio

In the original publication, the abundance of D-Ala was expressed relative to that of L-Ala as D/L-Ala ratio (%) that was calculated as D/L × 100. However, in most papers, the abundance of D-versus L-AAs is presented as D/L (not %) or as %D (D/(D+L) × 100) (e.g., McCarthy et al. 1998, Dittmar et al. 2001, Amon et al. 2001, Kaiser and Benner 2005). In order to prevent unnecessary confusion and for consistency, we now also use D/L (i.e. not %).

Correction for hydrolysis-induced racemization

In the original publication, it was assumed that formation of D-Ala from L-Ala by racemization during acid hydrolysis of the samples was negligibly low compared to the amount of D-Ala from bacteria. However, our analysis of D-Ala in biomass from axenic algal cultures yielded substantial D/L-Ala ratios of 0.015 to 0.018 (Table 4) and in all ^{15}N- and ^{13}C-labeling experiments so far we never measured excess ^{15}N or ^{13}C D/L-Ala ratios below 0.015, even in incubations of surface sediment slurries in which algal biomass was an order of magnitude higher than bacterial biomass (Chapter 3), which implies that D/L-Ala ratios of 0.015 to 0.018 resulted from racemization during hydrolysis. This was confirmed by a recent publication by Kaiser and Benner (2005) who found that liquid-phase hydrolysis of proteins and algal biomass in 6M HCl for 20 h at 110°C (same settings as used in present method) yielded D/L-Ala ratios between 0.017 and 0.019.

In addition, Amelung and Brodowski (2002) used deuterium-labeled HCl to determine hydrolysis-induced racemization in soil and litter samples which yielded D/L-Ala ratios due to racemization of 0.004-0.007 after hydrolysis for 12 h. Extrapolation to hydrolysis for 20 h assuming a constant racemization rate over time (Liardon et al. 1981, Erbe and Brückner 2000) yields values of 0.006-0.012. Taking into account possible other small methodological differences, these values are comparable with our values (Table 4) and those by Kaiser and Benner (2005). Altogether, D/L-Ala ratios resulting from racemization can be a substantial fraction of total bacterial D/L-Ala ratios (0.05 to > 0.1, see below), meaning that hydrolysis-induced racemization cannot be ignored in applications of the present method and hence needs to be corrected for.

Based on the values presented above, it seems that racemization during hydrolysis (in 6M HCl for 20 h at 110°C) of complex biological materials like microbial biomass and total organic matter in sediment results in D/L-Ala ratios of about 0.015 to 0.02 (i.e. 1.5 to 2% of L-Ala is converted into D-Ala). When results are presented as D/L-Ala ratios (concentrations or excess ^{15}N or ^{13}C), the most straightforward way is to present measured (i.e. uncorrected) ratios and to indicate the racemization background of 0.015-0.02 graphically (see for example figure 4 in Chapter 5). For calculation of the concentration or excess label in bacterial D-Ala, we used the following correction: X in bacterial D-Ala = [measured X in D-Ala] – 0.017 × [measured X in L-Ala], where X can be the concentration as well as excess ^{15}N or ^{13}C. 0.017 is used as the average of the 0.015-0.02 range for hydrolysis-induced racemization (see above).
The relative abundance of ^{15}N or ^{13}C in D-Ala can also be presented as $\delta^{15}N$ or $\delta^{13}C$ values, although the direct use of $\delta^{15}N$ or $\delta^{13}C$ did not prove very useful in applications of the method so far. It should however be possible to correct measured δ-values for D-Ala since the measured δ-value for D-Ala is the weighted average for D-Ala formed by racemization (concentration = 1.5 to 2% of L-Ala concentration and δ = δ of L-Ala) and the original bacterial D-Ala (concentration = measured total concentration – D-Ala from racemization).

Table 4. Measured D/L-Ala ratios for biomass from axenic cultures of some algae and cyanobacteria. Samples were processed and analyzed following the protocol presented in the original manuscript. Concentrations were derived from GC-c-IRMS analyses.

		D/L-Ala
Amphora	diatom	0.016
Odontella	diatom	0.016
Thalassiosira	diatom	0.018
Chlorella	green alga	0.015
Leptolyngbya	cyanobacterium	0.07
Synechococcus	cyanobacterium	0.11

Bacterial D/L-Ala ratios

In the original publication, available data pointed to a D/L-Ala ratio of ~ 0.05 (previously called 5%) for natural bacterial communities. However, some of the recent ^{15}N- and ^{13}C-labeling studies yielded excess ^{15}N and ^{13}C D/L-Ala ratios well over 0.05 with values up to 0.1-0.12 (Chapters 3, 4 and 5), indicating that D/L-Ala ratios of natural bacterial communities can actually be substantially higher than 0.05. Since D-Ala is primarily present in the cell wall component peptidoglycan, the D-Ala content and D/L-Ala ratio are directly linked to the thickness of the peptidoglycan layer which differs considerably between Gram negative (G–) versus Gram positive (G+) bacteria with a much thicker layer in G+ bacteria (Koch 1990, Madigan et al. 2000). Hence, G+ bacteria also have a higher D-Ala content and D/L-Ala ratio (see Table 2).

The bacterial community in aquatic environments is generally considered to be dominated by G– bacteria. However, the proportion G+ bacteria can actually be considerable, especially in deeper sediment (up to 30% of total bacterial pool), as indicated by microscopical observations by Moriarty and Hayward (1982) and by changes in the composition of the bacterial PLFA pool with increasing sediment depth (unpublished data D. van Oevelen) similar to changes in the bacterial PLFA pool composition with increasing depth in soils (Fierer et al. 2003). Given the substantially higher D/L-Ala ratio of G+ bacteria (values in Table 2 suggest 3-4 × higher), it seems feasible that a contribution of 10 to 30% G+ bacteria to the total active bacterial community can increase the D/L-Ala ratio of the total active bacterial community to well over 0.05.

Another potential cause for D/L-Ala ratios > 0.05 is the presence of cyanobacteria. Although cyanobacteria are classified as G–, their peptidoglycan layer is much thicker than that of other G– bacteria and has a higher degree of cross linking between the sugars strands (i.e. relatively rich in cross bridges containing D-Ala) (Hoiczyk and Hansel 2000). This indicates that the D-Ala content and D/L-Ala ratio of cyanobacteria is more similar to that of G+ bacteria than to that of other G– bacteria, which is confirmed by our measured D/L-Ala ratios for two cyanobacterial cultures (Table 4).

Additionally, some variation in bacterial D-Ala content and D/L-Ala ratios can also be expected within specific bacterial groups due to variation in the thickness of the cell wall and/or the ratio between cell walls and other biomass components which may for example be dependent on environmental conditions (nutritional status, temperature etc.).

Interpretation of results

The main use of the method is to quantify incorporation of ^{15}N (and ^{13}C) by bacteria and the bacterial contribution to total (microbial) label incorporation.

Total label incorporation by bacteria was originally calculated from excess label in D-Ala using a conversion factor of × 400 that was derived from measured concentrations of D-Ala per gram dry biomass in bacterial cultures (Table 2) and was consistent with D-Ala being ~ 5% of total Ala (this manuscript), Ala typically being ~ 10% of THAAs (Cowie and Hedges 1992) and THAAs contributing ~ 50% to total bacterial biomass (Simon and Azam 1989, Cowie and Hedges 1992) (20 × 10 × 2 = 400). This conversion still seems to be valid for studies where the bacterial D/L-Ala ratio is around 0.05 while in case of a higher bacterial D/L-Ala ratio (i.e. relatively high contributions by G+ bacteria and/or cyanobacteria) a smaller conversion factor should be used (e.g., × 200 for D/L-Ala ratio of 0.1). However, for precise estimates, both the bacterial D/L-Ala ratio and the measured excess label in D-Ala need to be corrected for hydrolysis-induced racemization (as presented above), which introduces some uncertainty. Moreover, additional uncertainty can be expected for example from variation in bacterial D-Ala content (see above) and from heterogeneous distribution of label over different biomass component. This uncertainty is illustrated by the variation in estimates of total ^{13}C incorporated in bacteria based on ^{13}C incorporation into D-Ala, THAAs and bacteria-specific PLFAs in Chapter 5. Therefore, it seems that conversion of excess label in D-Ala to

total excess label in bacteria should only be used to derive a rough estimate of total bacterial label incorporation.

Fortunately, the method also allows another approach to determine the bacterial contribution to total label incorporation, which involves comparison of label incorporation into D-Ala (representing bacteria) versus L-Ala (representing total (microbial) biomass). Unlike the example in the original manuscript (Fig. 5) where excess ^{15}N in D-Ala was first converted to excess ^{15}N in bacterial L-Ala, we now directly use measured excess ^{15}N or ^{13}C D/L-Ala ratios. When the bacterial contribution total label incorporation is negligible, the resulting excess label D/L-Ala ratio will be around racemization background (see for example Chapter 3). Conversely, when bacteria are the only organisms to incorporate label, the excess ^{15}N D/L-Ala ratio will be the same as the D/L-Ala ratio of the bacteria (0.05 to > 0.1, depending on the type of bacteria)(see for example Chapter 5). In case of substantial contributions by bacteria and other organisms (mostly algae and occasionally fauna), the excess label D/L-Ala ratio will be between 0.015-0.02 and 0.05-0.1. Translation of the measured excess label D/L-Ala ratio to the bacterial contribution to total label incorporation requires information on the D/L-Ala ratio of the bacteria involved. If this information cannot be derived from available data, the best option is to indicate a minimum and maximum estimated bacterial contribution (corresponding to a bacterial D/L-Ala ratio of 0.1 and 0.05 respectively).

In addition to label incorporation into D- and L-Ala, the method also allows analysis of label incorporation into other HAAs. In the original manuscript, we presented this feature as a new tool in studies on degradation of organic matter using the composition of the THAA pool as an indicator for the degree of organic matter degradation. Application of this tool indeed yielded some interesting results concerning the in situ degradation of ^{13}C-labeled bacterial remnants (Chapter 5). However, label incorporation into THAAs also proved to be a useful proxy for total (microbial) label incorporation since this pool makes up a large and relatively stable fraction of total (microbial) biomass (50-60% of bacterial biomass (Simon and Azam 1989, Cowie and Hedges 1992) and 60-80% of algal biomass (Cowie and Hedges 1992)) and hence requires only a small conversion to total label incorporation ($\times$ 1.2 to $\times$ 2). The ratio between excess label in THAAs versus that in the bulk sediment therefore provides a direct indication of the fraction of total

label in the sediment that was incorporated in (microbial) biomass (see Chapters 3 and 4 and Gribsholt et al. in prep.).

The best estimate of total bacterial label incorporation is probably obtained by combining the bacterial contribution derived from the excess label D/L-Ala ratio with the estimated total microbial incorporation from excess label in THAAs.

Example: updated interpretation of results from test experiment

The original interpretation of the results from the simple ^{15}N-labeling experiment presented in the original manuscript (surface sediment incubated with $^{15}NH_4^+$ and a ^{15}N-amino acid mixture for 6 h) that was performed to test the potential of the method yielded estimates of bacterial contributions to total microbial ^{15}N incorporation of 38% and 90% for incubations with $^{15}NH_4^+$ and the ^{15}N-amino acid mixture respectively (Fig. 5 and Table 3). The new interpretation of these data, taking into account the different points presented in this update, is as follows. The excess ^{15}N D/L-Ala ratio for the $^{15}NH_4^+$ incubations was 0.019, which is around racemization background (0.015-0.2), indicating that all ^{15}N-labeled D-Ala resulted from hydrolysis-induced racemization. This points to a negligible contribution by bacteria, indicating that ^{15}N incorporation was dominated by algae. Conversely, the excess ^{15}N D/L-Ala ratio for the incubations with the ^{15}N-amino acid mixture was 0.045, which is well above racemization background and indicates a substantial bacterial contribution to total microbial ^{15}N incorporation (between 34% and 85%, corresponding to bacterial D/L-Ala ratio of 0.1 and 0.05 respectively).

Acknowledgements

We thank Pieter van Rijswijk for helping with the laboratory work and bacterial cultures, Joop nieuwenhuize and Yvonne van der Maas for their help with the analytical work, Veronique Créach and Jody the Brouwer for providing some of the pure bacterial strains, and two anonymous reviewers for their useful comments.This research was supported by the EU (EUROTROPH, EVK3-CT-2000-00040) and a PIONEER grant of the Netherlands organization for scientific research (NWO, 833.02.002). This is publication 3556 of the Netherlands Institute of Ecology (NIOO-KNAW).

C H A P T E R 3

Incorporation of nitrogen and carbon from urea and amino acids by bacteria and algae in intertidal sediment

Bart Veuger and Jack J. Middelburg

In revision for *Aquatic Microbial Ecology*

ABSTRACT

We investigated the uptake and incorporation of nitrogen from urea and amino acids by the microbial community in surface sediment from an intertidal mudflat focusing on the relative contributions by bacteria versus algae to total microbial nitrogen incorporation and the (un)coupling between incorporation of nitrogen and carbon.

Dual-labeled (^{15}N and ^{13}C) urea and an amino acid mixture were added to surface sediment from two intertidal mudflats in the Scheldt Estuary (The Netherlands) and ^{15}N and ^{13}C were subsequently traced into the bulk sediment, total hydrolysable amino acids (THAAs) and the bacterial biomarker D-alanine (D-Ala) during a 24 h incubation period.

All added ^{15}N from urea and the amino acids was incorporated into microbial biomass within 24 h, with relatively rapid incorporation of ^{15}N from the amino acids. The bacterial contribution to total microbial ^{15}N incorporation (derived from ^{15}N incorporation into D-Ala) was large during the first 1 to 2 h of incubation but small to negligible after 24 h for both substrates, indicating that total ^{15}N incorporation was dominated by benthic microalgae that also dominated total microbial biomass in the sediment.

Comparison of ^{15}N versus ^{13}C incorporation into total microbial biomass (THAAs) for urea showed coupled incorporation of urea-derived nitrogen and carbon during the first 4 h (in daylight) but strong preferential incorporation of nitrogen after 24 h (overnight). Incorporation of nitrogen and carbon from the amino acids was partially uncoupled (^{13}C :^{15}N ratio of 1.5-2 versus ~ 4 for the added amino acid mixture), indicating that about half of the amino acids were directly taken up and incorporated into biomass while the other half was oxidized extracellularly to $^{15}NH_4^+$ that was subsequently taken up and used as nitrogen source for growth.

INTRODUCTION

Microbial uptake of dissolved nitrogen is an important process in aquatic systems since nitrogen is an essential element for microbial growth and can be a limiting factor for aquatic microbial production. Although traditional research typically focused on uptake of dissolved inorganic nitrogen (DIN), it is now clear that dissolved organic nitrogen (DON) can also be an important nitrogen source for the aquatic microbial community. Two important forms of DON are urea and amino acids, of which the latter can be present as individual amino acids or in a combined form (proteins, peptides). Although urea and amino acids generally make up no more than ~ 20% of the total aquatic DON pool, these labile compounds are relatively important nitrogen sources for the microbial community and can account for a large fraction of total microbial nitrogen uptake in coastal waters (Bronk 2002 and Chapter 1). At present, microbial nitrogen uptake has been studied extensively, especially by ^{15}N-labeling. However, some of its aspects are still unclear.

One aspect that has remained largely unresolved is the relative importance of algae versus bacteria, the two main groups involved in uptake of dissolved nitrogen. Method used to discriminate ^{15}N uptake by specific microbial groups include size fractionation (physical separation of relatively small bacteria from relatively large algae by filtration or flow cytometry) and the use of inhibitors to eliminate activity of a specific group (e.g. antibiotics) (e.g., Wheeler and Kirchman 1986, Lipschultz 1995, Middelburg and Nieuwenhuize 2000, Chapter 1). However, application of these methods in turbid waters and sediments is highly problematic, meaning that specific assessment of ^{15}N uptake by bacteria versus algae in these systems has so far been hampered by a lack of adequate methodology (see Chapter 2). Only recently, ^{15}N-labeling has been combined with analysis of ^{15}N incorporation into bacterial amino acids, which allows specific assessment of ^{15}N incorporation into bacterial biomass (Tobias et al. 2003 and Chapter 2).

Another understudied aspect of aquatic microbial nitrogen uptake is the (un)coupling between uptake and incorporation of nitrogen and carbon from organic compounds. Studies on algal cultures have shown that incorporation of urea-N results in uncoupling from urea-C (Price and Harrison 1988, Antia et al. 1991) while both coupled and uncoupled uptake of nitrogen and carbon is possible for amino acids (Algeus 1948, Stephens and North 1971, Palenik and Morel 1990,

Antia 1991). For natural aquatic microbial communities, uptake of N and C from organic compounds has been studied by analysis of uptake of labeled N and C from dual-labeled urea (Mulholland et al. 2004, Fan and Glibert 2005, Andersson et al. 2006) and amino acids (Schell 1974, Zehr et al. 1985, Mulholland et al. 2002, 2003, 2004, Andersson et al. 2006). In general, these studies found that amino acids can supply both N and C for microbial growth while urea serves as a source of N only. However, exceptions have been reported and the factors controlling uptake of N and C are still largely unclear. One important controlling factor may be the composition of the microbial community responsible for uptake and incorporation of the of the N and/or C. The recent introduction of a method for analysis of ^{15}N and ^{13}C incorporation by bacteria (Chapter 2) may help to resolve this point.

A third aspect of aquatic microbial nitrogen uptake is that work on this subject so far primarily involved studies on nitrogen uptake by the microbial community in the water column while microbial nitrogen uptake in sediments has remained largely unstudied. This is mainly due to methodological problems. In addition to problems with analysis of ^{15}N uptake by specific microbial groups (as mentioned above), analysis of total ^{15}N uptake by the benthic microbial community was hampered by the inability to differentiate between
^{15}N taken up by bacteria and algae versus other ^{15}N pools in the sediment such as the added ^{15}N-substrate bound to the sediment. However, by combining ^{15}N-labeling with analysis of ^{15}N incorporation into total hydrolysable amino acids (THAAs, representing total proteinaceous biomass) it is now possible to specifically trace ^{15}N incorporation into microbial biomass within sediments (Chapter 2).

In the present study, we investigated the uptake and incorporation of ^{15}N and ^{13}C from dual-labeled urea and amino acids by algae and bacteria in surface sediment from an intertidal mudflat. Analysis of ^{15}N and ^{13}C incorporation into THAAs, including the bacterial biomarker D-alanine (D-Ala) allowed us to clarify the relative contributions of bacteria versus algae to total microbial ^{15}N incorporation and the coupling between incorporation of N and C from urea and amino acids.

MATERIALS AND METHODS

Sediment collection

Sediment was collected from two intertidal mudflats in the midregion (salinity 20-25) of the turbid, nutrient rich and heterotrophic Scheldt Estuary (The Netherlands) (see Soetaert et al. 2006) during low tide at June 1st 2004. The first site was characterized by sediment patches covered by a diatom biofilm, sediment was sampled from these patches. The second site (Biezelingsche Ham,) was a mudflat ~ 5 km from the first site. At this site, no visible diatom biofilm was present, which was likely related to the high densities of diatom-grazing fauna at this site. At both sites, ~ 500 ml of surface sediment (upper ~ 10 mm) was collected as well as water from nearby intertidal pools. Sediment collection at the two sites and subsequent transportation of the sediment to the laboratory was done within 2 h. Hereafter, we refer to the sediment from the first and second site as "BMA+" and "BMA–" respectively, which reflects the relative difference in abundance of benthic microalgae (BMA) at the two sites.

Labeling and incubations

In the laboratory, sub samples (20 ml) of homogenized sediment were diluted with 20 ml water from the nearby intertidal pools in slurry bottles (100 ml, ø 35 mm). Incubations were started by addition of 1 ml of 0.8 mM ^{13}C-urea (Cambridge Isotope Laboratories, CLM-311, 99% ^{13}C) and 1 ml 0.8 mM ^{15}N-urea (Cambridge Isotope Laboratories, NLM-233, ≥ 98% ^{15}N) or 1 ml of 0.8 mM of a ^{13}C-labeled amino acid mixture (Cambridge Isotope Laboratories, CLM-1548, ≥ 98% ^{13}C) and 1 ml 0.8 mM of the same amino acid mixture, but ^{15}N-labeled (Cambridge Isotope Laboratories, NLM-2161, 96-99% ^{15}N). This amino acid mixture (hereafter referred to as "AA-mix") is an algal-derived mixture containing the following amino acids (with approximate mole % as indicated by the manufacturer): Ala (7%), Arg (7%), Asp (10%), Glu (10%), Gly (6%), His (2%), Ile (4%), Leu (10%), Lys (14%), Met (1%), Phe (4%), Pro (7%), Ser (4%), Thr (5%), Tyr (4%) and Val (5%). Added concentrations of labeled urea and dissolved free amino acids (DFAAs) were 5 to 8 times higher than ambient concentrations. After addition of the labeled substrates, samples were homogenized by gentle shaking and placed outdoors (around noon) under ambient light and temperature (~ 25°C). After shaking, the sediment quickly settled to form a homogenized layer (~ 2 cm thick). Bottles were left open during incubation. Incubations were terminated after 0.5, 1, 2, 4, 7 and 24 h of which the 24 h incubations included 7.5 h of darkness (between 10 h and 17.5 h). An

additional set of AA-mix incubations was terminated directly after addition of the labeled AA-mix, which resulted in an effective incubation period of 10-15 min. Incubations were terminated by transferring the slurries to 50 ml centrifuge tubes that were dipped in liquid nitrogen to stop microbial activity. Subsequently, thawed samples were centrifuged (10 min at 600 g) and supernatants were filtered (GF-6 and 0.2 µm cellulose), transferred to headspace vials (10 ml), capped, killed with 100 µl of a saturated HgCl solution and stored at 4°C for ^{13}C-DIC analysis (see below). Sediment pellets were frozen at -20°C and freeze dried for analysis of ^{15}N and ^{13}C in bulk sediment and HAAs (including D-Ala) (see below for details).

Analyses

^{15}N and ^{13}C in the bulk sediment was measured using a Fisons CN elemental analyzer coupled on-line to a Finnigan Delta S isotope ratio mass spectrometer (EA-IRMS). Samples for ^{13}C analysis were acidified to remove carbonates.

^{15}N and ^{13}C incorporation into hydrolysable amino acids (HAAs), including the bacterial biomarker D-alanine (D-Ala), was analyzed as described in Chapter 2. Briefly, samples (1 g) of freeze-dried sediment were washed with HCl (2 M) and Milli-Q water and subsequently hydrolyzed in HCl (6 M) at 110°C for 20 h. After purification by cation exchange chromatography, amino acids were derivatized with isopropanol (IP) and pentafluoropropionic anhydride (PFA) and samples were further purified by solvent extraction. The relative abundance of ^{15}N and ^{13}C (δ^{15}N and δ^{13}C) in the derivatized D- and L-amino acids were analyzed by gas-chromatography-combustion-isotope ratio mass spectrometry (GC-c-IRMS) during separate sessions for ^{15}N- and ^{13}C-analyses. Additional information on ^{13}C analysis can be found in Chapter 5. Because of time constraints, ^{13}C analyses were only performed for samples from 4 h and 24 h incubations. Unfortunately, only few samples from the BMA+ slurries incubated with urea could be analyzed for ^{15}N and ^{13}C enrichment of HAAs because part of these samples were lost during sample processing.

^{13}C analysis of pore water DIC was performed ~ 2 weeks after the experiment. 2 ml of the water in the headspace vials was replaced by a helium headspace. Samples were acidified (to convert all DIC to CO_2), shaken, and the headspace gas was analyzed by GC-IRMS.

A selection of samples was analyzed for concentrations of phospholipid-derived fatty acids (PLFAs) by GC to obtain an estimate of bacterial biomass in the sediment slurries (see Middelburg et al. 2000 and chapter 5). For analysis of ambient concentrations of urea and DFAAs, pore water was extracted from

unlabeled slurries by centrifugation (10 min at 600 g) and the resulting supernatant was filtered over GF/F and stored frozen (-20°C). Urea concentrations were measured by a standardized colorimetric technique (Middelburg and Nieuwenhuize 2000b) and DFAA concentrations by HPLC (Fitznar et al. 1999). For analysis of photosynthetic pigment concentrations, additional sediment samples were frozen, freeze dried, stored frozen (-20°C) and later analyzed by HPLC (Barranguet et al. 1997).

Data treatment

^{15}N and ^{13}C enrichments are expressed as excess ^{15}N and ^{13}C per gram dry sediment, which was calculated as: excess $X = [(at\%X_{sample} - at\%X_{control}) /100] \times$ [concentration N or C in sample]. At%X was calculated from δX as: $at\%X = [100 \times R_{standard} \times (\delta X_{sample} / 1000) +1] / [1 + R_{standard} \times (\delta X_{sample} / 1000) +1]$ and δX was calculated as: $\delta X (‰) = [(R_{sample} / R_{standard}) -1] \times 1000$. Here, X represents ^{15}N or ^{13}C. For ^{15}N, R= $^{15}N/^{14}N$ and atmospheric N_2 was used as standard. For ^{13}C, R = $^{13}C/^{12}C$ and Vienna Pee Dee Belemnite was used as standard. $\delta^{13}C$ values for the HAAs were corrected for addition of ^{12}C during derivatization as described in Chapter 5. Unlabeled sediment was used as control sample to determine the natural abundance of ^{15}N and ^{13}C.

D-Ala data were corrected for the formation of D-Ala from L-Ala by hydrolysis-induced racemization as described and discussed in the update of Chapter 2. Briefly, excess label (^{15}N or ^{13}C) in D-Ala was corrected for racemization by subtracting 1.7% of excess label in L-Ala from excess label in D-Ala. The same correction was used for D-Ala concentrations. Excess ^{15}N in D-Ala is also expressed relative to that in L-Ala as the excess ^{15}N D/L-Ala ratio that was calculated as: [excess ^{15}N in D-Ala] / [excess ^{15}N in L-Ala] using the measured excess ^{15}N in D-Ala (i.e. not corrected for racemization). Instead, the racemization background (0.015-0.02) is indicated graphically.

RESULTS

Sediment properties

The organic carbon (OC) content of the sediment was 2.0 ± 0.1 wt % (1.7 ± 0.1 mmol-C g^{-1}) for BMA+ sediment and 1.6 ± 0.1 wt % (1.3 ± 0.1 mmol-C g^{-1}) for BMA– sediment. The nitrogen content of the sediment was 0.26 ± 0.02 wt % (0.16 ± 0.05 mmol-C g^{-1}) for BMA+ sediment and 0.19 ± 0.02 wt % (0.13 ± 0.03 mmol-C g^{-1}) for BMA– sediment. The corresponding C : N ratio (moles OC : moles N) was 10 ± 2 for the sediment from both sites. THAA concentrations (summed concentrations of individual HAAs) were 100 ± 14 µmol AA g^{-1} in BMA+ sediment and 64 ± 6 µmol AA g^{-1} in BMA– sediments. THAA-C made up 24% and 19% of total OC in the BMA+ and BMA– sediment respectively and THAA-N made up 66% and 51% of total N in the BMA+ and BMA– sediment respectively. D-Ala concentrations (corrected for hydrolysis-induced racemization) were very similar for BMA+ and BMA– (0.29 ± 0.06 µmol g^{-1}) while D/L-Ala ratios (not corrected for racemization) were 0.038 ± 0.001 for BMA+ sediment and 0.050 ± 0.005 for BMA– sediment.

Microbial community composition

Bacterial biomass was estimated from concentrations of the bacteria-specific PLFAs i14:0, i15:0 and a15:0. Although PLFAs i16:0 and 18:1ω7c were also used as indicators of bacterial biomass in chapter 5, they were not included in the present study because analyses on the BPX column did not properly resolve i16:0 while 18:1ω7c was not used to avoid likely bias by the presence of 18:1ω7c in the abundantly present algae (Moodley et al. 2000). Estimates of total bacterial biomass were based on the assumption that C in i14:0, i15:0 and a15:0 makes up 12% of total bacterial PLFA-C and that total bacterial PLFA-C is 6% of total bacterial biomass-C (Middelburg et al. 2000). Resulting estimates were 24 and 22 µmol-C g^{-1} for BMA+ and BMA– sediment respectively.

Algal biomass was estimated from concentrations of chlorophyll *a* (Chl *a*) assuming a C : Chl *a* ratio of 50. Average Chl *a* concentrations were 118 µg g^{-1} for BMA+ sediment and 73 µg g^{-1} for BMA– sediment. Resulting estimates of total algal biomass were 492 µmol-C g^{-1} for BMA+ and 306 µmol-C g^{-1} for BMA–. In addition, the sediment showed a high abundance of the diatom pigment fucoxanthin with a fucoxanthin : Chl *a* ratio of ~ 0.25 for sediment from both sites and the diatom-specific PLFA 20:5ω3 made up a large fraction (18 ± 2 %) of the total PLFA pool in the sediment.

^{15}N in bulk sediment and THAAs

Addition of the ^{15}N-labeled substrates to the sediments resulted in clear ^{15}N enrichment of the bulk sediment and HAAs, including D-Ala, with $\delta^{15}N$ values op to ~ 250‰ (natural abundance was ~ 20‰). Trends in excess ^{15}N in bulk sediment and THAAs over time were very similar for BMA+ and BMA– slurries but showed a clear difference between slurries incubated with urea and those incubated with the AA-mix (Fig. 1). In the urea incubations (Fig. 1A), bulk excess ^{15}N after 0.5 h was ~ 40 nmol ^{15}N g^{-1} while excess ^{15}N in THAAs was negligibly low. During the following hours, both bulk ^{15}N and ^{15}N in THAAs increased, although precise trends are not completely clear. Over the whole 24 h, results for the BMA– slurries are described well by linear fits (3.4 nmol ^{15}N g^{-1} h^{-1} with R^2 of 0.90 for bulk ^{15}N and 2.2 nmol ^{15}N g^{-1} h^{-1} with R^2 of 0.97 for ^{15}N-THAAs). However, when only considering results for the first 7 h, it seems that the increase in excess ^{15}N in the bulk sediment and THAAs was relatively rapid during the first 2-4 h and then leveled off between 2-4 h and 7 h. The bulk excess ^{15}N in the BMA– sediment after 24 h (no data available for BMA+ sediment after 24 h) was similar to the total added amount of urea-^{15}N and excess ^{15}N in THAAs was 45% of bulk excess ^{15}N.

In the AA-mix incubations (Fig. 1B), bulk excess ^{15}N was high (~ 60 nmol ^{15}N g^{-1}) almost directly (10-15 min) after addition of the labeled AA-mix and remained at this level during the entire 24 h incubation period. This level corresponded to the total amount of AA-^{15}N added to the sediment. Conversely, excess ^{15}N in THAAs was initially very low, then rapidly increased during the first 4 h and leveled off to values that represented 48 - 52 % of bulk excess ^{15}N after 7 h and 57 - 76% after 24 h. The individual HAAs all showed a similar level of ^{15}N enrichment. The composition of the ^{15}N-labeled THAA pool was comparable for incubations with urea and the AA-mix and roughly reflected the composition of the complete THAA pool in the sediment (data not shown).

^{15}N in D-Ala

^{15}N incorporation into D-Ala was generally low with corresponding excess ^{15}N D/L-Ala ratios close to or at racemization background for incubations > 2 h (Fig. 2A) and lowest values for BMA+. Only incubations < 2 h yielded substantially higher excess ^{15}N D/L-Ala ratios (up to 0.08). For samples with an excess ^{15}N D/L-Ala ratio at racemization background, excess ^{15}N in D-Ala (after correction for racemization) could not be quantified. Therefore, trends in ^{15}N incorporation into D-Ala could only be determined for BMA– sediment (Fig. 2B). ^{15}N incorporation in the BMA– sediment incubated with the AA-mix appears to have been very rapid (~ 0.24 nmol ^{15}N g^{-1} h^{-1}) during the first 10-15 min while incorporation was substantially lower during the following 24 h (~ 0.001 nmol ^{15}N g^{-1} h^{-1}, $R^2 = 0.96$). Conversely, BMA– sediment incubated with urea showed a more or less linear increase in excess ^{15}N in D-Ala over time (~ 0.025 nmol ^{15}N g^{-1} h^{-1}, $R^2 = 0.67$).

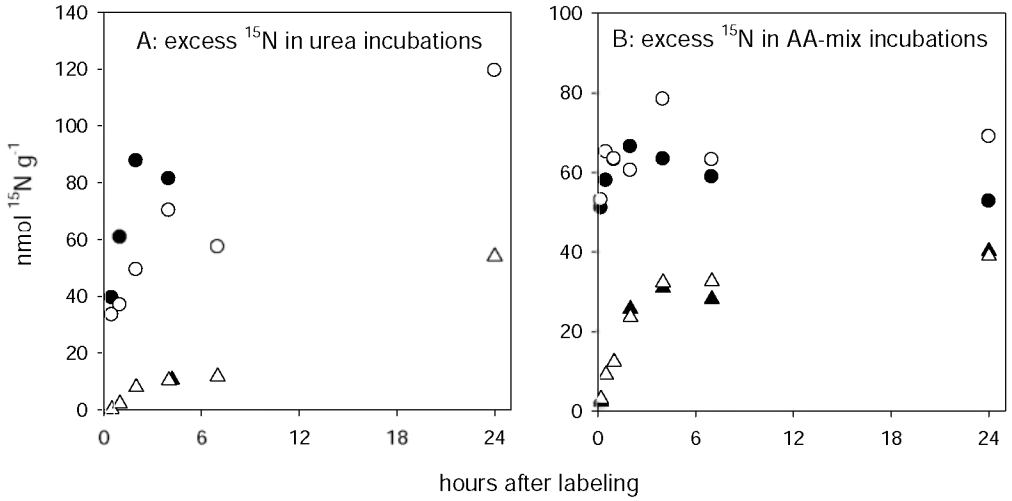


Figure 1: Excess ^{15}N in bulk sediment (circles) and THAAs (= summed excess ^{15}N in individual HAAs) (triangles) for BMA+ sediment (black symbols) and BMA– sediment (white symbols) incubated with urea (plot A) and the AA-mix (plot B).

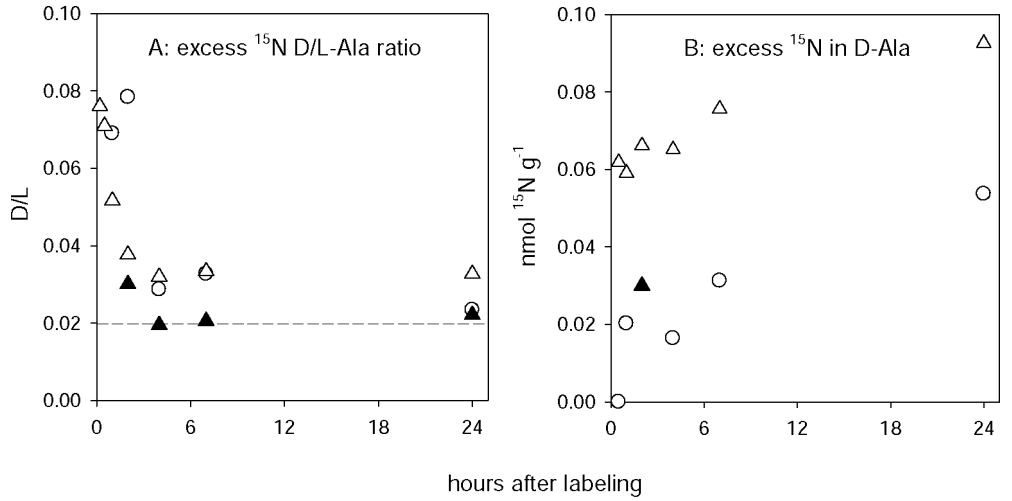


Figure 2. Excess ^{15}N D/L-Ala ratios (excess ^{15}N in D-Ala : excess ^{15}N in L-Ala) (plot A) and excess ^{15}N in D-Ala (plot B) for BMA+ sediment (black symbols) and BMA– sediment (white symbols) incubated with urea (circles) and the AA-mix (triangles). Excess ^{15}N in D-Ala (plot B) was corrected for hydrolysis-induced racemization. Excess ^{15}N D/L-Ala ratios (plot A) are not corrected for racemization, instead the racemization background (0.015-0.02) is indicated by the dashed line. No excess ^{15}N in D-Ala is presented for samples where excess ^{15}N D/L-Ala ratios were at racemization background. Results for BMA+ sediment incubated with urea are missing because these samples were lost during sample processing.

^{13}C in bulk sediment and HAAs

In the sediment incubated with urea, ^{13}C enrichment was low with no clear trend over time for bulk excess ^{13}C and a small excess ^{13}C in THAAs (~ 5 nmol ^{13}C g^{-1}) (Fig. 3A). Conversely, sediment incubated with the AA-mix did show substantial ^{13}C enrichment with trends over time similar to those for excess ^{15}N. Excess ^{13}C was about two times higher than excess ^{15}N in these incubations (Fig. 3B versus 1B). ^{13}C enrichment of D-Ala was below the limit of detection for urea incubations and very low for AA-mix incubations with excess ^{13}C D/L-Ala ratios close to racemization background. Therefore, excess ^{13}C in D-Ala could not be quantified properly.

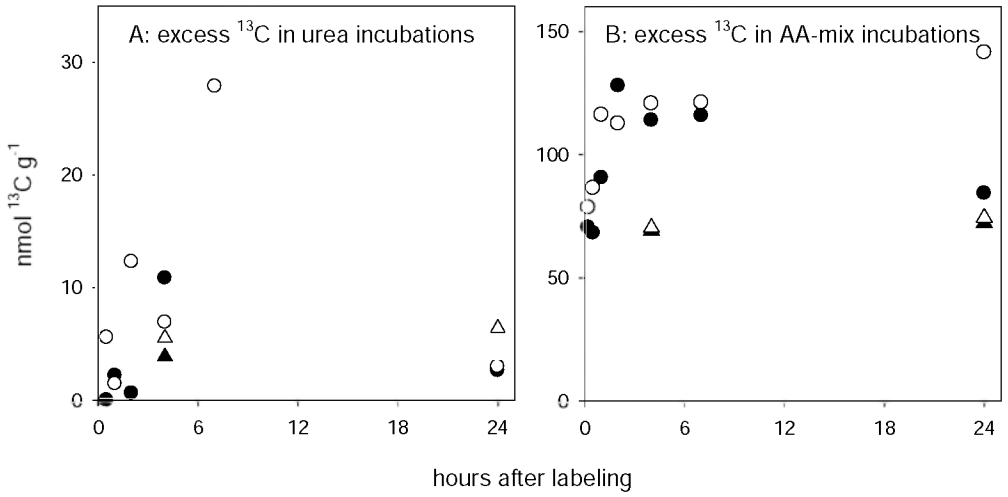


Figure 3: Excess ^{13}C in bulk sediment (circles) and THAAs (= summed excess ^{13}C in individual HAAs) (triangles) for BMA+ sediment (black symbols) and BMA– sediment (white symbols) incubated with urea (plot A) and the AA-mix (plot B).

^{15}N versus ^{13}C in bulk sediment and HAAs

For the incubations with the AA-mix, the excess ^{13}C : ^{15}N ratios for the bulk sediment were 1 to 1.5 during the first hour and 1.5 to 2 for the rest of the incubation period (Fig. 4A). The excess ^{13}C :^{15}N ratio for the THAAs (Fig. 4B) was similar to that for the bulk sediment (~ 2). For the urea incubations, excess ^{13}C : ^{15}N ratios for the bulk sediment ranged between 0.01 and 0.25 (with the exception of one point) and lowest values occurred after 24 h (Fig. 4A). The excess ^{13}C : ^{15}N ratio for THAAs (Fig. 4B) after 4 h was 0.4 to 0.5, which is higher than values for the bulk sediment and close to the ^{13}C : ^{15}N ratio of the added urea (0.5). After 24 h, the excess ^{13}C : ^{15}N ratio for the THAAs was substantially lower (0.1). No excess ^{13}C : ^{15}N ratio is presented for D-Ala since excess ^{13}C in D-Ala could not be quantified properly.

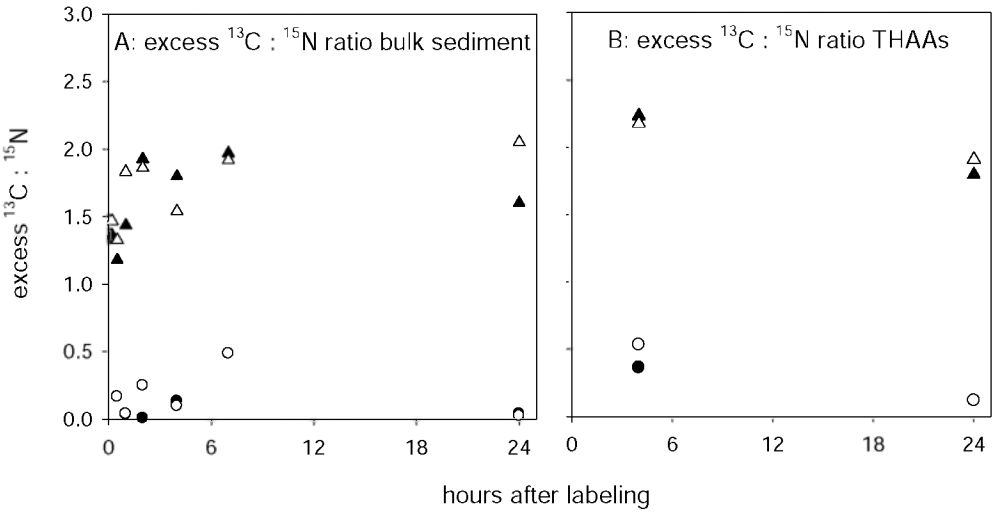


Figure 4. Excess ^{13}C : ^{15}N ratio of bulk sediment (plot A) and THAAs (plot B) for BMA+ sediment (black symbols) and BMA– sediment (white symbols) incubated with urea (circles) and the AA-mix (triangles). Excess ^{13}C:^{15}N ratio of added substrates was 0.5 for urea and ~ 4 for the AA-mix.

^{13}C-DIC production

For both substrates, part of the added ^{13}C was recovered as ^{13}C-DIC (Fig. 5). Unfortunately, it was not possible to quantify the total amount of ^{13}C that was recovered as ^{13}C-DIC because the experiment was performed in open bottles, which allowed loss of ^{13}C-DIC by diffusion. However, semi-quantitative interpretation of figure 5 shows that ^{13}C-DIC production was substantially higher in the incubations with urea than in those with the AA-mix. This difference becomes even larger when taking into account that the C-content of the AA-mix was about 4 times higher than that of urea. Highest excess ^{13}C-DIC values were reached after 4 to 7 h for urea and after 2 to 4 h for the AA-mix. Moreover, production ^{13}C-DIC from ^{13}C-urea was more rapid in BMA+ sediment than in BMA– sediment.

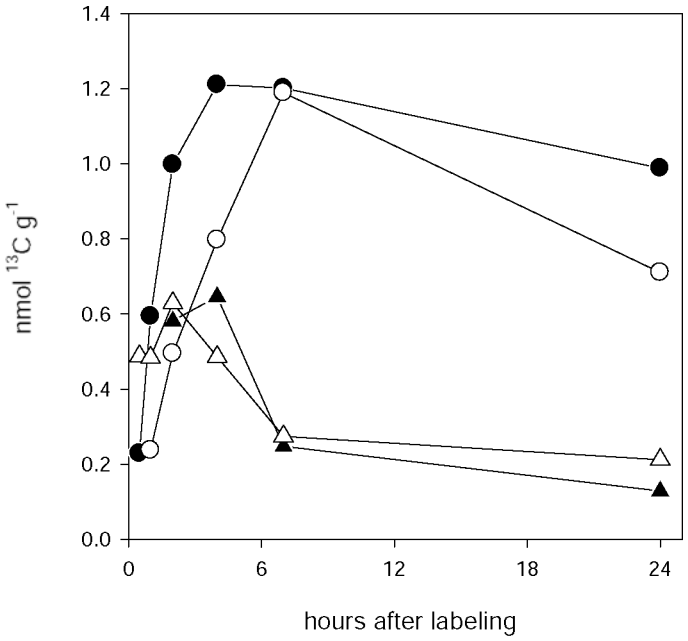


Figure 5. Excess ^{13}C-DIC produced per gram dry sediment for BMA+ sediment (black symbols) and BMA– sediment (white symbols) incubated with urea (circles) and the AA-mix (triangles).

DISCUSSION

Before discussing the results concerning excess ^{15}N and ^{13}C in the bulk sediment and hydrolysable amino acids (HAAs), it seems useful to first define these pools.
Excess label (^{15}N or ^{13}C) in the bulk sediment can include label incorporated in microbial biomass (see below), labeled compounds taken up in microbial cells (but not yet incorporated into biomass) as well as labeled compounds bound to the sediment (Rosenfeld 1979a+b, Henrichs and Sugai 1993) and/or dissolved in the pore water. For the present study, labeled compounds dissolved in the pore water were removed from the sediment by removal of the pore water after incubation.
HAAs are amino acids released from proteins and protein-like material by acid hydrolysis. With the method used in the present study, dissolvable material is removed from the sediment samples prior to hydrolysis, meaning that the measured total HAAs (THAAs, the sum of all individual HAAs) represent *particulate* proteinaceous material. Moreover, given the short time scale of the incubations, label incorporation into THAAs reflected incorporation into living microbial biomass (i.e. time scale is too short for substantial accumulation of labeled degradation products and/or label transfer to fauna via grazing). Since THAAs typically make up a large fraction (50 to 80%) of total microbial biomass (Simon and Azam 1989, Cowie and Hedges 1992) label incorporation into THAAs provides a good indication of label incorporation into total microbial biomass. One of the amino acids included in the THAA pool is D-alanine (D-Ala). Unlike the other HAAs that are common biomass constituents in all organisms, D-Ala is specific for bacteria. Therefore, label incorporation into D-Ala provides and indication of label incorporation into bacterial biomass (see Chapter 2).

Below, we first discuss results for ^{15}N in the bulk sediment and ^{15}N incorporation into total microbial biomass (represented by THAAs) for the incubations with urea versus those with the AA-mix. Thereafter, the discussion focuses on the relative importance of bacteria versus algae to the total microbial incorporation of ^{15}N from both substrates (based on ^{15}N incorporation into D-Ala). In the last sections we discuss the (un-)coupled behavior of ^{15}N and ^{13}C and relate this to the pathways for uptake and incorporation of carbon and nitrogen from urea and amino acids.

Bulk ^{15}N and total ^{15}N incorporation: Urea

The first interesting point from the urea incubations concerns the general trends in excess ^{15}N in total sediment and THAAs over time (Fig. 1A). The apparent leveling off of excess ^{15}N in the bulk sediment and THAAs between 2 h and 7 h suggests relatively rapid ^{15}N incorporation during the first 2 to 4 h and lower incorporation rates during the following hours. This decrease in incorporation rates may have been due to light limitation towards the end of the day. Light dependent uptake and incorporation of urea-^{15}N is consistent with the relatively high values after 24 h as the 24 h incubations included a second day light period during the following morning (17.5-24 h). Light-dependent nitrogen uptake and incorporation is likely for the present study since algae were important contributors to total ^{15}N incorporation (see discussion below). A diel rhythm in nitrogen uptake by phytoplankton has been reported for urea (McCarthy and Eppley 1972) as well as NO_3^- and NH_4^+ (Cochlan et al. 1991) and is consistent with the generally observed lower nitrogen uptake rates in the dark than in the light in algal cultures (Antia et al. 1991 and references therein), a microbial mat (Rondell et al. 2000) and estuarine waters (e.g., Middelburg and Nieuwenhuize 2000 and Chapter 1).

A second point from figure 1A concerns the difference between excess ^{15}N in THAAs and that in the bulk sediment. During the first few hours of incubation, ^{15}N incorporation into microbial biomass (represented by ^{15}N-THAAs) was very low while bulk excess ^{15}N was substantial. This indicates that early in the experiment, bulk excess ^{15}N mainly consisted of a ^{15}N pool bound to the sediment and/or taken up in microbial cells (but not yet incorporated into biomass). This pool may have included the added ^{15}N-urea while the uncoupled behavior of ^{15}N and ^{13}C (see discussion below) indicates that this pool also included a substantial fraction $^{15}NH_4^+$ produced by metabolization of the ^{15}N-urea.

The fraction of total ^{15}N in the sediment present in THAAs increased during the incubation period (13-16% after 4 h up to 45% after 24 h), which reflects the gradual incorporation of ^{15}N into microbial biomass. Given that all urea-derived ^{15}N was recovered in the bulk sediment after 24 h, these results indicate that 45% of total added urea-^{15}N had been incorporated into THAAs. This fraction is close to the typical THAA content of microbial biomass (50 to 80%, see Simon and Azam 1989, Cowie and Hedges 1992), indicating that a large fraction (60 to 90%) of the added urea-^{15}N had been incorporated into microbial biomass within 24 h.

Bulk ^{15}N and total ^{15}N incorporation: amino acids

Before discussing excess ^{15}N in bulk sediment and THAAs in the AA-mix incubations, it should be pointed out here that the added ^{15}N-labeled amino acids did not interfere with analysis of ^{15}N in the HAAs. Added ^{15}N-amino acids were present as free amino acids dissolved in the pore water or bound to the sediment. Dissolved free amino acids were removed from the sediment by removal of the pore water while bound amino acids were removed by washing the sediment samples with water and 2M HCl prior to hydrolysis (see Chapter 2). This effective removal of added ^{15}N-amino acids is confirmed by the very low excess ^{15}N in THAAs in the incubations that were stopped almost directly (10-15 min) after addition of the AA-mix (Fig. 1B).

Trends for ^{15}N in the bulk sediment and THAAs in the AA-mix incubations were rather different from those in the urea incubations. While bulk excess ^{15}N in the urea incubations showed a more or less continuous increase over the 24 h, bulk excess ^{15}N in the AA-mix incubations was already high after 10-15 min and remained at that level during the whole 24 h. This level of ~ 60 nmol ^{15}N g^{-1} corresponds to the total added amount of ^{15}N, which indicates that all added ^{15}N was rapidly bound to the sediment and/or taken up into microbial cells and/or incorporated into microbial biomass. Given that ^{15}N incorporation into microbial biomass (represented by ^{15}N-THAAs) was low during the first hour (Fig. 1B), the bulk ^{15}N in this period must have consisted mainly of ^{15}N bound to the sediment and/or ^{15}N taken up into microbial cells (but not yet incorporated). Like for the urea incubations, the sediment-bound and/or intracellular ^{15}N not only comprised intact ^{15}N-amino acids but also $^{15}NH_4^+$ resulting from extracellular oxidation of the ^{15}N-amino acids, which is indicated by the partly uncoupled behavior of ^{15}N and ^{13}C from the AA-mix (see discussion below).

The rapid increase in excess ^{15}N in THAAs (Fig. 1B) during the first 4 h indicates rapid incorporation of ^{15}N into microbial biomass. After 4 h, excess ^{15}N in THAAs was 41-49% of bulk excess ^{15}N, indicating that a large fraction (50-100%) of the added AA-^{15}N had been incorporated into microbial biomass after 4 h (see discussion above). The leveling off of excess ^{15}N in THAAs between 4 h and 7 h may thus have resulted from depletion of the available ^{15}N pool. However, excess ^{15}N in THAAs increased further between 7 h and 24 h, which resulted in excess ^{15}N in THAAs being an even larger fraction of bulk ^{15}N (57-76%) after 24 h. This indicates that the THAA content of the microbial biomass involved was high (well

over 50%), which is consistent with the large contribution by benthic microalgae to total ^{15}N incorporation (see next section) since the THAA content of algae (60-80%, Cowie and Hedges 1992) appears to be higher than that of bacteria (50-60%, Simon and Azam 1989, Cowie and Hedges 1992). This suggests that the leveling off of ^{15}N incorporation into THAAs between 4 h and 7 h was not only due to depletion of the available AA-^{15}N pool but may also have resulted from light limitation (see discussion above).

^{15}N incorporation: algae or bacteria ?

One of the objectives of the present study was to clarify the relative contributions of bacteria versus algae to total microbial nitrogen uptake and incorporation, a subject that has remained largely unresolved for a long time due to methodological limitations (see introduction and Chapter 2). Only recently, ^{15}N-labeling has been combined with analysis of ^{15}N incorporation into bacterial amino acids, which allows specific assessment of ^{15}N incorporation by bacteria (Tobias et al. 2003 and Chapter 2). The present study is one of the first in which analysis of ^{15}N incorporation into the bacterial biomarker D-Ala is used to clarify the bacterial contribution to total microbial nitrogen incorporation. The best indication of this bacterial contribution is provided by the excess ^{15}N D/L-Ala ratio (Fig. 2A). While D-Ala is specific for bacteria, L-Ala is a common protein amino acid that makes up a stable fraction of the THAA pool of all organisms. Therefore, the excess ^{15}N D/L-Ala ratio reflects incorporation of ^{15}N in bacteria (D-Ala) versus that in total microbial biomass (L-Ala). Interpretation of excess ^{15}N D/L-Ala ratios is as follows: when bacteria dominate ^{15}N incorporation, the excess ^{15}N D/L-Ala ratio for the sediment will be similar to the D/L-Ala ratio of bacterial biomass, which roughly ranges from 0.05 to 0.1 (see update Chapter 2). Conversely, when no ^{15}N is incorporated into bacteria, the excess ^{15}N D/L-Ala ratio will be 0.015-0.02, which represents ^{15}N-labeled D-Ala formed from ^{15}N-labeled L-Ala by racemization during sample hydrolysis (Amelung and Brodowski 2002, Kaiser and Benner 2005 and update of Chapter 2). Measured excess ^{15}N D/L-Ala ratios showed a clear difference between BMA+ and BMA– sediment (Fig. 2A). For BMA+ sediment (data for AA-mix incubations only), excess ^{15}N D/L-Ala ratios were around racemization background (0.015-0.02), indicating that the bacterial contribution to total microbial ^{15}N incorporation in these slurries was negligible and hence that ^{15}N incorporation was dominated by the benthic microalgae. The more elaborate data for the BMA– sediment showed somewhat higher excess ^{15}N D/L-Ala ratios than for

the BMA+ sediment and the excess ^{15}N D/L-Ala ratios of 0.05-0.08 for the first 1-2 h point to a substantial bacterial contribution to initial ^{15}N incorporation. However, the rapid decrease in excess ^{15}N D/L-Ala ratios during the following hours to values well below bacterial D/L-Ala ratios indicates that the relative contribution by bacteria had decreased strongly after 4 h.
The excess ^{15}N D/L-Ala ratio of 0.023 after 24 h for the BMA– sediment incubated with urea was even close to the racemization background (like values for the BMA+ sediment), indicating that the bacterial contribution to total urea-^{15}N incorporation after 24 h was negligible. Only for the BMA– sediment incubated with the AA-mix, excess ^{15}N D/L-Ala ratios after 24 h were still clearly above racemization background. Although precise quantification of the bacterial contribution is difficult due to uncertainty regarding the D/L-Ala ratio of the active bacterial community, it is possible to estimate a minimum and maximum contribution (assuming a bacterial D/L-Ala ratio of 0.1 and 0.05 respectively). Doing so for the excess ^{15}N D/L-Ala ratio of ~ 0.03 for the BMA– sediment incubated with the AA-mix yielded an estimated bacterial contribution between 12% and 33% of total microbial ^{15}N incorporation after 24 h.

Altogether, results show that incorporation of nitrogen from urea and amino acids was dominated by algae. The measured fucoxanthin : Chl *a* ratio of ~ 0.25 and the contribution of the diatom-specific PLFA 20:5ω3 to the total PLFA pool are consistent with values from diatom cultures (Dijkman and Kromkamp 2006), which indicates that the benthic microalgal community was dominated by diatoms and hence that nitrogen incorporation was also dominated by diatoms. This domination of total ^{15}N incorporation by algae is consistent with the estimates of algal biomass (300-500 µmol-C g^{-1}) that were an order of magnitude higher than those of bacterial biomass (22-24 µmol-C g^{-1}). The difference in estimated algal biomass between BMA+ and BMA– sediment was not as large as expected from the difference in visible diatom biofilms on top of the sediment. However, the relatively low algal biomass in the BMA– sediment resulted in a substantially larger bacterial contribution to total ^{15}N incorporation in this sediment.

These results have some important implications for studies that use total uptake of labeled amino acids (usually leucine) as a measure for bacterial production (e.g. Kirchman et al. 1985 and Chapter 1). One of the main assumptions underlying this method is that amino acids are specifically taken up by bacteria. However, results from the present study clearly show that amino acid uptake in surface sediment can

actually be dominated by algae. Substantial amino acid uptake by benthic microalgae is also reported by Linares and Sundbäck (2006). This large algal contribution to total amino acid uptake actually provides a feasible explanation for discrepancies between estimates of bacterial production in the sediment of an intertidal mudflat in the Scheldt Estuary where estimates based on leucine incorporation were an order of magnitude higher than those based on sediment oxygen consumption (van Oevelen et al. 2006c). Hence, our results indicate that great care should be taken when converting uptake of labeled amino acids to bacterial production in studies on surface sediments as well as other systems with relatively algal biomass (phototrophic biofilms, algal blooms in the water column, etc.).

^{15}N versus ^{13}C: urea

As already mentioned in the preceding discussion, results for urea-^{13}C were different from those for urea-^{15}N (Fig. 1 versus 3). This uncoupled behavior of ^{15}N and ^{13}C from urea is also reflected by the excess ^{13}C :^{15}N ratios that generally showed a substantially lower ^{13}C : ^{15}N ratio than that of the added urea (0.5), except for incorporation into THAAs after 4 h (Fig. 4). In addition, there was substantial production of ^{13}C-DIC in the urea incubations (Fig. 5). These results are comparable with those by Lund and Blackburn (1989) who measured production of ^{14}C-DIC after addition of ^{14}C-urea to a coastal marine sediment while no incorporation of ^{14}C occurred. Release of ^{13}C- or ^{14}C-DIC following addition of ^{13}C- or ^{14}C-urea was also observed for algal cultures (Price and Harrison 1988, Antia et al. 1991 and references therein), bacteria from estuarine waters (Jørgensen 2006) and a natural planktonic microbial community (Tamminen and Irmisch 1996). Moreover, uncoupled uptake of ^{15}N and ^{13}C from dual-labeled urea has been reported for coastal waters (Mulholland et al. 2004, Fan and Glibert 2005, Andersson et al. 2006). The uncoupling of C and N from urea is related to the urea metabolization pathway (Fig. 6). In order for bacteria and algae to use urea as a nitrogen source for growth, urea first needs to be broken down to NH_4^+ and DIC. Subsequently, the resulting NH_4^+ can be used to synthesize amino acids for production of proteinaceous biomass while the DIC may be used as carbon source for photosynthesis (Price and Harrison 1988, Antia et al. 1991). This urea metabolization pathway appears to be widespread since urease, the enzyme responsible for the breakdown of urea, is a common enzyme used by algae as well

as bacteria (Price and Harrison 1988, Mobley and Hausinger 1989, Antia et al. 1991, Zehr and Ward 2002).

Our results are consistent with the breakdown of urea into NH_4^+ and DIC. The excess ^{13}C : ^{15}N ratios for the bulk sediment during the first hours of incubations were already well below the ratio for the added urea. This indicates that the breakdown of urea into $^{15}NH_4^+$ and ^{13}C-DIC occurred rapidly, which is consistent with the rapid metabolization of urea reported for algal cultures (Price and Harrison 1988, Antia et al. 1991). During the first hours, only a small fraction of total added urea-^{15}N was incorporated into biomass (as indicated by ^{15}N-THAAs). Therefore, the relatively high excess ^{15}N (compared to ^{13}C) in the bulk sediment during this period must have been due to a relatively strong retention of $^{15}NH_4^+$ by binding to the sediment and/or by selective uptake into microbial cells (see preceding discussion). Contrary to the preferential retention of ^{15}N in the bulk sediment, the excess ^{13}C : ^{15}N ratio for the THAAs after 4 h (0.4-0.5) was very similar to that of the added urea (0.5), indicating that both $^{15}NH_4^+$ and ^{13}C-DIC were used as sources of nitrogen and carbon for growth respectively during the first hours of incubation. The strong decrease in excess ^{13}C : ^{15}N ratios for bulk sediment and THAAs between 4 h and 24 h, the period during which most of the added ^{15}N was incorporated into microbial biomass, may have been due to light limitation during this period (see preceding discussion). Since ^{13}C from ^{13}C-DIC could not be fixed into autotrophic biomass in the dark, the remaining ^{13}C-DIC was probably lost from the slurries by diffusion. Although incorporation of ^{15}N from $^{15}NH_4^+$ could also have been lower in the dark (see preceding discussion), $^{15}NH_4^+$ was probably retained relatively well in microbial cells and/or bound to the sediment and was therefore still available for incorporation in the light the following morning.

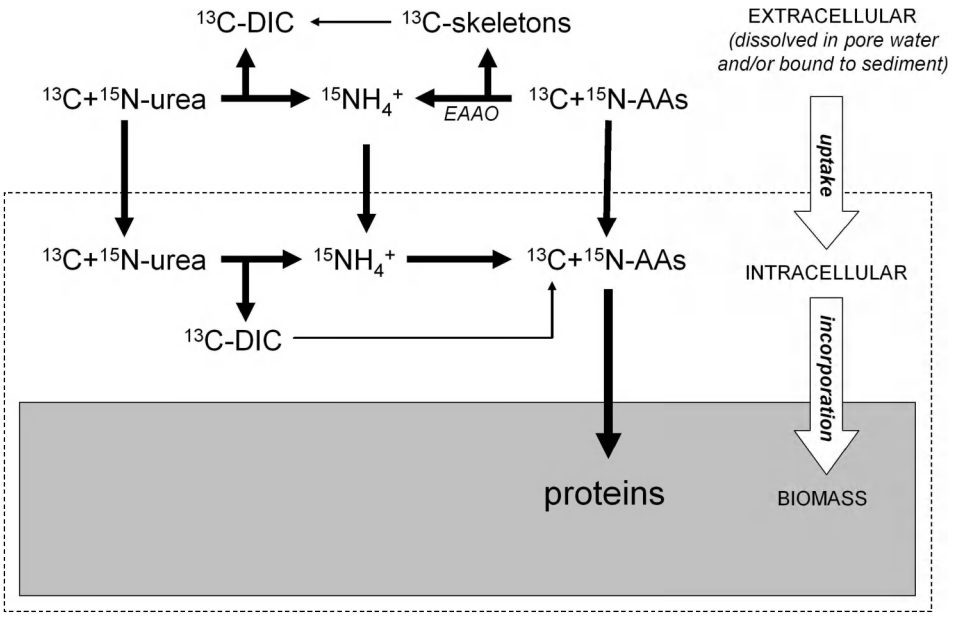


Figure 6. Schematization of pathways for uptake and incorporation of ^{15}N and ^{13}C from urea and amino acids into microbial biomass as measured in the present study. AAs = amino acids, EAAO = extracellular amino acid oxidation, DIC = dissolved inorganic carbon.

^{15}N versus ^{13}C: amino acids

Incubations with the AA-mix showed clear incorporation of both ^{15}N and ^{13}C. However, the excess ^{13}C : ^{15}N ratio of the bulk sediment and THAAs (1.5-2) was substantially lower than that of the added AA-mix (~ 4) which indicates preferential retention and incorporation of AA-^{15}N over AA-^{13}C. Moreover, the production of ^{13}C-DIC (Fig. 5) confirmed that part of the AA-^{13}C had been transformed to ^{13}C-DIC. This uncoupled behavior of nitrogen and carbon from amino acids is consistent with results from previous studies on algal cultures (Algeus 1948, Stephens and North 1971, Palenik and Morel 1990, Antia et al. 1991) and coastal waters (Schell 1974, Mulholland et al. 2002, 2003, Andersson et al. 2006) and conversion of amino acid carbon to DIC in a coastal sediment was reported by Christensen and Blackburn (1980).

Like for urea, the explanation for this uncoupling lies in the pathways for amino acid uptake and incorporation (Fig. 6). Incorporation of nitrogen and carbon from amino acids into microbial biomass can occur via three different pathways: 1) Direct uptake and incorporation: Amino acids are taken up from the surrounding water into microbial cells as complete molecules that form readily available building blocks for production of new biomass and therefore can be directly incorporated into proteinaceous biomass. 2) Direct uptake into cells followed by transformation to other amino acids when the composition of the available amino acid pool does not reflect that of microbial biomass (Stephens and North 1971). 3) Indirect uptake and incorporation via extracellular amino acid oxidation (EAAO). Amino acids are oxidized to NH_4^+, peroxide and C-skeletons outside the cells after which the NH_4^+ can be taken up and used as a nitrogen source for growth (Palenik and Morel 1990, Antia et al. 1991, Pantoja and Lee 1994, Mulholland et al. 1998).
These different pathways provide different potential explanations for the results from the present study. The relatively low ^{13}C : ^{15}N ratio for the bulk sediment and THAAs may theoretically have resulted from preferential incorporation of amino acids with a low C:N ratio such as Arg (C:N = 1.5), His and Gly (C:N = 2). However, this is unlikely because Arg, His and Gly only made up a small fraction (~ 15%) of the total added AA-mix while all amino acid-derived ^{15}N was incorporated into microbial biomass (see preceding discussion). Moreover, substantial transformation of added amino acids to those with a C:N ratio of 1.5 to 2 is very unlikely since the composition of the added (algal-derived) AA-mix was similar to that of microbial biomass (dominated by algae) (Cowie and Hedges 1992), meaning that the added AA-mix could directly be used for synthesis of new proteinaceous biomass. This was confirmed by the fact that all HAAs showed a similar level of ^{15}N enrichment ($\Delta\delta^{15}N$). The most likely explanation for the uncoupled incorporation of AA-^{13}C and AA-^{15}N is EAAO, which resulted in efficient uptake of $^{15}NH_4^+$ and subsequent incorporation of ^{15}N into biomass. The AA-C can theoretically also be incorporated into microbial biomass when the C-skeletons resulting from EAAO are used as a carbon source by heterotrophic bacteria and/or when the C-skeletons are remineralized to DIC which can subsequently be used a carbon source by autotrophic algae. However, we do not expect these pathways to have played an important role in the present study because the bacterial contribution to ^{13}C incorporation was probably similarly low as that to ^{15}N incorporation (i.e. substantial incorporation of ^{13}C from ^{13}C-skeletons by bacteria is unlikely) and because EAAO takes place extracellularly, where ^{13}C-DIC is likely to be lost by diffusion and/or dilution in a large background pool of ^{12}C-DIC (i.e. incorporation

of ^{13}C from ^{13}C-DIC by algae is also unlikely). Therefore, the most likely explanation for the observed ^{13}C incorporation is that, next to EAAO, part of the added AA-mix was directly taken up into cells and incorporated into biomass (pathway 1) which resulted in coupled incorporation of ^{13}C and ^{15}N. Given the ^{13}C : ^{15}N ratio of ~ 4 for the added AA-mix and the incorporated ^{13}C : ^{15}N ratio of 1.5-2, it seems that about half of the AA-^{15}N had been incorporated directly while the other half was incorporated via EAAO.

These results are consistent with previous reports on the importance of EAAO in water column studies (Pantoja and Lee 1994, Mulholland et al 1998, 2002, 2003, Andersson et al. 2006) and for the first time show the importance of EAAO in microbial nitrogen incorporation in sediments. Although extracellular oxidation of amino acids and subsequent resynthesis of amino acids inside the cells seems inefficient, it may actually be more economical than direct uptake of intact amino acids which requires more energy than uptake of NH_4^+ (Antia et al. 1991 and references therein). Moreover, EAAO may be a relatively efficient pathway for uptake and incorporation of amino acid nitrogen in sediments given the high cell densities (allowing efficient uptake of $^{15}NH_4^+$ resulting from EAAO) and the relatively high concentrations of amino acids (dissolved free and combined) in sediment pore waters. EAAO generally seems to be associated with algae as only one study reported EAAO activity in the bacterial size fraction (Pantoja and Lee 1994). However, even when only algae are capable of EAAO, bacteria may still be able to use EAAO products as nitrogen and carbon sources since these products are produced extracellularly. This may be especially relevant for systems like surface sediments and photosynthetic biofilms where bacteria and algae live in close association. Assessment of the role of EAAO in bacterial nitrogen uptake in the present study was not possible since nitrogen uptake and incorporation was dominated by algae.

Acknowledgements

We thank Pieter van Rijswijk for processing and analyzing the PLFA samples and Marco Houtekamer for his help with the GC-c-IRMS analyses.

C H A P T E R 4

Nitrogen incorporation and retention by bacteria, algae and fauna in a subtropical, intertidal sediment: An in situ ^{15}N-labeling study

Bart Veuger, Bradley D. Eyre, Damien Maher and Jack J. Middelburg

In revision for *Limnology and Oceanography*

ABSTRACT

We used an in situ ^{15}N-labeling approach to investigate nitrogen incorporation and retention by bacteria, benthic microalgae and fauna in the intertidal sediment of a subtropical, Australian estuary (Brunswick). The main experiment involved ^{15}N pulse labeling by injection of $^{15}NH_4^+$ into the sediment. After labeling, ^{15}N was traced into bulk sediment, total hydrolysable amino acids (THAAs, representing bulk proteinaceous biomass), the bacterial biomarker D-alanine and fauna during a 30-day sampling period. Additional experiments included short-term (24 h) incubations of sediment cores injected with different ^{15}N-labeled substrates and sediment core incubations for analysis of sediment-water fluxes of O_2, DIC, NH_4^+, NO_x^-, DON and N_2. ^{15}N was rapidly incorporated and strongly retained in microbial biomass (THAAs) during the 30-day sampling period, indicating very efficient recycling of ^{15}N by the benthic microbial community. Analysis of ^{15}N in D-alanine revealed a major bacterial contribution to total microbial incorporation and recycling of the ^{15}N. ^{15}N was also incorporated into fauna via grazing on ^{15}N-labeled microbial biomass, but this was a negligible fraction (< 1%) of total ^{15}N in the sediment. Results confirm the previously hypothesized immobilization of nitrogen in benthic microbial biomass and indicate that recycling of nitrogen by the microbial community within the sediment can be an important pathway supporting benthic microbial production, especially in nitrogen limited systems like the Brunswick Estuary.

INTRODUCTION

The sediments of subtropical estuaries with low nitrogen concentrations in the water column can act as a nitrogen sink for much of the year (e.g. Ferguson et al. 2004). The limited nitrogen availability in these systems results in strong competition between different microbial groups living in the sediment (heterotrophic bacteria, benthic microalgae (BMA) and cyanobacteria) that need dissolved nitrogenous substrates to support their growth as well as for catabolic processes such as nitrification and denitrification (Rysgaard et al. 1995, Risgaard-Petersen 2003, Sundbäck et al. 2004, Ferguson et al. 2004). Moreover, one would expect the benthic microbial community to be very economical with available nitrogen. This is reflected by results from studies on sediment-water nitrogen fluxes in subtropical Australian estuaries (Ferguson et al. 2004, Eyre and Ferguson 2005) as well as other coastal systems (Lomstein et al. 1998, Anderson et al. 2003, Cook et al. 2004, Sundbäck et al. 2004) that suggest a relatively strong retention of nitrogen in the sediment. This is thought to be due to incorporation of remineralized nitrogen by BMA and/or bacteria (Lomstein et al. 1998, Anderson et al. 2003, Cook et al. 2004, Ferguson et al. 2004, Sundbäck et al. 2004, Eyre and Ferguson 2005) and possibly by transfer of nitrogen to higher trophic levels (Eyre and Ferguson 2005). However, "traditional" measurements of sediment-water nitrogen fluxes only allow analysis of the net result of all nitrogen cycling processes in the sediment (i.e. the sediment remains a "black box"), meaning that elucidation of the numerous nitrogen transformation processes and nitrogen flows through (micro)organisms within the sediment requires an alternative approach.

A useful approach is stable isotope labeling with the nitrogen isotope ^{15}N. ^{15}N-labeling has previously been used to measure uptake of nitrogenous substrates by the microbial community in the water column (e.g., Dugdale and Goering 1967, McCarthy and Eppley 1972 and Chapter 1). However, applications in studies on sediments have so far been limited to studies on nitrogen transformation processes such as nitrification and denitrification (e.g. Rysgaard et al. 1993, Ottosen et al. 2001) or analysis of ^{15}N uptake by the whole sediment (e.g., Tobias et al. 2003, Gribsholt et al. 2005). Analysis of ^{15}N incorporation by the total benthic microbial community and by specific groups within the microbial community has long been hampered by methodological problems (see Chapter 2). Only recently, ^{15}N-labeling has been combined with analysis of ^{15}N incorporation into total hydrolysable amino acids (THAAs), which provides a measure for ^{15}N incorporation in total microbial

biomass (Chapter 2), and analysis of ^{15}N incorporation into bacteria-specific amino acids, which makes it possible to trace ^{15}N into bacteria (Tobias et al. 2003 and Chapter 2).

In the present study, ^{15}N-labeling was used to elucidate nitrogen flows through bacteria, BMA and fauna in a subtropical, intertidal sediment. A recently introduced method for analysis of ^{15}N in THAAs, including the bacterial biomarker D-alanine (D-Ala) (Chapter 2), was used to investigate ^{15}N incorporation and retention by the total benthic microbial community (THAAs) and the role of bacteria (D-Ala) herein.

MATERIALS AND METHODS

Location

The present study was undertaken in the Brunswick Estuary, a small, shallow, subtropical estuary in northern New South Wales, Australia (Fig. 1). Together with the smaller Simpsons river, the Brunswick river drains a catchment area of ~ 220 km^2 dominated by woodland and low intensity agriculture. The estuaries in this area can experience heavy rainfalls and flooding during the summer season (Eyre and Ferguson 2005). A small flood event occurred in the week prior to the sampling period with a peak of 56 m^3 d^{-1} (16 m^3 d^{-1} gauged) while river discharge was low, with gradual decrease, during the sampling period (8 ± 4 m^3 d^{-1}). The site for the present study was an intertidal mud bank in the upper estuary, ~ 50 m downstream from site RW sampled by Ferguson et al. (2003, 2004) and Eyre and Ferguson (2005) (Fig. 1). Average water column salinity at the test site was 8 ± 4 and water temperature during the field work period was ~ 29°C. More details on the Brunswick Estuary and the test site can be found in Ferguson et al. (2003, 2004) and Eyre and Ferguson (2005).

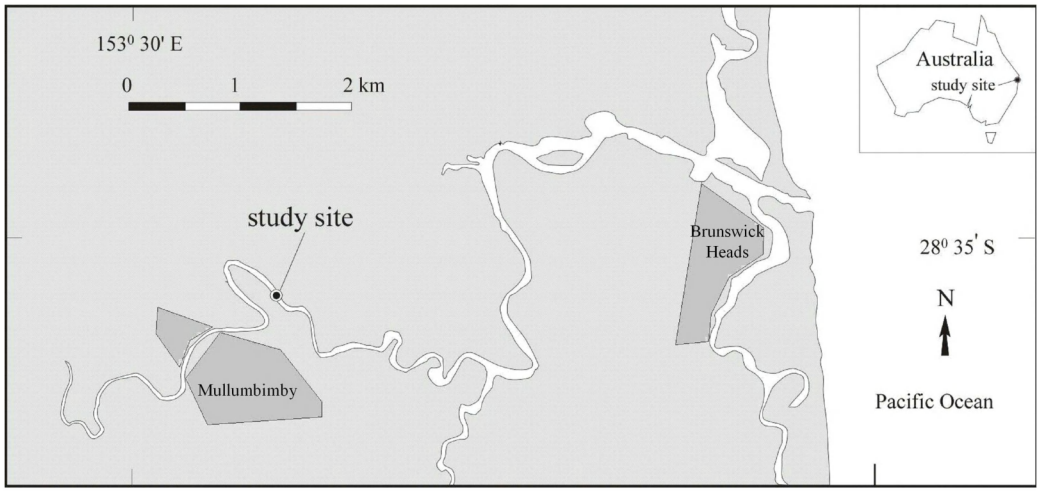


Figure 1. Location of the Brunswick Estuary and study site.

Benthic flux incubations

Three series of benthic flux incubations were performed in three consecutive weeks during the sampling period for the ^{15}N plots (corresponding to sampling days 1, 9 and 16, see below). Each week, three sediment cores (∅ 10 cm) were collected from the sediment surrounding the ^{15}N plots. Cores were transported to the laboratory within a few hours and placed in an incubator containing site water at in-situ temperature. Following a 24 h pre-equilibrium period, cores were incubated at the daily average in situ light (400 µE m^{-2} h^{-1}) and temperature (29 ± 2°C) over a 24 h dark-light cycle (10 h dark and 14 h light). Dissolved oxygen (DO) concentrations and pH were measured electro-chemically and samples for analysis of alkalinity and concentrations of NH_4^+, NO_x^-, total dissolved N (TDN) and N_2 were taken 3-4 times during the light and dark periods. Alkalinity, NH_4^+, NO_x^- and TDN samples were withdrawn with a plastic syringe and withdrawn water was replaced with site water from a gravity-feed reservoir. Samples were filtered (0.45 µm) and immediately frozen (-20°C), except alkalinity samples which were kept cold (10°C). Concentrations of NH_4^+, NO_x^- and TDN were measured colourmetrically using Lachat™ Flow Injection Analysis. Total DIN was calculated as NH_4^+ + NO_x^- and DON as TDN – DIN. N_2 samples were collected in triplicate by allowing water to flow, driven by the reservoir head, directly into 7 ml gas-tight glass vials with glass stoppers. Vials were allowed to overflow to minimize the introduction of bubbles. N_2 samples were poisoned with 20 µl of 5% $HgCl_2$ and stored submerged at about 1-2°C below ambient temperature. N_2 concentrations were measured using a modified membrane inlet mass spectrometer with O_2 removal (Eyre et al. 2002). Changes in alkalinity and pH were used to calculate fluxes of dissolved inorganic carbon (DIC). See Eyre and Ferguson (2005) for details on analytical procedures. Fluxes across the sediment-water interface were calculated by linear regression of the concentration data, corrected for the addition of replacement water, as a function of incubation time, core water volume and surface area. Net flux rates represent the net result of 10 h of dark fluxes and 14 h of light fluxes.

In situ ^{15}N plots

Two 50 × 50 × 4 cm aluminum frames were placed side by side on a flat section of the mud bank parallel to the river. Plots were labeled with $^{15}NH_4^+$ at low tide on February 5, 2005 by injecting a $^{15}NH_4^+$ solution in the upper 8 cm of the sediment using a 1 ml syringe with stainless steel needle (0.9 x 80 mm). Per plot, 361 injections with 0.5 ml $^{15}NH_4^+$ solution were made (1 injection per 6.3 cm^2). The $^{15}NH_4^+$ solution contained 0.6 g $(^{15}NH_4)_2SO_4$ dissolved in 400 ml Milli-Q water and

100 ml seawater from the mouth of the estuary (yielding a salinity similar to that of the river water at the test site). The $^{15}NH_4^+$ concentration in the label solution was 18 mmol l^{-1}, which resulted in a pore water $^{15}NH_4^+$ concentration of 320 µmol l^{-1}. During label injection and sampling, wooden walking boards were used to reduce impact on the surrounding sediment. Plots were sampled at low tide at 1, 2, 4, 9, 16 and 30 days after label injection by collecting 2-3 cores (∅ 5 cm) per plot. Resulting holes in the plots were filled with dummy cores containing sediment from outside the plots to prevent physical disturbance of the remaining sediment. Unlabeled control samples were taken from the sediment surrounding the plots. Cores were transported to the laboratory within 2 h after sampling and processed directly or frozen (-20°C) and processed later. Processing involved homogenization of sediment from the 0-8 cm layer of the cores after which subsamples were taken for analysis of ^{15}N in the bulk sediment and hydrolysable amino acids. Additional samples of surface sediment were taken by scraping off the upper 2 mm from intact cores (hereafter called the 0-0.2 cm layer). Sediment samples were stored frozen (-20°C) until oven drying (48 h at 60°C). At sampling days 2, 9 and 30, remaining sediment from the 0-8 cm layer was used for extraction of fauna that was also analyzed for ^{15}N-enrichment (see below).

^{15}N core incubations

Small sediment cores (∅ 2.5 cm, 8-12 cm deep) were collected from the sediment surrounding the ^{15}N plots at sampling day 9. Cores were transported to the laboratory within 2 h after sampling and placed in a water bath under the same temperature and light regime as used for the benthic flux incubations. Cores were left to settle overnight (in the dark, keeping the sediment surface submerged). The following day, cores were injected with $^{15}NH_4^+$, $^{15}NO_3^-$, ^{15}N-urea or a ^{15}N-amino acid mixture (Cambridge Isotope Laboratories, NLM-2161, hereafter called "AA-mix", see for composition) dissolved in 0.45 µm-filtered site water. For each substrate, 4 cores were labeled by 3 injections of 0.4 ml substrate solution per core (6 cm deep, using 1 ml syringe with 0.9 × 80 mm stainless steel needle). Injection resulted in addition of ~ 0.6 µmol ^{15}N in the upper 6 cm of the cores (~ 20 nmol ^{15}N ml^{-1} wet sediment) for $^{15}NH_4^+$, $^{15}NO_3^-$ and the ^{15}N-amino acid mixture and ~ 1.2 µmol ^{15}N for ^{15}N-urea. After injection, labeled cores together with a set of unlabeled control cores were incubated in a water bath under the same temperature and light regime as used for the benthic flux incubations. Cores were kept saturated with water by maintaining a small volume of water over the sediment. Incubations were

terminated after 6 h and 24 h (2 cores per incubation time per substrate) by freezing the 0-6 cm layer (-20°C). Later, samples were defrosted, homogenized and oven-dried (48 h at 60°C) after which subsamples were analyzed for ^{15}N enrichment of bulk sediment and hydrolysable amino acids (both in duplicate).

^{15}N Analyses

^{15}N in the bulk sediment was measured using a Fisons CN elemental analyzer coupled on-line to a Finnigan Delta S isotope ratio mass spectrometer (EA-IRMS) with a typical reproducibility of $\delta^{15}N$ within 0.4‰.

^{15}N in hydrolysable amino acids (HAAs), including D-Ala, was analyzed following the protocol described in Chapter 2. Briefly, samples (~ 1 g) of freeze-dried sediment were washed with HCl (2 M) and Milli-Q water and subsequently hydrolyzed in 6 M HCl at 110°C for 20 h. After purification by cation exchange chromatography, amino acids were derivatized with isopropanol (IP) and pentafluoropropionic anhydride (PFA) and samples were further purified by solvent extraction. Concentrations and relative abundance of ^{15}N for the derivatized D- and L-amino acids were analyzed by gas-chromatography-combustion-isotope ratio mass spectrometry (GC-c-IRMS) on a HP 6890 GC with a Thermo type III combustion interface and Thermo Delta Plus IRMS.

For analysis of ^{15}N in fauna, sediment from the upper 8 cm of 2-3 cores was sieved over a 500 µm sieve to collect particles > 500 µm (including larger animals). The organic fraction of the < 500 µm material was separated from inorganic particles by elutriation using water collected at the test site and concentrated on a 63 µm sieve. Animals from the > 500 µm and 63-500 µm fractions were hand picked using a microsope and rinsed with Milli-Q water. Larger animals were transferred to plastic vials (1-5 animals per vial) while smaller animals ("meiofauna") were pooled in tin "boats" (50-200 individuals for nematodes and 1-10 individuals for other groups). All fauna samples were oven dried (18 h at 50°C). Prior to ^{15}N analysis, subsamples from larger animals were transferred to tin boats. ^{15}N in the fauna samples was measured by EA-IRMS as for bulk sediment. The amount of faunal biomass-N used for EA-IRMS analysis was 2-20 µg for small animals and 5-150 µg-N for larger animals.

Data treatment

^{15}N data are presented as excess ^{15}N per gram dry sediment, which was calculated as: excess ^{15}N = [(at%^{15}N $_{sample}$ − at%^{15}N $_{control}$) /100] × [concentration N in sample] where at%^{15}N was calculated from $\delta^{15}N$ as: at%^{15}N = [100 × $R_{standard}$ × ($\delta^{15}N$ $_{sample}$ / 1000) +1] / [1 + $R_{standard}$ × ($\delta^{15}N$ $_{sample}$ / 1000) +1] with $\delta^{15}N$ (‰) = [(R_{sample} / $R_{standard}$) − 1] × 1000 and R = $^{15}N/^{14}N$. In addition, some ^{15}N data are presented as $\Delta\delta^{15}N$ values that were calculated as: $\Delta\delta^{15}N_{sample} = \delta^{15}N_{sample} - \delta^{15}N_{control\ (unlabeled)}$.

D-Ala data were corrected for the formation of D-Ala from L-Ala by hydrolysis-induced racemization as described and discussed in the update of Chapter 2. Briefly, excess ^{15}N in D-Ala was corrected for racemization by subtracting 1.7% of excess ^{15}N in L-Ala from excess ^{15}N in D-Ala. The same correction was used for D-Ala concentrations. Excess ^{15}N in D-Ala is also expressed relative to that in L-Ala as excess ^{15}N D/L-Ala ratios that were calculated as: [excess ^{15}N in D-Ala] / [excess ^{15}N in L-Ala] using the measured excess ^{15}N in D-Ala (i.e. not corrected for racemization). Instead, the racemization background (D/L-Ala ratio of 0.015-0.02) is indicated graphically.

Additional analyses

Total organic carbon and nitrogen content of the sediment was measured with the same elemental analyzer as used for bulk ^{15}N analysis after acidification of the samples for removal of carbonates. Separate sediment cores were collected and freeze dried for analysis of photosynthetic pigments by HPLC (Barranguet et al. 1997) and phospholipid-derived fatty acids (PLFAs, see Middelburg et al. (2000), Boschker (2004) and Chapter 5).

RESULTS

Bulk sediment properties

Sediment at the test site consisted of fine mud with a high plant litter content. The average nitrogen content of the bulk sediment was 0.24 ± 0.02% (weight %) and the organic carbon (OC) content was 3.7 ± 0.5%. The corresponding mole-based C : N ratio was 19 ± 1.4. Values were very similar for the whole 0-8 cm layer and the upper 0-0.2 cm layer. The average concentration of THAAs (summed concentrations of individual HAAs) was 47 ± 5 μmol g^{-1} (5.7 mg g^{-1}) with no significant difference between the whole 0-8 cm layer and the upper 0-0.2 cm layer. THAA-C was 7.6% of bulk OC and THAA-N was 28% of bulk N. Average D-Ala concentrations (corrected for D-Ala formed by hydrolysis-induced racemization) were 0.40 ± 0.04 μmol g^{-1} for the whole 0-8 cm layer and 0.35 ± 0.03 μmol g^{-1} for the upper 0-0.2 cm layer. Average D/L-Ala ratios (not corrected for racemization) were 0.071 ± 0.005 for the whole 0-8 cm layer and 0.063 ± 0.005 for the upper 0-0.2 cm layer.

Microbial community

HPLC analysis of photosynthetic pigments in the sediment showed high abundance of degradation products such as phaeophytin and phaeophorbide, causing poor peak separation which prohibited precise quantification of most pigments (data not shown). Chlorophyll *a* concentrations were 3 μg g^{-1} for the whole 0-8 cm layer (corresponding to ~ 250 mg m^{-2}) and 17 μg g^{-1} for the upper 0-0.2 cm. When assuming a C : Chl a ratio of 50, these values correspond to an estimated photosynthetic biomass of 14 ± 8 μmol-C g^{-1} for the whole 0-8 cm layer and 80 μmol-C g^{-1} for the upper 0-0.2 cm. The sediment also contained detectable, but not quantifiable, amounts of other pigments, including fucoxanthin (specific for diatoms, with highest abundance in the upper 0-0.2 cm layer) and zeaxanthin (specific for cyanobacteria). Note that estimates of total photosynthetic biomass above include cyanobacterial biomass.

Bacterial biomass was estimated from concentrations of the bacteria-specific PLFAs i14:0, i15:0, a15:0, i16:0 and 18:1ω7c assuming that these together make up 28% of total bacterial PLFAs and that PLFA-C makes up 6% of total bacterial-C (see Middelburg et al. 2000 and references therein). Resulting estimates of total bacterial biomass were 21 ± 11 μmol-C g^{-1} for the whole 0-8 cm layer and 46 μmol-C g^{-1} for the upper 0-0.2 cm. Note that these estimates concern heterotrophic bacteria only (i.e. cyanobacterial biomass not included).

Benthic fluxes

Benthic fluxes of DO and especially DIC showed considerable variation between the three series performed in three consecutive weeks (Fig. 2). DO showed a clear influx in the dark and efflux in the light, resulting in a very small average net efflux of DO from the sediment. DIC fluxes in the dark were always directed out of the sediment, with highest rates for week 1. DIC fluxes in the light were very different for all three weeks with a clear efflux in week 1, a clear influx in week 2 and a very small efflux in week 3. For the net DIC fluxes, this resulted in a clear efflux in week 1 and much lower effluxes for week 2 and 3. Nitrogen fluxes showed more consistent results for the three consecutive weeks. NH_4^+ showed high efflux rates in the dark and high influx rates in the light resulting in a relatively small net efflux. NO_3^- showed a net influx in both light and dark with very low rates for weeks 1 and 3. Fluxes of DON were highly variable with average values showing a net efflux of DON from the sediment. N_2 fluxes showed a consistent release of N_2 from the sediment in the dark as well as in the light.

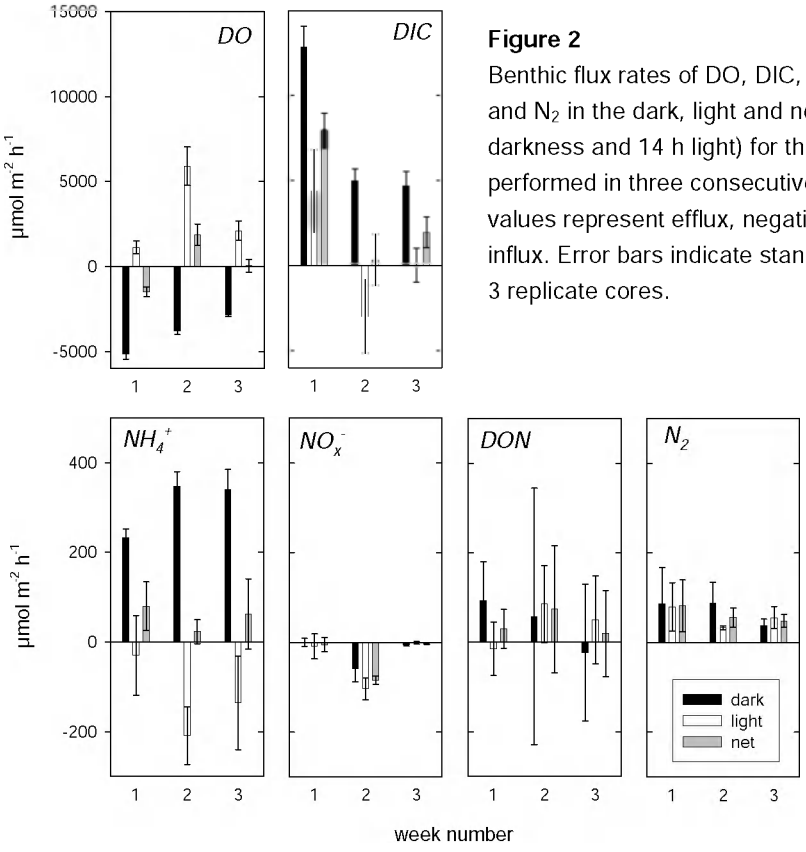


Figure 2
Benthic flux rates of DO, DIC, NH_4^+, NO_x^-, DON and N_2 in the dark, light and net (based on 10 h darkness and 14 h light) for three series performed in three consecutive weeks. Positive values represent efflux, negative values are influx. Error bars indicate standard deviation for 3 replicate cores.

^{15}N core incubations

Sediment cores showed moderate ^{15}N enrichment of the bulk sediment and HAAs following injection of the ^{15}N-labeled substrates with $\Delta\delta^{15}$N values up to ~ 50‰. Excess ^{15}N values for the bulk sediment were highest for the cores injected with ^{15}N-urea cores and lowest for those injected with $^{15}NH_4^+$ and $^{15}NO_3^-$ (Fig. 3A). Although these differences roughly reflect differences in added amounts of ^{15}N, values were relatively low for $^{15}NH_4^+$ and $^{15}NO_3^-$ cores. Moreover, bulk excess ^{15}N showed little to no difference between 6 h and 24 h of incubation.

Excess ^{15}N in THAAs (summed excess ^{15}N in individual HAAs) showed roughly the same relative differences between the four ^{15}N-substrates as bulk excess ^{15}N, except for relatively low values for ^{15}N-urea cores (Fig. 3B). Unlike bulk ^{15}N, excess ^{15}N in THAAs did show a difference between 6 h and 24 h with up to ~ 1.5 times higher values after 24 h. Excess ^{15}N in THAAs was 14-27% of bulk excess ^{15}N after 6 h and 19-35% after 24 h, with lowest values for cores injected with ^{15}N-urea (14-19%) and highest values for those injected with $^{15}NH_4^+$ (25-29%) and the ^{15}N-AA-mix (27-35%).

Trends in excess ^{15}N in D-Ala (Fig. 3C) for the four substrates after 6 h roughly matched those for excess ^{15}N in THAAs (including L-Ala) with a relatively high excess ^{15}N in cores injected with $^{15}NH_4^+$ and $^{15}NO_3^-$. However, unlike ^{15}N in THAAs, ^{15}N in D-Ala did not increase between 6 h and 24 h but actually decreased slightly for cores injected with $^{15}NO_3^-$, ^{15}N-urea and the ^{15}N-AA-mix. This resulted in a decrease in excess ^{15}N D/L-Ala ratios between 6 h and 24 h (Fig. 3D). After 6 h, average excess ^{15}N D/L-Ala ratios were very similar (0.09-0.11) for all four substrates while ratios were lower after 24 h with highest values for $^{15}NH_4^+$ cores (with considerable variation) and lowest values for the ^{15}N-AA-mix cores.

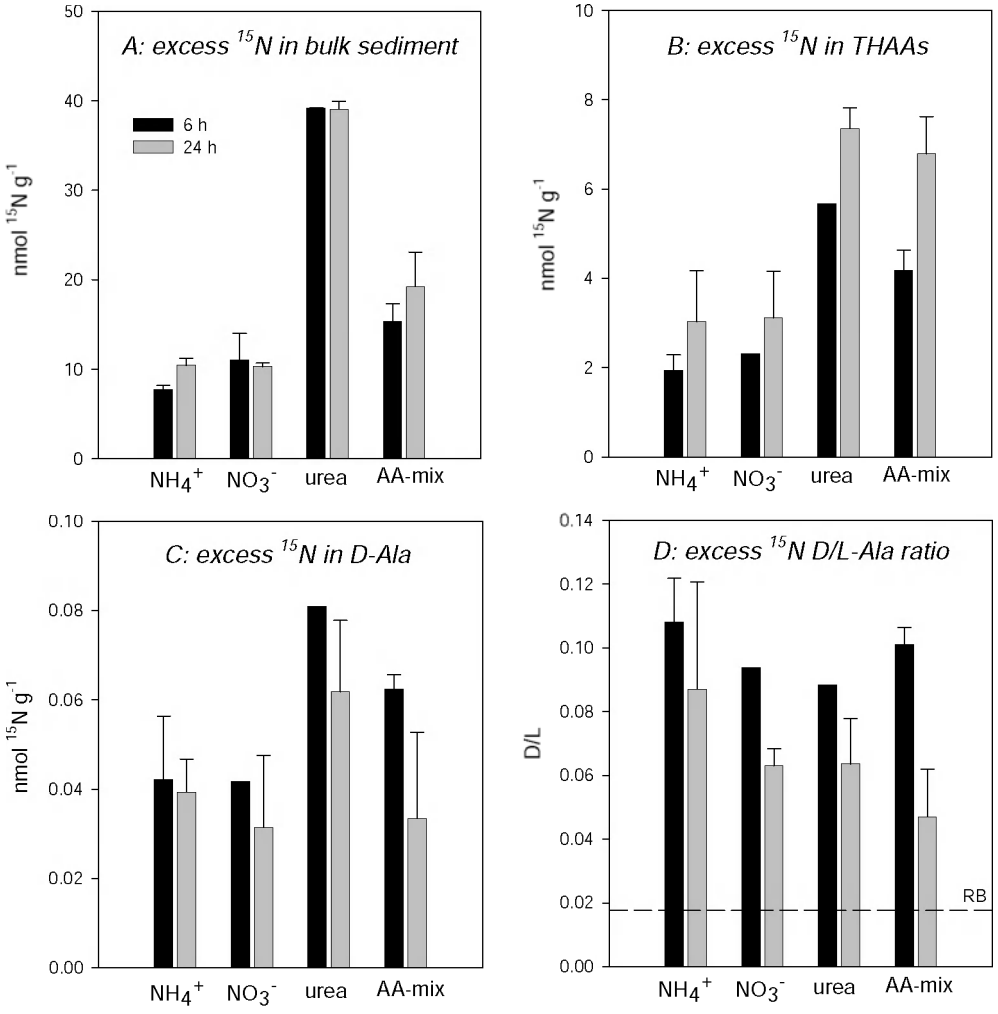


Figure 3. Excess ^{15}N in bulk sediment (A), total hydrolysable amino acids (THAAs) (B), D-Ala (corrected for hydrolysis-induced racemization) (C) and excess ^{15}N D/L-Ala ratio (not corrected for racemization) (D) for 0-6 cm layer from sediment cores incubated with $^{15}NH_4^+$, $^{15}NO_3^-$, ^{15}N-urea or a ^{15}N-AA-mix for 6 h and 24 h. Dashed line in plot D indicates racemization background (RB). Error bars represent standard deviation for two replicate cores. When no error bars are shown, only one sample was analyzed.

In situ ^{15}N plots: ^{15}N in bulk sediment

Injection of $^{15}NH_4^+$ into the sediment plots resulted in ^{15}N enrichment of the bulk sediment well above natural abundance with $\Delta\delta^{15}N$ values up to 400‰. Bulk excess ^{15}N was highest one day after injection with relatively high values for the upper 0-0.2 cm layer (254 nmol ^{15}N g^{-1} versus 86 nmol ^{15}N g^{-1} for the whole 0-8 cm layer) (Fig. 4A). Total excess ^{15}N recovered in the sediment after one day was ~ 50% of the total amount of injected ^{15}N. After day 1, bulk excess ^{15}N decreased during the 30-day sampling period for both layers. The 0-0.2 cm layer showed a steady exponential decrease (half life of ~ 9 days) while the decrease for the whole 0-8 cm layer was relatively rapid during the first 4 days and much slower after day 4 (resulting in an overall half life of ~ 28 days). After 30 days, bulk excess ^{15}N was very similar for both layers (~ 40 nmol ^{15}N g^{-1}).

In situ ^{15}N plots: ^{15}N in HAAs

Hydrolysable amino acids (HAAs) were also highly enriched in ^{15}N with $\Delta\delta^{15}N$ values of 100-700‰ for individual HAAs in the 0-0.2 cm layer and somewhat lower enrichments of 50-200‰ for the whole 0-8 cm layer. After day 1 (highest ^{15}N enrichment) excess ^{15}N in THAAs (Fig. 4B) in the 0-0.2 cm layer decreased exponentially with a very similar rate as for bulk excess ^{15}N (half life of ~ 9 days). Conversely, no such decrease occurred for excess ^{15}N in THAAs in the whole 0-8 cm layer (i.e. no net loss of ^{15}N from THAAs during the 30-day sampling period). For the 0-0.2 cm layer, excess ^{15}N in the THAAs was 43 ± 8 % of total excess ^{15}N in the bulk sediment with highest values (~ 50%) for day 16 and day 30. For the whole 0-8 cm layer, this fraction was relatively low at day 1 (17%) and rapidly increased to 29-37% for days 4 to 16 and 43 ± 1% at day 30.

Trends for excess ^{15}N in D-Ala over time (Fig. 4C) were very similar to those for the THAAs (including L-Ala). Corresponding excess ^{15}N D/L-Ala ratios for the 0-0.2 cm layer were stable at 0.04-0.05 throughout the whole sampling period while values for the 0-8 cm layer were relatively high at day 1 (0.076) and deceased during the following week to level off at 0.055-0.06 after day 9 (Fig. 4D).

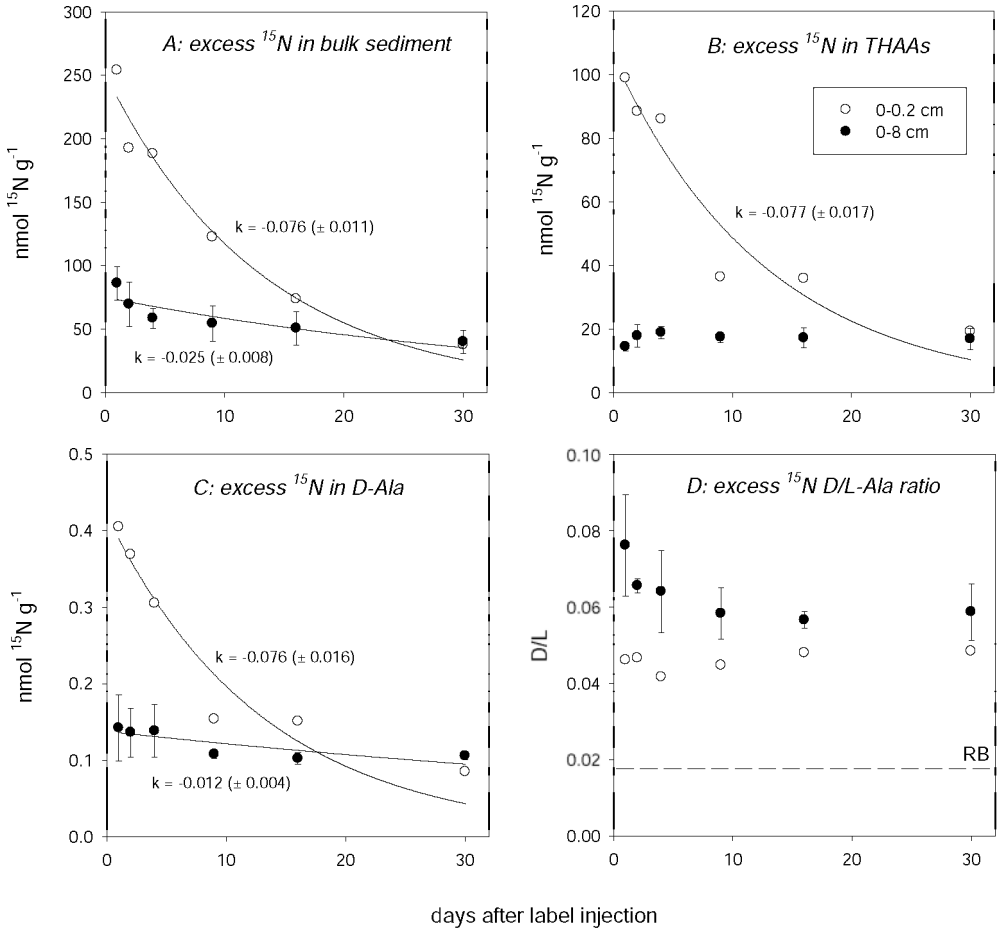


Figure 4. Excess ^{15}N in bulk sediment (A), THAAs (B) and D-Ala (corrected for hydrolysis-induced racemization) (C) and excess ^{15}N D/L-Ala ratios (not corrected for racemization) (D) for 0-0.2 cm layer and 0-8 cm layer from in situ ^{15}N plots. Error bars show standard deviation for replicate cores. Dashed line in plot D indicates racemization background (RB). Lines are exponential fits, k-values are corresponding loss rate constants (± standard error).

In situ ^{15}N *plots:* ^{15}N *in fauna*

Animals from various faunal groups were extracted from the sediment but densities of most groups were low. As a result, part of the fauna samples contained insufficient biomass for ^{15}N analysis while other samples (from different subgroups and/or sampling days) had to be pooled for proper ^{15}N analysis. The only groups that were abundantly present at all sampling days were nematodes and polychaetes. Nematodes dominated the 63-500 µm fraction with densities of 100-300 individuals core^{-1} and showed clear ^{15}N enrichment with highest $\Delta\delta^{15}N$ values after 9 days and $\Delta\delta^{15}N$ remaining high up to day 30 (Fig. 5A). The polychaetes *Notomastus estuarius* and *Simplisetia aequisetis* were extracted from the > 500 µm fraction at densities of 2-5 individuals core^{-1} (~ 30 g wet biomass m^{-2}). *Notomastus estuarius* showed lower $\Delta\delta^{15}N$ values than those for the nematodes but a similar trend over time (highest ^{15}N-enrichment after 9 days) (Fig. 5B). $\Delta\delta^{15}N$ values for *Simplisetia aequisetis* (Fig 5B) were very similar to those for the nematodes. However, the lack of data for day 9 and the substantial variation between samples from day 30 precludes assessment of a clear trend over time. Results for other faunal groups are summarized in Table 1. Although this dataset is too limited to reveal consistent trends over time, it does show that ^{15}N-enrichment of these animals was similar to that of the nematodes and polychaetes and that they remained well labeled up to 30 days after injection.

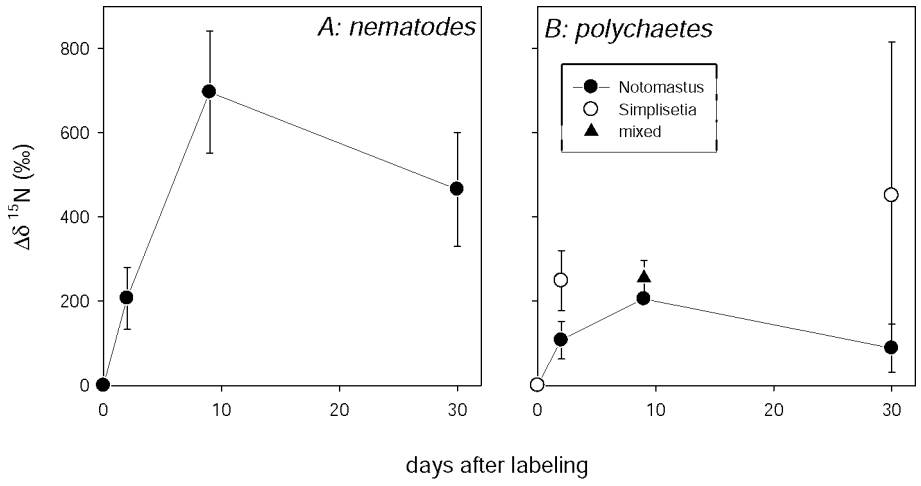


Figure 5. ^{15}N-enrichment ($\Delta\delta^{15}N$) of nematodes (A) and polychaetes (*Notomastus estuarius*, *Simplisetia aequisetis* and a mix of these two) (B). Error bars indicate standard deviation, n = 6-8 for nematodes and 1-3 for polychaetes.

Table 1. $\delta^{15}N$ values for fauna not included in figure 5 (natural abundance $\delta^{15}N$: 5-20‰). For comparison: $\delta^{15}N$ values of bulk sediment and THAAs were 60-160‰ and estimated $\delta^{15}N$ values for living microbial biomass were ~ 1000-1800‰.

				$\delta^{15}N$ (‰)		
Phylum	Class:Order	Family	Genus + species	day 2	day 9	day 30
63-500 µm fraction						
Arthropoda	Crustacea: Copepoda	Harpacticoida			110	21-53
Arthropoda	Crustacea: Ostracoda				194	
Annelida	Polychaeta	Nereididae + Spionidae		134		
Annelida	Oligochaeta				178	
Kinoryncha					36	
> 500 µm fraction						
Mollusca	Bivalvia	Galleomatidae	Arthritica helmsi (mussel)	991		174
Annelida	Polychaeta	Eunicidae		38-64		
Arthropoda	Crustacea: Decapoda	Ocypodidae	Macrophthalmus sp. (crab)			261
Annelida	Polychaeta (mixed)					462

DISCUSSION

Benthic fluxes

Benthic fluxes in the Brunswick Estuary and other subtropical Australian estuaries have already been studied and discussed extensively (e.g. Ferguson et al. 2003, 2004, Eyre and Ferguson 2005, 2006). Therefore, the present discussion primarily deals with those aspects that are relevant for interpretation of the ^{15}N-labeling work.

The benthic fluxes of DO and DIC provide information on the overall metabolism of the sediment. The net efflux of DIC from the sediment indicates that the sediment was net heterotrophic (i.e. DIC release from organic matter degradation exceeded DIC consumption for primary production). In addition, the fact that DO fluxes were quite well balanced while DIC fluxes were not, suggests that organic matter was partly degraded via anaerobic pathways. These results are consistent with those from more extensive studies on the benthic metabolism of the Brunswick Estuary (Ferguson et al. 2003, 2004, Eyre and Ferguson 2005).

The benthic nitrogen fluxes provide some insight in benthic nitrogen cycling. In general, measured nitrogen fluxes (Fig. 2) were comparable with those previously measured for the upper Brunswick Estuary (Eyre and Ferguson 2005). The strong efflux of NH_4^+ in the dark most likely consisted of NH_4^+ produced during organic matter degradation. In the light, this production likely continued, but simultaneous with NH_4^+ uptake, which resulted in a net influx of NH_4^+ in the light. The light dependence of the NH_4^+ uptake indicates that this influx was due to uptake by phototrophs (BMA and/or cyanobacteria). The influx of NO_3^- in the light as well as the dark indicates steady consumption in the sediment. This may have included uptake of NO_3^- as a N source for microbial growth (both algae and bacteria) as well as the use of NO_3^- for denitrification ($NO_3^- \rightarrow N_2$). The latter is consistent with the continuous efflux of N_2. However, average efflux rates for N_2 were about two times higher than influx rates of NO_3^-, indicating that denitrification not only used NO_3^- from the water column, but also NO_3^- produced in the sediment via nitrification ($NH_4^+ \rightarrow NO_3^-$) using NH_4^+ produced during organic matter degradation. The net efflux of DON probably involved loss of DON produced during organic matter degradation and possibly grazing of BMA. The strong variation in DON fluxes may partly have been due the indirect way in which DON fluxes were calculated ($DON = TDN - [NH_4^+ + NO_3^-]$).

During this study, the sediment was a net source of dissolved nitrogen to the water column, which contrasts with the general concept of sediments in the subtropical Brunswick Estuary being a sink for nitrogen (Ferguson et al. 2004, Eyre and Ferguson 2005). However, the heterotrophic sediments in the upper Brunswick Estuary have been found to be a DN source before, in particular following flood events (like in the present study) that stimulate phytoplankton blooms in the water column which results in increased deposition of phytodetritus to the sediments and subsequent release of dissolved nitrogen to the overlying water (Eyre and Ferguson 2006).

In previous benthic flux studies, fluxes of remineralized nitrogen have been compared with those of remineralized carbon in the form of a remineralized C:N ratio. Comparison of this ratio with that of the organic matter being degraded often yielded relatively high remineralized C:N ratios, suggesting a relatively strong retention of remineralized nitrogen within the sediment (Ferguson et al. 2004, Eyre and Ferguson 2005). Similar results were also obtained for other coastal sediments (e.g., Lomstein et al. 1998, Anderson et al. 2003, Cook et al. 2004, Sundbäck et al. 2004). For the present study, comparison of the efflux of remineralized carbon (net DIC flux) with that of remineralized nitrogen (net DIN + N_2 flux) yielded remineralized C:N ratios of 33, 7 and 12 for weeks 1, 2 and 3 respectively (17 on average). These values are similar to the C:N ratio of the total organic matter in the bulk sediment (19) which consisted of bacteria (C:N ~ 5), algae (C:N ~ 7 to 20), bacterial- and algal remnants (C:N higher than that of living organisms) and material from plants and trees surrounding the estuary (C:N of 25-500, Cowie and Hedges 1992). Therefore, it seems that effluxes of remineralized carbon and nitrogen were more or less balanced during the sampling period. This absence of preferential retention of nitrogen within the sediment may have been due to increased nitrogen availability following the small flood event in the week before the start of the sampling period.

^{15}N core incubations: total ^{15}N incorporation

Core incubations with the different ^{15}N-labeled substrates were performed to investigate the potential importance of DIN (NH_4^+ and NO_3^-) and labile DON (urea and amino acids) as nitrogen sources for the microbial community, focusing on the relative contributions by bacteria versus BMA to total microbial incorporation of the nitrogen from these substrates. It was assumed that ^{15}N incorporation in these short term core incubations primarily involved direct incorporation of ^{15}N from the added ^{15}N-substrates by the microbial community (bacteria and/or BMA). ^{15}N incorporation into faunal biomass was considered negligible given that this mainly occurs indirectly via grazing of ^{15}N-labeled microbial biomass which causes a delay of more than 24 h (see ^{15}N plots).

Excess ^{15}N in the bulk sediment (Fig. 3A) can include added ^{15}N-substrates (dissolved in pore water and/or bound to the sediment) and ^{15}N incorporated in microbial biomass as well as possible ^{15}N-compounds formed by degradation of ^{15}N-labeled biomass, although the latter was probably not very relevant for the short-term core incubations. The differences in bulk excess ^{15}N between cores injected with different ^{15}N-substrates roughly reflect differences in added amounts of ^{15}N since two times more ^{15}N was added to the urea cores than to the other cores. The relatively low bulk excess ^{15}N for NO_3^- and NH_4^+ cores (in comparison to values for the AA-mix cores and 50% of those for the urea cores) may have been due to loss of some $^{15}N_2$ from the sediment by nitrification-denitrification.

The best indication of total ^{15}N incorporation into benthic microbial biomass is provided by ^{15}N incorporation into THAAs as these represent proteinaceous biomass, a substantial fraction of total microbial biomass. In the ^{15}N cores, excess ^{15}N in THAAs was 14-27% of bulk excess ^{15}N after 6 h and 19-35% after 24 h. Assuming that THAAs make up about 50-60% of total bacterial biomass (Simon and Azam 1989, Cowie and Hedges 1992) and 60-80% of algal biomass (Cowie and Hedges 1992) (i.e. in case of full incorporation of ^{15}N into microbial biomass, excess ^{15}N in THAAs should be 50-80% of bulk excess ^{15}N), these values suggest that a substantial fraction (roughly half) of the added ^{15}N had been incorporated into microbial biomass after 24 h. Moreover, the relatively high fraction of bulk ^{15}N incorporated into THAAs for cores injected with the ^{15}N-AA-mix and $^{15}NH_4^+$ and the relatively low values for cores injected with ^{15}N-urea and $^{15}NO_3^-$ are consistent with the general microbial preference for amino acids and NH_4^+ as nitrogen sources (e.g., McCarthy et al. 1977, Kirchman 1994 and Chapter 1). However, differences between the ^{15}N-substrates were only small and may have included bias from differences between uptake versus incorporation, adsorption to the sediment,

conversion to other forms of nitrogen and combinations of these points. Therefore, the main point here is that the four substrates were used as nitrogen source by the benthic microbial community to a similar extent, which confirms that the microbial community was nitrogen limited (i.e. all available nitrogen was rapidly incorporated) even though benthic nitrogen fluxes suggested that the system was not as nitrogen limited as it can be (see above).

^{15}N core incubations: ^{15}N in bacteria

Studies on benthic nitrogen cycling in shallow coastal systems tend to focus on BMA as the main microbial group responsible for uptake and incorporation of dissolved nitrogen. Given that BMA are generally concentrated at the sediment surface, they may well be the main group responsible for uptake and incorporation of nitrogen from the overlying water. However, bacteria are also an abundant microbial group in terms of biomass (as confirmed by the similar biomass estimates for bacteria and BMA in the present study) and they also need nitrogen to support their growth. Therefore, bacteria should also be taken into account when investigating microbial nitrogen uptake and incorporation, especially in studies like the present one that includes deeper sediment where bacteria are relatively abundant. Moreover, the bacterial contribution to total microbial nitrogen incorporation may be relatively important in subtropical, mesotrophic systems like the Brunswick Estuary in comparison to that in temperate, eutrophic systems such as the Scheldt Estuary (see chapters 2, 3 and 5) since the benthic microbial community in these systems is relatively dependent on nitrogen from organic matter degradation within the sediment and because bacterial abundance and activity are relatively high in (sub)tropical sediments (Alongi 1994).

For the present study, ^{15}N incorporation by bacteria is represented by ^{15}N incorporation into the bacterial biomarker D-Ala. The best indication of the bacterial contribution to total microbial ^{15}N incorporation is provided by the ratio between excess ^{15}N in D-Ala versus that in L-Ala, a common protein amino acid present in all organism. Excess ^{15}N D/L-Ala ratios for the ^{15}N core incubations (Fig. 3D) were well above the racemization background (i.e. ^{15}N in D-Ala represented ^{15}N incorporated in bacteria) and within the range of bacterial D/L-Ala ratios (see update of Chapter 2). Highest excess ^{15}N D/L-Ala ratios (0.1-0.12) occurred after 6 h and are amongst the highest values measured in ^{15}N- and ^{13}C-labeling studies so far. These high D/L-Ala ratios indicate a substantial contribution by Gram positive (G+)

bacteria and/or cyanobacteria to total microbial ^{15}N incorporation during the first 6 h of incubation since both have a relatively high D/L-Ala ratio (related to their cell wall structure, see update of Chapter 2). G+ bacteria have been shown to contribute up to 30% of the total bacterial community in deeper, anaerobic sediment from a subtropical Australian coastal embayment (Moriarty and Hayward 1982), indicating that they may have been equally abundant in the sampled 0-6 cm layer of the ^{15}N-cores. The presence of cyanobacteria in the Brunswick sediment is also likely given the high temperatures and nitrogen limited conditions and was confirmed by the presence of the cyanobacterial pigment zeaxanthin. Unfortunately, the quantitative contribution of cyanobacteria to total photosynthetic biomass remains unclear since zeaxanthin concentrations could not be quantified properly. Although available data do not allow quantitative assessment of the contributions by these different bacterial groups, the high excess ^{15}N D/L-Ala ratios clearly show that bacteria dominated the initial incorporation of ^{15}N from all ^{15}N-substrates (probably with a substantial contribution by G+ and/or cyanobacteria). The subsequent rapid decrease in excess ^{15}N D/L-Ala ratios between 6 h and 24 h was considerable (Fig. 3D), especially for the cores injected with the ^{15}N-AA-mix, and was the combined effect of a loss of ^{15}N from D-Ala (Fig. 3C) and a continued increase of excess ^{15}N in THAAs, including L-Ala (Fig. 3B). The net loss of ^{15}N from D-Ala between 6 h and 24 h indicates rapid turnover of the bacteria that dominated ^{15}N incorporation during the first 6 h. Such rapid turnover of bacterial biomass is consistent with bacterial turnover times of < 1 day reported for (sub)tropical intertidal sediments (Alongi 1994). The continued increase of excess ^{15}N in THAAs between 6 h and 24 h indicates net ^{15}N incorporation into total microbial biomass up to 24 h. Given the net loss of ^{15}N from bacteria with a high D/L-Ala ratio, this implies that bacteria with a lower D/L-Ala ratio (likely Gram negatives (G–), see update of Chapter 2) and/or BMA (corresponding to D/L-Ala ratio around racemization background) accounted for this continued ^{15}N incorporation. Moreover, it seems that these groups were characterized by slower ^{15}N incorporation and/or biomass turnover. Although this uncertainty about the contributions by G– bacteria and/or BMA prohibits precise quantification of the bacterial contribution to total microbial ^{15}N incorporation, it is possible to estimate a minimal and maximal bacterial contribution (corresponding to bacterial D/L-Ala ratios of 0.1 and 0.05 respectively). This yields bacterial contributions of 80-100% for $^{15}NH_4^+$ cores, 55-100% for cores injected with $^{15}NO_3^-$ and ^{15}N-urea and 35-100% for the ^{15}N-AA-mix cores over 24 h. Therefore, the measured excess ^{15}N D/L-Ala ratios point to a major bacterial contribution to total microbial ^{15}N incorporation.

In situ ^{15}N plots: methodological aspects

Labeling of the in situ sediment with $^{15}NH_4^+$ was undertaken to investigate incorporation of nitrogen from pore water NH_4^+ into bacteria and BMA directly in the field and the subsequent fate of the incorporated nitrogen during the following days and weeks, including a potential transfer to fauna. $^{15}NH_4^+$ was selected as ^{15}N-substrate since NH_4^+ is an abundant form of dissolved nitrogen in the sediment pore water (e.g. Ferguson et al. 2004) and because of its central role in benthic nitrogen cycling and microbial nitrogen uptake.

One day after injection of the $^{15}NH_4^+$, ~ 50% of total added ^{15}N was recovered in the bulk sediment, indicating that the other ~ 50% had been lost from the sediment during the first 24 h. Most of this loss probably involved loss of $^{15}NH_4^+$ to the overlying water since the plots were submerged within 1 h after injection. Additional losses may have included loss of ^{15}N to sediment outside the plots and/or to sediment below the 8 cm sampling depth as well as loss of $^{15}N_2$ resulting from coupled nitrification-denitrification. However, these initial losses did not interfere with the aim of the study since it only deals with the ^{15}N that was retained in the upper 8 cm of the sediment plots. Another technical point concerns the difference in excess ^{15}N between the 0-0.2 cm layer and the whole 0-8 cm layer (Fig. 4A-C) which mainly reflects a relatively high initial $^{15}NH_4^+$ concentration in the surface sediment due to upwelling of $^{15}NH_4^+$-rich water during injection (directly from injection holes and indirectly through worm burrows etc.).

Although ambient concentrations of NH_4^+ in the pore water were not measured for the present study, the added $^{15}NH_4^+$ concentration of ~ 300 µM was within the range of concentrations of free NH_4^+ (100-500 µM) and freeze lysable NH_4^+ (100-1000 µM) reported for Brunswick sediments (Ferguson et al. 2004) while total ambient NH_4^+ concentrations (including NH_4^+ bound to the sediment) were probably even higher. Moreover, the sediment also contained other forms of bioavailable nitrogen, including NO_x^- and DON as well as nitrogen in microbial biomass (see discussion below), where concentrations of the latter were an order of magnitude higher than the added $^{15}NH_4^+$ concentration. Therefore, addition of $^{15}NH_4^+$ did not cause a major increase in total bioavailable nitrogen in the sediment, meaning that measured ^{15}N fluxes can be considered as representative for ambient nitrogen fluxes.

In situ ^{15}N plots: total ^{15}N incorporation

The strong and rapid incorporation of ^{15}N in THAAs (Fig. 4B) indicates substantial incorporation of ^{15}N into microbial biomass (see preceding discussion of ^{15}N-core incubation for details on interpretation). Assuming that THAAs make up 50-80% of total microbial biomass microbial (Simon and Azam 1989, Cowie and Hedges 1992), the excess ^{15}N THAA : bulk ratios of 33% and 46% after 4 days for the 0-8 cm layer and 0-0.2 cm layer respectively indicates that a large fraction (about 40% to 90%) of bulk ^{15}N was present in microbial biomass. After 30 days, the excess ^{15}N THAA : bulk ratio had increased even further to 43% for the 0-8 cm layer and 51% for the 0-0.2 cm layer, which corresponds to about 50% to 100% of bulk ^{15}N being present in microbial biomass. Given that ^{15}N was probably mainly present in bacteria (see next section) that appear to have a somewhat lower THAA content (50-60%, Simon and Azam 1989, Cowie and Hedges 1992) than algae (60-80%, Cowie and Hedges 1992) the highest estimates above are probably the most realistic (i.e. the majority of total ^{15}N in the sediment was present in microbial biomass). The remaining fraction of total ^{15}N that was not present in microbial biomass probably consisted of added $^{15}NH_4^+$ (especially during the first few days) and possibly some ^{15}N-labeled material (PON, DON, DIN) resulting from degradation of ^{15}N-labeled microbial biomass (see below).

The most striking point from figure 4 is the strong retention of ^{15}N in the whole 0-8 cm layer, especially in THAAs (Fig. 4B). Theoretically, this strong retention may have been due to retention in the organisms that were responsible for the initial incorporation of ^{15}N from $^{15}NH_4^+$. However, this is unlikely given the rapid turnover of bacteria and BMA in (sub)tropical sediments like those in the Brunswick Estuary (Alongi 1994, Ferguson et al. 2004) which likely resulted in rapid loss of ^{15}N from the original ^{15}N-labeled microbial biomass. Another theoretical explanation for the strong retention of ^{15}N in THAAs is accumulation of ^{15}N-labeled microbial remnants (cell components that escaped degradation). However, accumulation of a substantial pool of ^{15}N-labeled remnants is unlikely for the present study given the high temperatures and microbial activity which likely resulted in rapid degradation of the majority of dead microbial biomass. This was confirmed by the fact that the composition of the ^{15}N-labeled THAA pool was stable during the whole 30-day sampling period (data not shown) while a change in its composition would be expected in case of accumulation of a substantial pool of labeled microbial remnants (see Chapter 5). Therefore, the most likely explanation for the strong retention of ^{15}N in THAAs is retention of ^{15}N in living microbial

biomass due to uptake of ^{15}N-DON and/or ^{15}N-DIN released during degradation of ^{15}N-labeled biomass and subsequent incorporation into new microbial biomass (i.e. recycling of ^{15}N). The fact that there was no net loss of ^{15}N from THAAs during the 30-day sampling period suggest that recycling efficiency was near 100%. This very high efficiency may not be sustained over longer periods of time (months to years) since nitrogen can be lost from the sediment during periods of increased disturbance of the sediment, for example due to bioturbation by fauna or periodical flood events (Eyre and Ferguson 2006) or during periods with a net decrease of microbial biomass. However, the ^{15}N results clearly show that recycling of nitrogen by the microbial community within the sediment can serve as a nitrogen sink (without net accumulation of nitrogen) during a period of several weeks or even months. This confirms the previously hypothesized immobilization of nitrogen in microbial biomass based on the relatively low release of remineralized nitrogen in benthic flux studies for the Brunswick Estuary (Ferguson et al. 2004, Eyre and Ferguson 2005) and other coastal sediments (e.g., Lomstein et al. 1998, Anderson et al. 2003, Cook et al. 2004, Sundbäck et al. 2004) and indicates that recycling of nitrogen within the sediment can be an important pathway supporting benthic microbial production, especially in nitrogen limited systems like the Brunswick Estuary.

Contrary to the strong retention of ^{15}N in the whole 0-8 cm layer, there was a clear decrease of excess ^{15}N in the 0-0.2 cm layer. This decrease may have been due to loss of dissolved ^{15}N-compounds and/or ^{15}N-labeled biomass from the sediment surface to the overlying water as well as due to deposition of new (unlabeled) sediment on top of the sediment plots. The latter was confirmed by the observed continuous deposition of sediment on the frames surrounding the plots. This deposition effectively "buried" the original (labeled) 0-0.2 cm layer, resulting in a gradual decrease in excess ^{15}N in the upper 2 mm of the sediment over time.

In situ ^{15}N plots: ^{15}N in bacteria

Different microbial groups may have been involved in the incorporation and recycling of ^{15}N in the in situ sediment, including BMA (with a D/L-Ala ratio around racemization background), heterotrophic bacteria (D/L-Ala ratio of 0.05-0.1, depending on the proportions of G– versus G+ bacteria) and cyanobacteria (D/L-Ala ratio of 0.07-0.1) (see discussion ^{15}N cores and update of Chapter 5). A fourth group that may be relevant in experiments like our ^{15}N in situ plots is the benthic fauna (with a D/L-Ala ratio around racemization background). However, a

quick "back of the envelope" calculation showed that excess ^{15}N in fauna was only a negligible fraction of ^{15}N in total benthic biomass (see next section), meaning that total ^{15}N-labeled benthic biomass was dominated by bacteria and/or algae. Despite the uncertainty regarding the bacterial groups involved in ^{15}N incorporation, the excess ^{15}N D/L-Ala ratios (Fig. 4D) provided valuable information about the bacterial contribution to total microbial incorporation and retention of the ^{15}N. The high excess ^{15}N D/L-Ala ratio for the 0-8 cm layer one day after labeling (~ 0.08) was similar to that measured in the cores incubated with $^{15}NH_4^+$ after 24 h (Fig. 3D). This high value indicates that total (microbial) ^{15}N incorporation during the first day was dominated by bacteria, probably with a large contribution by G+ bacteria and/or cyanobacteria (see discussion ^{15}N cores). The decrease in excess ^{15}N D/L-Ala ratio for the 0-8 cm layer up to day 9 while excess ^{15}N in total (microbial) biomass (THAAs) remained stable suggests a net transfer of ^{15}N to bacteria with a lower D/L-Ala ratio and/or BMA during recycling of the ^{15}N. As excess ^{15}N D/L-Ala ratios leveled off at ~ 0.05 for the 0-8 cm layer after day 9 and were stable at ~ 0.06 for the 0-0.2 cm layer, which is still within the range of bacterial D/L-Ala ratios, these values point to a major bacterial contribution to total microbial ^{15}N incorporation. Estimation of a minimum and maximum bacterial contribution (corresponding to bacterial D/L-Ala ratios of 0.1 and 0.05 respectively, see discussion ^{15}N cores) yielded a bacterial contribution between 40% and 100%, leaving a contribution by BMA between 0% and 60%. Although available data do not allow further elucidation, we suspect that the actual bacterial contribution was closer to 100% than to 40%. A large contribution by BMA seems counterintuitive, especially for the whole 0-8 cm layer, because active BMA are typically concentrated at the sediment surface while bacteria are present throughout the whole 0-8 cm, meaning that bacteria likely predominated in most of the 0-8 cm layer. Although our biomass estimates yielded roughly similar estimates for phototrophs (based on Chl *a* concentrations) and heterotrophic bacteria (based on bacteria-specific PLFA concentrations), these estimates may not reflect the biomass of BMA versus bacteria due to the presence of cyanobacteria which are included in phototrophic biomass and not in bacterial biomass. Additional bias may have resulted from the presence of Chl *a* in dead organic matter such as plant litter next to Chl *a* in active BMA. Another point that contradicts a large contribution by BMA is that bacteria are generally considered to mediate organic matter degradation and to be better at utilizing nutrients at low concentrations (Goldman and Dennett 2001, Kirchman

1994 and references therein). Since ^{15}N incorporation during the last three weeks of the sampling period mainly involved recycling of ^{15}N that was gradually released during degradation of ^{15}N-labeled biomass (i.e. low concentrations), one would expect ^{15}N incorporation during this period to have been dominated by bacteria.

In situ ^{15}N plots: ^{15}N in fauna

^{15}N was traced into fauna because faunal biomass may serve as a temporary sink for bacterial- and/or algal-derived nitrogen (via grazing) since the turnover of faunal biomass is considerably slower than that of microbial biomass. Moreover, transfer of nitrogen from microbial biomass to grazers is the first step in a potential nitrogen-flow to higher tropic levels.

Extraction and ^{15}N-analysis of fauna had an experimental character since little was known about faunal densities in the sediment and the required numbers of individuals for ^{15}N analysis of small fauna such as nematodes. Recovered faunal densities may have been somewhat lower than actual densities in the sediment due to the relative coarse extraction method that was used. However, recovered polychaetes densities are within the range of densities reported for subtidal sediment in the upper Brunswick Estuary (Eyre and Ferguson 2005) and recovered nematode densities are comparable with densities reported for other intertidal sediments (e.g. Steyaert et al. 2001), suggesting that recovered densities did not severely underestimate actual densities.

The clear ^{15}N enrichment of animals from different faunal groups with $\Delta\delta^{15}N$ values in the same range as those for bulk sediment and HAAs (Fig. 5 and Table 1) indicates substantial incorporation of ^{15}N into fauna. Moreover, the delay in ^{15}N enrichment of faunal biomass compared to that of the bulk sediment and THAAs confirms that ^{15}N incorporation into fauna did not occur via direct incorporation of ^{15}N from $^{15}NH_4^+$ but indirectly via grazing on ^{15}N-labeled microbial biomass.

In order to determine the contribution of ^{15}N-labeled microbial biomass to the total nitrogenous diet of the animals from figure 5, a simple isotope model (Hamilton et al. 2004) was used to simulate the dynamics of $\Delta\delta^{15}N$ values for microbial- and faunal biomass under the non steady-state situation in the present experiment. This approach is described in detail for an in situ ^{13}C-labeling study similar to the present study by van Oevelen et al. (2006b). $\Delta\delta^{15}N$ values for living microbial biomass were estimated from those for the HAAs by calculating the fraction of THAAs in the whole sediment present in living microbial biomass (derived from biomass estimates of phototrophs (Chl *a*) + heterotrophic bacteria (PLFAs)). This

indicated that ~ 7% of THAAs was present in microbial biomass, meaning that $\Delta\delta^{15}N$ values for HAAs in living biomass were ~ 14 times higher than those for the HAAs in the whole sediment (i.e. $\Delta\delta^{15}N$ for "living" HAAs was ~ 1000-1800‰). Comparison of microbial- and faunal $\Delta\delta^{15}N$ values, taking into account their dynamics over time, yielded an estimated dependence on ^{15}N-labeled microbial biomass (likely dominated by bacteria) of 42 ± 12% for nematodes, 12 ± 4% for *Notomastus estuarius* and 37 ± 12% for *Simplisetia aequisetis*. The low contribution for *Notomastus* is comparable with previously reported low (< 20%) dependence on bacterial carbon for various faunal groups (see van Oevelen et al. 2006b and references therein) while values for *Simplisetia* and especially those for the nematodes are relatively high. This relatively high contribution may have been due to preferential incorporation of microbial nitrogen over carbon and/or due to the relatively high bacterial biomass in (sub)tropical sediments like in the present study (Alongi 1994). However, results are too limited for further discussion.

In order to clarify the importance of the ^{15}N transfer to fauna as part of the total ^{15}N budget, we made a quick "back of the envelope" calculation of total excess ^{15}N incorporated into faunal biomass using measured densities of nematodes and polychaetes and nitrogen contents of 50 ng-N individual^{-1} for nematodes and ~ 100 µg-N individual^{-1} for polychaetes (derived directly from EA-IRMS analyses). Resulting estimates were 0.2-0.5 µmol ^{15}N plot^{-1} in nematodes and ~ 2 µmol ^{15}N plot^{-1} in the polychaetes. Together, these estimates are ~ 0.2% of total excess ^{15}N in the plots. Even when taking into account the ^{15}N in other faunal groups (Table 1) and the possible underestimate of actual densities, it seems that only a very small fraction (< 1%) of total microbial ^{15}N was transferred to faunal biomass via grazing. This is consistent with results from other studies in which only a small fraction (typically no more than 20-25%) of bacterial production is consumed by fauna (Kemp 1990, van Oevelen et al. 2006a). The very low values for the present study may be related to the high temperatures and high organic matter content of the sediment which results in very high bacterial biomass and productivity relative to faunal biomass and grazing rates (Alongi 1994).

Altogether, the ^{15}N-fauna results show that, even though ^{15}N-labeled microbial biomass was a moderate to substantial part of the faunal diet, ^{15}N incorporated into faunal biomass was only a very small fraction of total ^{15}N in biomass. These results suggest, at least for the present study, a minor role for nitrogen flows through fauna in total benthic nitrogen cycling.

Conclusions

The combination of ^{15}N-labeling with analysis of ^{15}N incorporation into HAAs, including D-Ala, allowed us to clarify the nitrogen flows within the sediment of the upper Brunswick Estuary. Results revealed a strong retention of nitrogen within the sediment due to very efficient recycling of nitrogen by the benthic microbial community with a major contribution by bacteria and a negligible role for fauna. These finding also indicate that recycling of nitrogen within sediments can be an important pathway supporting benthic microbial production, especially in nitrogen limited systems like the Brunswick Estuary.

Acknowledgments

We thank Lexie Walker for doing the fauna extractions, Iain Alexander for the nutrient analysis, alkalinity titrations and N_2/Ar analysis, Peter van Breugel for analysis of ^{15}N in bulk sediment and fauna by EA-IRMS, Marco Houtekamer for his help with the GC-c-IRMS analyses, Nicole Dijkman for the PLFA extractions, Cobie Kleppe for the pigment analyses and Dick van Oevelen for the model simulations of ^{15}N in fauna. This research was supported by the Netherlands Organisation for Scientific Research (PIONIER 833.02.2006) and an ARC Discovery Grant (DP0342956).

C H A P T E R 5

Fate of peptidoglycan in an intertidal sediment: An in situ ^{13}C-labeling study

Bart Veuger, Dick van Oevelen, Henricus T.S. Boschker and Jack J. Middelburg

Limnology and Oceanography 51 (2006): 1572-1580

ABSTRACT

We investigated the fate of peptidoglycan, a bacterial cell wall component, in sediment by ^{13}C-labeling the bacterial community of an intertidal mudflat and subsequently tracing the fate of ^{13}C in D-alanine (D-Ala, a bacterial biomarker specific for peptidoglycan), bacteria-specific phospholipid-derived fatty acids (PLFAs, specific for cell membranes of living bacteria) and total hydrolysable amino acids (THAAs, representing bulk proteinaceous material) over a 4.5-month period in situ. Results showed a relatively slow loss of ^{13}C from D-Ala (half lives of 20-67 days) compared with that from bacteria-specific PLFAs (half lives of 13-33 days) during the sampling period and a relatively strong retention of ^{13}C in D-Ala compared with the other compounds after 4.5 months. This provides direct in situ evidence for relative accumulation of peptidoglycan during reworking and degradation of bacterial biomass in sediments. Degradation also resulted in compositional changes of the ^{13}C-THAA pool, including increased relative abundances of glycine, serine and proline.

INTRODUCTION

Bacteria play a central role in ecological and biogeochemical processes in marine sediments. Not only do bacteria dominate degradation of organic matter but bacterial biomass itself is also an important active pool of organic carbon (OC) in the sediment. Bacterial numbers in sediments are relatively invariant (Schmidt et al. 1998) because bacterial production is balanced by loss of bacterial biomass by processes such as cell death (e.g., viral lysis) and consumption of bacteria by bacterial grazers. Bacterial cell death produces bacterial remnants consisting of a variety of components that are prone to degradation. Some of these components will be readily degraded, whereas others are more resistant to degradation. One of the more refractory components is peptidoglycan, a unique constituent of bacterial cell walls. Laboratory studies on the degradation of peptidoglycan in bacterial cultures (Jørgensen et al. 2003) and seawater samples (Nagata et al. 2003) showed that peptidoglycan is degraded slower than proteins and can be characterized as semilabile. Peptidoglycan contains D-amino acids (D-AAs) which are specific for peptidoglycan. The abundance of these D-AAs in aquatic systems is usually reported relative to the abundance of their respective L-stereoisomers as D/L-AA ratios where L-AAs are common protein amino acids occurring in all organisms. Studies on the abundance of D-AAs in seawater dissolved organic matter (DOM) revealed high D/L-AA ratios (McCarthy et al. 1998) and increasing D/L-AA ratios with increasing degradation (Dittmar et al. 2001, Amon et al. 2001) suggesting a relative accumulation of peptidoglycan. Similarly, studies on the abundance of D-AAs in marine sediments showed increasing D/L-AA ratios with increasing depth, which has also been attributed to selective preservation of peptidoglycan during organic matter degradation (Pedersen et al. 2001, Grutters et al. 2002). However, these D/L-AA ratios typically reflect the net result of reworking and degradation of organic matter over long periods of time (10^1-10^4 years) and do not provide direct information on production and degradability of peptidoglycan. Conversely, laboratory studies of the degradation of peptidoglycan in vitro allow direct assessment of degradation rates, but resulting rates cannot directly be translated to degradation in the field since in situ degradation is dependent on various additional processes such as interactions with other compounds and/or particles (Borch and Kirchman 1999, Arnarson and Keil 2005). Moreover, retention of bacterial biomass and remnants, including peptidoglycan,

in the field is not only determined by degradation, but also by other processes such as grazing and resuspension.

This study aims to clarify the fate of bacterial carbon, with particular interest on peptidoglycan, in an intertidal sediment. To do this, we linked the two existing lines of research (degradation of peptidoglycan in vitro versus analysis of D-AA concentrations and D/L-AA ratios in situ) by in situ ^{13}C-labeling of the bacterial community of an intertidal sediment and subsequent analysis of the fate of the ^{13}C-labeled bacterial biomass during a 4.5-month sampling period. Comparison of the fate of ^{13}C in the bacterial biomarker D-alanine (D-Ala, representing peptidoglycan) with that of ^{13}C in bacteria-specific phospholipid-derived fatty acids (PLFAs, representing living bacteria) and ^{13}C in total hydrolysable amino acids (THAAs, representing total proteinaceous material) allowed us to investigate the fate of peptidoglycan in comparison to other components from ambient bacteria under in situ conditions.

MATERIALS AND METHODS

Experimental setup

The material presented in this paper is part of a larger ^{13}C-labeling study (Van Oevelen et al. 2006a). In May 2003, two 0.25 m^2 sediment plots were selected at a silty part of the Molenplaat intertidal mudflat in the turbid, nutrient rich and heterotrophic Scheldt estuary (The Netherlands). Plots were confined by steel frames (8 cm deep) and the upper 10 cm of the sediment was injected with ^{13}C-glucose one injection per 6.25 cm^2 with ~ 0.4 ml of a ^{13}C-glucose solution (24 mmol ^{13}C l^{-1}) per injection) at days 0, 2, 3 and 4, resulting in a ^{13}C flux of 15.3 mmol ^{13}C m^{-2} per injection event. During the label addition period and in the days, weeks and months after label addition (up to 136 days) plots were sampled regularly by taking sediment cores (∅ 50 mm). Cores were sliced into three layers (0-2 cm, 2-5 cm and 5-10 cm) that were analyzed for total C and ^{13}C in the bulk sediment, pore water DIC, PLFAs and benthic fauna. Further details on the experimental setup and analytical procedures can be found in Van Oevelen et al. (2006a). In addition to these analyses, a selection of sediment samples from the 0-2 cm layer of plot 2 and the 2-5 cm layer of plots 1 and 2 were analyzed for ^{13}C in hydrolysable amino acids (HAAs) including bacterial biomarker D-Ala. Since this paper is largely based on the ^{13}C-HAA data, it only deals with the layers that were analyzed for ^{13}C in HAAs.

PLFA analyses

Lipids were extracted from 3 g of dry sediment in chloroform-methanol-water using a modified Bligh and Dyer method and fractionated on silica gel into different classes by polarity. The most polar fraction containing the PLFAs was derivatized by mild methanolysis yielding fatty acid methyl esters (FAMEs) that were analyzed by gas-chromatography-combustion-isotope ratio mass spectrometry (GC-c-IRMS), see Middelburg et al. (2000) and Boschker (2004) for details. Although PLFA analyses included a wide range of PLFAs, this paper primarily deals with results for bacteria-specific PLFAs i14:0, i15:0, a15:0, i16:0 and 18:1ω7c.

HAA analyses

^{13}C in HAAs, including D-Ala, was analyzed by GC-c-IRMS. Sample preparation and analyses were according to the protocol presented in Chapter 2. Briefly, samples (1 g) of freeze-dried sediment were washed with HCl (2 M) and Milli-Q water (removing dissolvable HAAs), followed by hydrolysis in HCl (6 M) at 110°C for 20 h. After purification by cation exchange chromatography, amino acids were derivatized with isopropanol (IP) and pentafluoropropionic anhydride (PFA) and samples were further purified by solvent extraction. Derivatized D- and L-amino acids were separated by gas chromatography using a Chirasil-L-Val column.

Concentrations were calculated from separate analyses with flame ionization detection (GC-FID) or directly from GC-c-IRMS analyses. A selection of samples was also analyzed with a quadrupole mass spectrometer (GC-MS) to verify peak identity and purity.

^{13}C abundance was measured by GC-c-IRMS and expressed as $\delta^{13}C$: $\delta^{13}C$ (‰) = $[(R_{sample} / R_{VPDB}) - 1] \times 1000$ where $R = {}^{13}C/{}^{12}C$ and $R_{VPDB} = 0.0112372$ (VPDB = Vienna Pee Dee Belemnite). During derivatization, extra (unlabeled) C atoms are added to the original amino acids, which changes their $\delta^{13}C$ (Silfer et al. 1991, Pelz et al. 1998). It is possible to correct for the effect of the added C (Silfer et al. 1991, Pelz et al. 1998) using the following mass-balance equation: $\delta^{13}C_{AA} = [\delta^{13}C_{DAA} \times (C_{AA} + C_{IP} + C_{PFA})] - [\delta^{13}C_{IP+PFA} \times (C_{IP} + C_{PFA})] / C_{AA}$, where C_{AA} = number of C atoms in the original amino acid, $_{DAA}$ = derivatized amino acid, C_{IP} = number of C atoms added by esterification with IP and C_{PFA} = number of C atoms added by acylation with PFA. However, the change in $\delta^{13}C$ during derivatization is also influenced by additional kinetic fractionation during acylation (addition of C from PFA). The effect of this fractionation depends on the %C derived from PFA (i.e. the number of added C atoms from PFA relative to the number of C atoms in the original amino acid). As this effect is not included in the mass balance equation, it requires empirical correction. This was done by measuring the $\delta^{13}C$ of three amino acid standards (D-Ala, D-Glu and D-Ser) before and after derivatization on an elemental analyzer coupled to an IRMS (EA-IRMS), which allowed us to determine empirical $\delta^{13}C_{IP+PFA}$ values for these three amino acids. These values showed a strong linear correlation with the %C from PFA, which is similar to results by Silfer et al. (1991). Subsequently, this correlation was used to determine empirical $\delta^{13}C_{IP+PFA}$ values for the other amino acids which were then used to calculate their original $\delta^{13}C$ values using the mass balance equation. Empirical $\delta^{13}C_{IP+PFA}$ values ranged between -52 and -45‰, which is lighter than those in e.g. Silfer et al. (1991) because we used PFA for acylation, which contains more C

than the trifluoroacetic anhydride (TFA) used by Silfer et al. (1991) and because the used PFA was very depleted in ^{13}C ($\delta^{13}C$: -55‰, measured by EA-IRMS).

Data treatment

$\delta^{13}C$ values for the different ^{13}C pools were calculated by the formula presented above for the HAAs. Subsequently, $\delta^{13}C$ values were used to calculate $\Delta\delta^{13}C$: $\Delta\delta^{13}C_{sample}$ (‰) = $\delta^{13}C_{\ sample}$ - $\delta^{13}C_{\ control\ sample\ (unlabeled)}$ and the atom percentage of ^{13}C: $at\%^{13}C = [100 \times R_{\ VPDB} \times ((\delta^{13}C_{\ sample} / 1000) + 1)] / [1 + R_{\ VPDB} \times ((\delta^{13}C_{\ sample} / 1000) + 1)]$, which was used to calculate excess ^{13}C (absolute amount of incorporated ^{13}C): excess $^{13}C = [(at\%^{13}C_{\ sample} - at\%^{13}C_{\ control}) / 100] \times$ AA-C concentration $_{sample}$. Concentrations of carbon and excess ^{13}C for the different OC pools are all expressed in moles C or ^{13}C per gram dry sediment.

D-Ala data were corrected for the formation of D-Ala from L-Ala by racemization during acid hydrolysis. Measured concentrations and excess ^{13}C values for D-Ala were corrected by subtracting 1.7% from concentrations and excess ^{13}C for L-Ala where 1.7% is based on the 1.5-2% range reported for liquid phase hydrolysis of proteins and microbial biomass (see Kaiser and Benner 2005 and update of Chapter 2).

Abundance of ^{13}C in D-Ala is also presented relative to that of ^{13}C in L-Ala as excess ^{13}C D/L-Ala ratios that were calculated as excess ^{13}C in D-Ala / excess ^{13}C in L-Ala using the measured excess ^{13}C in D-Ala (i.e., not corrected for hydrolysis-induced racemization). Instead, the racemization background (0.015-0.02) is indicated graphically. D/L-Ala ratios for concentrations were calculated the same way (i.e., also not corrected for racemization).

Loss rate constants for loss of ^{13}C from the different compounds after the ^{13}C-glucose injection period were determined by fitting the data with an exponential model by minimizing the sum of squared model–data deviations. Standard errors of the model parameters were estimated with the non-linear regression method in the free software package R (www.r-project.org).

RESULTS

Sediment organic carbon pools

Total OC in the sediment was ~ 400 ± 30 µmol-C g^{-1} for the 0-2 cm layer and ~ 600 ± 100 µmol-C g^{-1} for the 2-5 cm layers. A considerable fraction of total OC consisted of HAA-C with THAA-C concentrations of 94 ± 33 µmol C g^{-1} for the 0-2 cm layer and 54 ± 57 µmol C g^{-1} for the 2-5 cm layers. D-Ala was a stable fraction of the THAA pool with concentrations (corrected for racemization) of 0.35 ± 0.15 µmol C g^{-1} for the 0-2 cm layer and 0.19 ± 0.23 µmol C g^{-1} for the 2-5 cm layers and D/L-Ala ratios of 0.05-0.07 for all samples (not corrected for racemization). Bacterial biomass in the sediment plots was derived from concentrations of bacteria-specific PLFAs i14:0, i15:0, a15:0, i16:0 and 18:1ω7c. Summed concentration of these five bacteria-specific PLFAs were 292 ± 172 nmol C g^{-1} for the 0-2 cm layer and 77 ± 84 nmol C g^{-1} for the 2-5 cm layers. Assuming that bacteria-specific PLFAs make up 28% of total bacterial PLFAs and that total bacterial PLFA-C makes up 6% of total bacterial-C (Middelburg et al. 2000) yielded estimates of total bacterial-C of 27 ± 16 µmol-C g^{-1} for the 0-2 cm layer and 7 ± 7 µmol-C g^{-1} for the 2-5 cm layers. Concentrations of bacteria-specific PLFAs as well as THAAs and D-Ala were rather variable (data not shown), most likely reflecting spatial variability in (microbial) biomass. In addition to bacteria, fauna were an important pool of OC. Benthic macrofauna biomass (dominated by the bivalve *Macoma balthica* and the polychaetes *Heteromastus filiformis* and *Pygospio elegans*) in the upper 10 cm was 1624 mmol-C m^{-2} (13 µmol-C g^{-1} dry sediment) while meiofauna biomass (dominated by nematodes and foraminifera) was smaller at 188 mmol-C m^{-2} (1.5 µmol-C g^{-1} dry sediment) (see Van Oevelen et al. 2006a).

^{13}C in bacteria-specific PLFAs

Bacteria-specific PLFAs showed clear ^{13}C enrichment following the injection of ^{13}C-glucose with $\delta^{13}C$ values (up to 700‰) well above natural abundance $\delta^{13}C$ (-21 ± 2‰). Trends in excess ^{13}C values for the five individual bacteria-specific PLFAs were very similar and data are therefore presented as summed excess ^{13}C in the five bacteria-specific PLFAs (Fig. 1). Although 18:1ω7c can also be present in certain algae (Moodley et al. 2000), ^{13}C incorporation dynamics for 18:1ω7c were very similar to that for the other bacteria-specific PLFAs and different from that for diatom PLFA 20:5ω3 (data not shown), indicating that ^{13}C-labeled 18:1ω7c was bacterial. Excess ^{13}C in bacteria-specific PLFAs was highest at days 6 and 8 (directly

after the ^{13}C-glucose injection period) with highest values for the 0-2 cm layer. Average summed excess ^{13}C in the five bacteria-specific PLFAs for the period between days 6 and 18 (representing freshly labeled bacteria) was used to estimate excess ^{13}C incorporated in total bacterial biomass. Using the same conversion factors as for estimates of total bacterial biomass (see previous paragraph) yielded estimates of 107 nmol ^{13}C g^{-1} for the 0-2 cm layer and 18-23 nmol ^{13}C g^{-1} for the 2-5 cm layers.

After the labeling period, excess ^{13}C in the bacteria-specific PLFAs decreased exponentially with a rate constant of 0.053 (5.3% day^{-1}) for the 0-2 cm layer and 0.021-0.022 for the 2-5 cm layers. Corresponding half lives (ln(2)/k) were 13 days and 31-33 days respectively. At the end of the 136-day sampling period, ^{13}C-enrichment of the bacteria-specific PLFAs had decreased to near natural abundance ($\Delta\delta^{13}$C < 10‰). Corresponding excess ^{13}C values were 0.1% of the original excess ^{13}C in bacteria-specific PLFAs in the period directly after ^{13}C-glucose injection (day 6-18) for the 0-2 cm layer and 0.5-2% for the 2-5 cm layers. In addition to the bacteria-specific PLFAs, ^{13}C enrichment of diatom marker 20:5ω3 and fauna marker 20:4ω6 were examined to verify that ^{13}C glucose labeling specifically tagged bacteria. This was confirmed by $\Delta\delta^{13}$C values for both these markers that were an order magnitude lower than those for the bacteria-specific PLFAs (data not shown).

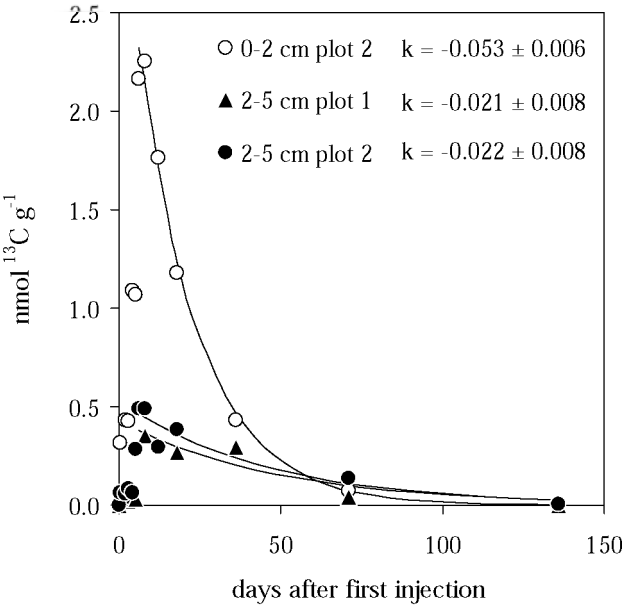


Figure 1
Excess ^{13}C in bacteria-specific PLFAs (summed excess ^{13}C in i14:0, i15:0, a15:0, i16:0 and 18:1ω7c). Lines are exponential fits for data between day 6 and day 136 (after label injection period). k-values are loss rate constants (± standard error).

^{13}C in THAAs

Hydrolysable amino acids (HAAs) were enriched in ^{13}C well above natural abundance (corrected natural abundance $\delta^{13}C$ values for individual HAAs ranged from -8‰ to -38‰) with corresponding $\Delta\delta^{13}C$ values up to 100-150‰. Trends for excess ^{13}C in the different individual HAAs were very similar, except for D-Ala which is presented separately below. Therefore, we only present excess ^{13}C in THAAs (i.e. summed excess ^{13}C in individual HAAs) (Fig. 2). Average excess ^{13}C in THAAs in the period between day 6 and day 18 was used to estimate excess ^{13}C incorporated in total bacterial biomass assuming that THAA-C makes up ~ 50% of total bacterial-C (Cowie and Hedges 1992; Madigan et al. 2000). Resulting estimates of ^{13}C incorporation into total bacterial biomass were 168 nmol ^{13}C g^{-1} for the 0-2 cm layer and 38-48 nmol ^{13}C g^{-1} for the 2-5 cm layers.

After the ^{13}C-glucose injection period, excess ^{13}C in THAAs in the 0-2 cm layer decreased exponentially with a rate constant of 0.030 (half life of 23 days) while excess ^{13}C in THAAs in the 2-5 cm layers remained at a stable level up to day 71 and showed a strong decrease between day 71 and day 136 (Fig. 2). At day 136, excess ^{13}C in THAAs was below the limit of detection for two of the three layers. Only the 2-5 cm layer of plot 2 still contained traces of excess ^{13}C in all HAAs (totaling 1.7 nmol ^{13}C g^{-1}) which corresponded to 9% of the original excess ^{13}C in THAAs in the period directly after ^{13}C-glucose injection (day 6-18). The relative composition of the ^{13}C-THAA pool in this layer after 136 days was clearly different from its composition during the first 71 days of the experiment with increased relative abundance of glycine (Gly), serine (Ser) and proline (Pro), decreased relative abundance of tyrosine (Tyr), threonine + valine (Thr+Val), leucine (Leu), isoleucine (Ile), L-alanine (L-Ala) and aspartic acid (Asp) and similar abundance of lysine (Lys) and glutamic acid + phenylalanine (Phe) (Fig. 3).

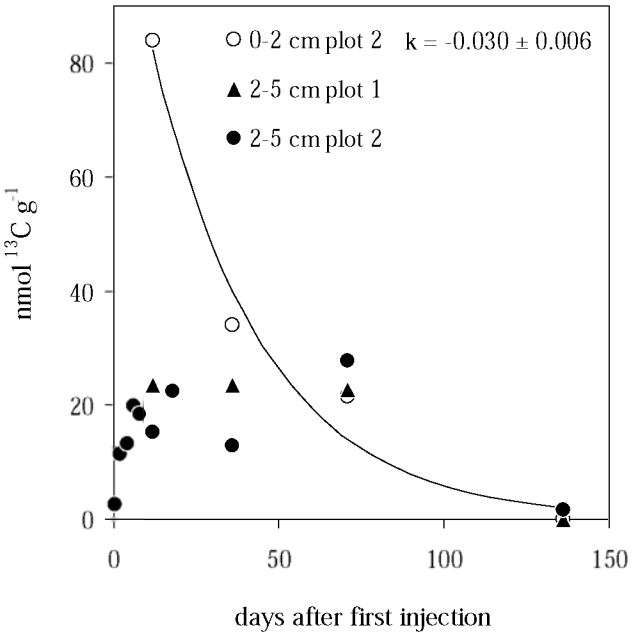


Figure 2
Excess ^{13}C in THAAs (summed excess ^{13}C in individual HAAs). Line is exponential fit for data between day 6 and day 136 (after label injection period) for 0-2 cm layer of plot 2. k-value is loss rate constant (± standard error).

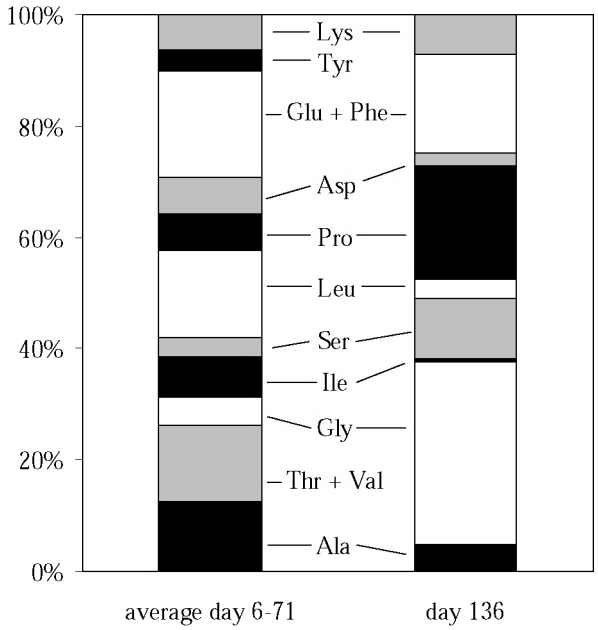


Figure 3
Composition of ^{13}C-THAA pool (relative contributions of excess ^{13}C in individual HAAs to summed excess ^{13}C in THAAs) for period between day 6 and day 71 versus day 136. All amino acids are L-AAs. Glu+Phe and Thr+Val are combined since their GC-c-IRMS peaks were not sufficiently separated.

^{13}C in D-Ala

Timing of ^{13}C incorporation into D-Ala during the label injection period was very similar to that for the other HAAs and the bacteria-specific PLFAs with $\Delta\delta^{13}C$ values within the range of values for the other HAAs (up to 140‰ with natural abundance $\delta^{13}C$ of -10 ± 7‰). Excess ^{13}C D/L-Ala ratios (not corrected for racemization) directly after the ^{13}C-glucose injection period were 0.05-0.07 for the 2-5 cm layers and 0.09 for the 0-2 cm layer (Fig. 4). Like for the bacteria-specific PLFAs and THAAs, excess ^{13}C in D-Ala for the period between day 6 and day 18 (corrected for racemization) was used to estimate excess ^{13}C incorporated in total bacterial biomass assuming: (1) a bacterial D/L-Ala ratio of 0.05 (0.07 - racemization background of ~ 0.02). (2) Ala being ~ 10% of the THAA pool (Cowie and Hedges 1992; Keil et al. 2000). (3) THAA-C being ~ 50% of total bacterial C (see previous section). Resulting estimates were 324 nmol ^{13}C g^{-1} for the 0-2 cm layer and 40-80 nmol ^{13}C g^{-1} for the 2-5 cm layers.

During the weeks and months after the ^{13}C-glucose injection period, excess ^{13}C in D-Ala decreased exponentially with a rate constant of 0.035 (half life of 20 days) for the 0-2 cm layer and 0.010-0.012 (half life of 58-67 days) for the 2-5 cm layers (Fig. 5). This more rapid decrease of excess ^{13}C in D-Ala relative to that of excess ^{13}C in other HAAs, including L-Ala, resulted in a decrease in excess ^{13}C D/L-Ala ratios between days 12 and 71 (Fig. 4).

After 136 days, excess ^{13}C in D-Ala was 4% of the original excess ^{13}C in D-Ala in the period directly after ^{13}C-glucose injection (day 6-18) for the 0-2 cm layer and 18-27% for the 2-5 cm layers. The excess ^{13}C D/L-Ala ratio for the 2-5 cm layer of plot 2, the only layer for which excess ^{13}C was present in L-Ala, was 0.22 (not corrected for racemization) (Fig. 4). Excess ^{13}C in D-Ala relative to that in THAAs (not shown in Fig. 3) increased from 0.5% for the period up to day 71 to 1.0% at day 136. This increase was smaller than the ~ 4 × increase in excess ^{13}C D/L-Ala ratio (Fig. 4) since the relative abundance of ^{13}C L-Ala in the ^{13}C-THAA pool actually decreased ~ 2 × between day 71 and 136 (Fig. 3).

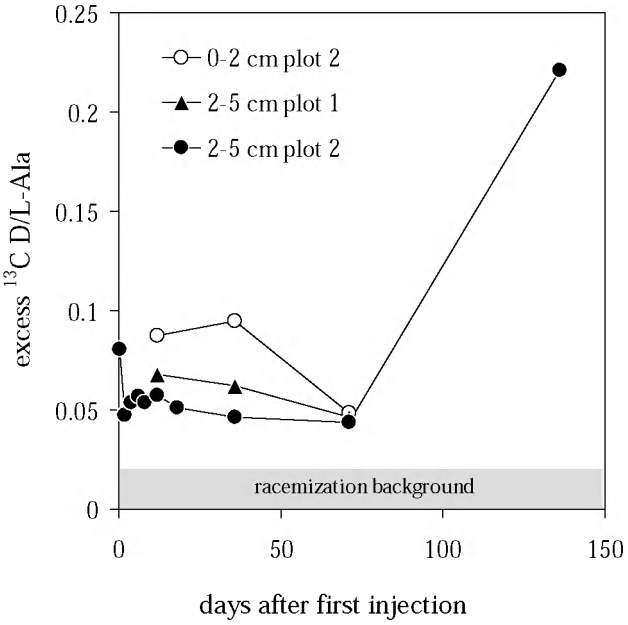


Figure 4
Excess ^{13}C D/L-Ala ratios (excess ^{13}C in D-Ala / excess ^{13}C in L-Ala), uncorrected for racemization. Racemization background (0.015-0.02) is indicated by grey box.

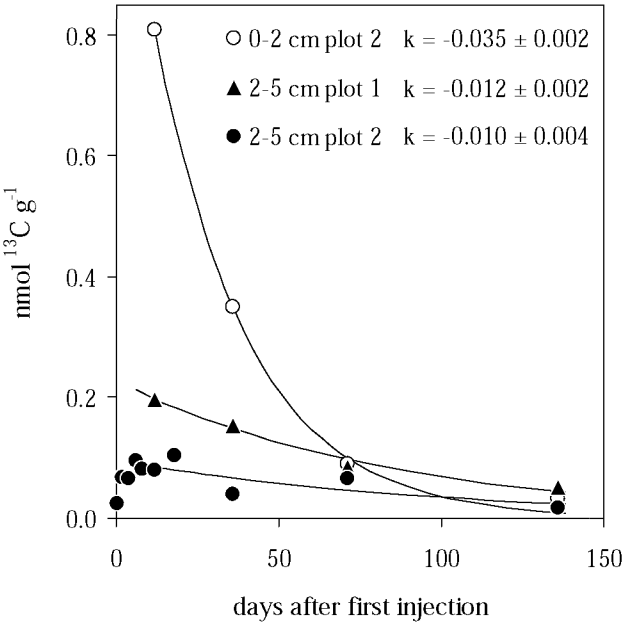


Figure 5
Excess ^{13}C in D-Ala (corrected for racemization). Lines are exponential fits for data between day 6 and day 136 (after label injection period). k-values are loss rate constants (± standard error).

DISCUSSION

The data presented in this paper are part of a comprehensive in situ ^{13}C-labeling experiment investigating the fate of bacterial carbon in an intertidal sediment. The main data concerning total ^{13}C and total bacterial ^{13}C for the whole upper 10 cm layer of both sediment plots are presented in a companion paper (Van Oevelen et al. 2006a) that focuses at modeling the ^{13}C results to quantify loss processes for ^{13}C from bacterial biomass and the sediment. In addition, a separate paper was dedicated to detailed analysis of the transfer of ^{13}C from bacteria to fauna via grazing (Van Oevelen et al. 2006b). Van Oevelen et al. (2006a) documents strong loss of bacterial ^{13}C from the sediment during the 4.5-month sampling period. Loss processes included grazing (~ 25% of total loss), exchange processes such as resuspension (~ 10% of total loss) and cell death due to viral lysis, programmed cell death and other potential causes (~ 65% of total loss). Cell death resulted in production of bacterial remnants. Degradation of these remnants produced ^{13}C-DOC of which part was lost from the sediment (either directly as ^{13}C-DOC or indirectly as ^{13}C-DIC following respiration) while another part of the ^{13}C-DOC was recycled back to bacterial biomass (Van Oevelen et al. 2006a). As a result of these different loss processes, bacterial ^{13}C had disappeared from the sediment almost completely after 4.5 months.

The material presented here adds to that in the companion papers by focusing at the fate of different ^{13}C-labeled compounds from the ^{13}C-labeled bacterial biomass during the weeks and months after the ^{13}C-glucose injection period. Before discussing the results, it seems useful to clarify some points that should be considered during interpretation of the data: 1) Since $\Delta\delta^{13}C$ values represent changes in ratios between ^{13}C and ^{12}C, $\Delta\delta^{13}C$ values for the ^{13}C-labeled bacteria may have been "diluted" by production of new, unlabeled bacterial biomass. Therefore, our results are all presented as excess ^{13}C (i.e. absolute amounts of ^{13}C), which is not biased by such dilution and therefore represents the true fate of the ^{13}C-labeled compounds from bacteria that were labeled during the ^{13}C-glucose injection period. 2) PLFAs are turned over within days after cell death (Parkes 1987, Moodley et al. 2000). Therefore, at the experimental time scale of weeks to months, ^{13}C-labeled bacteria-specific PLFAs can be considered specific for living bacteria. Conversely, HAAs, including D-Ala, can remain present in the sediment as remnants for considerably longer periods of time. Therefore, ^{13}C-THAAs and ^{13}C D-Ala were not specific for living (bacterial) biomass. 3) HAAs included in

this study, except D-Ala, are common protein amino acids that are abundant in proteinaceous biomass of all organisms and are therefore not specific for bacteria. However, during the first 2-3 weeks of the experiment, ^{13}C was predominantly present in bacterial biomass, meaning that during this period, ^{13}C-THAAs represented bacterial proteinaceous biomass. During the following weeks to months, a substantial fraction of ^{13}C was transferred to non-bacterial biomass, meaning that ^{13}C-THAA was no longer specific for bacteria but instead represented total benthic proteinaceous material. 4) Sediment samples for analysis of ^{13}C in HAAs were washed with water and 2 M HCl prior to hydrolysis, which removed dissolved HAAs (see Chapter 2). Therefore, ^{13}C-THAAs and ^{13}C-D-Ala reflect particulate HAAs only (living biomass + particulate remnants).

In the following paragraphs we first discuss incorporation of ^{13}C from ^{13}C-glucose into the different bacterial components and resulting estimates of total bacterial ^{13}C. Subsequently, we focus on the fate of the different ^{13}C-labeled compounds, distinguishing between bacterial ^{13}C (^{13}C in bacteria-specific PLFAs and D-Ala) and ^{13}C in total benthic proteinaceous biomass (^{13}C-THAAs). The last section deals with retention of ^{13}C-D-Ala in the sediment and its consequences for the accumulation potential of peptidoglycan.

^{13}C incorporation and estimates of total bacterial excess ^{13}C

^{13}C incorporation into the different bacterial cell components confirmed that ^{13}C incorporation from ^{13}C-glucose was initially dominated by bacteria. First, the substantially lower ^{13}C enrichment of biomarker PLFAs for other groups of organisms (algae and fauna) indicated that total ^{13}C incorporation into these groups was substantially lower than that into bacteria. Second, the excess ^{13}C D/L-Ala ratios of 0.05-0.08 during the ^{13}C-glucose injection period (Fig. 4) are within the range of D/L-Ala ratios for bacterial biomass (see Chapter 2). In case of substantial ^{13}C incorporation by organisms other than bacteria (algae and/or fauna) excess ^{13}C D/L-Ala ratios would have been lower since ^{13}C incorporation by non-bacterial organisms results in ^{13}C enrichment of L-Ala only.

Although the use of biomarkers in stable isotope labeling studies is primarily a semi-quantitative tool, compound-specific results can also be used for estimating label incorporation into total biomass. Excess ^{13}C incorporated in total bacterial biomass was estimated from excess ^{13}C in the two different bacterial compounds (bacteria-specific PLFAs and D-Ala). In addition, a third estimate could be made

from excess ^{13}C in THAAs since this pool was also predominantly bacterial during the first 2-3 weeks of the experiment (as already discussed). This estimate is a useful addition since THAAs represent a much larger fraction of total biomass than the two bacterial biomarkers and hence requires only a small conversion step. Estimates of total bacterial excess ^{13}C were in reasonable agreement given the magnitude of the conversion steps from bacteria-specific PLFAs and D-Ala to total bacterial C and the variation and uncertainty inherent to this kind of conversions. This variation and uncertainty includes dependence on bacterial cell size, community composition and environmental conditions as well as potential heterogeneity in distribution of ^{13}C between different cell components. Moreover, additional bias may have resulted from differences in the turnover of the different ^{13}C-labeled bacterial compounds (as discussed below) during the first 2-3 weeks of the experiment.

Fate of ^{13}C in bacteria-specific PLFAs and D-Ala

Both bacterial biomarkers showed substantial loss of ^{13}C during the months after the ^{13}C-glucose injection period, indicating rapid loss of ^{13}C from bacterial biomass. The substantially shorter half lives for the 0-2 cm layer in comparison to the 2-5 cm layers (Fig. 1 and 5) point to a more rapid turnover of bacterial biomass in the 0-2 cm layer. However, the relatively strong loss of ^{13}C from the 0-2 cm layer probably also included a relatively strong loss by resuspension. Although modeling results showed that resuspension accounted for only ~ 10% of total loss of bacterial ^{13}C from the whole 0-10 cm layer (Van Oevelen et al. 2006a), this fraction may well have been substantially higher for the 0-2 cm layer alone given its direct exposure to the overlying water.

Although trends for excess ^{13}C in the two bacterial biomarkers were roughly similar, the consistent ~ 2 × longer half lives for D-Ala show that ^{13}C-D-Ala was retained in the sediment longer than the ^{13}C-bacteria-specific PLFAs (Fig. 1 and 5). This was also evident from the substantially larger fraction of original excess ^{13}C in D-Ala (day 6-18) still present in D-Ala after 136 days (18-27% for the 2-5 cm layers) in comparison to the much lower fraction ^{13}C left in bacteria-specific PLFAs (≤ 2%). The relatively strong retention of ^{13}C in D-Ala could simply have resulted from more rapid degradation of PLFAs (cell membranes) than D-Ala (cell wall peptidoglycan) alone, but may also reflect a difference in recycling efficiency for the two compounds. This recycling involves reincorporation of ^{13}C from the ^{13}C-DOC pool resulting from degradation of ^{13}C-bacterial remnants into new

bacterial biomass, either by direct reincorporation of intact dissolved ^{13}C-PLFAs and ^{13}C-amino acids or by new production from other ^{13}C-DOC compounds. Modeling results indicated that about one-third of all released ^{13}C-DOC was reincorporated into new bacterial biomass (Van Oevelen et al. 2006a). Although recycling may have resulted in reincorporation of ^{13}C in D-Ala (and other HAAs) as well as in bacteria-specific PLFAs, recycling of ^{13}C-amino acids may well have been more efficient than recycling of ^{13}C-PLFAs since dissolved amino acids also play an important role as a nitrogen source to the benthic microbial community, including bacteria (Chapters 2, 3 and 4). Since nitrogen is generally a limiting element for microbial production and given the high microbial affinity for dissolved amino acids as a source of nitrogen (Chapter 1), recycling of nitrogenous compounds is likely to be more efficient than for non-nitrogenous compounds such as PLFAs.

Irrespective of the precise mechanisms for retention, our results clearly show a relatively strong retention of ^{13}C in D-Ala and hence confirm the potential for accumulation of peptidoglycan in sediments, as discussed in more detail below.

Fate of ^{13}C in THAAs

The fate of ^{13}C in THAA is discussed separately from the bacterial biomarkers since ^{13}C-THAAs represented ^{13}C in total benthic proteinaceous material, which was different from bacterial biomass and remnants alone. Trends in excess ^{13}C in the THAAs were clearly different for the 0-2 cm versus 2-5 cm layers (Fig. 2). The decrease of ^{13}C in the 0-2 cm layer occurred at a rate similar to that for ^{13}C in D-Ala (half life of ~ 20 days), indicating that, for this layer, degradation and subsequent loss of ^{13}C from bulk proteinaceous biomass (^{13}C-THAAs) was similar to that for peptidoglycan (D-Ala). Conversely, the lack of such a decrease in excess ^{13}C in THAAs for the 2-5 cm layers up to day 71 indicates that there was little to no loss of ^{13}C from total proteinaceous biomass. This stronger retention of ^{13}C in THAAs (including L-Ala) than ^{13}C in the bacterial biomarkers, as reflected in a decrease in excess ^{13}C D/L-Ala ratios (Fig. 5), might imply a lower degradability of the THAAs. However, this seems unlikely since peptidoglycan (D-Ala) has been found to be more refractory than proteins that make up a large fraction of the THAA pool (Jørgensen et al. 2003, Nagata et al. 2003). Moreover, the relative composition of the ^{13}C-THAA pool did not change up to day 71, which strongly suggests that, during this period, the ^{13}C-THAA pool was not subject to substantial degradation (see discussion below for details). Therefore, the strong retention of ^{13}C in THAAs

while ^{13}C was rapidly lost from bacterial biomass is more likely explained by a substantial transfer of ^{13}C from bacteria to other organisms.

Bacterial-derived ^{13}C may have been incorporated by benthic microalgae (BMA), either via uptake of ^{13}C-DOM resulting from degradation of ^{13}C-labeled bacterial remnants or via uptake of ^{13}C-DIC resulting from respiration. However, substantial transfer of ^{13}C to BMA seems unlikely given that BMA predominate in the photic zone, i.e. the upper few millimeters, of the sediment, which is only a small fraction of the sediment layers analyzed in this study. Moreover, recycling of DOM in the upper millimeters of intertidal sediment is much less efficient than that deeper in the sediment due to diffusional losses of DOM to the overlying water. The negligible role of ^{13}C in BMA (mainly diatoms) in this study was confirmed by the low ^{13}C enrichment for diatom-specific PLFA 20:5ω3.

Alternatively, the strong retention of ^{13}C in THAAs and the associated decrease in excess ^{13}C D/L-Ala ratios may have resulted from a ^{13}C transfer from bacteria to fauna via grazing. The clear ^{13}C enrichment of the fauna following ^{13}C labeling of bacterial biomass showed that substantial faunal grazing at ^{13}C labeled bacteria did occur (Van Oevelen et al. 2006a+b). Since turnover of (macro)faunal biomass is considerably slower than that of bacterial biomass, transfer of bacterial C to fauna serves as a potential mechanism for retention of bacterial-derived C in the sediment over longer periods of time. However, at day 136 there was no excess ^{13}C in THAAs for two of the three sampled layers while a small fraction (9% of original ^{13}C in THAAs for day 6-18) was left in the 2-5 cm layer of plot 2. These results indicate that, like for bacterial biomass, only a minor fraction of the original ^{13}C was retained in total benthic proteinaceous material over the 4.5 month sampling period.

The relative composition of the ^{13}C-THAA pool for the 2-5 cm layer of plot 2 also showed some interesting changes (Fig. 3). These changes, especially the increased relative abundance of Gly and Ser and decreased relative abundance of Leu and Ile, are consistent with observed changes during degradation of total organic matter in sediments, (e.g., Cowie and Hedges 1992, Dauwe et al. 1999, Keil et al. 2000, Pantoja and Lee 2003). Therefore, results indicate that the remaining ^{13}C-labeled material at day 136 had been subject to substantial degradation, meaning that this material likely consisted of remnants of ^{13}C labeled bacterial and/or faunal biomass rather than living biomass. As our THAA analyses only included particulate HAAs (dissolved material was removed by washing the sediment prior to hydrolysis), the remnants remaining after 136 days must have consisted of particulate biomass components and/or smaller molecules associated with larger molecules and/or

particles by mechanisms similar to those reported by Borch and Kirchman (1999) and Arnarson and Keil (2005). Therefore, the relative accumulation of Gly, Ser and Pro likely resulted from their presence in larger molecules and/or particles that were relative resistant to degradation. The relative accumulation of Gly during degradation of total organic matter in sediments is usually attributed to its presence in refractory diatom cell walls and/or peptidoglycan (Dauwe and Middelburg 1998, Keil et al. 2000), of which the latter might have been relevant for this study. However, the relative accumulation of ^{13}C-Gly was actually ~ 4 × stronger than that for ^{13}C-D-Ala (specific for peptidoglycan), indicating that the relative accumulation of ^{13}C-Gly could not have resulted from its presence in peptidoglycan alone. Similarly, the relative accumulation of ^{13}C-Ser and ^{13}C-Pro was also stronger (~ 2 ×) than for ^{13}C-D-Ala. Hence, these results suggest that the relative accumulation of Gly, Ser, and Pro was due to their presence in other compounds and/or particles that were more refractory than peptidoglycan. An alternative explanation for the relative accumulation of Gly and Ser is the formation of degradation products that are relatively rich in "simple" amino acids such as Gly and Ser. This is similar to the production of non-protein amino acids (γ-ABA en β-Ala) as degradation products (Lee and Cronin 1982, Cowie and Hedges 1994, Keil et al. 2000) and is supported by results from Keil and Fogel (2001) who found natural abundance δ^{13}C values for Gly to be different from those for other HAAs in marine organic matter and attributed this to relatively intensive microbial reworking of Gly. Results from Ziegler and Fogel (2003) suggest that similar mechanisms may apply for Ser. Conversely, Pro is a large, more complex, secondary amino acid and therefore less likely to accumulate as degradation product like Gly and Ser. The relatively refractory character of Pro, which also showed in Chapter 2, might be related to its molecular structure (including a cyclic side group).

Accumulation potential of peptidoglycan

Although most bacterial ^{13}C was lost during the 4.5 months in situ, results clearly showed a ~ 2 × slower loss of ^{13}C from D-Ala than from bacteria-specific PLFAs during the sampling period and a relatively high excess ^{13}C in D-Ala in comparison to that in the bacteria-specific PLFAs and THAAs in the remaining leftovers at the end of the sampling period (day 136). This relatively strong retention of D-Ala (i.e., peptidoglycan) is consistent with the relatively low degradability of peptidoglycan in laboratory experiments (Jørgensen et al. 2003, Nagata et al. 2003). Hence, results show that the potential for accumulation of peptidoglycan is also present during

reworking and degradation of bacterial biomass in the highly dynamic upper centimeters of an intertidal sediment. This provides direct evidence that increasing D/L-AA ratios with increasing time and/or depth, as reported for sediment OM (Pedersen et al. 2001, Grutters et al. 2002) and seawater DOM (Amon et al. 2001, Dittmar et al. 2001) as well as for OM in soils (Brodowski et al. 2004), can indeed result from the relative accumulation of peptidoglycan (rich in D-AAs) relative to bulk proteinaceous material (L-AAs).

Peptidoglycan is only small fraction of total bacterial biomass and our ^{13}C results showed that only part of the peptidoglycan from bacterial biomass was retained in the sediment. However the relative accumulation of peptidoglycan over longer periods of time (≥ 10 years) combined with potential reworking of sediment OM by bacteria might result in a substantial accumulation of peptidoglycan. This makes peptidoglycan a potentially important contributor to total sediment OM just like it is thought to be an important contributor to total DOM in seawater (McCarthy et al. 1998, Amon et al. 2001, Dittmar et al. 2001).

The contribution of peptidoglycan to total OM in the intertidal sediment in the present study was estimated from measured concentrations of D-Ala and total OC. Peptidoglycan concentrations were estimated from D-Ala concentrations assuming that C in D-Ala makes up ~ 10% of total peptidoglycan-C, which was based on the typical composition of peptidoglycan (equal amounts of D-Ala, L-Ala, D-Glu, DAP and the two sugar derivatives NAG and NAM) (Madigan et al. 2000). Resulting estimates showed that peptidoglycan-C was only a minor fraction (~ 1%) of total OC in the sediment. This low contribution is consistent with other HAA-based estimates by Keil et al. (2000), Pedersen et al. (2001) and Grutters et al. (2002) who also found that, despite its relatively refractory character, peptidoglycan was only a minor fraction of total sediment organic matter.

Acknowledgements

Cynthia van der Voorn, Ria Bos, Pieter van Rijswijk, Gert Weststrate, Ko Verschuure, and Anton Tramper provided invaluable assistance during field and laboratory work. We thank Marco Houtekamer for helping with the GC-c-IRMS work, Henrik Andersson for statistical help and Karline Soetaert for her scientific input.

Liz Canuel and two anonymous reviewers are thanked for their constructive feedback. This research was partially supported by the Netherlands Organization of Scientific Research (PIONIER 833.02.2002).This is publication 3770 of the Netherlands Institute of Ecology (NIOO-KNAW).

THESIS SUMMARY

Nitrogen flows through bacteria and algae in aquatic microbial communities are an important part of the nitrogen cycle, which plays a central role in aquatic ecosystems. However, work on uptake and retention of nitrogen in bacteria versus algae in natural microbial communities has long been hampered by a lack of adequate methodology, especially in turbid waters and sediments. This thesis deals with the development, validation and application of a new method for analysis of nitrogen and carbon flows through bacteria and algae in aquatic microbial communities. The method concerns analysis of incorporation of the stable isotopes ^{15}N and ^{13}C in hydrolysable amino acids (HAAs), including the bacterial biomarker D-alanine (D-Ala) in combination with stable isotope labeling. As the applications of the method presented in this thesis are rather diverse, this thesis is best summarized chapter by chapter.

Chapter 1 is a "traditional" ^{15}N-labeling study in which water samples from Randers Fjord (Denmark) were incubated with different ^{15}N-labeled substrates to investigate uptake of these substrates by the whole microbial community in the water column. Results confirmed the potential importance of dissolved *organic* nitrogen as a nitrogen source for the planktonic microbial community next to dissolved *inorganic* nitrogen. Moreover, results illustrate the problems with traditional methods used to discriminate between ^{15}N incorporation by bacteria versus algae and hence stress the need for a new method.

This new method is presented in **Chapter 2** which focuses on the analytical aspects of the method and presents some first results from a simple ^{15}N-labeling experiment with surface sediment from an intertidal mudflat in the Scheldt Estuary that illustrates the potential of the method. Chapter 2 is followed by an **update** which deals with how to interpret results obtained with the method. This update is based on application of the method in various ^{15}N- and ^{13}C-labeling studies as well as analysis of D-Ala in algal cultures. This update discusses the formation of D-Ala from L-Ala by hydrolysis-induced racemization during sample processing and introduces a simple way to correct measured concentrations and excess label in D-Ala for this racemization. Moreover, additional information on the D-Ala content

and related D/L-Ala ratio of bacteria is presented and its implications for interpretation of results obtained with the method are discussed.

Chapter 3 presents one of the first studies in which the method was applied. Dual-labeled (^{15}N and ^{13}C) urea and amino acids, two favourable nitrogen sources for aquatic microbes, were added to surface sediment from two intertidal mudflats in the Scheldt Estuary and incorporation of ^{15}N and ^{13}C into total microbial biomass (THAAs) and bacteria (D-Ala) was measured at several time points during a 24 h incubation period. All ^{15}N from urea and the amino acids was incorporated into microbial biomass within 24 h. The bacterial contribution to total microbial ^{15}N incorporation (derived from ^{15}N incorporation into D-Ala) was large during the first 1 to 2 h of incubation but small to negligible after 24 h for both substrates, indicating that total ^{15}N incorporation was dominated by benthic microalgae that also dominated microbial biomass in the sediment. Comparison of incorporation of ^{15}N versus ^{13}C into total microbial biomass (THAAs) for urea showed coupled incorporation of urea-derived nitrogen and carbon during the first 4 h (in daylight) but strong preferential incorporation of nitrogen after 24 h (overnight). Incorporation of nitrogen and carbon from the amino acids was partially uncoupled (with relative strong incorporation of ^{15}N), indicating that about half of the amino acids were directly taken up while the other half was taken up via the extracellular amino acid oxidation pathway.

In **Chapter 4**, the method is used to investigate the incorporation and retention of nitrogen by bacteria and algae in the sediment of the subtropical Brunswick Estuary (Australia). The main experiment involved in situ pulse labeling of an intertidal sediment by injection of $^{15}NH_4^+$ and subsequent analysis of incorporation and retention of ^{15}N in the bulk sediment, THAAs, D-Ala and fauna during a 30-day sampling period. The ^{15}N was rapidly incorporated and strongly retained in microbial biomass (THAAs), which indicated very efficient recycling of ^{15}N by the benthic microbial community. Analysis of ^{15}N in D-Ala revealed a major bacterial contribution to total microbial incorporation and recycling of the ^{15}N. ^{15}N was also incorporated into fauna via grazing on ^{15}N-labeled microbial biomass, but this was a negligible fraction (< 1%) of total ^{15}N in the sediment. Results confirm the previously hypothesized immobilization of nitrogen in benthic microbial biomass and indicate that recycling of nitrogen by the microbial community within the sediment can be an important pathway supporting benthic microbial production, especially in nitrogen limited systems like the Brunswick Estuary.

Unlike the other chapters, **Chapter 5** does not involve nitrogen. Instead, the method is used to investigate the fate of peptidoglycan, a bacterial cell wall component, in intertidal sediment. This work is part of a larger study in which the bacterial community of an intertidal mudflat in the Scheldt Estuary was labeled with ^{13}C (by injection of ^{13}C-glucose into the sediment) in order to investigate the fate of bacterial carbon in this sediment. This chapter involves comparison of the fate of ^{13}C in D-Ala (specific for peptidoglycan), THAAs (representing bulk proteinaceous material) and bacteria-specific PLFAs (representing cell membranes of living bacteria) over a 4.5-month period in situ. Results revealed relatively strong retention of ^{13}C in THAAs relative to that in living bacteria (PLFAs) with strongest retention in D-Ala. This provided direct in situ evidence for relative accumulation of peptidoglycan during reworking and degradation of bacterial biomass in sediments.

The applications of the method presented in this thesis as well as those in other ^{15}N- and/or ^{13}C-labeling studies (see publication list) served as a thorough validation of the method. Analysis of incorporation of ^{15}N and ^{13}C in D-Ala proved to be a valuable tool in ^{15}N- and ^{13}C-labeling studies to trace incorporation and retention of the label in bacterial biomass. While incorporation of label into D-Ala alone can be used to estimate total bacterial label incorporation, the most useful information was derived from comparison of label incorporation into D-Ala versus that in L-Ala (one of the common protein amino acids present in all organisms). This excess label D/L-Ala ratio provides a direct indication of the bacterial contribution to total (microbial) label incorporation. In addition to D- and L-Ala, analysis of label incorporation into the other HAAs also proved to be very useful. Firstly, because THAAs make up a large fraction of total biomass, label incorporation into THAAs provides a direct indication of label incorporation into total biomass. Secondly, the composition of the labeled THAA pool can be used as an indicator for the degree of degradation of the labeled material.

Altogether, the method proved to be very suitable for its originally intended application (analysis of ^{15}N incorporation by bacteria). Together with the additional features like analysis of ^{13}C in bacteria and ^{15}N and ^{13}C in total (microbial) biomass, this makes the method a very useful, versatile new tool for studies on flows of nitrogen and carbon through bacteria and algae in aquatic microbial communities.

SAMENVATTING
VAN DIT PROEFSCHRIFT

De opname en retentie van stikstof door bacteriën en algen in aquatische microbiële gemeenschappen is een belangrijk onderdeel van de stikstofcyclus, welke weer een belangrijke rol speelt in aquatische ecosystemen. Onderzoek aan de opname en retentie van stikstof door bacteriën versus algen in natuurlijke microbiële gemeenschappen, met name in troebele wateren en sedimenten, werd lange tijd sterk beperkt door een gebrek aan geschikte methodologie. Dit proefschrift gaat over de ontwikkeling, validatie en toepassing van een nieuwe methode voor de analyse van opname en retentie van stikstof en koolstof door bacteriën en algen in aquatische microbiële gemeenschappen. Deze methode omvat de analyse van incorporatie van de stabiele isotopen ^{15}N en ^{13}C in hydrolyseerbare aminozuren (HAAs) inclusief de bacteriële biomarker D-alanine (D-Ala) gecombineerd met stabiele isotoop labeling. Omdat de toepassingen van de methode zoals die in dit proefschrift worden gepresenteerd nogal divers zijn worden de hoofdstukken hieronder één voor één samengevat.

Hoofdstuk 1 is een voorbeeld van een "traditionele" ^{15}N-labeling studie waarin watermonsters uit de Deense Randers Fjord werden geincubeerd met verschillende ^{15}N-gelabelde substraten om de opname van deze substraten door de totale microbiële gemeenschap in het water van Randers Fjord te onderzoeken. De resultaten bevestigen het belang van opgelost *organisch* stikstof als stikstofbron voor de planktonische microbiële gemeenschap naast opgelost *anorganisch* stikstof. Daarnaast illustreren de resultaten de problemen met traditionele methodes voor het onderscheiden van ^{15}N opname door bacteriën versus algen en benadrukken daarmee de behoefte aan een nieuwe methode.

Deze nieuwe methode wordt gepresenteerd in **Hoofdstuk 2.** Hierin ligt de nadruk op de analytische aspecten van de methode en worden de resultaten van een eenvoudig ^{15}N-labeling experiment met sediment van een intergetijdeplaat in de Westerschelde gepresenteerd die de potentie van de methode illustreren. Hoofdstuk 2 wordt gevolgd door een **update** van het oorspronkelijke manuscript

welke dieper in gaat op de interpretatie van met de methode verkregen resultaten. Dit is gebaseerd op toepassingen van de methode in verschillende ^{15}N- en/of ^{13}C-labeling studies en de analyse van D-Ala in een aantal algencultures. Deze update behandeld de vorming van D-Ala uit L-Ala door racemizatie tijdens hydrolyse van de monsters en hoe hiervoor te corrigeren bij berekening van D-Ala concentraties en label incorporatie in D-Ala. Daarnaast gaat de update dieper in op (de variatie in) het D-Ala gehalte en de D/L-Ala ratio van bacteriën en de implicaties daarvan voor interpretatie van met de methode verkregen resultaten.

In **Hoofdstuk 3** wordt één van de eerste experimenten waarin de nieuwe methode werd toegepast gepresenteerd. Dubbel gelabelde (^{15}N en ^{13}C) urea en een aminozuurmix werden toegevoegd aan oppervlaktesediment afkomstig van twee intergetijdeplaten in de Westerschelde en de incorporatie van ^{15}N en ^{13}C in totale microbiële biomassa (THAAs) en bacteriën (D-Ala) werd gemeten op verschillende tijdstippen over een periode van 24 uur. Resultaten lieten zien dat alle ^{15}N van zowel urea als de aminozuurmix werd geïncorporeerd in microbiële biomassa binnen 24 uur. De bijdrage van bacteriën aan de totale incorporatie (afgeleid uit ^{15}N incorporatie in D-Ala) was groot gedurende de eerste 1 à 2 uur maar klein tot verwaarloosbaar na 24 uur. Dit duidt er op dat totale ^{15}N opname werd gedomineerd door benthische microalgen die in dit sediment ook een groot deel van de totale microbiële biomassa uitmaakten. Vergelijking van ^{15}N- en ^{13}C incorporatie in totale microbiële biomassa liet voor de urea incubaties een gekoppelde incorporatie van ^{15}N en ^{13}C zien gedurende de eerste 4 uur (in daglicht) terwijl er na 24 uur sprake was van een sterke preferentiële incorporatie van ^{15}N. In de aminozuurincubaties was sprake van een gedeeltelijk ontkoppelde incorporatie van ^{15}N en ^{13}C (relatief sterke incorporatie van ^{15}N) wat er op duidt dat ongeveer de helft van de aminozuren in z'n geheel werd geïncorporeerd in microbiële biomassa terwijl van de andere helft alleen de ^{15}N werd geïncorporeerd na extracellulaire oxidatie van de aminozuren.

In **Hoofdstuk 4** wordt de methode gebruikt om de incorporatie en retentie van stikstof door bacteriën en algen in het sediment van het subtropische Brunswick estuarium (Australië) te onderzoeken. Het hoofdexperiment omvatte de injectie van $^{15}NH_4^+$ in een intergetijdesediment in situ waarna incorporatie en retentie van de ^{15}N werd gemeten in het totale sediment, THAAs, D-Ala en fauna gedurende een periode van 30 dagen. De ^{15}N werd snel opgenomen in microbiële biomassa (THAAs) en bleef hierin aanwezig zonder enig verlies gedurende de volledige

bemonsteringsperiode wat er op duidt dat de ^{15}N zeer efficiënt werd hergebruikt door de benthische microbiële gemeenschap. Analyse van ^{15}N in D-Ala liet een belangrijke bijdrage zien van bacteriën aan de totale microbiële ^{15}N incorporatie en het hergebruikt van de ^{15}N. ^{15}N werd ook geincorporeerd in fauna als gevolg van de consumptie van ^{15}N-gelabelde bacteriën en/of algen. De ^{15}N in fauna was echter maar een minimale fractie (< 1%) van het totale ^{15}N in het sediment. De resultaten van deze studie bevestigen de eerder al veronderstelde immobilisatie van stikstof in benthische microbiële biomassa en duiden er op dat hergebruik van stikstof door de microbiële gemeenschap in het sediment een belangrijke stikstofbron kan zijn voor benthische microbiële productie, met name in stikstofgelimiteerde systemen als het Brunswick estuarium.

In **Hoofdstuk 5** gaat het niet langer om stikstof, maar wordt de methode gebruikt om de retentie van peptidoglycaan, een bacteriële celwandcomponent, in intergetijdesediment te onderzoeken. Dit werk is onderdeel van een groter experiment waarin bacteriën in het sediment van een intergetijdeplaat in de Westerschelde werden gelabeld met ^{13}C (door middel van injectie van ^{13}C-glucose in het sediment) om het lot van bacteriële koolstof te onderzoeken. In dit hoofdstuk gaat het om vergelijking van de retentie van ^{13}C in D-Ala (specifiek voor peptidoglycaan), THAAs (totale proteïne biomassa) en bacteriespecifieke PLFAs (phospholipid-derived fatty acids, vetzuren specifiek voor celmembranen van levende bacteriën) in het sediment gedurende een periode van 4½ maand in situ. Resultaten lieten een relatief sterke retentie van ^{13}C in D-Ala ten opzichte van andere HAAs en de PLFAs zien wat duidt op een relatieve accumulatie van peptidoglycaan gedurende de afbraak van bacteriële biomassa in sedimenten.

De toepassingen van de methode in de verschillende studies zoals die worden gepresenteerd in dit proefschrift en een aantal andere studies (zie publicatielijst) vormen samen een grondige validatie van de methode. Analyse van ^{15}N en ^{13}C incorporatie in D-Ala heeft zich bewezen als een waardevolle methode voor het meten van incorporatie en retentie van label in bacteriële biomassa in ^{15}N- en ^{13}C-labeling studies. Hoewel analyse van label incorporatie in D-Ala alleen al kan worden gebruikt voor het schatten van totale bacteriële label incorporatie werd de meest nuttige informatie verkregen door vergelijking van label incorporatie in D-Ala met dat in L-Ala (een van de “gewone” HAAs aanwezig in alle organismen). Uitgedrukt als excess label D/L-Ala ratio geeft dit een directe indicatie van de bacteriële bijdrage aan de totale microbiële label incorporatie.

Naast D- en L-Ala is de analyse van label in andere HAAs ook erg nuttig gebleken. Ten eerste omdat deze HAAs samen (THAAs) een groot deel van de totale microbiële biomassa vormen en label incorporatie in THAAs zodoende een goede indicatie geeft van label incorporatie in totale microbiële biomassa. Ten tweede kan de relatieve samenstelling van de gelabelde THAA pool worden gebruikt als indicator voor de mate van afbraak van het gelabelde materiaal.

Alles bij elkaar heeft de methode bewezen zeer geschikt te zijn voor zijn oorspronkelijke toepassing (analyse van ^{15}N incorporatie door bacteriën). Samen met aanvullende mogelijkheden als analyse van ^{13}C in bacteriën en ^{15}N en ^{13}C in totale microbiële biomassa maakt dit de methode een zeer bruikbaar en breed toepasbaar stuk gereedschap voor studies aan de opname en retentie van stikstof en koolstof door bacteriën en algen in aquatische microbiële gemeenschappen.

REFERENCES

Admiraal, W., C. Riaux-Gobin and R.W.P.M. Laane. 1987. Interactions of ammonium, nitrate, and D- and L-amino acids in the nitrogen assimilation of two species of estuarine benthic diatoms. Mar. Ecol. Prog. Ser. 40: 267-273.

Algeus, S. 1948. The deanimation of glycocoll by green algae. Physiologia Plantarum 1: 382-386.

Alongi, D.M. 1994. The role of bacteria in nutrient recycling in tropical mangrove and other coastal benthic ecosystems. Hydrobiologia 285: 19-32.

Amelung, W. and X. Zhang. 2001. Determination of amino acid enantiomers in soils. Soil Biology and Biochemistry 33: 553-562.

Amelung, W. and S. Brodowski. 2002. In vitro quantification of hydrolysis-induced racemization of amino acid enantiomers in environmental samples using deuterium labeling and electron-impact ionization mass spectrometry. Analytical Chemistry 74: 3239-3246.

Amon, R.W.M., H.P. Fitznar and R. Benner. 2001. Linkages among bioreactivity, chemical composition and diagenetic state of marine dissolved organic matter. Limnol. Oceanogr. 46: 287-297.

Anderson, I.C., K.J. McGlathery and A.C. Tyler. 2003. Microbial mediation of 'reactive' nitrogen transformations in a temperate lagoon. Mar. Ecol. Prog. Ser. 246: 73-84.

Andersson, M., P. van Rijswijk and J.J. Middelburg. 2006. Uptake of dissolved inorganic nitrogen, urea and amino acids in the Scheldt estuary: a comparison of organic C and N uptake. Aquatic Microbial Ecology 44(3) (*in press*).

Antia, N.J., P.J. Harrison, L.Oliveira. 1991. The role of dissolved organic nitrogen in phytoplankton nutrition, cell biology and ecology. Phycologia 30: 1-89.

Arnarson, T.S., and R.G. Keil. 2005. Influence of organic-mineral aggregates on microbial degradation of the dinoflagellate *Scrippsiella trochoidea*. Geochim. et Cosmochim. Acta 69: 2111-2117.

Barranguet, C., P.M.J. Herman and J.J. Sinke. 1997. Microphytobenthos biomass and community composition studied by pigment biomarkers: importance and fate in the carbon cycle of a tidal flat. Journal of Sea Research 38: 59-70.

Benner R. 2002. Chemical composition and reactivity. In: Hansell, D.A., Carlson, C.A. (Eds). Biogeochemistry of Marine Dissolved Organic Matter. Academic Press, San Diego, pp 59-90.

Berg G.M., P.M. Glibert, M.W. Lomas and M.A. Burford. 1997. Organic nitrogen uptake and growth by the chrysophyte *Aureococcus anophagefferens* during a brown tide event. Marine Biology 129: 377-387.

Berg, G.M., P.M. Glibert, N.O.G. Jørgensen, M. Balode and I. Purina. 2001. Variability in inorganic and organic nitrogen uptake associated with riverine nutrient input in the Gulf of Riga, Baltic Sea. Estuaries 24: 204-214.

Berman, T. and D.A. Bronk. 2003. Dissolved organic nitrogen: a dynamic participant in aquatic ecosystems. Aquatic Microbial Ecology 31: 279-305.

Boschker, H.T.S. and J.J. Middelburg. 2002. Stable isotopes and biomarkers in microbial ecology. FEMS Microbiol. Ecol. 40: 85-95.

Boschker H.T.S., S.C. Nold, P. Wellsbury, D. Bos, W. de Graaf, R. Pel, R.J. Parkes and T.E. Cappenberg. 1998. Direct linking of microbial populations to specific biogeochemical processes by ^{13}C-labelling of biomarkers. Nature 392: 801-805.

Boschker H.T.S. 2004. Linking microbial community structure and functioning: stable isotope (^{13}C) labeling in combination with PLFA analysis, p. 1673-1688. In G.A. Kowalchuk, F.J. de Bruijn, I.M. Head, A.D. Akkermans and J.D. van Elsas, [eds.], Molecular Microbial Ecology Manual II. Kluwer Academic Publishers.

Borch, N.H., and D.L. Kirchman. 1999. Protection of protein from bacterial degradation by submicron particles. Aquat. Microb. Ecol. 16: 265-272.

Brenna, T.J., T.N. Corso, H.J. Tobias and R.J. Caimi. 1997. High-precision continuous-flow isotope ratio mass spectrometry. Mass Spectrometry Reviews 16: 227-258.

Brodowski, S., W. Amelung, I. Lobe and C.C. Du Preez. 2004. Losses and biogeochemical cycling of soil organic nitrogen with prolonged arable cropping in the South African Highveld – evidence from D- and L-amino acids. Biogeochemistry 71: 17-42.

Bronk, D.A. and P.M. Glibert. 1993. Application of a ^{15}N tracer method to the study of dissolved organic nitrogen uptake during spring and summer in Chesapeake Bay. Marine Biology 115: 501-508.

Bronk, D.A. 2002. Dynamics of DON. In: Hansell, D.A., Carlson, C.A. (Eds). Biogeochemistry of Marine Dissolved Organic Matter. Academic Press, San Diego, pp 153-247.

Capone, D.G. 2000. The marine microbial nitrogen cycle. *In* Kirchman, D.L. (ed.) Microbial ecology of the oceans. Wiley-Liss. ISBN 0-471-29993-6

Caraco, N.F. and J.J. Cole. 2002. The importance of organic nitrogen production in aquatic systems: a landscape perspective. p 263-283. In: S.E.G. Findlay and R.L. Sinsabaugh (eds.). Aquatic ecosystems: Interactivity of dissolved organic matter. Academic.

Christensen, D. and T.H. Blackburn. 1980. Turnover of tracer (^{14}C, ^{3}H labelled) alanine in inshore marine sediments. Marine Biology 58: 97-103.

Cochlan, W.P., P.J. Harrison and K.L. Denman. 1991. Diel periodicity of nitrogen uptake by marine phytoplankton in nitrate-rich environments. Limnol. Oceanogr. 36 (8): 1689-1700.

Coffin, R.B. 1989. Bacterial uptake of dissolved free and combined amino acids in estuarine waters. Limnol. Oceanogr. 34: 531-542.

Cook, P.L.M., A.T. Revill, E.C.V. Butler and B.D. Eyre. 2004. Carbon and nitrogen cycling on intertidal mudflats of a temperate Australian estuary. II. Nitrogen cycling. Mar. Ecol. Prog. Ser. 280: 39-54.

Cook, P.L.M., B. Veuger, S. Böer and J.J. Middelburg. Effect of nutrient availability on carbon and nitrogen incorporation and flows through benthic algae and bacteria in nearshore sediment. *to be submitted.*

Cowie, G.L. and J.I. Hedges. 1992. Sources and reactivities of amino acids in a coastal marine environment. Limnol. Oceanogr. 37(4): 703-724.

Cowie, G.L., J.I. Hedges and S.E. Calvert. 1992. Sources and relative reactivities of amino acids, neutral sugars, and lignin in an intermittently anoxic marine environment. Geochim. Cosmochim. Acta 56: 1963-1978.

Cowie, G.L., and J.I. Hedges. 1994. Biochemical indicators of diagenetic alterations in natural organic matter mixtures. Nature 369: 304-307.

Créach, V., F. Lucas, C. Deleu, G. Bertu and A. Mariotti. 1999. Combination of biomolecular and stable isotope techniques to determine the origin of organic matter used by bacterial communities: application to sediment. Journal of Microbiological Methods 38: 43-52.

Dauwe, B. and J.J. Middelburg. 1998. Amino acids and hexosamines as indicators of organic matter degradation state in North Sea sediments. Limnol. Oceanogr. 43: 782-798.

Dauwe, B., J.J. Middelburg, P.M.J. Herman and C.H.R. Heip. 1999. Linking diagenetic alteration of amino acids and bulk organic matter reactivity. Limnol. Oceanogr. 44: 1809-1814.

Dijkman, N.A. and J.C. Kromkamp. 2006. Phospholipid-derived fatty acids as chemotaxonomic markers for phytoplankton and application to derive phytoplankton composition in the Scheldt estuary (Belgium & the Netherlands). Mar. Ecol. Prog. Ser. in press.

Dittmar, T., H.P. Fitznar and G. Kattner. 2001. Origin and biogeochemical cycling of organic nitrogen in the eastern Arctic Ocean as evident from D- and L-amino acids. Geochim. et Cosmochim. Acta 65 (22): 4103-4114.

Dugdale, R.C. and J.J. Goering. 1967. Uptake of new and regenerated forms of nitrogen in primary productivity. Limnol. Oceanogr. 12(2): 196-206.

Erbe, T. and H. Brückner. 2000. Studies on the optical isomerisation of dietary amino acids in vinegar and aqueous acetic acid. Eur. Food Res. Technol. 211 (1): 6-12.

Erbe, T. 1999. Die Quantifizierung von Aminosäurenisomeren in Lebensmitteln mittels chiraler Gaschromatographie-Massenspektrometrie im Hinblick auf die Relevanz und die Entstehungsmechanismen von D-Aminosäuren. Ph.D. thesis. Univ. of Gießen. [URL: http://geb.uni-giessen.de/geb/volltexte/2000/215/]

Evrard, V., P.L.M. Cook, B. Veuger and J.J. Middelburg. "The fate of C and N in a subtidal sandy sediment: A ^{13}C and ^{15}N pulse-chase approach". *In preparation.*

Eyre, B.D., S. Rysgaard, T. Dalsgaard, P.B. Christensen. 2002. Comparison of isotope pairing and N_2:Ar methods for measuring sediment denitrification – assumptions, modifications, and implications. Estuaries 25: 1077-1087.

Eyre, B.D. and A.J.P. Ferguson. 2005. Benthic metabolism and nitrogen cycling in a subtropical east Australian estuary (Brunswick): Temporal variability and controlling factors. Limnol. Oceanogr 50(1): 81-96.

Eyre, B.D. and A.J.P. Ferguson. 2006. Impact of a flood event on benthic and pelagic coupling in a sub-tropical east Australian estuary (Brunswick). Estuarine, Coastal and Shelf Science 66: 11-122.

Fan, C. and P.M. Glibert. 2005. Effects of light on nitrogen and carbon uptake during a *Prorocentrum minimum* bloom. Harmful Algae 4: 629-641.

Fantle, M.S., A.I. Dittel, S.M. Schwalm, C.E. Epifanio and M.L. Fogel. 1999. A food web analysis of the juvenile blue crab, *Callinectes sapidus*, using stable isotopes in whole animals and individual amino acids. Oecologia 120: 416-426.

Ferguson, A.J.P., B.D. Eyre and J.M.Gay. 2003. Organic matter and benthic metabolism in euphotic sediments along shallow sub-tropical estuaries, northern New South Wales, Australia. Aquatic Microbial Ecology 33: 137-154.

Ferguson, A.J.P., B.D. Eyre and J.M.Gay. 2004. Benthic nutrient fluxes in euphotic sediments along shallow sub-tropical estuaries, northern New South Wales, Australia. Aquatic Microbial Ecology 37: 219-235.

Fierer, N., J.P. Schimel and P.A. Holden. 2003. Variations in microbial community composition through two soil depth profiles. Soil Biology and Biochemistry 35: 167-176.

Fitznar, H.P., J.M.Lobbes and G. Kattner. 1999. Determination of enantiomeric amino acids with high-performance liquid chromatography and pre-column derivatisation with o-phthaldialdehyde and N-isobutyrylcysteine in seawater and fossil samples (mollusks). Journal of Chromatography A 832: 123-132.

Flynn, K.J. and I. Butler. 1986. Nitrogen sources for the growth of marine microalgae: role of dissolved free amino acids. Mar. Ecol. Prog. Ser. 34: 281-304.

Glaser, B. and W. Amelung. 2002. Determination of ^{13}C natural abundance of amino acid enantiomers in soil: methodological considerations and first results. Rapid Commun. Mass Spectrom. 16: 891-898.

Goldman, J.C. and M.R. Dennett. 2000. Growth of marine bacteria in batch and continuous culture under carbon and nitrogen limitation. Limnology and Oceanography 45: 789-800.

Goldman, J.C. and M.R. Dennett. 2001. Rapid nitrogen uptake by marine bacteria. Limnol. Oceanogr. 46(5): 1195-1198.

Granum, E. and S.M. Myklestad. 1999. Effects of NH_4^+ assimilation on dark carbon fixation and β-1,3-glucan metabolism in the marine diatom skeletonema costatum (bacillariophyceae). Journal of Phycology 35: 1191-1199.

Grasshoff, K., K. Kremling and M. Ehrhardt (Eds.). 1999. Methods of Seawater Analysis. WILEY-VCH, Weinheim, pp 203–205.

Gribsholt, B., H.T.S. Boschker, E. Struyf, M. Andersson, A. Tramper, L. De Brabandere, S. van Damme, N. Brion, P. Meire, F. Dehairs, J.J. Middelburg and C.H.R. Heip. 2005. Nitrogen processing in a tidal freshwater marsh: A whole-ecosystem ^{15}N labeling study. Limnol. Oceanogr. 50(6): 1945-1959.

Gribsholt, B., B. Veuger, A. Tramper, H.T.S. Boschker and J.J. Middelburg. Long-term ^{15}N retention in a tidal freshwater marsh fringing the nutrient rich Scheldt River, Belgium: a pulse-chase ^{15}N labeling experiment. *In preparation*

Griffiths, H. 1998. Stable isotopes. Integration of biological, ecological and geochemical processes. BIOS, Oxford. ISBN: 1-85996-135-5

Grutters M., W. van Raaphorst, E. Epping, J. Helder, J.W. de Leeuw, D.P. Glavin and J. Bada. 2002. Preservation of amino acids from in situ-produced bacterial cell wall peptidoglycans in northeastern Atlantic continental margin sediments. Limnol. Oceanogr. 47 (5): 1521-1524.

Hamilton, S.K., J.L. Tank, D.E. Raikow, E.R. Siler, N.J. Dorn and N.E. Leonard. 2004. The role of instream vs allochthonous N in stream food webs: Modeling the results of an isotope addition experiment. Journal of the North American Benthological Society 23: 429-448.

Hansell, D.A., Carlson, C.A. (Eds). 2002. Biogeochemistry of Marine Dissolved Organic Matter. Academic Press, San Diego.

Henrichs, S.M. and S.F. Sugai. 1993. Adsorption of amino acids and glucose by sediments of Resurrection Bay, Alaska, USA: Functional grou effects. Geochimica et Cosmochimica Acta 57: 823-835.

Herbert, R.A. 1999. Nitrogen cycling in coastal marine ecosystems. FEMS Microbiology Reviews 23: 563-590.

Hoiczyk, E. and A. Hansel. 2000. Cyanobacterial cell walls: news from an unusual prokaryotic envelope. Journal of Bacteriology 182 (5): 1191-1199.

Jørgensen, N.O.G., R. Stepanauskas, A.G.U. Pedersen, M. Hansen and O. Nybroe. 2003. Occurrence and degradation of peptidoglycan in aquatic environments. FEMS Microbiol. Ecol. 46: 269-280.

Jørgensen, N.O.G. 2006. Uptake of urea by estuarine bacteria. Aquatic Microbial Ecology 42: 227-242.

Kaiser, K., and R. Benner. 2005. Hydrolysis-induced racemization of amino acids. Limnol. Oceanogr.: Methods 3: 318-325.

Kanda, J., E.A. Laws, T. Saino and A. Hattori. 1987. An evaluation of isotope dilution from conventional data sets of ^{15}N uptake experiments. Journal of Plankton Research 9: 79-90.

Keil, R.G. and D.L. Kirchman. 1993. Dissolved combined amino acids: chemical form and utilization by marine-bacteria. Limnology and Oceanography 38: 1256-1270.

Keil, R.G., E. Tsamakis and J.I. Hedges. 2000. Early diagenesis of particulate animo acids in marine systems. *In* G.A. Goodfriend, M.J. Collins, M.L. Fogel, S.E. Macko and J.F. Wehmiller [eds.], Perspectives in amino acid and protein geochemistry. Oxford University Press. pp 69-82.

Keil, R.G. and M.L. Fogel. 2001. Reworking of amino acids in marine sediments: Stable carbon isotopic composition of amino acids in sediments along the Washington coast. Limnol. Oceanogr. 46 (1): 14-23.

Kemp. P.F. 1990. The fate of benthic bacterial production. Aquatic Sciences 2: 109-124.

Kirchman, D., E.K. K'nees and R. Hodson. 1985. Leucine incorporation and its potential as a measure of protein synthesis by bacteria in natural aquatic systems. Applied and Environmental Microbiology 49 (3): 599-607.

Kirchman, D.L. 1994. The uptake of inorganic nutrients by heterotrophic bacteria. Microbial Ecology. 28: 255-271.

Kirchman, D.L. 2000. Uptake and regeneration of inorganic nutrients by marine heterotrophic bacteria. *In* Kirchman, D.L. (ed.) Microbial ecology of the oceans. Wiley-Liss. ISBN 0-471-29993-6

Koch, A.L. 1990. Growth and form of the bacterial cell wall. American Scientist 78: 327-341.

Kroer, N., N.O.G. Jørgensen and R.B. Coffin. 1994. Utilization of dissolved nitrogen by heterotrophic bacterioplankton: a comparison of three ecosystems. Applied and Environmental Microbiology. 60 (11): 4116-4123.

Lajtha, K. and R.H. Michener. 1994. Stable isotopes in ecology and environmental science. Blackwell, Oxford. ISBN 0-632-03154-9

Lee, C., and C. Cronin. 1982. The vertical flux of particulate organic nitrogen in the sea: Decomposition of amino acids in the Peru upwelling area and the equatorial Atlantic. J. Mar. Res. 41: 227-251.

Lee, S. and J.A. Fuhrman. 1987. Relationships between biovolume and biomass of naturally derived marine bacterioplankton. Applied and Environmental Microbiology 53: 1298-1303.

Liardon, R. S. Ledermann and U. Ott. 1981. Determination of D-amino acids by deuterium labelling and selected ion monitoring. Journal of Chromatography 203: 385-395.

Linares, F. and K. Sundbäck. 2006. Uptake of dissolved free amino acids (DFAA) by microphytobenthic communities. Aquatic Microbial Ecology 42: 175-186.

Lipschultz, F. 1995. Nitrogen-specific uptake rates of marine phytoplankton isolated from natural populations of particles by flow cytometry. Mar. Ecol. Prog. Ser. 123: 245-258.

Lomstein, B.A., A.G.U. Jensen, J.W. Hansen, J.B. Andreasen, L.S. Hansen, J. Berntsen and H. Kunzendorf. 1998. Budgets of sediment nitrogen and carbon cycling in the shallow water of Knebel Vig, Denmark. Aquatic Microbial Ecology 14: 69-80.

Luna, G.M., E. Manini and R. Danovaro. 2002. Large fraction of dead and inactive bacteria in coastal marine sediments: comparison of protocols for determination and ecological significance. Applied and Environmental Microbiology 68(7): 3509-3513.

Lund, B.A. and T.H. Blackburn. 1989. Urea turnover in a coastal marine sediment measured by a ^{14}C-urea short term incubation. J. Microb. Methods 9: 297-308.

MacGregor, B.J. V. Bruchert, S. Fleischer and R. Amann. 2002. Isolation of small-subunit rRNA for stable isotope characterization. Environmental Microbiology 4 (8): 451-464.

Macko, S.A., M.E. Uhle, M.H. Engel and V. Andrusevich. 1997. Stable nitrogen isotope analysis of amino acid enantiomers by gas chromatography/combustion/isotope ratio mass spectrometry. Anal. Chem. 69: 926-929.

Madigan, M.T., J.M. Martinko and J. Parker. 2000. Brock Biology of microorganisms. 9th ed. Prentice-Hall.

McCarthy J.J. and R.W. Eppley. 1972. A comparison of chemical, isotopic, and enzymatic methods for measuring nitrogen assimilation of marine phytoplankton. Limnol. Oceanogr. 17 (3): 371-382.

McCarthy, J.J., W.R. Taylor and J.L. Taft. 1977. Nitrogenous nutrition of the plankton in the Chesapeake Bay. 1. Nutrient availability and phytoplankton preferences. Limnology and Oceanography 22: 996-1011.

McCarthy, M.D., J.I. Hedges and R. Benner. 1998. Major bacterial contribution to marine dissolved organic nitrogen. Science 281: 231-234.

McClelland, J.W. and J.P. Montoya. 2002. Trophic relationships and the nitrogen isotopic composition of amino acids in plankton. Ecology 83 (8): 2173-2180.

McClelland, J.W., C.M. Holl and J.P. Montoya. 2003. Relating low $\delta^{15}N$ values of zooplankton to N_2-fixation in the tropical North Atlantic: insights provided by stable isotope ratios of amino acids. Deep Sea Research I 50: 849-861.

Middelboe, M., N.H. Borch and D.L. Kirchman. 1995. Bacterial utilization of dissolved free amino acids, dissolved combined amino acids and ammonium in the Delaware Bay estuary: effects of carbon and nitrogen limitation. Mar. Ecol. Prog. Ser. 128: 109-120.

Middelburg, J.J., C. Barranguet, H.T.S. Boschker, P.M.J. Herman, T. Moens and C.H.R. Heip. 2000. The fate of intertidal microphytobenthos carbon: An in situ ^{13}C-labeling study. Limnol. Oceanogr. 45 (6): 1224-1234.

Middelburg, J.J., and J. Nieuwenhuize. 2000a. Uptake of dissolved inorganic nitrogen in turbid, tidal estuaries. Marine Ecology Progress Series 192, 79-88.

Middelburg, J.J. and J. Nieuwenhuize. 2000b. Nitrogen uptake by heterotrophic bacteria and phytoplankton in the nitrate-rich Thames estuary. Mar. Ecol. Prog. Ser. 203: 13-21.

Middelburg, J.J., and J. Nieuwenhuize. 2001. Nitrogen isotope tracing of dissolved inorganic nitrogen behaviour in tidal estuaries. Estuarine, Coastal and Shelf Science 53: 385-391.

Mobley, H.L.T. and R.P. Hausinger. 1989. Microbial ureases: significance, regulation and molecular characterization. Microbiological reviews 53(1): 85-108.

Moodley, L., H.T.S. Boschker, J.J. Middelburg, R. Pel, P.M.J. Herman, E. de Deckere and C.H.R. Heip. 2000. Ecological significance of benthic foraminifera: ^{13}C labeling experiments. Mar. Ecol. Prog. Ser. 202: 289-295.

Moriarty, D.J.W. and A.C. Hayward. 1982. Ultrastructure of bacteria and the proportion of Gram-negative bacteria in marine sediments. Microb. Ecol, 8: 1-14.

Mulholland, M.R., P.M. Glibert, G.M. Berg, L. Van Heukelem, S. Pantoja and C. Lee. 1998. Extracellular amino acid oxidation by microplankton: a cross-ecosystem comparison. Aquat. Microb. Ecol. 15: 141-152.

Mulholland, M.R., C.J. Gobler and C. Lee. 2002. Peptide hydrolysis, amino acid oxidation, and nitrogen uptake in communities seasonally dominated by *Aureococcus anophagefferens*. Limnol. Oceanogr. 47 (4): 1094-1108.

Mulholland, M.R., C. Lee and P.M. Glibert. 2003. Extracellular enzyme activity and uptake of carbon and nitrogen along an estuarine salinity and nutrient gradient. Mar. Ecol. Prog. Ser. 258: 3-17.

Mulholland, M.R., Boneillo, G. and Minor, E. C. 2004. A comparison of N and C uptake during brown tide (*Aureococcus anophagefferens*) blooms from two coastal bays on the east coast of the USA. Harmful Algae 3: 361-376.

Nagata, T., B. Meon and D.L. Kirchman. 2003. Microbial degradation of peptidoglycan in seawater. Limnol. Oceanogr. 48 (2): 745-754.

Nielsen, K., N. Risgaard-Petersen, B. Somod, S. Rysgaard and T. Bergo. 2001. Nitrogen and phosphorus retention estimated independently by flux measurements and dynamic modeling in the estuary, Randers Fjord, Denmark. Marine Ecology Progress Series 219: 25-40.

Nieuwenhuize, J., Y.E.M. Maas and J.J. Middelburg. 1994. Rapid analysis of organic-carbon and nitrogen in particulate materials. Marine Chemistry 45: 217-224.

Oremland, R.S. and D.G. Capone. 1988. Use of specific inhibitors in biogeochemistry and microbial ecology. Advances in Microbial Ecology 10; 285-383.

Ottosen, L.D.M., N. Risgaard-Petersen, L.P. Nielsen and T. Dalsgaard. 2001. Denitrification in exposed intertidal mud-flats, measured with a new ^{15}N-ammonium spray technique. Mar. Ecol. Prog. Ser. 209: 35-42.

Palenik, B. and F.M.M. Morel. 1990. Amino acid utilization by marine phytoplankton: A novel mechanism. Limnol. Oceanogr. 35(2): 260-269.

Pantoja, S. and C. Lee. 1994. Cell-surface oxidation of amino acids in seawater. Limnol. Oceanogr. 39(7): 1718-1726.

Pantoja, S. and C. Lee. 2003. Amino acid remineralization and organic matter lability in Chilean coastal sediments. Organic Geochemistry 34: 1047-1056.

Parkes, R.J., 1987. Analysis of microbial communities within sediments using biomarkers, p. 147-177. *In* M. Hetcher, R.T.G. Gray and J.G. Jones [eds.], Ecology of microbial communities. Cambridge University Press.

Pedersen, A.G.U., T.R. Thomsen, B.A. Lomstein and N.O.G. Jørgensen. 2001. Bacterial influence on amino acid enantiomerisation in a coastal marine sediment. Limnol. Oceanogr. 46 (6): 1358-1369.

Pelz, O., L.A. Cifuentes, B.T. Hammer, C.A. Kelley and R.B. Coffin. 1998. Tracing the assimilation of organic compounds using δ^{13}C analysis of unique amino acids in the bacterial peptidoglycan cell wall. FEMS Microbiol. Ecol. 25: 229-240.

Porter, K.G. and T.S. Feig. 1980. The use of DAPI for identifying and counting aquatic microflora. Limnology and Oceanography 25: 943-948.

Price, N.M. and P.J. Harrison. 1988. Uptake of urea C and urea N by the coastal marine diatom *Thalassiosira pseudonana*. Limnol. Oceanogr. 33(4): 528-537.

Risgaard-Petersen, N. 2003. Coupled nitrification-denitrification in autotrophic and heterotrophic estuarine sediments: On the influence of benthic microalgae. Limnol. Oceanogr. 48(1): 93-105.

Rondell, J.B., K.W. Finster and B.A. Lomstein. 2000. Urea and DON uptake by a *Lyngbya gracialis* dominated microbial mat: a controlled laboratory experiment. Aquatic Microbial Ecology 21: 169-175.

Rosenfeld, J.K. 1979. Ammonium adsorption in nearshore anoxic sediments. Limnol. Oceanogr. 24: 356-364.

Rosenfeld, J.K. 1979. Amino acid diagenesis and adsorption in nearshore anoxic sediments. Limnol. Oceanogr. 24: 1014-1021.

Rysgaard, S., N. Risgaard-Petersen, L.P. Nielsen and N.P. Revsbech. 1993. Nitrification and denitrification in lake and estuarine sediments measured by the ^{15}N dilution technique and isotope pairing. Applied and Environmental Microbiology 59(7): 2093-2098.

Rysgaard, S., P.B. Christensen and L.P. Nielsen. 1995. Seasonal variation in nitrification and denitrifiction in estuarine sediment colonized by benthic microalgae and bioturbating infauna. Mar. Ecol. Prog. Ser. 126: 111-121.

Schell, D.M. 1974. Uptake and regeneration of free amino acids in marine waters of Southeast Alaska. Limnol. Oceanogr. 19 (2): 260-270.

Schmidt, J.L., J.W. Deming, P.A. Jumars and R.G. Keil. 1998. Constancy of bacterial abundance in surficial marine sediments. Limnol. Oceanogr. 43: 976-982.

Seitzinger, S.P., R.W. Sanders and R. Styles. 2002. Bioavailability of DON from natural and anthropogenic sources to estuarine plankton. Limnology and Oceanography 47: 353-366.

Silfer, J.A., M.H. Engel, S.A. Macko and E.J. Jumeau. 1991. Stable carbon isotope analysis of amino acid enantiomers by conventional isotope ratio mass spectrometry and combined with gas chromatography/isotope ratio mass spectrometry. Anal. Chem. 63: 370-374.

Simon, M. and F. Azam. 1989. Protein content and protein synthesis rates of planktonic marine bacteria. Marine Ecology Progress Series 51: 201-213.

Sinninghe Damsté, J.S. et al. 2004. The rise of the rhizosolenid diatoms. Science 304: 584-587.

Smith, D.C. and F. Azam. 1992. A simple, economical method for measuring bacterial protein synthesis rates in seawater using 3H-leucine. Marine Microbial Food Webs 6 (2): 107-114.

Soetaert, K., J.J. Middelburg, C. Heip, P. Meire, S. van Damme and T. Maris. 2006. Long-term change in dissolved inorganic nutrients in the heterotrophic Scheldt estuary (Belgium, The Netherlands). Limnol. Oceanogr. 51: 409-423.

Sonesson, A., L. Larsson, A. Fox, G. Westerdahl and G. Odham. 1988. Determination of environmental levels of peptidoglycan and lipopolysaccharide using gas chromatography with negative-ion chemical-ionization mass spectrometry utilizing bacterial amino acids and hydroxy fatty acids as biomarkers. Journal of Chromatography 431: 1-15.

Stepanauskas, R., N.O.G. Jørgensen, O.R. Eigaard, A. Zvikas, L.J. Tranvik and L. Leonardson. 2002. Summer inputs of riverine nutrients to the Baltic Sea: bioavailability and eutrophication relevance. Ecological Monographs 72: 579-597.

Stephens, G.C. and B.B. North. 1971. Extrusion of carbon accompanying uptake of amino acids by marine phytoplankters. Limnol. Oceanogr. 16 (5): 752-757.

Steyaert, M. P.M.J. Herman, T. Moens, J. Widdows and M. Vincx. 2001. Tidal migration of nematodes on an estuarine tidal flat (the Molenplaat, Schelde Estuary, SW Netherlands). Mar. Ecol. Prog. Ser. 224: 299-304.

Sundbäck, K., F. Linares, F. Larson, A. Wulff and A. Engelsen. 2004. Benthic nitrogen fluxes along a depth gradient in a microtidal fjord: The role of denitrification and microphytobenthos. Limnol. Oceanogr. 49(4): 1095-1107.

Tamminen, T. and A. Irmisch. 1996. Urea uptake kinetics of a midsummer planktonic community on the SW coast of Finland. Mar. Ecol. Prog. Ser. 130: 201-211.

Tank, J.L., J.L. Meyer, D.M. Sanzone, P.J. Mulholland, J.R. Webster, B.J. Peterson, W.M. Wollheim and N.E. Leonard. 2000. Analysis of nitrogen cycling in a forest stream during autumn using a ^{15}N-tracer addition. Limnol. Oceanogr. 45(5): 1013-1029.

Tobias, C., A. Giblin, J. McClelland, J. Tucker and B. Peterson. 2003. Sediment DIN fluxes and preferential recycling of benthic microalgal nitrogen in a shallow macrotidal estuary. Mar. Ecol. Prog. Ser. 257: 25-36.

Tunlid, A. and G. Odham. 1983. Capillary gas chromatography using electron capture or selected ion monitoring detection for the determination of muramic acid, diaminopimelic acid and the ratio of D/L-alanine in bacteria. Journal of Microbiological Methods 1: 63-76.

Tunlid, A., G. Odham, R.H. Findlay and D.C. White. 1985. Precision and sensitivity of the measurement of ^{15}N enrichment in D-alanine from bacterial cell walls using positive/negative ion mass spectrometry. Journal of Microbiological Methods 3: 237-245.

Ueda, K., S.L. Morgan, A. Fox, J. Gilbart, A. Sonesson, L. Larsson, G. Odham. 1989. D-Alanine as a chemical marker for the determination of streptococcal cell wall levels in mammalian tissues by gas chromatography/negative ion chemical ionisation mass spectrometry. Anal. Chem. 61: 265-270.

Uhle, M.E., S.A. Macko, H.J. Spero, M.H. Engel and D.W. Lea. 1997. Sources of carbon and nitrogen in modern planktonic foraminifera: the role of algal symbionts as determined by bulk and compound specific stable isotope analyses. Org. Geochem. 27: 103-113.

Van Oevelen, D., J. J. Middelburg, K. Soetaert, and L.Moodley. 2006a. The fate of bacterial carbon in an intertidal sediment: Modeling an in situ isotope tracer experiment. Limnol. Oceanogr. 51(3): 1302-1314.

Van Oevelen, D., L. Moodley, K. Soetaert, and J. J. Middelburg. 2006b. The trophic significance of bacterial carbon in a marine intertidal sediment: Results of an in situ stable isotope labeling study. Limnol. Oceanogr. 51 (5): 2349-2359.

Van Oevelen, D., K. Soetaert, J.J. Middelburg, P.M.J. Herman, L. Moodley, I. Hamels, T. Moens and C.H.R. Heip. 2006c. Carbon flows through a benthic food web: Integrating biomass, isotope and tracer data. Journal of Marine Research 64: 453-482.

Veuger, B., J.J. Middelburg, H.T.S. Boschker, J. Nieuwenhuize, P. van Rijswijk, E.J. Rochelle-Newall and N. Navarro. 2004. Microbial uptake of dissolved organic and inorganic nitrogen in Randers Fjord. Estuarine, Coastal and Shelf Science 61 (3): 507-515. (Chapter 1)

Veuger, B., J.J. Middelburg, H.T.S. Boschker and M. Houtekamer. 2005. Analysis of ^{15}N incorporation into D-alanine: a new method for tracing nitrogen uptake by bacteria. Limnol. Oceanogr. Methods 3: 230-240. (Chapter 2)

Veuger, B., D. van Oevelen, H.T.S. Boschker and J.J. Middelburg. 2006. Fate of peptidoglycan in an intertidal sediment: an in situ ^{13}C-labeling study. Limnology and Oceanography 51 (4): 1572-1580. (Chapter 5)

Veuger, B., B.D. Eyre, D. Maher and J.J. Middelburg. Nitrogen incorporation and retention by bacteria, algae and fauna in a subtropical, intertidal sediment: An in situ ^{15}N-labeling study. *In revision for Limnology and Oceanography* (Chapter 4)

Veuger, B and J.J. Middelburg. Incorporation of nitrogen and carbon from urea and amino acids by bacteria and algae in interidal sediment. *In revision for Aquatic Microbial Ecology* (Chapter 3)

Wheeler, P.A. and D.L. Kirchman. 1986. Utilization of inorganic and organic nitrogen by bacteria in marine systems. Limnol. Oceanogr. 31 (5): 998-1009.

Zehr JP, Axler RP, Goldman CR (1985) Heterotrophic mineralization of amino acid nitrogen in subalpine Castle Lake, California. Marine Chemistry 16: 343-350

Zehr, J.P. and B.B. Ward. 2002. Nitrogen cycling in the ocean: New perspectives on processes and paradigms. Applied and Environmental Microbiology 68 (3): 1015-1024.

Ziegler, S.E., and M.L. Fogel. 2003. Seasonal and diel relationships between the isotopic compositions of dissolved and particulate organic matter in freshwater ecosystems. Biogeochemistry 64: 25-52.

CURRICULUM VITAE

Bart Veuger (born May 23rd, 1979 in Purmerend) studied biology at the University of Amsterdam (1997-2001) where he specialized in aquatic eco(toxico)logy by working on the use of benthic waterfleas as water quality test organisms and on the production of extracellular polymeric substances during the development of photosynthetic freshwater biofilms. From November 2001 until April 2006, he worked as a PhD-student at the Netherlands Institute of Ecology (NIOO-KNAW), Centre for Estuarine and Marine Ecology (CEME) in Yerseke, which resulted in this PhD-thesis. After his PhD-work, he stayed at the CEME as a post-doctoral researcher continuing the line of work started during his PhD-period.

PUBLICATIONS by Bart Veuger

2004

Veuger, B., J.J. Middelburg, H.T.S. Boschker, J. Nieuwenhuize, P. van Rijswijk, E.J. Rochelle-Newall and N. Navarro. 2004. Microbial uptake of dissolved organic and inorganic nitrogen in Randers Fjord. *Estuarine, Coastal and Shelf Science 61(3): 507-515* (Chapter 1)

C. Barranguet, S.A.M. van Beusekom, **B. Veuger**, T.R. Neu, E.M.M. Manders, J.J. Sinke and W. Admiraal 2004 Studying undisturbed autotrophic biofilms: still a technical challenge. *Aquatic Microbial Ecology 34: 1-9*

2005

Veuger, B., J.J. Middelburg, H.T.S. Boschker and M. Houtekamer. 2005. Analysis of ^{15}N incorporation into D-alanine: a new method for tracing nitrogen uptake by bacteria. *Limnology and Oceanography: Methods 3: 230-240* (Chapter 2)

Barranguet, C., **B. Veuger**, S.A.M. van Beusekom, P. Marvan, J.J. Sinke and W. Admiraal. 2005. Divergent composition of algal-bacterial biofilms developing under various external factors. *European Journal of Phycology 40: 1-8*

2006

Veuger, B., D. van Oevelen, H.T.S. Boschker and J.J. Middelburg. 2006. Fate of peptidoglycan in an intertidal sediment: an in situ ^{13}C-labeling study. *Limnology and Oceanography 51(4): 1572-1580* (Chapter 5)

Veuger, B. 2006. A new method for tracing flows of nitrogen and carbon through bacteria and algae in aquatic microbial communities: Analysis of ^{15}N- and ^{13}C-incorporation into D-alanine and other hydrolysable amino acids. PhD thesis, Utrecht University.

Dekker, T., G.D. Greve, T.L. ter Laak, M.E.Y. Boivin, **B. Veuger**, G. Gortzak, S. Dumfries, S.M.G. Lücker, M.H.S. Kraak, W. Admiraal and H.G. van der Geest. 2006. Development and application of a sediment toxicity test using the benthic cladoceran Chydorus sphaericus. *Environmental Pollution 140: 231-238*

Under construction

Veuger, B., B.D. Eyre, D. Maher and J.J. Middelburg. Nitrogen incorporation and retention by bacteria, algae and fauna in a subtropical, intertidal sediment: An in situ ^{15}N-labeling study. *In revision for Limnology and Oceanography* (Chapter 4)

Veuger, B and J.J. Middelburg. Incorporation of nitrogen and carbon from urea and amino acids by bacteria and algae in interidal sediment. *In revision for Aquatic Microbial Ecology* (Chapter 3)

Cook, P.L.M., **B. Veuger**, S. Böer and J.J. Middelburg. Effect of nutrient availability on carbon and nitrogen incorporation and flows through benthic algae and bacteria in nearshore sediment. *To be submitted.*

Evrard, V., P.L.M. Cook, **B. Veuger** and J.J. Middelburg. "The fate of C and N in a subtidal sandy sediment: A ^{13}C and ^{15}N pulse-chase approach". *In preparation.*

Gribsholt, B., **B. Veuger**, A. Tramper, H.T.S. Boschker and J.J. Middelburg. Long-term ^{15}N retention in a tidal freshwater marsh fringing the nutrient rich Scheldt River, Belgium: a pulse-chase ^{15}N labeling experiment. *In preparation.*

For up to date information regarding my work have a look at
http://www.nioo.knaw.nl/ppages/bveuger
or send me an e-mail (b.veuger@nioo.knaw.nl)

ACKNOWLEDGEMENTS

In addition to the acknowledgements in the individual chapters I thank the following people for their contributions to the work presented in this thesis:

Jack Middelburg for being my PhD-supervisor giving me the freedom to do things my own way while keeping an eye on the big picture and giving me an occasional push in the right direction.

Eric Boschker for his help with starting up the method, especially the GC-c-IRMS part, and his contributions to chapters 1, 2 and 5.

Marco Houtekamer for his valuable help with the GC-c-IRMS analyses and other lab work.

Dick van Oevelen for the opportunity to analyze some of his samples and the subsequent stimulating cooperation that resulted in chapter 5.

Pieter van Rijswijk for his indispensable help with all kinds of lab work.

Perran Cook, Victor Evrard, Britta Gribsholt and Karel van den Meersche for daring to include me and "my method" into their work.

The other (former) CEME-scientists for stimulating discussions, presentations, cooperations, advice, etc.

Joop Nieuwenhuize, Peter van Breugel, Coby Kleppe, Jan Sinke en Yvonne van der Maas for their help in the lab and for doing all kinds of analyses.

Brad Eyre, Damien Maher, Lexie Walker and the other people at the Centre for Coastal Biogeochemistry, Southern Cross University, Lismore, Australia for the opportunity to work with you. The field work in the Brunswick Estuary was a great experience and I think our results turned out really nice.

Christiane Barranguet for introducing me to Jack at the CEME when I was looking for a PhD-job.

Anke for translating the short summary into german.

Carlo Heip for acting as my co-promotor.

Ron Benner, Jaap Sinninghe Damsté, Gerhard Herndl, Wim Admiraal and Riks Laanbroek for being members of my PhD-committee.