Decomposition of Organic Matter in the Littoral Sediments of a Lake

H.T.S. Boschker

Promotor: dr. A.J.B. Zehnder
hoogleraar in de microbiologie

Co-promotor: dr. T.E. Cappenberg
senior onderzoeker microbiële ecologie, NIOO, Yerseke

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Aerial view of the littoral system of Lake Gooimeer. The sampling transect used is just above the middle of the picture. Camera is looking to the west.

Front page: The reed bed of Lake Gooimeer, The Netherlands. Lake-side view in autumn.

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Stellingen

1. De afbraak van sedimentair organisch materiaal wordt in de rietkraag van het Gooimeer niet beïnvloed door adsorptie aan de minerale matrix van het sediment. Dit in tegenstelling tot wat algemeen in mariene sedimenten wordt gevonden [1].

Dit proefschrift

[1] Keil et al. (1994) Sorptive preservation of labile organic matter in marine sediments. Nature 370: 549-551

2. De moleculaire struktuur van humus is zo complex [1], dat de vraag gesteld kan worden of er wel sprake is van enige struktuur. Aangezien humus wel afbreekbaar is, zullen er micro-organismen bestaan die in het bezit zijn van enzymsystemen met een struktuur aspecifiek werkingsmechanisme.

[1] Hayes et al. (1989) Structures of humic substances: the emergence of 'forms'. In: Hayes et al (Eds) Humic substances II. Wiley New York: 689-733..

3. De door Benner et al. gevonden mineralisatie van lignine onder anaerobe omstandigheden is mogelijk een artefact van de gebruikte methode, gezien het lage percentage dat gemineraliseerd werd [1] en de gedeeltelijk niet-specifieke markering van het door hen gebruikte ligno-cellulose [2, 3].

[1] Benner et al. (1984) Anaerobic biodegradation of the lignin and polysaccharide components of lignocellulose and synthetic lignin by sediment microflora. Appl. Environ. Microbiol. 47: 998-1004.
[2] Benner et al. (1984) Preparation, characterization, and microbial degradation of specifically radiolabeled [^{14}C]lignocelluloses from marine and freshwater macrophytes. Appl. Environ. Microbiol. 47: 381-389.
[3] Crawford and Crawford (1976) Microbial degradation of lignocellulose: the lignin component. Appl. Environ. Microbiol. 31: 714-717.

4. Het gebruik van enzymactivitieiten, zoals gemeten met artificiële substraten, als schatting voor de koolstofmineralisatiesnelheid in natuurlijke systemen [1,2] geeft een grote overschatting van de werkelijke snelheden.

Dit proefschrift

Poremba (1995) Hydrolytic enzymatic activity in deep-sea sediments. FEMS Microbiol. Ecol. 16, 213-222.
Middelboe et al. (1995) Attached and free-living bacteria: production and polymer hydrolysis during diatom bloom. Microb. Ecol. 29: 231-248.

5. De afbraak van riet strooisel is traag vergeleken met de duur van een promotie aanstelling, maar was gedurende de afgelopen jaren vergelijkbaar met de afbraaksnelheid van arbeidsvoorwaarden voor nieuwe promovendi.

6. Onderzoek doen is als een regenboog. Vol enthousiasme gaat men op weg naar de pot met goud. Maar na een interessante reis blijkt deze zich toch altijd weer achter de horizon te bevinden.

7. Bij het doel van natuurbeheer moeten vragen gesteld worden als mooie gevarieerde overgangszones, met rietkraag, wilgen en populieren, worden omgezet in een riet monoculture uitsluitend om de rietzanger beter te beschermen (Gooimeer, 1990/1991).

8. Er is een discrepantie tussen de algemene inzet van vaccins voor ziektebestijding bij de mens en het verbieden van preventieve vaccinatie tegen de varkenspest.

9. Uit een doorsnee avondje zappen blijkt dat vooral praten over sport en daaraan verwante zaken als sport moeten worden gezien.

10. De trend om de tuin te beschouwen als het verlengstuk van de woonkamer heeft mede tot gevolg dat tuincentra steeds meer op meubelzaken gaan lijken.

11. Voor microbiologen die aan afbraakprocessen werken is het toch mogelijk om opbouwend bezig te zijn.

H.T.S. Boschker
Stellingen behorend bij het proefschrift "Decomposition of organic matter in the littoral sediments of a lake".
Wageningen, 1997

Voorwoord

Het onderzoek voor dit proefschrift is tussen 1989 en 1994 uitgevoerd op het Nederlands Instituut voor Oecologisch Onderzoek - Centrum voor Limnologie (NIOO/CL) te Nieuwersluis. De studie beschreven in dit proefschrift is het gevolg een eerste, prille samenwerking met eerst één en vervolgens twee instituten, welke nu de andere centra in het NIOO vormen. Aangezien deze drie instituten aquatisch en terrestrisch onderzoek doen, leek het een voordehand liggend en uitdagend idee om gezamelijk onderzoek te verrichten op het raakvlak van deze werkterreinen: de overgang tussen water en land.

Onderzoek doen in de rietkraag van het Gooimeer viel in eerste instantie niet mee. Staand tussen het riet werd het zicht beperkt tot zo'n twee meter. Keek je vervolgens naar beneden dan leek het daar zo heterogeen te zijn, dat de vraag opkwam hoe hier ooit zinnige data uit te extraheren. Verder bleek het systeem zo veranderlijk dat elk bezoek aan het veld weer nieuwe verrassingen bracht. Soms was alle rietstrooisel, waaraan onderzoek gedaan moest worden, plotsklaps van het sediment verdwenen, of meldde er zich een rietmaaier die wel trek had in de onderzoeksplots. Terugkijkend is het toch allemaal goed verlopen en is het mogelijk gebleken om de koolstof dynamiek van dit complexe systeem te onderzoeken.

Tom Cappenberg wil ik bedanken voor het scheppen van een omgeving waarin in grote vrijheid onderzoek gedaan kon worden en het zelden ontbrak aan financiële mogelijkheden. Mijn promotor, Alex Zehnder, ben ik erkentelijk voor zijn kritische bijdrage aan het onderzoek en de manuscripten. Tevens is hij een belangrijke stimulans geweest voor een brede kijk op de microbiologie, zodat ik niet in het sediment ben blijven steken. Mede door de prettige samenwerking met mijn beide lotgenoten in goede en slechte tijden Kerst Buis en Mathieu Staring is het allemaal zo goed verlopen. Ten Dekkers heeft een belangrijk deel van de analyses uitgevoerd, mijn dank daarvoor. Steven Visser wil ik bedanken voor alle ritjes naar het Gooimeer en de assistentie in het veld. Hoewel ik weinig ingewikkelde, fijn mechanische apparatuur heb weten te bedenken waarop zij zich eens echt lekker uit konden leven, wil ik Hennie Uitenhout, Piet Schouten, Tinus Röling en Petra Polman bedanken voor hun technische steun en de fraaie 'gadgets'. Special thanks to my student from Sweden, Stefan Bertilsson; whithout his seemingly endless energy several chapters would have been much shorter and less comprehensive. Mardie Hack wil ik natuurlijk bedanken als steun en toeverlaat buiten de normale werktijden. Riks Laanbroek en Marten Hemminga ben ik erkentelijk voor de tijd die ik heb gekregen om dit proefschrift af te ronden. En tenslotte bedank ik alle collega's in Nieuwersluis, Yerseke en Wageningen voor de gezellige werksfeer en hun bijdragen aan dit proefschrift.

Het onderzoek werd gefinancierd door de Programma Comissie Basisonderzoek Bodem (PCBB), Wageningen, en het Rijksinstituut voor Intergraal waterbeheer en Zuivering Afvalwater (RIZA), Lelystad, die ik daarvoor zeer erkentelijk ben.

Yerseke, maart 1997

Contents

Chapter 1

Introduction

This thesis deals with the decomposition of organic matter in littoral sediments of lakes. Special attention was given to the initial step in the decomposition of polysaccharides that form a major component of macrophyte litter produced in these systems. This initial step, an extracellular enzymatic hydrolysis, is generally regarded as the rate limiting step in the decomposition of biopolymers in natural systems. The study site selected was a reed bed that covers most of the upper littoral zone of Lake Gooimeer, The Netherlands.

Littoral systems of lakes

The littoral is the transition zone between land and water, and therefore combines characteristics of terrestrial and aquatic ecosystems. Aquatic since it has water-saturated, mostly anaerobic sediments for at least part of the year, and terrestrial since there is a macrophyte vegetation present. On the land side of the littoral this vegetation exists of helophytes, plants that root in the sediment and have emergent stems and leaves. Dominant emergent plant species in temperate climate zones are grasses, like common reed (*Phragmites australis*), or other plants, such as cat-tails (*Typha* spp) and rushes (*Scirpus* spp). These plants generally grow in monospecific stands and have an extensive rooting system, which is used for winter survival. These vegetations are highly productive, but are generally not grazed directly by herbivores due to the low food quality of the biomass. Instead, it is thought that most of the above-ground production eventually becomes available to microbial decomposition after the plants have died back in autumn (Dykyjová & Kvet; Mann 1988; Westlake 1963).

Littoral systems and wetlands in general have only recently gained more attention as an endangered type of ecosystem (Mitsh & Gosselink 1993). There has been a reduction

in the macrophyte cover in many littoral systems. Even a common species like *Phragmites australis* appears to decline in Europe, which is caused by a combination of a number of environmental changes like deterioration of sediment conditions as a result of eutrophication, water level changes, and destruction of habitat (Ostendorp 1989). Wetlands also play an important role in the cycling of atmospheric trace gasses (Conrad 1995). They are for example the largest single natural source of the greenhouse gas methane (IPCC 1994).

Previous lack of interest has been partially caused by the complex nature of littoral ecosystems. First of all, besides emergent macrophytes a substantial number of other primary producers are potentially important as organic carbon sources for the food web and for the sediment. A high primary production of submerged macrophytes is characteristic for most littorals, and in addition to phytoplankton, other algal sources like epiphytes and benthic algae are present. Furthermore, terrestrial material can be transported to the littoral by run-off or winds (Wetzel 1992). A second complicating factor in studying wetlands is that roots of macrophytes have a major influence on sedimentary microbial processes. Wetland plants may release oxygen and organic matter into their rhizosphere, and compete with microbes for nutrients like nitrogen and phosphorus (Mitsh & Gosselink 1989; Reddy & Patrick 1984). The small-scale three-dimensional structure of the rooting system and its surrounding rhizosphere makes it difficult to study in terms of organic matter turnover and electron acceptor consumption. Fluctuations in water level causing shifts in sedimentary redox conditions also add to the complexity of microbial processes in wetland sediments.

Microbial decomposition of organic matter in sediments

Organic matter in sediments mainly exists of a mixture of polymeric compounds, which are either of direct biological origin (biopolymers) or derived from partially degraded and re-polymerised material (geopolymers or humus). Microbial decomposition of these polymers is a step-wise process (Fig. 1). The initial step is a extracellular enzymatic hydrolysis to smaller units, which can then be taken up by micro-organisms. Under oxic and denitrifying conditions, these smaller units are directly mineralised to CO_2 (Fig. 1A). In water-logged littoral sediments, oxygen will only be found in the top few millimetres of the sediment and in the direct vicinity of macrophyte roots. Under anoxic conditions, the intermediates are first fermented to mainly short chain fatty acids, especially acetate, and to H_2/CO_2. These fermentation products are finally mineralised by a number of processes, like metal reduction, sulphate reduction and methanogenesis, depending on the electron acceptors available (Fig. 1B). Most work on the processes in Fig. 1 has been directed either to the degradation of bulk sediment organic matter and

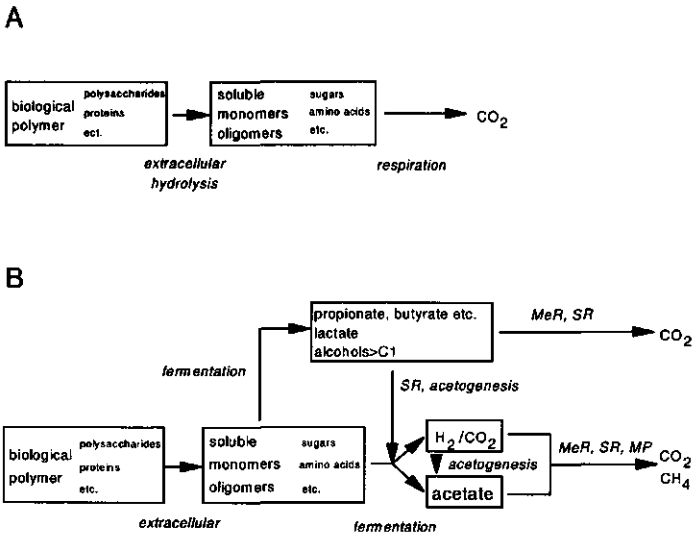

Figure 1. Schematic presentation of the processes and intermediates involved in the decomposition of organic matter in sediments under oxic and denitrifying conditions (A), and under anoxic conditions (B). MeR stands for metal reduction, SR for sulphate reduction and MP for methane production or methanogenesis. Adapted from Parkes (1987).

simple organic compounds (hydrolysis and fermentation products), or to electron acceptor consumption, and these processes are relatively well understood at least in unvegetated sediment (Capone & Kiene 1988; Nedwell 1984; Jørgensen 1983).

Although the initial, enzymatic decomposition is generally thought to be the rate determining step in the mineralization of organic matter (Billen 1982; Meyer-Reil 1986; King 1986, Cappenberg 1988), there is little direct information on the rates and intermediates involved in the decomposition of specific biopolymers in natural systems like sediments (Henrichs 1992). This is partially due to a lack of methods. Several approaches have been used to study the initial decomposition of polymeric material. The activities of extracellular enzymes in environmental samples are often assessed by adding an artificial substrate at a saturating concentration (Burns 1978; King 1986; Sinsabaugh et al. 1991). The measured enzyme activity should be regarded as a potential value; the actual concentration of the natural substrate, its availability to the enzymes, or its interactions with other polymers in the sediment organic matter are not incorporated. Some of these problems can be circumvented by using specifically radio-labelled polymers (Benner et al. 1984a; Cunningham & Wetzel 1989; Schink & Zeikus 1982) or by following changes in the composition of the sedimentary matter itself (Benner et al. 1990; Wilson et al. 1986b). Another approach is the use of radiolabelled compounds to study the turnover of the free pool of intermediates that are formed during initial

decomposition (Gocke et al. 1981; King & Klug 1982; Sawyer & King 1993). Problems with this last approach are that (i) the identity of the important intermediates is not known, (ii) not all possible intermediates will be readily available in labelled form, and (iii) the free bioavailable concentration of the intermediates, which is also needed, might be hard to assess (Dawson & Liebezeit 1981; Gocke et al. 1981; Henrichs 1992).

A further difficulty in studying initial decomposition of the sediment organic matter is that its exact polymeric composition is seldom known. The composition of organic matter in sediment is normally seen as very complex. Sediment organic matter can be subjected to various hydrolysis, extraction, and decomposition procedures. Although the resulting products of these procedures can be analysed by a suit of analytical methods to determine for example the composition of carbohydrates, amino acids, lipids and lignin phenols, there generally remains a substantial part (60% or more) of the organic matter which does not belong to those categories of compounds (Cowie & Hedges 1992; Kemp & Johnston 1979). Furthermore, these methods produce no information on how these monomeric compounds are chemically bound in the polymeric matrix of the sediment. The type of chemical bounding is important since it determines which types of microbial enzymes are needed for its degradation and bioavailability. Types of bounding have been studied with the use of specific microbial enzymes. Proteinase, for example, has been used by Mayer et al. (1995) to quantify biologically reactive protein in marine sediments and their results show that only a small part (<30%) of the total amino acids are present as protein or polypeptides.

Sediments of the upper littoral are interesting sites to study initial decomposition processes. They are covered by a dense vegetation of mostly a single plant species, which is most likely also a major carbon source for the sediment. Furthermore, the organic litter that is produced by these plants has a well defined biopolymeric composition. Micro-organisms and enzymes capable of degrading these biopolymers have been studied extensively during the last decades, though not in complex systems like sediments.

Polymers in plant litters and their decomposition

During senecense of the plants, most of the cell content is transported to the rhizome for winter survival and regrowth in the next season. What remains of the above-ground biomass is mainly cell wall material, which is to a large extent made of lignocellulose and has a relatively simple polymeric composition (Benner et al. 1984a). Lignocellulose is made of a variable combination of lignin, cellulose and a class of polysaccharides collectively called hemicelluloses. Cellulose is the backbone of the plant cell wall and is found as crystalline microfibrills in which cellulose molecules are found in parallel bundles. Hemicellulose molecules are situated between the cellulose microfibrills,

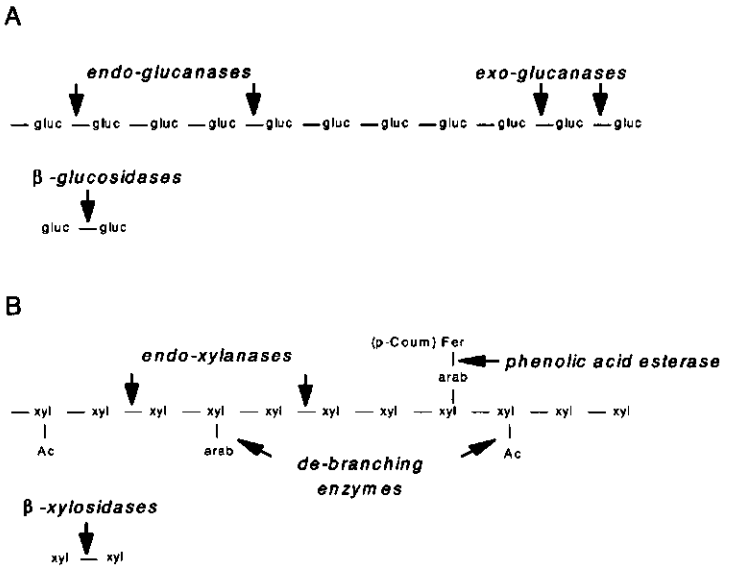

Figure 2. Composition of the two major polysaccharides in grass litters, cellulose (A) and arabino-xylan (B), and the enzymes involved in their decomposition. Glucose (gluc) in cellulose and xylose (xyl) in arabino-xylan are (1-4)-β bound, and arabinose (arab) is (1-3)-α or sometimes (1-2)-α bound to the xylan back bone. Xylan also contains O-acetyl side groups (Ac). De-branching enzymes involved in the decomposition of arabino-xylan are α-arabinosidase and O-acetyl esterase. The phenolic acids ferulic acid (Fer) and *p*-coumaric acid (*p*-Coum), which are esterified to some of the arabinose side-groups, are removed by feruloyl and *p*-coumaroyl estarases, respectively.

connecting the microfibrills as a cement. During secondary cell wall growth, lignin is laid down in the hemicellulose layers of the cell wall as a three-dimensional network. Since lignin is hydrophobic, this results in a loss of water from the plant cell wall and a closing of the space between the cellulose. The different polymers form a composite in which they are closely linked by physical and chemical bonds (Bacic et al. 1988, Terashima et al 1993). The cellulose microfibrills are approximately 3 nm wide and leave a space of 4 nm for lignin and hemicelluloses (Terashima et al 1993).

In grasses, the cell wall typically contains about 40% cellulose, depending on the species and on the plant organs analysed (Bacic et al 1988). Cellulose is made of a linear chain of glucose molecules and is microbially degraded by a complex of extracellular enzymes, which exists of at least three enzyme types (Fig. 2A). Endo-glucanases randomly split cellulose, whereas exo-glucanases act on the end of a cellulose chain releasing cellobiose or sometimes glucose. Finally, β-glucosidases split cellobiose into two glucose molecules. The enzymes act synergistically in the decomposition of cellulose to cellobiose and glucose. With the exception of Archea, the capability to degrade cellulose is widely found in all groups of micro-organisms, which is to be expected since

cellulose is by mass the most important organic compound in extant biomass on Earth. A large number of microbial isolates have been described that can degrade crystalline cellulose completely. In addition, there are numerous strains that can only grow on partially degraded, amorphous cellulose or on decomposition products of cellulose like cello-oligomers and glucose (Ljungdahl & Eriksson 1985; Béguin & Aubert 1994).

Hemicelluloses are a diverse group of polysaccharides. In grasses, mainly arabino-xylan is found, with minor amounts of other hemicelluloses like xyloglucans and (1-3,1-4)-β-glucan (Bacic et al., 1988). Arabino-xylan exists of a linear xylose chain with side groups of arabinose, acetate and some glucoronic acid (Fig. 2B). Enzymes involved in its decomposition are: i) endo-xylanases, which randomly split the xylose backbone, ii) β-xylosidases which split xylobiose in two xyloses, and iii) so-called 'de-branching enzymes' in this case mainly arabinases and O-acetyl esterases, which remove side groups (Fig. 2B). These enzymes act synergistic in the decomposition of arabino-xylan. The ability to degrade arabino-xylan has a similar distribution among microbes as for cellulose (Biely 1993; Wong et al. 1988). Major carbohydrates that are formed when all enzymes are present are xylose, arabinose and xylobiose. If on the other hand only endo-xylanases are active, a variety of branched xylo-oligomers are formed in addition to the normally formed products (Puls & Schuseil 1993; Kormelink & Wolaver 1993). In grasses, some arabinose side groups are covalently bound to phenolic groups and possibly also to lignin (Bacic 1988; Terashima et al. 1993).

Lignin is a non-polysaccharide polyphenol and is considered to be the microbially most resistant component of vascular plant cell walls. It is made of a number of phenols, which are randomly polymerised to a heterogeneous, three-dimensional structure that has not yet been characterised completely (Terashima et al. 1993). Lignin is degraded by a number of bacteria and fungi. Most studies have centred on so-called white-rot fungi, especially on *Phanerochaete chrysosporium*, which use a number of phenol oxidases (lignin peroxidase, manganese dependent lignin peroxidase and laccase) in combination with other enzymes to extracellular release lignin fragments which are subsequently mineralised to CO_2 (Hatakka 1994; Reddy & Dsouza 1994). Streptomycetes have gained most attention within the Bacteria, and they seem to possess a lignase system which is different from white-rot fungi. Lignin is only partially degraded to CO_2 and mainly released as a soluble, high-molecular-weight ligno-carbohydrate complex (Kirk & Farrell 1987). It has been suggested that this is one of the processes contributing to the formation of dissolved humic acids in sediments and lakes (Moran & Hodson 1990; Trigo & Ball 1994). All of the described lignases are oxidases that directly or indirectly depend on the use of oxygen as an electron acceptor. Although there are some reports of modifications and release of dissolved components from lignin by anaerobic cultures (Bernard-Vailhé 1995; Ziomek & Williams 1989), no strains have been described that degrade lignin under these conditions to CO_2. Studies using ^{14}C-labelled lignins suggest that lignin is not or

only slowly degraded in anaerobic sediments compared to rates under anoxic conditions (Akin & Benner 1988; Benner et al 1984b; Odier & Monties 1983).

The different polymers in cell walls of plants are closely linked both physically and chemically. It is therefore believed that this internal linkage affects the decomposition of the individual polymers. Since lignin is the microbially most resistant polymer, it is thought that this polymer limits the decomposition rate of the polysaccharides and of the cell wall (Valiela et al. 1984). Although the ability to degrade all the polymers in lignocellulose can be found in a single strain, for instance in white-rot fungi, it seems more likely that in natural environments the decomposition of plant cell wall will be done by a consortium of micro-organisms that perform different steps in the total mineralization of the material to CO_2 (Ljungdahl & Eriksson 1985).

The above description of polymers in plant litter is rather specific for grasses like common reed. Other potentially important sources of organic matter in littoral sediments can have a very different polymeric composition. Cellulose is generally found in macrophytes and algae, but different types of hemicelluloses are present in other plant genera than grasses (Bacic et al 1988). Submerged macrophyte litters and especially algae generally contain higher amounts of protein (Wetzel 1983); a polymer that is rather scarce in reed litter (C/N ratio in standing reed litter > 80). Lignin is not found in algae and in relatively low amounts in submerged macrophytes (de Leeuw & Largeau 1993; Wetzel 1983). The source of the sediment organic matter will therefore determine which biopolymers are present in a sediment and, furthermore, which enzymes are needed for its degradation.

Outline of the thesis

The decomposition of organic matter was studied in the littoral sediments of Lake Gooimeer in the centre of The Netherlands. On the land side, the littoral is covered by a almost monospecific stand of common reed, *Phragmites australis*. A further description of the site can be found in Chapter 2.

At the start of this study, very little ecological information was available for the littoral of Lake Gooimeer. Data on several general aspects of sedimentary carbon cycling were not available, but were necessary for an implimentation of a detailed study on the initial decomposition of biopolymers in sediments. Since a number of different alga and macrophyte primary producers can occur in littoral systems that have a greatly different biopolymeric composition, the relative importance of carbon sources other than reed litter was studied with the use of stable carbon isotope and carbohydrate compositions (Chapter 2). In Chapter 3, a carbon budget was made for the reed bed in which mineralization was compared to other processes that affected sediment organic matter like the input of fresh

reed litter and sediment erosion. In addition, effects of environmental factors such as temperature, variation in water level, and litter dynamics on carbon mineralization rates were studied.

The remainder of the thesis deals with the initial, decomposition of organic matter and more specifically with polysaccharides in plant litters and sediments. Polysaccharides were chosen as the main study object because they form the major part of reed litter. Furthermore, methods for analysing polysaccharides and their degradation products (sugars) in natural systems became available when this work was started. Polysaccharides were probably also more important for carbon mineralization than the refractory lignin component of reed litter. First, extracellular enzyme activities were studied as these catalyse the initial decomposition of biopolymers to smaller fragments. A method was developed to measure extracellular enzyme activities involved in polysaccharide hydrolysis by using artificial, fluorochrome labelled substrates (Chapter 4). This method was further used to study temporal and spatial variability of enzyme activities during two years in the littoral sediments (Chapter 5).

Although the enzyme assays are easy to use and allow information from a large number of samples to be gathered and compared, they give little information on the actual *in situ* hydrolysis rates of natural occurring polysaccharides in sediments. This is mainly caused by the use of an artificial substrate that may not be representative of the form and availability of the natural substrates. Therefore, a new method was developed to measure hydrolysis rates of naturally occuring polysaccharides in sediments. This new method is based on the selective inhibition of microbial uptake of hydrolysis products by toluene without affecting the extracellular decomposition of polysaccharides. The accumulation of hydrolysis products in time was subsequently followed. This approach was thoroughly tested (Chapter 6) and subsequently used to study initial decomposition of individual polysaccharides in reed litter and sediments (Chapter 7).

Finally, implications of these studies are discussed in view of general functioning of littoral zones and the mechanisms of polymer decomposition in natural systems (Chapter 8).

Chapter 2

Sources of Organic Carbon in the Littoral of Lake Gooimeer as indicated by Stable Carbon Isotope and Carbohydrate Compositions.

H.T.S. Boschker, E.M.J. Dekkers, R. Pel & T.E. Cappenberg

Biogeochem. 29: 89-105 (1995)

Abstract. The relative importance of potential carbon sources in the littoral of Lake Gooimeer, a lake in the centre of the Netherlands, was studied using a combination of $^{13}C/^{12}C$-ratio analysis and carbohydrate composition analysis. The littoral is covered on the land side by a 80 m wide *Phragmites australis* bed. Potentially important sources were macrophyte litter, seston and benthic algae. Samples of carbon sources, sediments and benthic macrofauna from inside and outside the bed were analysed for their $^{13}C/^{12}C$-ratio and some for their carbohydrate composition. Results indicate that inside the bed, macrophyte litter was the main source of carbon for both the sediment organic matter and the benthic macrofauna, and that algal material was of minor importance. Outside the bed, production by benthic algae was the main carbon source, with seston as a second source. No macrophyte derived material could be detected outside the reed bed.

Abbreviations: DOC (Dissolved Organic Carbon), SOM (Sediment Organic Matter).

Introduction

In littoral systems of lakes, a number of primary producers might be important as organic carbon sources for the food web and the sediment organic matter. A high production of both submerged and emergent macrophytes is characteristic for most littorals, and in addition to phytoplankton, other algal sources like epiphytes and benthic algae are present. Also, terrestrial material can be transported to the littoral by run-off or winds (Wetzel 1992). Information on the relative importance of these carbon sources is important to our understanding of the structure and functioning of wetlands and of littoral regions in particular.

A number of techniques have been applied to trace the flow of carbon in ecosystems. The $^{13}C/^{12}C$-ratio in organic matter has been used extensively to trace carbon sources in sediments and in food webs (Fry & Sherr 1984; Peterson & Fry 1987; Gearing 1991). The method is based on differences in $^{13}C/^{12}C$-ratios among primary producers caused by differences in CO_2 fixation metabolism and CO_2 source. Emergent C3 plants have a ratio of around -27 o/oo ($\delta^{13}C$ notation; see method section for definition) whereas C4 plants are relatively enriched (-13 o/oo). Phytoplankton of lakes is variable and ranged in a number of lakes from -17 o/oo to -45 o/oo (Siller 1979; Rau 1980; LaZerte 1983; Estep & Vigg 1985; Takahashi et al. 1990). The isotopic fractionation is in general small during the assimilation of food by heterotrophic organisms and the same applies for diagenetic processes acting on the bulk organic matter in sediments. This means that both heterotrophic organisms and sediment organic matter have a ratio close to their carbon source. By comparing $^{13}C/^{12}C$-ratios of potential carbon sources with ratios in sediments and/or heterotrophic organisms, conclusions can be drawn about the relative importance of these sources in an ecosystem. A prerequisite is that the potential carbon sources have a different $^{13}C/^{12}C$-ratio.

Problems arise when more than two major carbon sources are present in the system. In that case, intermediate $^{13}C/^{12}C$-ratios of sediments or organisms can originate from a number of different mixtures of the carbon sources. Conclusions can only be drawn unequivocally if sediments or animals have $^{13}C/^{12}C$-ratios comparable to one of the extremes in the range spanned by the carbon sources. In most ecosystems, littoral zones included, there are more than two carbon sources present. Therefore, combinations of the $^{13}C/^{12}C$-ratio method with other stable isotope or chemical composition-derived parameters have been used to increase the resolution of the analysis (Hedges & Parker 1976; Fry & Sherr 1984; Peterson et al. 1985).

This study is part of a project on the decomposition of organic matter in a reed bed covering a section of the littoral of Lake Gooimeer. The purpose of the study was to determine the most important sources of organic matter for the sediment from inside and

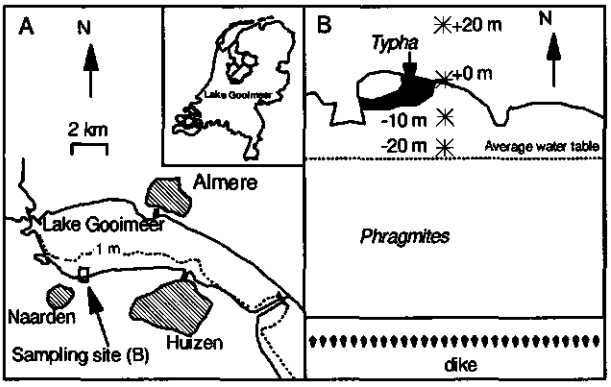

Fig. 1. Map of Lake Gooimeer (A) showing the sampling site (B); small map (A) shows location of Lake Gooimeer in the Netherlands. Sampling points (B) for stable carbon isotope and carbohydrate measurements are indicated by stars and by their distance from the edge of the reed bed. Average water depth at the lake side of the reed bed was about 20 cm and at point 20 m about 30 cm. Nearby cities are indicated by striped areas.

outside the bed. To answer this question, stable carbon isotope measurements were combined with determinations of the neutral carbohydrate composition. The latter measurement can be used to discriminate between algal and macrophyte sources in sediments (Cowie & Hedges 1984a). Results indicate that inside the reed bed, primarily macrophyte derived material was present in the sediment and algal material was of minor importance. Outside the bed, no macrophyte material could be detected and benthic algae production was the most important source, followed by sestonic material.

Description of sampling site and methods

Sampling site. The 'Gooimeer' is a very shallow lake (mean depth of 3.6 m, surface area 24 km^2) with a sandy sediment in the centre of the Netherlands (Fig. 1, De Haan et al. 1993). On the southern shore, there is a reed bed of approximately 80 m wide and 4 km long, with *Phragmites australis* (Cav.) Trin. Ex Steudel (common reed) as the dominant macrophyte species and some patches of *Typha augustifolia* L. (cat-tail) on the lake side of the bed. Outside the bed some *Potamogeton pectinatus* L. plants were present from May to July and the sediment was covered with benthic algae during the whole sampling period. The bed is bordered on the land side by a dike. Sedimentary organic matter is accumulating inside the bed and increases in concentration from <0.5% at the lake side to 1% at 10 m and to 30% at 20 m inside the bed.

The lake is eutrophic, with summer chlorophyll-a concentrations of about 60 μg l^{-1}. The phytoplankton was dominated by the cyanobacterium *Oscillatoria agardhii* Gom. during most of the year. Green algae were present in varying numbers throughout the year, and diatoms bloomed in early spring (H.L. Hoogveld, NIE-CL, pers. comm.). The pH varied between 7.5 in winter and 8.8 in summer.

Horizontal gradients in the water column. During 1990-1991, overlaying water was collected in a gradient from inside to outside the reed bed at fixed sampling points marked with sticks. Care was taken to sample the water phase without resuspending the sediment. Water samples were first sieved over 150 μm to remove larger floating particles and zooplankton; the latter was present in high numbers inside the reed bed especially during summer and obscured possible gradients in seston originating from the open water. The material that passed the sieve contained mainly phytoplankton and some detritus. The organic matter concentration in the seston (<150 μm) was measured by filtering 200 to 500 ml through a Whatman GF/F glass-fibre filter. The filter was dried overnight at 105 °C and organic matter on the filter was determined as the subsequent loss of weight after 4 hours at 550 °C. DOC in the filtrate was determined using photochemical (UV) oxidation with persulfate (Schreurs 1979).

Sampling and sample treatment. Samples for stable carbon isotope ratio and carbohydrate composition measurements were mainly collected during two periods: (i) from October 1990 to August 1991, when samples were taken of potential carbon sources, sediments and macrofauna from 20 m inside (-20 m) the bed, and (ii) from October 1992 to March 1993, when samples were taken from inside (-20 m) and outside (20 m) the bed. Sediments from -10 m and +0 m were sampled for carbohydrate analysis on several occasions. Furthermore, monocultures of a green algae (*Scenedesmus obligus*) and a cyanobacterium (*Limnotrix limnetica*), both grown under light limitation in continues cultures, were analysed for carbohydrates.

Emergent macrophytes were sampled at the end of the growing season when plants started to become yellow. *Potamogeton pectinatus* was not sampled because densities were low and it was only present during a short period of the year. Macrophyte litter was taken by hand from the top layer of the sediment and gently washed to remove sediment particles. SOM was sampled from the top 1 cm of three sediment cores (id 7 cm). For SOM samples from outside the bed, sand was removed by repeatedly shaking the sediment sample with water and decanting the SOM suspension; SOM was concentrated by centrifugation. Seston was collected by centrifugation of a 25 l water sample taken 20 m outside the reed bed. Centrifugation was performed with a Sorvall SR-5C centrifuge equipped with a continues flow angle rotor (Sorvall SS-34/KSB system) at 10,000 rpm and a flow of 5 l/min (Sorvall Instruments, du Pont, Wilmington, Del.). More than 90 % of the particles were removed with this method, as determined by seston analysis (see above) before and after centrifugation. Seston samples for stable isotope and carbohydrate

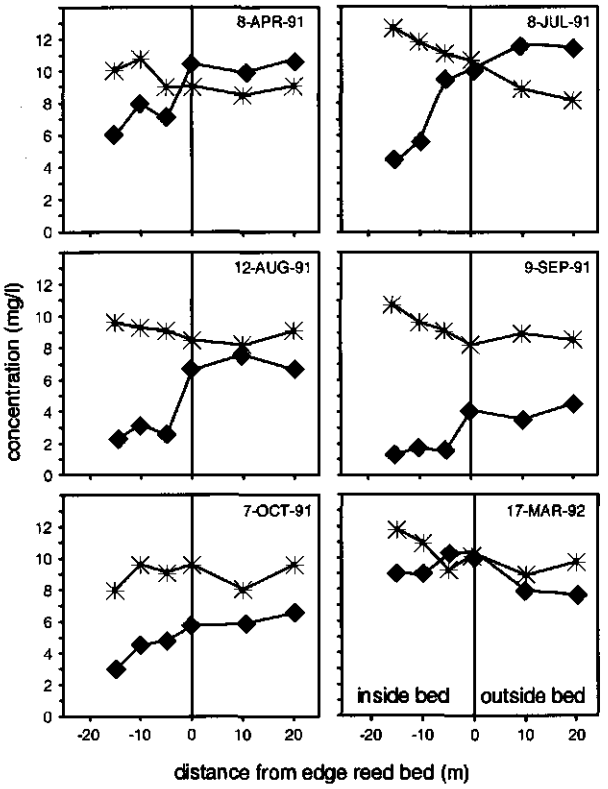

Fig. 2. Horizontal gradient measurements of seston (mg OM/l <150 µm, ◆) and DOC (mg C/l, ✳) in the water phase of the littoral on several dates.

measurements were not sieved over 150 µm as for the horizontal gradient study, since samples were taken outside the bed and contained neither zooplankton nor course macrophyte litter. Seston and SOM samples were pre-treated overnight with 0.25 N HCl to remove any carbonate minerals present. Macrofauna was sorted by hand from sediment material collected on a 0.7 mm sieve. Only dominant macrofauna species were collected and were grouped as chironomids, oligochetes, *Asellus* sp, leeches (Hirudinea) or Heteroptera. All samples were lyophilised and ground to a fine powder before further analysis.

Stable carbon isotope measurements. After combustion of the organic carbon to CO_2, stable carbon ratios were determined on a VG SIRA2 mass spectrometer (VG MassLab, UK) for the first sampling period and on an Europa 20-20 mass spectrometer (Europa Scientific, UK) for the second period. All carbon isotope data are given in the

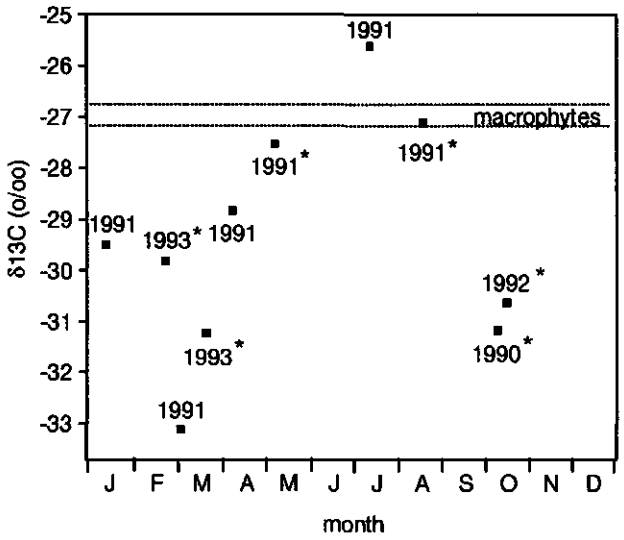

Fig. 3. Seston $\delta^{13}C$ values through the year. Black squares represent results from one seston sample analysed in duplicate. For comparison, values for the two sampling periods are plotted on a one year scale. Years next to the $\delta^{13}C$ values indicate when samples were taken. Years labelled with stars show when sediment and macrofauna were sampled for $\delta^{13}C$ analysis. The range of $\delta^{13}C$ values for macrophytes is indicated by dotted lines.

$\delta^{13}C$ notation:

$$\delta^{13}C\ (o/oo) = (R_{sample}/R_{standard} - 1) \times 1000,$$

where R is the $^{13}C/^{12}C$-ratio and the standard is PeeDee Belemite. The mean ± SD for the difference between duplicate subsamples was 0.19 o/oo ± 0.11 (n=60).

Carbohydrate composition. The neutral carbohydrate composition of potential carbon sources and sediment samples was determined in duplicate after acid hydrolysis (H_2SO_4) according to Cowie and Hedges (1984b). Carbohydrates in the hydrolysates were determined with a Dionex 2000i/SP ion chromatograph. A Dionex PA1 column, with Milli- Q water as eluent (flow rate: 1 ml/min), was used to separate carbohydrates. Prior to use, the column was preconditioned with 100 mM NaOH plus 25 mM NaAcetate (flow rate: 1 ml/min) for one hour to reduce retention times. To optimise the detection, a post column addition of 1.6 M NaOH (flow rate: 0.3 ml/min) was performed using an AMMS II anion-micromembrane reactor (Haginaka et al. 1989). Carbohydrates were detected with a Dionex PED detector in the pulsed amperometric mode. A gold electrode with an $AgCl_2/Cl$ reference electrode was used, and pulsed amperometric wave forms were optimised according to LaCourse and Johnson (1991). Hydrolysates were analysed for

fucose, arabinose, rhamnose, galactose, glucose, xylose, mannose and ribose (listed in order increasing retention time) using external standards. No other compounds were identified. The detection limit was between 5 and 15 nM, depending on the compound analysed. The carbohydrate hydrolysis efficiency was checked with cellulose (MN300, Hachery Nagel & Co). The glucose yield from cellulose was 83.0% ± 0.7 (n=2). No corrections for hydrolysis efficiency were made.

Results

Gradient measurements in the overlaying water (Fig. 2) showed that seston concentrations were lower inside the reed bed than outside on all sampling dates except on 17-March-92. DOC gradients were less evident; only on 8-July-91 and on 9-September-91 DOC concentrations were clearly higher inside the reed bed. On the other dates DOC concentration tended to be higher (< 2 mg C/l) inside the bed, but differences were small compared to variability in the DOC determination (± 1 mg C/l).

Seston, sampled 20 m outside the reed bed for stable carbon isotope and carbohydrate analysis, was examined by microscope and contained mainly phytoplankton with little detritus and no zooplankton. Stable carbon isotope measurements of seston (Fig. 3) showed a pattern with more negative values in the winter period and values comparable or somewhat enriched to the macrophytes during summer. In March, a dip in the $^{13}C/^{12}C$-ratios of seston was found, which coincided with a bloom of diatoms (see Method section). Seston $^{13}C/^{12}C$-ratios for both sampling periods (1990-1991 and 1992-1993) were in agreement with each other during the winter period; they were clearly depleted in ^{13}C compared to the macrophytes and showed the lowest value in March. *Phragmites australis* and *Typha augustifolia* had similar ratios of -27.0 o/oo ± 0.15 (mean for all samples) indicating a C3 type of CO_2 fixation metabolism for both species.

Sediment organic matter and macrofauna were sampled on six dates (Fig. 3): four during the winter period when seston and macrophytes had clearly different $\delta^{13}C$ values, and two during summer when seston and reed had similar values. Although, there was an overlap between the range in $\delta^{13}C$ for litter and SOM from inside the bed and the range for macrophytes, the data for litter and SOM were somewhat shifted to more negative ratios (on average -0.7 o/oo, Fig. 4). SOM from outside the reed bed (+20 m) had variable but clearly higher $\delta^{13}C$ values than could be explained by either a seston or a macrophyte source. Coarse recognisable litter was never found on the sediment outside the reed bed.

Stable carbon isotope values for macrofauna from inside the bed (Fig. 4) did not vary much between sampling dates (mean values ± SD, -25.5 o/oo ± 0.9). Values for macrofauna inside the bed were comparable to seston in summer, macrophytes and litter. There was no correlation (r^2 = 0.09) between $\delta^{13}C$ values of seston and $\delta^{13}C$ values of

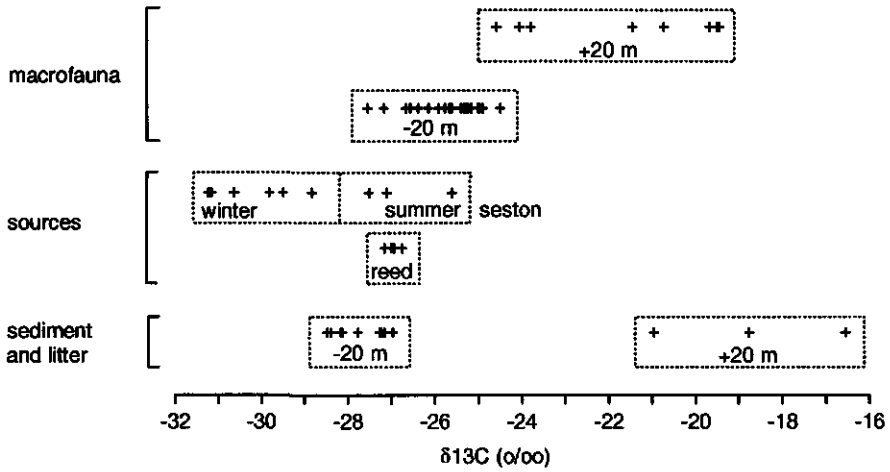

Fig. 4. Summary of all $\delta^{13}C$ data of potential carbon sources, sediments and macrofauna at 20 m inside (-20 m) and 20 m outside the reed bed (+20 m). Symbols represent results from one sample analysed in duplicate.

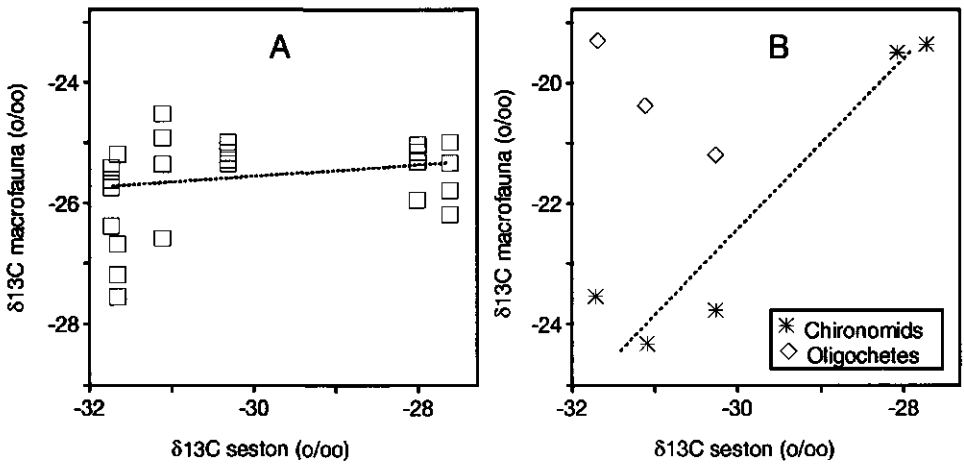

Fig. 5. Plot of $\delta^{13}C$ values of macrofauna from 20 m inside the reed bed (A) and 20 m outside the reed bed (B) against seston from the same sampling date. Lines show results of linear regression analysis (A: animals=-21.7+0.1 x seston, r^2 = 0.09; B: animals=15.3+1.3 x seston, r^2 = 0.90); for B) only chironomid data were included in the regression although data are very sparse.

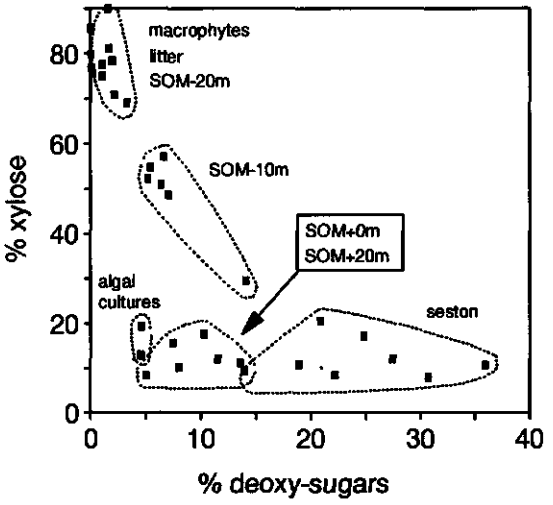

Fig. 6. Plot of weight % xylose against weight % deoxy-sugars (fucose+rhamnose) calculated on a glucose free basis in hydrolysates of sources, litter and SOM. Also shown are results from two monocultures of algae.

macrofauna from inside the bed (Fig. 5a). Macrofauna from outside was clearly enriched in ^{13}C and fell between seston in summer and SOM from +20 m. Although data from outside the bed were too sparse for a reliable correlation analysis, ^{13}C values of chironomids seemed to correlate with values of seston (Fig. 5b). Outside the bed (Fig. 5b) the two sampled macrofauna groups appeared to behave differently; inside (Fig. 5a) no such differences were found between the five dominant groups and all macrofauna data were pooled for correlation analysis.

Results of carbohydrate analysis are shown in Appendix 1. Xylose and deoxy-sugars (fucose+rhamnose) showed the highest variability between different types of samples. A plot of xylose against deoxy-sugars, as percentages on a glucose free basis (total carbohydrates minus glucose), gave good graphical separation between macrophyte and algae based materials (Fig. 6). Glucose was excluded in the calculations for Fig. 6 because its dominance in all samples tends to obscure differences among the less abundant carbohydrates (Cowie & Hedges 1984a). Macrophytes, litter and SOM from -20 m had high % xylose and low % deoxy-sugars (Fig. 6). Monocultures of algae, SOM from outside the bed and seston had low % xylose compared to macrophytes. The % deoxy-sugars was higher in seston than in algae and SOM from outside. This might have been due to differences in algal populations, since % deoxy-sugars can vary between 0 and 63% depending on the species analysed (Haug & Myklestad 1976; Vaidya & Mehta 1989; Brown 1991). SOM from -10 m had intermediate % xylose, which suggested the presence of a mixture of algae and macrophyte derived material.

Discussion

In this study, three main potential sources of carbon for the sediment were considered: litter of emergent macrophytes, seston and benthic algae. All three sources were visually abundant in the littoral. The gradient study showed consistently lower seston concentrations inside the reed bed, which suggested that the bed acted as a sink for sestonic material. The importance of seston as a carbon source for the reed bed sediment could not be estimated from these gradients. The gradient study also suggested a small flux of DOC to the open water. Input of terrestrial material was probably not important, since the land side of the system was bordered by a dike that prevented direct the transport of terrestrial material to the littoral. Submerged macrophytes with epiphytic algae attached to them show a high productivity in some littoral systems (Wetzel 1992), but were almost absent and restricted to a short period in summer in the littoral of Lake Gooimeer. Therefore, submerged macrophyte derived material was not considered to be an important source of organic carbon.

We used two parameters to trace carbon inside the littoral of lake Gooimeer: $^{13}C/^{12}C$-ratios and neutral carbohydrate compositions. It is important to notice that these methods are not equivalent. With the $^{13}C/^{12}C$-ratio method all carbon is taken into account, whereas the carbohydrate composition method considers only the carbohydrate fraction in the sample. The carbohydrate fraction of macrophytes and litter (total carbohydrate yield of 45 to 60%, this study) is higher than that of algae sources (5 to 25%, this study). The percentage total carbohydrates will also change during decomposition, since polysaccharides in macrophyte litters are selectively faster degraded then lignin (Wilson et al. 1986b; Benner at al. 1987).

The carbohydrate measurements showed that emergent macrophytes, litter and SOM from -20 m had a comparable composition with high percentages of xylose and low percentages of deoxy-sugars, which are characteristic for macrophyte-derived material (Cowie & Hedges 1984a; Moers et al. 1990), and had a composition clearly different from algae, seston and sediments from outside the bed (Fig. 6). The $\delta^{13}C$ values of sediment and litter from inside the bed were on average slightly lighter (-0.7 o/oo) but in the same range as the macrophytes (Fig. 4). Small differences between sediment $^{13}C/^{12}C$-ratios and their likely carbon sources have been noted before (Fry & Sherr 1984) and in our case this might have been caused by the selective decomposition of the isotopically heavier polysaccharides in macrophyte litters resulting in an enrichment of the lighter lignin (Benner et al. 1987). In summary, the carbohydrate analysis indicated that SOM at 20 m inside the reed bed was dominated by macrophyte-derived material with little or no algal derived material present. The $\delta^{13}C$ values of SOM determinations were in agreement with this indication.

Inside the bed, stable carbon ratios for macrofauna ranged from similar to clearly enriched compared to macrophytes. On average, macrofauna from inside the bed was 1.5 o/oo heavier than the macrophytes, which is in the range of fractionation between animals and their carbon source of ± 2 o/oo found in literature (Fry & Sherr 1984). Seston in summer had $\delta^{13}C$ values comparable or somewhat enriched to macrophytes, but in winter seston ratios were clearly depleted in ^{13}C compared to the macrophytes. This variation in sestonic $\delta^{13}C$ did not correlate with the ratios of the macrofauna from inside the bed, which suggested that seston was of little importance as a carbon source. An alternative explanation for the macrofauna ratios inside the bed might be that a mixture of seston and benthic algae was used as food source in addition to the abundant litter. However, production by benthic algae inside the bed was not likely to be high, because they would be heavily shaded by the macrophytes. In August, less than 10 % of the incident light intensity reached the sediment; the remainder was absorbed by the vegetation (data not shown, similar to Rodewald-Rudescu 1974; Roos & Meulemans 1987).

Outside the reed bed, $\delta^{13}C$ values of SOM were higher than could be explained by either macrophytes or seston, which meant that another source or sources of carbon were present. We believe that this other source was most likely material produced by benthic micro-algae, which were visually present on the sediment of this shallow water during the whole sampling period. The low % xylose of the SOM outside the bed also indicates that algal material dominated and that little macrophyte derived material was present (Haug & Myklestad 1976; Cowie & Hedges 1984a; Vaidya & Mehta 1989; Brown 1991). Recent measurements with oxygen micro-electrodes have shown a high primary production in the top layer of the sediment from outside the bed (K. Buis, NIE-CL, pers. comm.). The presence of isotopically heavy material derived from C4 macrophytes was not likely, since the dominant species in the littoral system were of the C3 type and carbohydrate compositions showed that little macrophyte derived was present outside the reed bed.

Outside the bed, macrofauna had $\delta^{13}C$ values similar to or lower than SOM. Since carbohydrate analysis suggested that macrophyte material was neither present in the SOM outside the bed nor in the seston, the macrofauna outside the bed probably used a mixture of SOM and seston as food. Based on our $\delta^{13}C$ data, a simple two source mixing model (formula on page 216 of Gearing 1991) with SOM and seston as sources, suggested that chironomids used 35% to 50% seston in their food and oligochetes 5% to 20% during the second sampling period.

Carbohydrate composition measurements showed a gradient in the SOM, with macrophyte derived material dominating at 20 m inside the bed and algal material dominating outside the bed. Algal material also appeared to be important at 10 m inside the bed, although the standing stock of macrophytes at this point was similar to 20 m inside the bed (data not shown). It seemed that most of this standing stock, which will eventually die and enter the sediment, did not remain at -10 m but was transported

elsewhere. Macrophyte material could not be detected outside the reed bed, which meant that there was little transport of macrophyte litter towards the open water or that the material was diluted beyond the detection limit of the methods used.

LaZerte (1983) showed that in Lake Memphremagog approximately 40 to 50% of the organic carbon in the pelagic sediment was terrestrial, macrophyte derived material, whereas up to 100 % was terrestrial in littoral sediments. Similarly, results of Rau (1980) indicated that terrestrial material was important as food sources for certain aquatic insects species in the subalpine Findley Lake. Both lakes are situated in a relatively large forested watershed compared to the area of the lake. In the littoral of Lake Gooimeer, terrestrial or emergent macrophyte influence on the sediment organic carbon was only present inside the reed bed and no macrophyte material could be detected outside the bed. The surface ratio between the macrophyte covered area and the lake is an additional important factor in determining the importance of macrophyte derived material in lakes. For Lake Gooimeer, this ratio is rather small, about 0.025.

Our results suggested that production by benthic algae was the primary carbon source outside the reed bed. A recent study showed that benthic microalgae production is also an important component in the food web of a salt marsh (Sullivan & Moncreiff 1990). Based on feeding habits and $\delta^{13}C$ values, Rau (1980) suggested that periphyton (algae that grow attached to a substrate) might be a food source for a mayfly larvae in Findley Lake. These data show that benthic microalgae production should also be considered in studies on carbon flow in other littoral systems.

In conclusion, combined results of stable carbon isotope and carbohydrate composition measurements suggest that mainly macrophyte-derived material was present in the sediment at 20 m inside the reed bed of Lake Gooimeer, and that macrophyte-derived material was probably also the most important food source for benthic macrofauna. Outside the reed bed, in the open part of the littoral, benthic algae production was of primary importance as source of carbon, with a variable contribution of seston depending on the macrofauna group.

Acknowledgements

This work was supported by grants from the Netherlands Integrated Soil Research Programme and from the Institute for Inland Water Management and Waste Water Treatment. We thank K. Schwenck and K. Siewertsen for the supply of the algal cultures and the Institute for Soil Fertility Research (IB-DLO, Haren, The Netherlands) for analysing the first series of stable carbon isotope samples.

Appendix 1. Carbohydrate composition of selected samples from the littoral of Lake Gooimeer. Showing percentage of organic matter explained by carbohydrates and concentrations of individual compounds

Sample	Date	% of OM	% of total carbohydrates							
			fucose	arabinose	rhamnose	galactose	glucose	xylose	mannose	ribose
Macrophytes										
Phragmites(1)	01-Oct-90	54.1	(2)	6.4		2.5	54.0	36.7	0.4	
Phragmites(1)	01-Oct-90	54.8		5.3	0.9	3.0	48.5	42.3	0.1	
Typha	01-Oct-90	58.3		5.5	0.9	3.1	55.0	35.4		
Macrophyte litter										
-20 m	06-Oct-92	60.8		6.8		2.7	54.9	34.2	1.3	0.1
-20 m	22-Mar-93	51.0	0.1	6.9		3.7	54.9	34.3	0.2	
-10 m	06-Oct-92	46.6		6.2		2.7	55.6	34.2	1.3	
-10 m	22-Mar-93	50.2		4.2		2.4	54.5	38.9		
Algal cultures										
Limnotrix		5.6	0.2	0.8	3.2	32.0	28.7	13.8	21.3	
Chlamydomonas		11.3	1.0	0.6	1.9	37.8	37.5	8.2	12.9	
Seston										
	14-Jan-91	16.5		2.0	7.0	6.6	66.7	6.8	7.7	3.2
	08-Apr-91	12.9	1.3	1.6	5.1	10.3	68.6	3.6	7.5	2.1
	06-May-91	19.9	1.5	0.2	6.1	9.1	68.7	2.8	8.4	3.2
	08-Jul-91	23.8	1.5	0.2	5.0	10.0	58.3	4.2	17.9	3.0
	17-Mar-92	15.1	5.7	2.9	7.2	14.9	55.4	5.0	8.9	
	06-Oct-92	7.1	4.3	1.6	20.4	22.9	36.6	7.3	6.9	
	23-Feb-93	14.2	1.5	1.8	7.0	9.8	68.8	5.8	5.3	
	22-Mar-93	19.0	1.4		10.2	10.7	64.7	3.0	6.1	4.0
Sediments (0-1 cm)										
-20 m	01-Oct-90	38.4		7.6	0.6	4.7	42.6	43.6	0.9	0.1
-20 m	08-Jul-91	22.6		5.5	0.5	2.8	53.8	35.6	1.8	
-20 m	23-Feb-93	45.1		7.7	1.4	4.3	54.0	28.6	4.0	
-20 m	19-Apr-93	22.6		5.2	0.5	3.0	57.5	31.6	2.1	
-10 m	01-Oct-90	14.2		4.4	3.2	6.5	51.9	27.5	6.5	
-10 m	14-Jan-91	8.1		7.7	3.9	7.7	45.0	31.1	4.5	
-10 m	06-May-91	17.2	2.0	5.6	4.0	8.8	61.1	12.5	6.0	
-10 m	08-Jul-91	22.1	0.6	8.3	2.1	7.2	50.6	26.5	4.8	
-10 m	06-Oct-92	18.9	1.1	7.1	2.5	8.1	50.2	25.0	5.9	
-10 m	23-Feb-93	19.8	0.6	7.8	2.2	6.7	50.6	27.9	4.3	
+0 m	06-Oct-92	24.4	0.7	2.6	2.3	7.8	64.3	3.7	14.9	3.7
+0 m	23-Feb-93	13.6	0.9	2.7	3.8	10.7	58.8	6.7	16.2	0.2
+0 m	22-Mar-93	18.5	0.6	1.3	3.4	9.3	68.0	4.2	11.3	2.0
+20 m	06-Oct-92	15.6	0.1	2.6	2.0	8.4	60.4	3.4	23.0	
+20 m	23-Feb-93	13.2	0.6	2.4	3.0	10.9	54.1	7.4	19.5	2.1
+20 m	22-Mar-93	16.2	0.7	0.8	4.2	10.3	66.5	4.0	9.5	4.1

(1): The *Phragmites* sample was analyzed on two occasions

(2): Blanc enteries were not detected

Chapter 3

Mineralization of Organic Matter in the Littoral Sediments of Lake Gooimeer, The Netherlands

H.T.S. Boschker, E.M.J. Dekkers & T.E. Cappenberg

Submitted to Freshwater Biology

SUMMARY

1. Factors have been determined that govern the decomposition of reed litter and mineralization of sediment organic matter in the littoral of Lake Gooimeer, The Netherlands. Mineralization in sediments was determined by direct microcalorimetry.

2. Mineralization rates in sediments were mainly determined by i) temperature, ii) water level induced changes in oxic or anoxic conditions in the sediment, and iii) changes in sediment organic matter by input of fresh litter and removal by erosion of the sediment.

3. The percentage water in reed litter was an additional factor in the decomposition of the material. Standing reed litter was decomposed only very slowly because of its low water content, whereas fallen litter always had an optimal water content.

4. Mineralization explained only 25 to 30% of the total removal of sediment organic matter. Transport due to erosion during spring storms was the most important process, explaining 60 to 70%.

Introduction

Wetlands are characterised by a substantial primary production of emergent vascular plants (Wetzel 1992). Due to the low food quality of the vascular plant material, direct grazing of live plants by animals is generally low and most of the produced material eventually enters the sediment as plant litter (Mann 1988). Reed beds, fringing shores of lakes are a important type of wetland and are mostly dominated by a limited number of plant species like *Phragmites australis* (common reed) and *Typha* spp. (cat-tail). Most research on reed beds has been directed to the vegetation or the degradation of litter. Processes in reed bed sediment, as such, have received little attention (Andersen 1981), in contrast to the amount of studies on tidal marine wetlands like salt marshes and mangroves (e.g. Howard 1993; Howes et al. 1985; Nyman & DeLaune 1991; Teal & Kanwisher 1961). In this article, we present results from experiments and field measurements on the factors that governed mineralization of organic matter in a gradient through the littoral of Lake Gooimeer, a shallow lake in the centre of the Netherlands. In addition, the importance of mineralization in the sedimentary carbon cycle will be discussed.

Materials and Methods

Description of sampling site. The 'Gooimeer' is a very shallow, eutrophic lake (mean depth of 3.6 m, surface area 24 km^2) with a sandy sediment situated in the centre of the Netherlands (Boschker et al. 1995b). On the southern shore, there is a reed bed of approximately 80 m wide and 4 km long, with *Phragmites australis* (Cav.) Trin. Ex Steudel (common reed) as the dominant macrophyte species and some patches of *Typha augustifolia* L. (cat-tail) on the lake side of the bed. The average water depth at the lake side of the reed bed was approximately 30 cm in summer and 10 cm in winter. This difference is caused by manipulations by the water authorities. Wind also had a large impact on the water level inside the reed bed. Shifts in wind direction and strength during storm depressions caused changes in the water level of up to 1 m within a day.

Three sampling points were compared in this study: one at 20 m inside the bed where organic matter was clearly accumulating (≈30% organic matter (OM)), one at 10 m inside the bed where organic matter of the sediment was still relatively low (≈1% OM) and a reference site at 20 m outside the bed (<0.5% OM). A similar pattern of organic matter content was found in other sections of the reed bed.

Production of reed litter. Production of reed litter was estimated from the standing crop at the end of the growing season shortly after plants started to turn brown and before most

leaves were shed. Standing shoots were counted in 4 replicate plots of 0.25 to 0.5 m^2 area, and between 15 and 25 stems were harvested randomly in every plot. Numbers of leaves and internodes were counted, and leaves and stems were separately dried at 105 °C until stable weight. Most stems had already shed some of their lower leaves, and corrections were made using the average leave weight of the remaining leaves. Leaves were shed shortly after plants died down. Although stems remained standing longer, no or only a small number (estimated at less then 1%) of dead stems from the last season were still found standing at the end of the next growing season. This suggested that the standing crop at the end of the growing season entered the sediment almost completely during the following year and, therefore, can be used as the yearly above ground production of litter.

Decomposition of reed litter. Standing and fallen reed litter were collected by hand inside the bed and cut into 1 to 2 cm pieces. Standing litter was handled as fast as possible and was always kept in closed bottles to avoid changes in water content. For short term incubations, approximately 2 g wet standing litter or 5 g wet fallen litter was incubated in 250 ml serum bottles sealed with butyl-rubber stoppers fitted to a butyl-rubber septum through which gas samples for carbon dioxide analysis were taken. To study the effect of aerobic or anaerobic conditions on the decomposition of reed litter, long term incubations were carried out in 500 ml serum bottles. These contained 0.5 g dry matter of standing litter, 10 ml artificial lake water (Boschker et al. 1995a) for aerobic and 100 ml for anaerobic incubations, 130 µg PO_4-P and a small inoculum from the sediment inside the bed. The bottles were incubated for 70 d at 15 °C. Aerobic incubations received less water to avoid diffusion limitation of oxygen from the head space. Inorganic nitrogen (0.8 mg N) was added as nitrate for the aerobic and as ammonium for the anaerobic incubations. Anaerobic incubations were flushed with oxygen-free-nitrogen gas. Accumulation of carbon dioxide and methane in the headspace, and dissolved inorganic carbon were followed in time.

Carbon dioxide was measured in the head space of incubation bottles using an Infra Red Gas Absorbance Analyser (IRGA) BINOS 1 (Leybold-Heraeus). A flow of nitrogen gas (1 $l.min^{-1}$) was first led through the reference cell of the IRGA and subsequently through the measuring cell. Between the two cells, gas samples (0.2 ml) could be injected into the gas flow. IRGA response was followed on a strip chart recorder and peak height was used to calculate carbon dioxide concentrations. A 25 mM $BaCO_3$ suspension in Milli-Q was used for standardisation: 0 to 1 ml of this suspension was added to a 8 ml scintillation vial, the vial was immediately closed with a Hungate septum (Bellco Inc.) and 0.2 ml 1 N HCl was injected. After an equilibration period, the headspace was sampled for carbon dioxide. Dissolved inorganic carbon in the water phase of the incubation bottle was determined in 1 ml samples, which were treated the same way as the $BaCO_3$ standard.

Methane was measured in the head space of anaerobic incubations using a Packard model 428 equipped with a Chrompack Q column and a flame ionisation detector. The carrier gas was helium at a flow rate of 20 $ml \cdot min^{-1}$.

Sediment organic matter. Sediment cores were taken by drilling a Perspex cylinder (7 cm internal diameter) with a sharpened cutting edge into the sediment by hand. Cores were sliced in the laboratory, living roots and rhizomes were removed as far as possible, and slices were homogenised in a Warren blender. The dry matter (DM) content was measured after drying overnight at 105 °C and the organic matter (OM) content was determined as the subsequent loss of weight after 4 hours at 550 °C. On several occasions, the carbon content of reed litter and sediment organic matter was determined with a Carlo Erba elemental analyser (model 1106), which showed that approximately 50% (weight/weight) of the OM was carbon.

Carbon mineralization rates in sediment. Mineralization rates were calculated from heat production rates as measured by direct microcalorimetry (Graf 1987; Gustafsson 1987) with a Thermometric Thermal Activity Monitor 2277.

Duplicate or triplicate minicores were taken at all three sampling sites with stainless steel cores of 2.1 cm internal diameter. A special procedure was used to transfer the top 3 to 4 cm of the sediment cores to microcalorimetry ampoules (25 ml). The top layer was transfered to a small subcore and the bottom of the subcore was closed with a stainless steel plate. The subcore fitted exactly into the measuring ampoule. This procedure minimised sediment disturbance leaving existing vertical gradients intact. All manipulations were done in an anaerobic glove box (filled with N_2 and 1.5% H_2; Coy Laboratory Products Inc.) to minimise inclusion of air in the anaerobic parts of the sediment, since this would cause an additional heat production from the oxidation of reduced substances by oxygen. Ampoules were closed outside the glove box, thus creating an aerobic headspace above the sediment. Exposure of the sediment surface to anaerobic conditions in the glove box was minimised to less then 3 min. Heat production was determined as an average during at least 4 h after a stabilisation period of 2 to 4 h. Measurements were made at *in situ* temperature except if temperatures were below 4 °C, which was the minimum setting of the microcalorimeter. Temperatures below 0 °C were not encounted during the sampling period.

Carbon mineralization rates were calculated from the heat production by the following formula:

$$R_c = \frac{86.4 \times Q}{(Q_{comb} \times a_{ampl})} \qquad (1)$$

Where R_c is the carbon mineralization rate (mg $C \cdot m^{-2} \cdot d^{-1}$), Q is the heat production as measured by microcalorimetry ($\mu W = \mu J \cdot s^{-1}$), Q_{comb} is the specific heat of combustion for sediment organic matter (40 $kJ \cdot g\ C^{-1}$ (Graf 1987)), and a_{ampl} is the area of the sediment in the ampoule (0.000346 m^2). The factor of 86.4 is used to correct for differences in units.

To test the method, an experimental specific heat of combustion was determined in two types of experiments, where both heat production and carbon mineralization could be linked. First, freshly sampled reed litter was incubated under aerobic conditions in microcalorimetry ampoules, and carbon dioxide production (determined as described under 'Decomposition of reed litter') and heat production of the material were determined. In a second type of experiment, microcosms were used to mimic sediments in the reed bed. These contained a sediment (5 cm) and water layer (5 cm, artificial lake water) in 1 l beakers. Two types of microcosms were made: one with low organic loading, which contained a layer of organic low sediment from outside the reed bed, and the other with high organic loading, which was similar except that extra standing reed litter (10 g DM) was mixed through the sediment layer. The water phase was aerated by bubbling with a gentle flow of air. After 1, 20 and 40 d incubation at 15 °C, the water phase of two high and one low organic incubation was removed and three mini cores were taken from each incubation for microcalorimetry. After microcalorimetry, sediment from the measuring ampoules was combined with remaining sediment in the incubations and homogenised in a Warren blender. The dry matter and organic matter content was measured as above. The decrease in sediment organic matter was corrected for DOC increases in the water phase, which were probably caused by an efflux of DOC from the sediment. DOC was determined using photochemical (UV) oxidation with persulphate (Schreurs 1979). The specific heat of combustion was calculated from the loss of organic matter in the microcosms and the heat production as measured by microcalorimetry.

Results and Discussion

Decomposition of reed litter. Environmental factors that may govern litter decomposition rates were studied in short and long term litter incubations. Standing reed litter showed no detectable or only a low carbon dioxide production rate compared to litter sampled from the sediment surface (Fig. 1). Addition of extra water stimulated carbon dioxide production of standing litter (Fig. 2) up to rates found for wet, fallen litter, which contained between 70 and 90% H_2O. This suggested that the low water content of standing litter in the field was the main cause for the low carbon dioxide production rate and that reed litter is mainly mineralised once it has fallen to the sediment. This is in contrast to data from salt marshes, where decomposition of standing *Spartina alterniflora* litter is also important, which is probably caused by frequent tidal wetting (Newell et al.

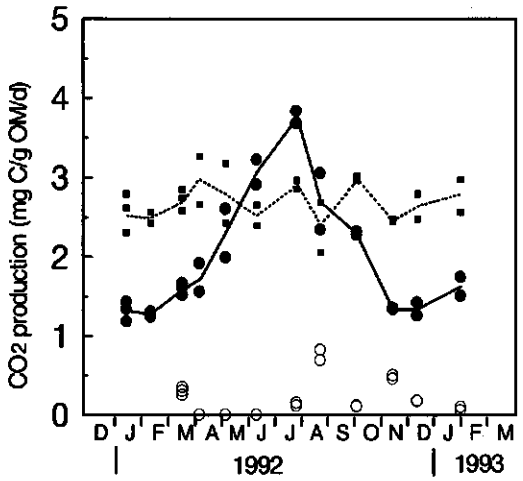

Figure 1. The CO2 production rates of fallen (black circles) and standing reed litter (open circles) measured at the in situ temperature in 1992-1993. Also shown is the CO_2 production rate of fallen litter corrected to 15 °C (black squares) using a Q^{10} of 1.91 (see results and discussion).

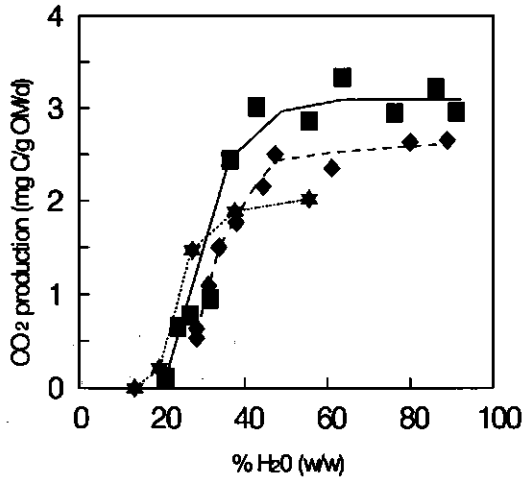

Figure 2. Effect of water concentration of CO_2 production from standing litter sampled on different dates and measured at *in situ* temperatures: (stars) 11 June 1992 (18 °C), (squares) 27 Juli 1992 (19 °C), (diamonds) 24 Aug 1992 (17 °C). Temperature corrected rates (Q^{10} = 1.91) at higher water contents are similar to fallen litter, except for the lower value of 11 June 1992 which had a very low *in situ* water content.

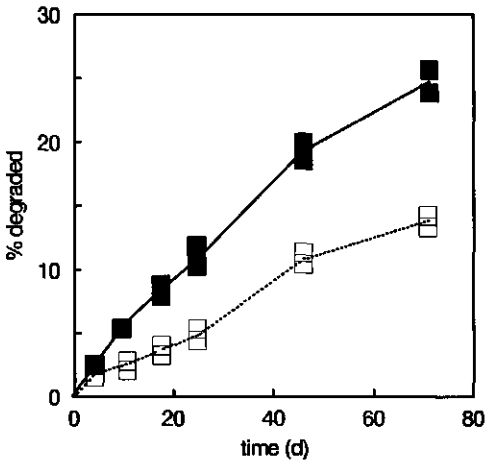

Figure 3. Effect of aerobic (black squares) or anaerobic conditions (open squares) on the degradation of reed litter determined at 15 °C.

1985,1989).

Carbon dioxide production rates from fallen reed litter were higher in summer than in winter (Fig. 1). The effect of temperature on carbon dioxide production was determined in a separate experiment and a Q^{10} of 1.91 ± 0.11 was found, which is in the range found in literature for organic matter decomposition (Andersen 1978; Middelburg et al. 1996; Newell et al. 1985; Teal & Kanwisher 1961). Correcting carbon dioxide production rates at *in situ* temperatures to 15 °C with this relationship, resulted in an almost constant activity as shown in Fig. 1. This suggested that temperature was the main factor determining the mineralization of fallen litter and that its reactivity did not change throughout the year.

Degradation data from the long term litter incubations used to study effects of different electron acceptors (Fig. 3) fitted well to pseudo first order type of kinetics. Degradation rates for aerobic conditions (0.0040 ± 0.0002 d^{-1}) were approximately two times faster than rates for anaerobic conditions (0.0021 ± 0.0001 d^{-1}). Similar ratios between aerobic and anaerobic degradation have been found for other grasses (Godschalk & Wetzel 1978; Benner et al. 1984b). The aerobic rate constant from this experiment compared well with the one, calculated from the temperature corrected carbon dioxide production of fallen reed litter (-0.0052 ± 0.0005 d^{-1}; Fig. 1). Aerobic decomposition rates are in the same range as in other studies for *Phragmites* litter (Andersen 1978; Mason & Byrant 1975; Polunin 1982; Tanaka 1991) or other grasses (Webster & Benfield 1986).

Sediment organic matter. Seasonal variation in the amount of sediment organic matter was determined at three sites in the littoral of Lake Gooimeer. A profile of DM and OM content of the high organic matter site, 20 m inside the bed showed that organic matter

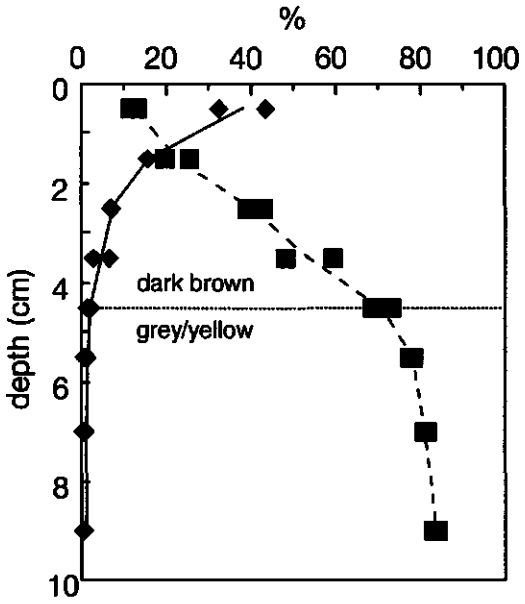

Figure 4. Depth profile of percentage sediment dry matter (squares) and organic matter (diamonds) at 20 m inside the reed bed of Lake Gooimeer, 6 May 1993. The sediment colour changed from dark brown to greyish yellow between 4 and 5 cm depth.

mainly accumulated in the top 4 to 5 cm of the sediment (Fig. 4). Below this layer the sediment consisted of almost pure sand; the base material in the system. A colour change from dark brown to greyish yellow, which marked the transition, enabled us to sample the accumulation horizon through the sampling period and thereby to detect changes in the accumulated sediment organic matter at the high organic matter site (Fig. 5A). Although duplicate cores gave rather scattered results especially in winter 1991 - 1992, the general trend was that organic matter started to accumulate in the sediment during autumn. This coincided with the die down of the macrophytes and was therefore probably caused by the input of fresh reed litter. Organic matter peaked in January to February, after which it abruptly decreased to similar values as the previous spring. Decomposition of reed litter could only explain a small fraction (maximal 8%, using the aerobic rate (Fig. 3)) of the organic matter removed. In both years, the abrupt changes coincided with the passing of a storm depression, causing high water inside the bed and fast changes in water level. It seems therefore more likely that high water flow rates in combination with high landward winds caused erosion of the sediment surface and thereby transport of organic matter to elsewhere. Net transport as calculated from the sudden decrease in organic matter was between 500 and 750 g $C.m^{-2}$ in spring 1992 and approximately 450 g $C.m^{-2}$ in 1993. Especially in 1992 transport was so high that it can not be explained by the annual fresh

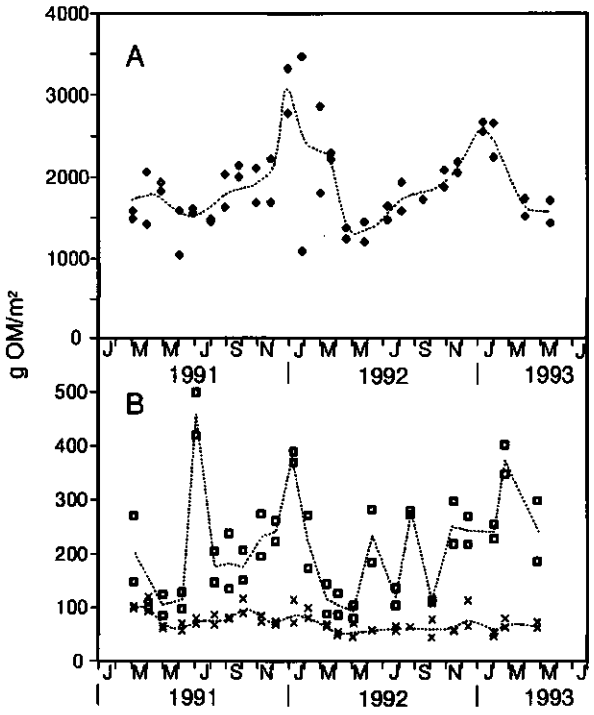

Figure 5. Seasonal variation in the littoral sediment organic matter contents of Lake Gooimeer from 1991 to 1993. Figure 5A shows data from the dark brown, top layer of the sediment at 20 inside the bed (Fig. 4). Data in Figure 5B are for 10 m inside the bed (squares) and 20 m outside the bed (stars) were determined over the 0 to 1 cm horizon.

litter production (Table 1), but probably also includes some older material produced in previous years. We have no data to suggest where the material was transported to. But during the event in spring 1992, banks of reed litter accumulated at the landside of the bed, which suggested that at least part of the material was transported inward.

The two other sites showed a slightly higher organic matter content in only the top centimetre of the sediment, and amounts organic matter are, therefore, given for this horizon (Fig. 5B). The site outside the bed had the lowest amount of organic matter with little variation during the years. Organic matter at 10 m inside the bed was variable during the sampling period.

Pieczynska (1986) also showed that a sharp decrease occurs in sediment surface covered by coarse litter in a littoral during spring, but did not quantify transport in terms of carbon removed. Litter transport seems to occur mainly in spring, which might be explained by a combination of factors: i) litter input has been high during winter resulting in a clear accumulation of litter and ii) erosion protection of the sediment by the vegetation is low, since approximately half of the dead stems from last season had already

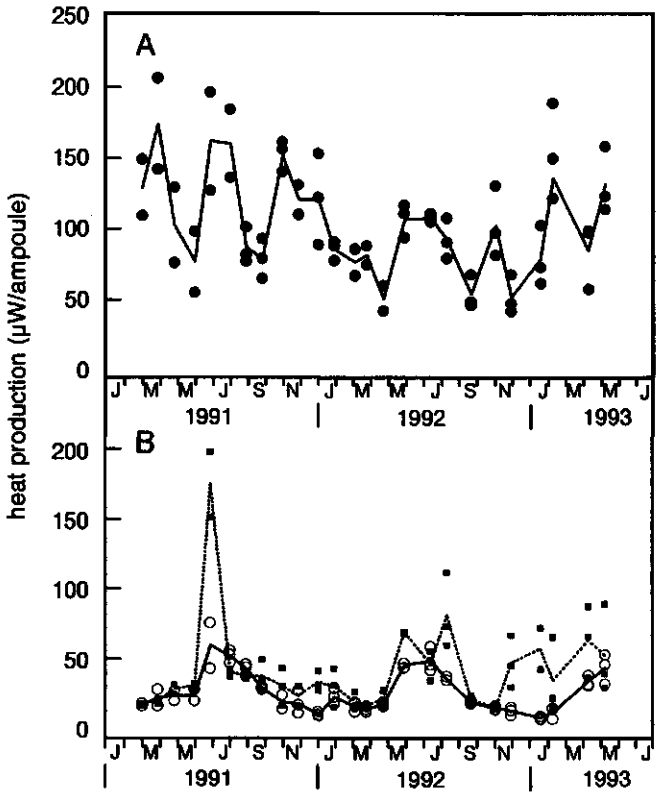

Figure 6. Seasonal variation in sediment heat production (µW per ampoule of 3.46 cm^2 area) as measured by microcalorimetry at *in situ* temperatures. Figure 6A, 20 m inside the reed bed of Lake Gooimeer; Figure 6B, 10 m inside the bed (squares) and 20 m outside (circles).

fallen to the sediment and new shoots have not been formed yet. The observation that sheltered parts of a reed bed tend to accumulate more organic matter than parts exposed to waves (Weisner 1987), also suggests that erosion is an important process at the more exposed parts.

Carbon mineralization in the sediment. Carbon mineralization rates were determined by microcalorimetry at *in situ* temperatures. As shown in Fig. 6, sediment heat production was almost always higher at the high organic matter site (20 m inside the bed) than at the two sites with low organic matter (10 m inside and outside the bed). Outside the bed, activity followed fluctuations in temperature, with higher activities in summer and lower in winter. Activities at 10 m inside the bed were mostly in the range of those found outside the bed. There were a few exceptions in summer when activities at 10 m reached those at the high organic matter site at 20 m inside the bed. Also during winter activities at 10 m inside the bed tended to be higher than outside. This was especially true during

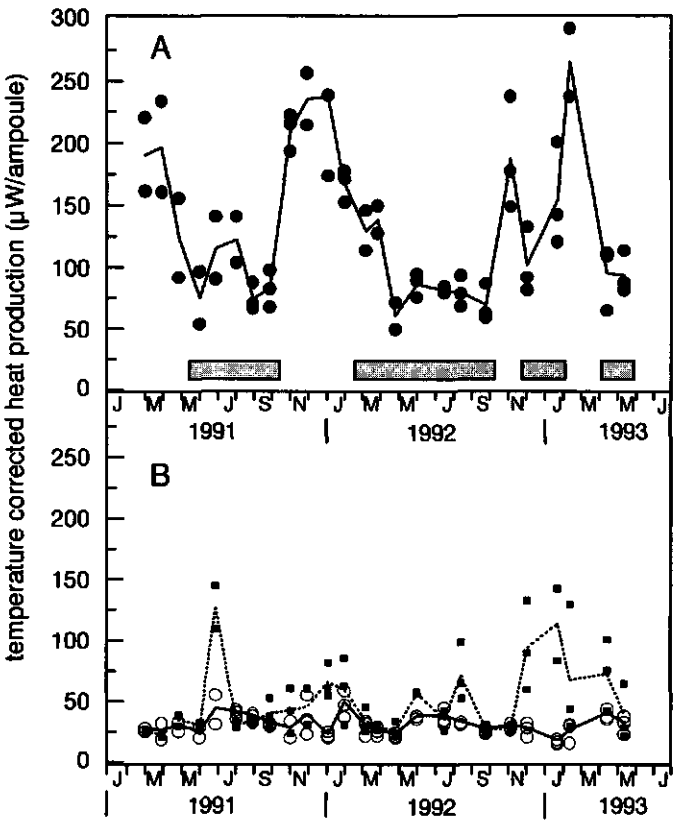

Figure 7. Seasonal variation in sediment heat production (µW per ampoule of 3.46 cm^2 area) as measured by microcalorimetry with temperature corrected to 15 °C using a Q^{10} of 1.91. Figure 7A, 20 m inside the reed bed of Lake Gooimeer; Figure 7B, 10 m in side the bed (squares) and 20 m outside (circles). Grey bars at the bottom of Figure 7A show when there was water over the sediment at 20 m inside the bed. The other two sites always remained submerged.

the last winter, when activities became higher after die down of the reed plants and became similar again to outside the bed during spring. The pattern of activities at 20 m inside the bed was complex, with higher activities in both summer and winter than in spring and autumn. This suggested that there were additional factors besides temperature that governed carbon mineralization rates at 10 m and especially at 20 m inside the bed.

Heat productions measured at *in situ* temperatures were corrected to 15 °C (Fig. 7) using the temperature activity relationship that was found for reed litter (see before). For the site outside the bed, temperature corrected data were almost constant through the whole sampling period, which strongly suggested that temperature was indeed the main factor that determined fluctuations in carbon mineralization at this site. Heat productions at 10 m inside the bed were again mostly similar to outside the bed, with higher activities

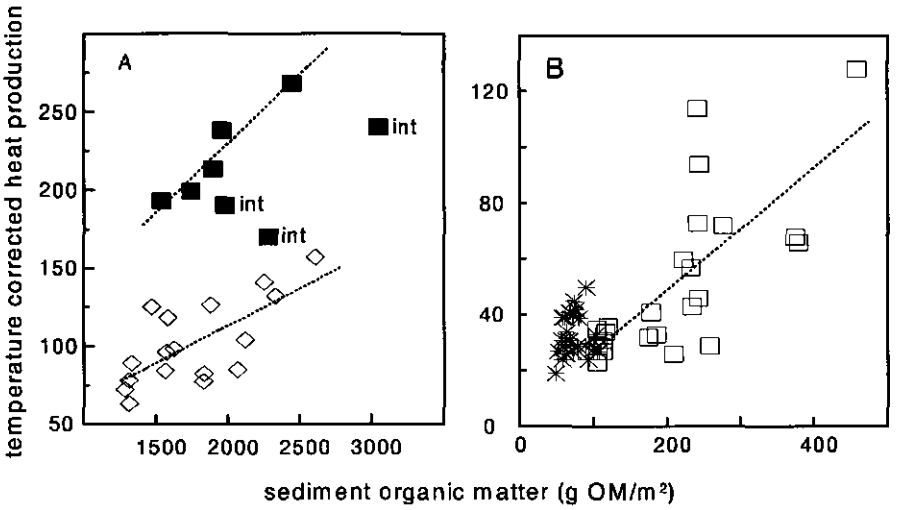

Figure 8. Relation between average temperature-corrected (15 °C) heat production and average organic matter content of sediments. Results for 20 m inside the bed are presented in Figure 8A and are split between dates when overlaying water present (squares) or absent (diamonds). Points labelled with 'int' are considered to be intermediate points (see results and discussion). Figure 8B shows results for 10 m inside (squares) and 20 outside the bed (stars), where the sediment was always covered by overlaying water. Also shown are linear regression lines for the different data sets.

on several occasions in summer and throughout winter. At 20 m inside the bed, temperature corrected activities showed a bimodal pattern, with high activities in winter and low in summer. In Fig. 7 is also shown when there was water above the sediment at 20 m inside the bed. The inundation pattern almost coincided with the heat production pattern, with high activities without overlying water present and lower with water. We suggest that the bimodal pattern in mineralization rates was to a large extent caused by a better aeration of the sediment during winter due to an, on average, lower water level, which would result in a shift from anaerobic to aerobic decomposition of sediment organic matter. As shown before, the aerobic decomposition of reed litter was faster then under anaerobic conditions (Fig. 3).

The amount of sediment organic matter inside the bed was variable, as shown before, due to the input of fresh reed litter at the end of the growing season and the erosion of the sediment in spring and this might also affect mineralization rates. In Fig. 8, the relationship between temperature corrected heat production and sediment organic matter is shown for all sites. Outside the bed, no correlation ($r = 0.12$) could be detected between activity and organic matter content, which was probably due to the low variation in both parameters. For the low organic matter site at 10 m inside the bed, higher activities were clearly related to organic matter increases ($r = 0.742$, $p > 0.99$, linear regression

coefficient = 0.22 ± 0.04 $\mu W \cdot g\ OM^{-1} \cdot m^{-2}$ = 0.0028 ± 0.0005 d^{-1}). At 20 m inside the bed, a situation with and without overlaying water could be distinguished. With overlaying water present there was a clear correlation between the two parameters (r = 0.71, p > 0.99, regression coefficient = 0.00059 ± 0.00002 d^{-1}). Without overlaying water, there was no significant correlation if all points were considered (r = 0.48). This is probably caused by a less direct relationship between our observation that water did not reach the point at 20 m inside the bed and the microbial processes that took place in the sediment. It is possible that on certain sampling dates we did not observe water on the sediment, but that part of the sediment top layer was still water saturated and therefore not completely oxic. If only the outer five point with high activity are taken as truly oxic, a significant correlation is found (r = 0.93, p > 0.99, regression coefficient = 0.00110 ± 0.00002 d^{-1}).

Our regression data suggested that decomposition with water on the sediment was approximately 2 times slower than without water and that this was due to a shift from aerobic to anaerobic processes. The same relation in decomposition rates was found between anaerobic and aerobic litter incubations (Fig. 3). Nyman and DeLaune (1991) found that lower water levels stimulates carbon mineralization up to a factor of 5 in a range of wetlands. However, the absolute rates of sediment organic matter decomposition as derived from the regressions in Figure 8 were approximately a factor of 4 lower than expected from the decomposition experiment. These lower absolute rates may be caused by less favourable conditions in the sediment or, more likely, by the presence of older less reactive material in the sediment organic matter in addition to fresh litter. The reactivity of the organic matter at 10 m inside the bed was intermediate those found in the aerobic and anaerobic litter decomposition experiments. This suggested that the highest activities at 10 m inside the bed were the result of i) the input of only relatively fresh reed litter, or ii) a mixture of material from deeper inside the bed with the more reactive algal material derived from either sedimentation of seston or benthic, micro-algae production (Boschker et al. 1995b). The sediment organic matter outside the bed, which existed primarily of algal derived material, had an even higher reactivity of 0.0059 ± 0.0015 d^{-1}.

To convert the heat production rates as measured by microcalorimetry to actual carbon mineralization rates a specific heat of combustion is needed (see formula 1). For the aerobic mineralization of reed litter we found an experimental factor of -38.2 ± 2.2 $kJ \cdot g\ C^{-1}$ which is similar to the heat of combustion of -38 $kJ \cdot g\ C^{-1}$ that is generally found for plant derived materials (Straskraba 1968). Factors determined with the sediment microcosms were not different after 20 and 40 d of incubation, and also not between low and high organic systems. On average, a heat of combustion of 44.9 ± 3.5 $kJ \cdot g\ C^{-1}$ was found for the microcosms, which is also similar to the theoretically expected value. This all suggests that a factor of approximately -40 $kJ \cdot g\ C^{-1}$ can be used to convert our measured heat production data to actual carbon mineralization rates (see also Graf 1987). Using this factor on our field data, carbon mineralization varied between 320 and 1090

mg $C \cdot m^{-2} \cdot d^{-1}$ for the high organic matter site and between 100 and 1090 mg $C \cdot m^{-2} \cdot d^{-1}$ for the low organic site inside the bed. Mineralization rates were lower outside the bed with a range of 60 to 380 mg $C \cdot m^{-2} \cdot d^{-1}$. Mineralization rates for the high organic matter site are on the lower side of the range found for a Phragmites swamp (Andersen 1981) and ii) for salt marshes (Howes et al. 1985; Morris & Whiting 1986; Nymann & DeLaune 1991; Smith et al. 1983; Teal & Kanwisher 1961). Maximum rates reported by these authors are up to 4 times higher than our data, which might be caused by the high transport of organic matter to elsewhere in the reed bed of Lake Gooimeer. This process removed organic matter, that would otherwise be available for mineralization by micro-organisms.

In conclusion, factors that affected mineralization rates in the sediments of the littoral zone of Lake Gooimeer were in order of importance: i) temperature, ii) water level induced changes in oxic or anoxic conditions in the sediment, and iii) changes in sediment organic matter by input and removal of organic matter. Since most wetlands will be subjected to variation in the same environmental factors, we suggest that these factors will have general importance. Other factors, like nutrients and pH, may be locally important. These were either stable or optimal in the littoral of the eutrophic Lake Gooimeer (pH 7 to 7.5, high nutrient concentrations).

Carbon budget. To summarise our measurements, a carbon budget for the sediment at 20 m inside the bed is presented in Table 1. The budget is based on data from September 1991 to September 1992, since this was the only complete litter cycle recorded. Results were similar if data from April 1991 to April 1992 or from April 1992 to April 1993 were used (not shown). No budget is presented for the two other sites, because it has been shown that the input of seston and production by benthic algae play an important but not quantified role (Boschker et al. 1995b). Several processes in the budget have not been discussed before in this paper and need additional explanation.

On the input side of the budget, we only determined the above-ground reed litter production, but common reed also has an extensive root system. Estimating the below-ground production litter of common reed is difficult because of its number of different root types and an extended rhizome system, which can be found to a depth of 1 m in the sediment (Haslam 1972, Rodewal-Rodescu 1974). Instead, we used literature data to estimate the below-ground production from our above-ground production data. The ratio between below to above ground biomass of common reed is rather variable and is influenced by the nutrient status of the system. Eutrophic stands generally have a relatively low ratio ranging from 0.5 to 2.5 (Boar at al. 1989; van der Linden 1980; Schierup 1978), which gives a below-ground biomass of 340 to 1690 g $C.m^{-2}$ for the Lake Gooimeer reed bed. The average life span of rhizomes is estimated at 3 to 6 years (Haslam 1972), which gives a production of 60 to 560 $gC \cdot m^{-2} \cdot y^{-1}$. Others have found below-ground productivities in a similar range (van der Linden 1980; Schierup 1978). It is

Table 1. A carbon budget for the sediment 20 m inside the reed bed of Lake Gooimeer for the period September 1991 to September 1992.

Processes	g $C \cdot m^{-2} \cdot y^{-1}$
Inputs	
Above ground production[1]	675 ± 150
Below ground production	ND[4]
Sedimentation of seston[3]	negl[5]
Benthic algal production[3]	negl
Total inputs	675 ± 150
Outputs	
Decomposition of standing litter	negl
Mineralization	250 ± 25
Net transport spring	500 to 750
DOC efflux[3]	negl
Preservation[2]	35
Total outputs	785 to 1035
Deficit	-110 to -360

[1] A standing crop of 675 ± 150 g $C.m^{-2}$ was determined at the end of the growing season in October 1991, which was similar to 1990 and 1992. These standing stocks are in the range as found by others in similar ecosystems (Boar et al. (1989); van der Linden (1980); Mason & Byrant (1975); Schierup (1978); Weisner (1987)).

[2] Calculated from the average sediment organic matter in spring (1700 ± 300 g $OM.m^{-2}$ (Figure 5A)) and from the minimum age of the vegetation as known from aerial photos (25 years).

[3] Based on data presented in Boschker et al. 1995b.

[4] Not Determined, see discussion.

[5] Negligible

not clear how much of this production actually enters the sediment organic matter, since it will be partially respired by the rhizome or translocated to other parts of the plant. Translocation is especially likely for the non-structural carbohydrate reserve material in the rhizome, which represents between 40 and 60 % of the rhizome dry weight (Rodewald-Rodescu 1974). Therefore, a best estimate of below-ground litter production ranges from 25 to 340 g $C \cdot m^{-2} \cdot y^{-1}$, which is not different from the estimated deficit of the carbon budget. An other complicating factor in estimating the importance of rhizome derived material in the budget, is that the carbon balance (Tab. 1) only accounts for the

top 3 to 5 cm of the sediment since mineralization, transport and burial were also determined over this horizon. The rhizome/root system can be found to a depth of 1 m or more (Haslam 1972, Rodewal-Rodescu 1974) and it is unlikely that the deeper material will influence carbon dynamics in the top layers of the sediment.

It should be noticed that the estimated net transport data are based on abrupt changes in sedimentary organic carbon in spring. It is possible that some movement of organic matter also took place in other periods, and net transport might therefore be underestimated. The fast changes in both organic matter and mineralization rates, which were observed at 10 m inside the bed, indeed suggested that some transport of organic matter occurred during summer. Unfortunately, transport figure in Table 1 is based on the very variable organic matter data in winter and spring 1992, this is the main cause for the wide range of the estimated transport and the high, variable missing part of the budget. In addition, the high transport suggested that some older material was also removed, which would overestimate yearly transport data. Differences between duplicate organic matter data were less extreme in spring 1993 and transport accounted for only approximately 450 g $C \cdot m^{-2} \cdot y^{-1}$. If a budget from april 1992 to april 1993 is calculated with this lower transport figure, the missing part is approximately -100 g $C \cdot m^{-2} \cdot y^{-1}$.

Taking all uncertainties into account, the carbon budget suggests that net transport of litter was the main loss process accounting for 60 to 70% of the total output. This high removal rate for organic matter due to erosion probably is the main factor for the relatively low contribution of mineralization (25 to 30% of the output) and for the very low burial rate (less than 4%).

In a recently published partial budget for a reed bed, Gessner et al. (1996) specifically stated that they assumed that transport of reed litter between littoral and pelagic systems was negligible: an assumption that in view of our present work no longer can be made. We do not know of other carbon budgets for similar freshwater wetlands. But budgets for marine and estuarine tidal wetlands, like mangroves and salt marshes, suggest that transport is a very variable process. In mangroves, export of litter can sometimes explain 90 % of the litter losses (Boto & Bunt 1981; Flores-Verdugo et al. 1987; Twilley 1985; Robertson & Daniel 1989). Sediment budget studies for salt-marshes suggest that a substantial part of the macrophyte production is available for transport (Jackson et al. 1986; Nyman et al. 1995; Smith et al. 1983). However, direct measurements of carbon exchange between the salt marsh and the open water indicate a variation of transport between net import and a net export of particulate organic carbon. Both export and import fluxes are although mostly rather small compared to the macrophyte production on the marsh (Childers 1994; Dame 1994; Nixon 1980). As in our system, storms seem to play an important role in marine wetlands with respect to transport of organic matter (Dame 1982; Hackney & Bishop 1981; Roman & Daiber 1989). However, these storm events are seldom covered by studies in which carbon exchange is directly measured.

Acknowledgements

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Chapter 4

A Sensitive Method Using 4-Methylumbelliferyl-β-Cellobiose as a Substrate to Measure (1,4)-β-Glucanase Activity in Sediments

H.T.S. Boschker and T.E. Cappenberg

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Abstract. A sensitive method to measure (1,4)-β-glucanase activity in organic matter-rich sediments using 4-methylumbelliferyl-β-cellobiose as a substrate, is described. β-Glucosidases, which were also able to hydrolyse this substrate, were inhibited with D-glucono-δ-lactone. The produced 4-methylumbelliferone was recovered quantitatively out of the sediment by extraction with 80% ethanol. An inhibition experiment with known substrates or inhibitors suggested that at least 59% of the measured activity could be explained by enzymes of the exo-(1,4)-β-glucanase type and that the contribution of endo-(1,4)-β-glucanases was minor. Results of the inhibition experiment also suggested that the measured activity was of bacterial origin in the sediment used. First results of field measurements are given for sediments from the reed bed of Lake Gooimeer.

Introduction

The major input of organic matter (OM) to the sediment of lakes is macrophyte- or algae-derived material. This material is mainly polymeric and needs to be degraded extracellularly to smaller units before it can be taken up by microorganisms. The polymeric material is hydrolyzed by enzymes released by or on the outside of microorganisms, and this process is generally regarded as the rate-limiting step in the mineralization of complex organic material (Billen 1982; King 1986). Many studies that address the importance of extracellular enzymes in the decomposition of organic matter can be found (Burns 1983; Sinsabaugh et al. 1991).

Cellulose is by mass the most important polymeric compound in macrophytes and is degraded by a complex of enzymes. In the cellulase system, three types of enzymes which cooperate in the degradation of cellulose to cellobiose and glucose are distinguished: endo-(1,4)-β-glucanases, which randomly split cellulose; exo-(1,4)-β-glucanases, which release cellobiose or sometimes glucose from the end of a cellulose chain; and β-glucosidases, which split cellobiose into two glucose molecules (Ljungdahl & Eriksson 1985; Walker & Wilson. 1991).

Fluorogenic substrates have been used for some time to assay extracellular enzymes in aquatic ecosystems (Hoppe 1983; Somville & Billen 1983). A sensitive method to measure β-glucosidase activity directly in sediments has been described that uses an artificial substrate: 4-methylumbelliferyl-β-glucose (MUF-glu) (King 1986; Meyer-Reil 1986a). MUF-glu is split by the β-glucosidases present in the sediment sample into glucose and 4-methylumbelliferone (MUF). The produced MUF is measured fluorometrically down to nanomolar concentrations, which results in a sensitive method with short incubation times. To our knowledge, no sensitive method for sediments has been described yet to measure the activity of the two enzyme types that act directly on the polymeric cellulose: endo-(1,4)-β-glucanases and exo-(1,4)-β-glucanases. Such a method might be of interest because the measured activities would be more closely associated with the presence of cellulose than is the case with β-glucosidases.

Here we describe the development and testing of a method to measure (1,4)-β-glucosidase activity in sediment samples by using MUF-β-cellobiose (MUF-cel) as a substrate. The principles of the method have been published by Desphande at al. (1984; 1988), who use *p*-nitrophenyl-β-cellobiose to assay exo-(1,4)-β-glucosidases in purified cellulases. β-Glucosidases, which also showed activity towards p-nitrophenyl-β-cellobiose, were specifically inhibited with D-glucono-δ-lactone (GLN). We also present a general experimental procedure for measuring enzymatic activities in sediment with MUF-labelled substrates. In this procedure the produced MUF is quantitatively extracted from the sediment with 80% ethanol.

Materials and Methods

Sampling sites. Sediments used in this study were collected at different sites inside and at 10 m outside the 80 m wide reed bed on the southern shore of Lake Gooimeer. Lake Gooimeer is very shallow (mean depth, 3.6 m) with a sandy sediment and is situated in the center of the Netherlands. Common reed (*Phragmites australis* (Cav.) Trin. ex Steudel) is the dominant plant species in the bed. Inside the bed, OM is accumulating. The OM content of the sediment increases from <1% at the lake side of the bed to 30% on the land side. Because of the high OM content and the yearly input of fresh reed litter, containing about 40% cellulose, it is likely that cellulolytic enzymes play an important role in the decomposition of OM in these sediments.

Sediments. For the testing of the enzyme assay, sediment from the top 3 cm was collected and slurried for 30 s in a Waring blender after the addition of some water (sediment/water [wt/wt] ratio = 5). To determine profiles of enzyme activities, sediment cores from inside and outside the bed were taken by drilling a perspex cylinder (7 cm inside diameter) with a sharpened cutting edge into the sediment by hand. In the laboratory, cores were sliced with 1-cm intervals over the top 7 cm and with 2-cm intervals below. Slices were mixed in a Waring blender. Sediments were slurried because of the presence of course reed litter fragments that otherwise could not be included in the assay. Living plant parts were removed as far as possible before slurrying.

The dry matter (DM) content of sediment samples was measured after an overnight drying at 105 °C, and OM content was determined as the subsequent loss of weight after 4 h at 550 °C.

Chemicals and solutions. All substrates and other chemicals were purchased from Sigma, except for cellulose MN300, which was from Hachery Nagel & Co., and cellodextrin mixture I, which was from Merck. According to batch analyses by Merck, cellodextrins mixture I contained 0.9% glucose, 14.3% cellobiose, 25.5% cellotriose, 30.2% cellotetraose, 16.3% cellopentaose, and 3.9% cellohexaose. To remove low-molecular-weight compounds, methylcellulose of low viscosity (MC-low) and, carboxymethyl celluloses of medium (CMC-medium) and low (CMC-low) viscosities were repeatedly suspended in 80% ethanol and centrifuged down. After this treatment, the washed material was dried at 40 °C. GLN was dissolved in a 0.1 M phosphate buffer and adjusted to pH 7.0. All other compounds were dissolved in water.

Enzyme assays. Between 0.1 and 0.3 ml of sediment slurry was added to a 15 ml centrifuge tube. Reactions were started by adding 1 ml of MUF-glu or MUF-cel at the desired concentration (final pH, 7.0 to 7.5) and tubes were incubated on a rotary shaker at 20 °C. Incubations with MUF-glu took between 5 and 20 min, and those with MUF-cel took between 0.5 and 1.5 h depending on the sediment used and the purpose of the experiment. Reactions were stopped by the addition of 5 ml 96% ethanol, mixing thoroughly, and putting the tubes

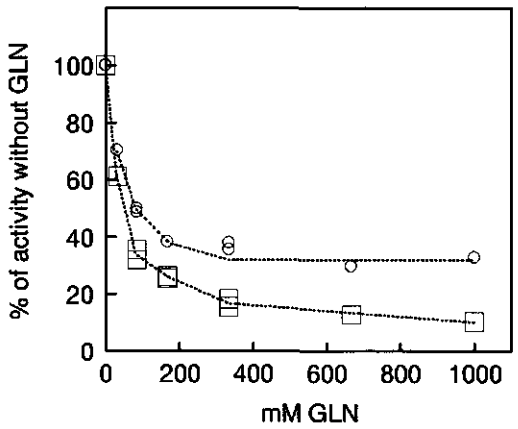

Figure 1. The effect of GLN on the MUF-production from MUF-glu (squares) and MUF-cel (circles). Above 400 mM GLN no interference from β-glucosidases in the hydrolysis of MUF-cel was observed. Sediment (16% OM) was collected from the top 3 cm on 6 February 1990. All points represent duplicates. SD's in the lower part of the figure were smaller than the symbols.

on ice. Particles were removed by centrifugation at 6,000 rpm for 20 min (-2 °C) and the supernatant was decanted into another tube containing 1 ml of 1 M glycine-NaOH buffer, pH 11. From this point, the samples were stable for at least 2 h.

To measure MUF concentration, 3 ml of the sample was added to an UV-cuvette, and fluorescence was measured on an Aminco-Bowman spectrofluorometer at an excitation wavelength of 365 nm and an emission wavelength of 455 nm. For blanks, MUF-glu and MUF-cel additions were made shortly after 5 ml of 96% ethanol was added to the centrifuge tube containing the sediment sample. After the blank fluorescence was read, MUF fluorescence was calibrated by adding 200 µl of 50 µM MUF standard to the cuvette containing the blank, after which fluorescence was measured again. At the wavelength settings used, MUF-glu, and MUF-cel incubations and free MUF showed maximum fluorescence. Blanks for both MUF-glu and MUF-cel incubations had low and similar fluorescence signals, which suggested that increased fluorescence in the incubations was due to free MUF.

Characterization of enzyme activities. Inhibition experiments were conducted to characterize the enzyme activities measured with MUF-glu and MUF-cel plus 500mM GLN (MUF-cel+GLN). Seven known substrates or inhibitors of β-glucosidases or β-glucanases (Table 1) (Rasmussen et al. 1988; Walker & Wilson. 1991; Wood & McCrae. 1982) were tested for their inhibiting capacities on enzymatic activities against MUF-glu or MUF-cel+GLN. Additions were made shortly before the MUF-labelled substrate was added. Multiple regression analysis with dummy variables was used to test for statistically significant effects of the different additions.

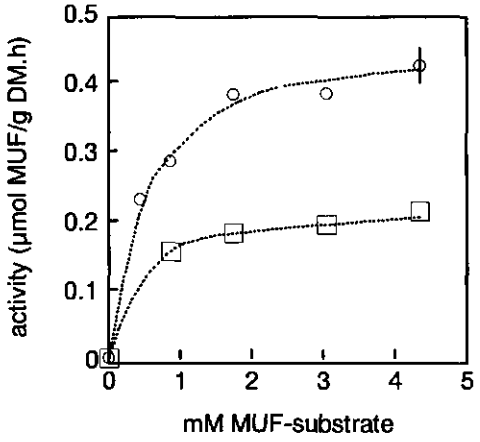

Figure 2. Relation between the substrate concentration and the activity against MUF-glu (circles) and MUF-cel+GLN (squares). Both enzyme systems were saturated at 2 mM. Sediment (16% OM) was collected from the top 3 cm on 6 February 1990. (Error bars are one SD; when SD was smaller then symbol, no bar is used).

Results

Testing the application of MUF-cel+GLN in sediment. A problem associated with the use of MUF labelled substrates in sediments is that the produced MUF in the assay adsorbs onto the sediment (King 1986). Preliminary experiments revealed that when no further measures were taken, only 9 to 15% of a standard MUF addition was recovered from a sediment slurry having an OM content of 15%. Although it is possible to correct for recovery by determining it in a separate experiment (King 1986), the low and variable recovery gave inaccurate results. Therefore, several methods were tested to extract MUF quantitatively from of the sediment. The extractants used were buffers of pH 2 (0.2 M glycine-NaCl-HCl) or 11 (0.2 M glycine-NaOH), 1% Triton X-100 in water, 80% methanol and 80% ethanol. Extraction with ethanol resulted in a recovery of 88% (standaard deviation (SD) = 5, n = 6). In these extracts, MUF fluorescence was linear up to a concentration of 10 µM.

The effect of GLN, a specific inhibitor of β-glucosidases (Desphande et al. 1984; Deshpande et al. 1988, Wood & McCrae 1982), on the MUF production from MUF-glu and MUF-cel is shown in Fig. 1. While the activity against MUF-glu was low and still went down at higher concentrations of GLN, activity against MUF-cel levelled off at about 35% with GLN concentrations of 400 mM or higher. We concluded that at GLN concentrations above 400 mM, β-glucosidases did not significantly hydrolyze MUF-cel. A concentration of 500 mM GLN was chosen for further incubations with MUF-cel (referred to as MUF-cel+GLN). GLN precipitated when the 5 ml of ethanol was added for MUF extraction, but this had no effect on the recovery of MUF.

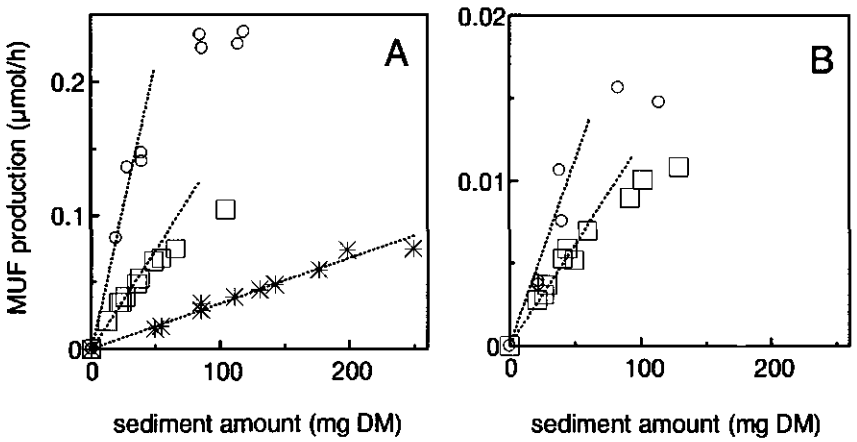

Figure 3. Effect of the sediment amount used in the assay on the measured MUF production from MUF-glu (A) and MUF-cel (B). The sediment with <1% OM (stars) was from outside the reed bed; the other two were from different locations inside the bed. For the two sediments (6% OM, squares; 16% OM, circles) from inside the bed only 50 mg DM or less could be used before activity deviated from linearity with the amount of sediment. For the sediment with <1% OM no activity against MUF-cel+GLN was detected. Sediments were collected from the top 3 cm on 1 June 1990.

The MUF production from MUF-glu was linear in time for at least 30 min and for MUF-cel+GLN it was linear for at least 2 h (data not shown). A single incubation time of 5 to 15 min for MUF-glu and of 0.5 to 1.5 h for MUF-cel+GLN was used in further experiments and was long enough to produce a MUF fluorescence signal of 4 to 30 times the blank fluorescence. Maximum activity for both substrates was reached at a substrate concentration of 2 mM (Fig. 2). Unless otherwise stated, further measurements were carried out at a MUF-glu and a MUF-cel concentration of 3.3 mM. MUF production was only linear with the amount of sediment in the assay when very small amounts of sediment were used (< 50 mg of DM [Fig. 3]). These figures also show that there was an inverse relationship between the OM content of the sediment and the maximum amount of sediment that could be used before MUF production deviated from linearity with the amount of sediment in the assay. Heat sterilized sediment (2 h at 120 °C) showed no detectable activity (< 1% of fresh sediment) for both substrates. Therefore, it was concluded that the activities were of a biological or enzymatical origin.

Characterization of enzyme activities. The enzyme activities measured with MUF-glu and MUF-cel+GLN were characterized by performing inhibition experiments using 7 known substrates or inhibitors of β-glucosidases and endo- or exo-glucanases (Table 1). At a substrate concentration of 1.5 mM, patterns of inhibition were clearly different for MUF-glu and MUF-cel+GLN. Cellobiose and glucose were inhibiting activity only against MUF-glu, while MC-low inhibited activity only against MUF-cel+GLN. Activity against both substrates

Table 1. Effects of additions on the enzymatic activity against MUF-glu and MUF-cel+GLN.

Addition	Concentration (g/l)	% Activity[b] of control (mean [SD])		
		MUF-glu	MUF-cel+GLN	
		(1.5 mM)	1.5 mM	0.3 mM
Control		100 (6)	100 (7)	100 (3)
Cellulose	10	102 (2)	100 (2)	
CMC-medium	10	72 (12)*	90 (10)*	
CMC-low	25	48 (9)*	86 (11)*	73 (2)*
MC-low	25	100 (12)	73 (6)*	66 (5)*
Cellodextrins	10	65 (5)*	72 (2)*	41 (1)*
Cellobiose	10	75 (6)*	101 (4)	
Glucose	10	59 (16)*	100 (7)	

[a] Sediment (16% OM) was collected from the top 3 cm on 27 September 1990.

[b] *, significantly ($p = 0.95$) different from control.

was significantly inhibited by a cellodextrin mixture, CMC-low, and CMC-medium. Cellulose did not inhibit activity in either case, which was not expected. However, since cellulose was the only solid compound tested, a longer preincubation time might be needed before an effect is obtained. The percent inhibition by all additions was higher at a concentration of 0.3 mM MUF-cel+GLN than at 1.5 mM. This was especially true for the cellodextrin mixture, which reduced the activity to 41% of the control level.

Example of field measurements. The results of an application of the method are shown in Fig. 4; enzyme activities against MUF-glu and MUF-cel+GLN were measured in sediment cores taken inside and outside the reed bed of Lake Gooimeer. Inside the bed, activities for both enzymes were highest in the top layer of the sediment, suggesting a main input of fresh material from above, probably in the form of dead reed litter. The secondary peak in the enzyme activities between 3 and 4 cm of depth might be caused by an input of material from the *Phragmites* roots that show a maximum standing stock at this depth (data not shown). Outside the bed, β-glucosidase activities were lower than inside and no exo-(1,4)-β-glucanase activity could be detected.

Discussion

Testing the application of MUF-cel+GLN in sediment. An advantage of the method described here is that the produced MUF is quantitatively recovered from the sediment, using

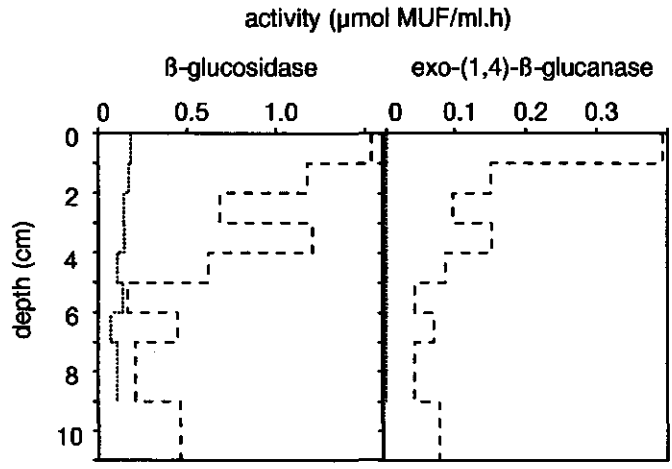

Figure 4. Enzyme activities in two cores, one from inside the reed bed (striped line) and one from outside (stippled line), sampled on 22 May 1990. In the core from outside the bed no exo-(1,4)-β-glucanase activity was detected. All measurements are in duplicate. SD was less then 10% of the measured activity.

an extraction with 80% ethanol. This means that there is no need for an internal standardization as described by King (1986), who used MUF-glu to measure β-glucosidase activity in marine sediments. We also have used this extraction procedure with similar results for other MUF labelled substrates like MUF-xylose and MUF-butyrate (no data shown).

The presented method was sensitive; incubation times of only 0.5 to 1.5 h were sufficient to produce a well-measurable MUF fluorescence. No inhibition or induction of enzyme activity occurred during this incubation period, since MUF production was linear with time. A limitation was that only a small amount of sediment could be used in the assay before hydrolysis of the substrates deviated from linearity with the amount of sediment. This small amount of sediment implies that the method was sensitive to the heterogeneity in the sample. For the type of sediments used in this study, which contained course litter fragments, the method could be applied only to well mixed samples. The organic content of the sediments was high, which was an important factor in the amount of sediment that could be used. For other types of sediments, MUF labelled substrate analogs have been used with larger amounts of sediment (King 1986) or even whole cores (Meyer-Reil 1986a).

In this study it was found that a GLN concentration of 400 mM or higher was effective in inhibiting the hydrolysis of MUF-cel by β-glucosidases in sediments. A much lower concentration of GLN (0.6 to 3.0 mM) is sufficient to inhibit a β-glucosidase from the fungus *Trichoderma reesei* (Desphande et al. 1984; Deshpande et al. 1988). It is possible that GLN was less available in sediments because of adsorption and therefore was a less effective inhibitor. An alternative explanation might be found in a combination of the acidic nature of

GLN and of a difference in pH between our study conditions (pH 7.0 to 7.5) and those of Desphande et al. (1984, 1988), who use a pH of 5. It is likely that the undissociated form of the acidic GLN is the inhibiting species for β-glucosidases and this species is present at a 100 times lower concentration at pH 7 than at pH 5.

Characterization of enzyme activities. Because sediments probably contain a large variety of enzyme types, we tried to characterize the activities against MUF-glu and MUF-cel+GLN in inhibition experiments with known inhibitors or substrates of cellulolytic enzymes. MUF-glu is a selective substrate for β-glucosidases (Desphande et al. 1984; Somville 1984). This is in agreement with our finding of the almost complete inhibition of MUF-glu activity by GLN, a specific inhibitor of β-glucosidases (Desphande et al. 1984; Deshpande et al. 1988; Wood & McCrae 1982), indicating that MUF-glu was mainly hydrolyzed by β-glucosidases. Activity against MUF-cel+GLN showed an inhibition pattern that was clearly different from that of activity against MUF-glu (Table 1) and was therefore not of the β-glucosidase type.

Of the two other types of cellulolytic enzymes, endo-(1,4)-β-glucanases act on the glucoside linkages along the cellulose chain while exo-(1,4)-β-glucanases act on the end groups of the cellulose molecule (Ljungdahl & Eriksson 1985; Walker & Wilson 1991). We found that the activity against MUF-cel+GLN was more sensitive for inhibition by the cellodextrin mixture then by CMC-medium, CMC-low, or MC-low. The cellodextrin mixture has far more end groups per gram of material than CMC-medium, CMC-low, and MC-low, meaning that at least 59% of the measured activity could be explained best by an enzyme sensitive to end groups of cellulose or similar compounds: an exo-(1,4)-β-glucanase type of enzyme. Data in the literature on purified cellulolytic enzymes agree with these results. Although, several endo-(1,4)-β-glucanases are able to split MUF-cel (Chernoglazov et al. 1989; Van Tilbeurgh & Claeyssens 1985) or *p*-nitrophenyl-β-cellobiose (Desphande et al. 1984; Schellhorn & Forsberg 1984), a similar substrate, most of them show no detectable activity on these substrates (Bhat et al 1990; Gilkes et al. 1984; Van Tilbeurgh et al. 1982), and if activities are found they are lower than with exo-(1,4)-β-glucanases (Desphande et al. 1984). Maybe MUF labelled longer cellodextrins (Bhat et al. 1990; Van Tilbeurgh et al. 1982) than MUF-cel could be used to measure endo-(1,4)-β-glucanase activity in sediments, but these substances are very expensive or not commercially available.

We found that MUF-cel+GLN activity was not inhibited by high concentrations (10 g/l) of both glucose and cellobiose, the primary products of cellulose decomposition. Most cellulases of bacterial origin are resistant to inhibition by glucose (Murray 1987; Ng &. Zeikus 1981; Shinmyo et al. 1979) and some are even unaffected by cellobiose (Murray 1987; Shinmyo et al. 1979). On the other hand, inhibition by both products at concentrations used in this study is described for all studied fungal cellulases (Murray 1987; Ng & Zeikus 1981; Walker & Wilson 1991; Wood & McCrea 1978). This suggests that fungal cellulases were of minor importance compared with bacterial cellulases in the sediment of the reed bed.

Similarly, Benner et al. (1986) showed, by using size fractionation and antibiotic treatments, that bacteria are predominant in the decomposition of ^{14}C-labelled lignocellulose in both freshwater and marine sediments.

Application of the method. There are not many other assays known for measuring exo-(1,4)-β-glucanase activity directly in sediments. Glucose production from microcrystalline cellulose has been used to determine exo-(1,4)-β-glucanase activity in sediments (Sinsabaugh et al. 1990) and soils (McClaugherty & A.E. Linkens. 1990), but incubation times are very long (18 to 24 h), and toluene is added to prevent uptake of glucose and induction of enzyme activity during this long period. Although toluene is inhibitory to β-glucosidases (King 1986), the effect of toluene on exo-(1,4)-β-glucanases was not determined in these studies. *p*-Nitrophenyl-β-cellobiose was used by Sinsabaugh and Linkins (1990) to measure exo-(1,4)-β-glucanase activity with incubation times of 4 to 6 h. But their assay was probably not selective for exo-(1,4)-β-glucanases, since β-glucosidases are not inhibited. In this study, inhibition experiments with GLN showed that 65% of the activity measured with MUF-cel could be explained by β-glucosidases.

In contrast to cellulose, the natural polymeric and particulate substrate of cellulases, MUF-cel is an oligomeric and dissolved substrate. It is therefore very unlikely that the activities measured with MUF-cel are actual, *in situ* rates of cellulose hydrolysis or exo-(1,4)-β-glucanase activities. Furthermore, since MUF-cel was used at a saturating concentration, the measured activities should be seen as potential activities (King 1986). Despite these limitations, we believe that the method can be used to compare activities at different sampling sites (Fig. 4) and to measure changes in activity with time. Also, the effects of various additions on the cellulolytic activities in sediment can be studied to characterize the enzymes present in sediments (Fig. 2 and Table 1). These measurements give valuable information about the microbial contributions to a particular ecosystem.

The method described here will be used in our laboratory to study extracellular enzymes activities and their roles in the initial steps of organic matter decomposition in the reed bed of Lake Gooimeer.

Acknowledgments

This work was supported by grants from the Netherlands Integrated Soil Research Programme and from the Institute for Inland Water Management and Waste Water Treatment. We thank A.J.B. Zehnder for his comments on the earlier versions of the manuscript.

Chapter 5

Patterns of extracellular enzyme activities in littoral sediments of Lake Gooimeer, The Netherlands

H.T.S. Boschker and T.E. Cappenberg

Submitted to FEMS Microbiol. Ecol.

Abstract. Seasonal variation in enzymatic activities against protein and several polysaccharides together with general hydrolytic activity was studied in a gradient of sediments through the littoral zone of Lake Gooimeer, The Netherlands. Absolute activities were determined by organic matter input or content, and β-glucosidase activities were among the highest ever detected in sediments. Patterns of activities normalized to general hydrolytic activity were different between inside a reed bed and outside the bed, which was related to the polymeric composition of the dominant carbon sources at these sites. This suggests a new approach to study available sources of organic matter in natural systems by measuring relative enzyme activities. Exo-glucanase activities, as measured with methylumbelliferyl-cellobiose in combination with gluconolactone as an inhibitor of β-glucosidases, showed the highest discrimination between carbon sources in the studied system.

Introduction

The major input of organic matter to sediments of lakes is macrophyte or algal derived material. This material is mainly polymeric and needs to be hydrolysed extracellularly by enzymes to smaller units before it can be taken up by micro-organisms (Billen 1982, King 1986). Since there are differences in the polymeric composition of organic matter produced by the major groups of primary producers, it is likely that different sets of extracellular enzymes are produced by the microbial populations that degrade these materials. We recently have shown that there is a gradient in sedimentary carbon sources in the littoral zone of Lake Gooimeer, with macrophyte derived material dominating inside a reed bed, and algal or cyanobacterial sources outside the bed (Boschker et al. 1995b). Macrophyte litters are mainly made of cellulose, hemicelluloses like xylan, and lignin and are low in protein. Algae and cyanobacteria, however, have a far higher protein content and a lower polysaccharide content, with a different composition than plant litters (Cowie & Hedges 1984a). In addition to the composition of the sediment organic matter, enzyme activities in sediments are probably also affected by inputs of organic matter to the sediments (Boetius & Lochte 1994; Meyer-Reil 1986b).

In the present work, we have studied seasonal variation of number enzymatic activities in a gradient of sediments through the littoral zone of Lake Gooimeer. Sampling points choosen in this gradient showed differences in both organic matter content and source. Enzymatic activities against protein and several polysaccharides were measured with fluorogenic, methylumbelliferyl-labelled substrates (King 1986; Meyer-Reil 1986a; Boschker & Cappenberg 1994). Results show that there are indeed major differences in patterns of enzymatic activities between sites, which can be explained well by the polymeric composition of the dominant carbon sources. Absolute activities were mostly related to sedimentary organic matter content or input.

Materials and Methods

Sampling site. Lake Gooimeer is very shallow (mean depth of 3.6 m) with a sandy sediment and is situated in the centre of the Netherlands (Boschker et al. 1995b). On its southern shore is an approximately 80 m wide reed bed with common reed (*Phragmites australis* (Cav.) Trin. ex Steudel) as the dominant plant species. Inside the bed organic matter (OM) is accumulating. The OM content of the sediment increases from <1% at the lake side of the bed to 30% on the land side.

Sediments were sampled 20 m outside the reed bed, and 10 and 20 m inside the bed. From spring 1991 to spring 1993 cores were taken by drilling a polycarbonate cylinder (7 cm

internal diameter) with a sharpened cutting edge into the sediment by hand. In the laboratory, the top centimetre was collected and homogenised in a Warring blender. Sediments were slurried because of the presence of course reed litter fragments, which otherwise could not be included in the assay. Living plant parts were removed as far as possible before homogenisation.

The dry matter (DM) content of sediment was measured after drying overnight at 105 °C and organic matter (OM) content was determined as the subsequent loss of weight after 4 hours at 550 °C.

Extracellular enzyme assays. The following substrates (Sigma) were used to determine extracellular enzyme activities: MUF-β-glucose (β-glucosidase), MUF-α-glucose (α-glucosidase), MUF-cellobiose with gluconolactone as inhibitor of β-glucosidases (exo-glucanase), MUF-xylose (β-xylosidase), MUF-mannose (mannosidase), MUF-guanidine (endo-peptidase) and MUF-butyrate (esterase). Substrates were added to a final concentration of 3.3 mM, except for MUF-butyrate (0.3 mM). All activities were substrate saturated at the concentrations used. Assays were conducted according to Boschker and Cappenberg (1994). In short, 1 ml substrate was added to 0.1 to 0.3 g of wet homogenised sediment. After incubation at 20°C, 4 ml of ethanol was added to extract the produced methylumbelliferyl and sediment was removed by centrifugation. Incubation time (5 min to 2 h) was varied between substrates and sediment source to produce a well measurable fluorescence signal as measured on an Amico-Bowman spectrafluorometer. In the final step of this procedure, a pH 11 buffer is added to stop enzymatic activities and to enhance MUF fluorescence. Unfortunately, MUF-guanidine and MUF-butyrate were not stable at this high pH. Therefore a pH 7.5 phosphorus buffer was used for these substrates and samples were kept on ice till fluorescence was measured.

Addition experiments. Effects of the addition of different organic substrates on enzyme activities were studied for sediments sampled outside the reed bed. Cores (internal diameter 5 cm) were taken from outside the reed bed on 18 October 1993. The following substrates were mixed through the top centimetre of replicate cores: milled reed litter (< 2 mm, 0.45 g OM), lake sampled seston (0.1 g OM), cellulose (0.5 g OM, Hachery Nagel & Co. MN300) and casein (0.5 g OM, Merck) and none (Control). Organic matter content in the top-layer of the sediment was increased 2 (seston) to 5 times (casein) by the additions. Cores were incubated in the dark at 15 °C for 0, 10 and 25 days. Seston was collected from 100 l of lake water by continues centrifugation and consisted mainly of the cyanobacterium *Oscillatoria agardhii* Gom.. All additions were dried at 105 °C before use, which also destroyed all enzymatic activities associated with the additions. To characterise the organic additions, carbohydrate composition was measured after acid hydrolysis by HPLC in combination with electro-chemical detection (Boschker et al. 1995b) and carbon to nitrogen ratios were determined on a Carlo Erba elemental analyser (model 1106).

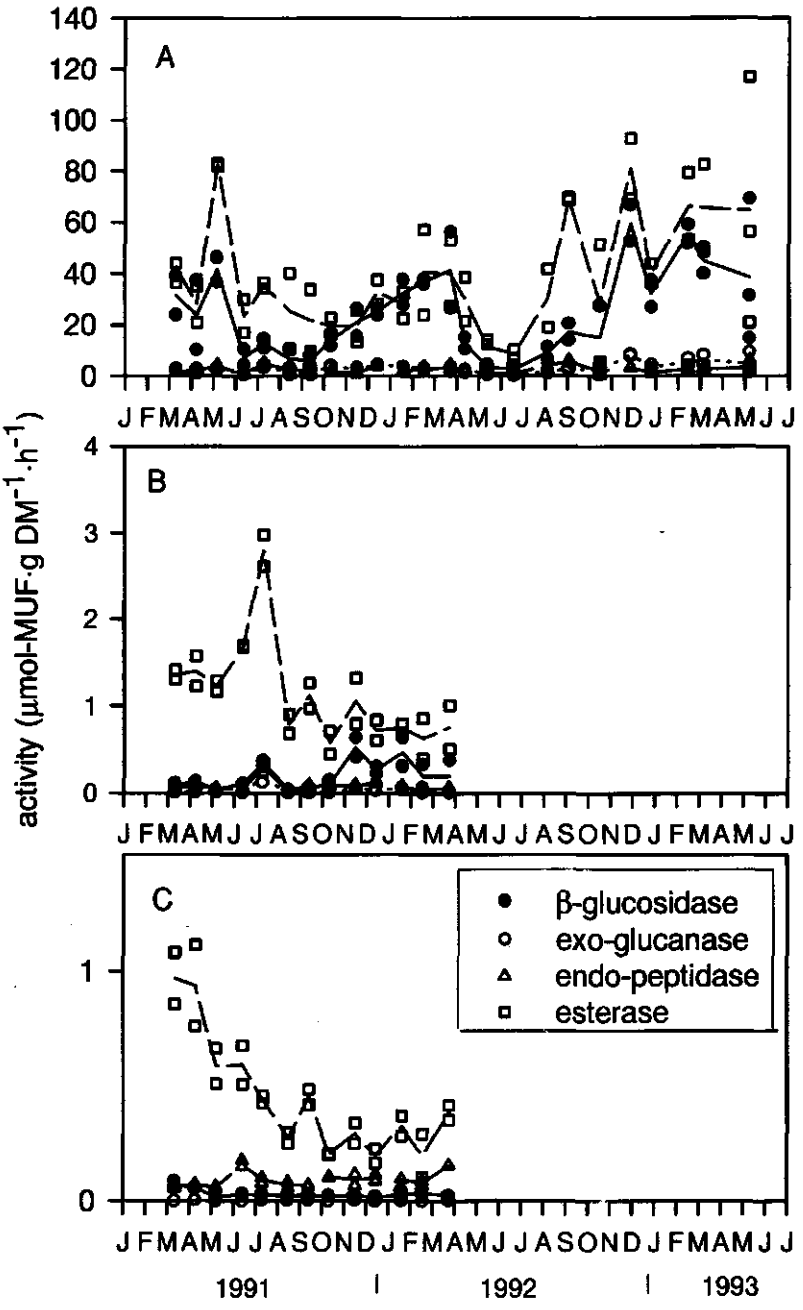

Figure 1. Seasonal variation in extracellular enzyme activities in sediments (0-1 cm) at 20 m (A) and 10 m inside (B), and 20 m outside (C) the reed bed of Lake Gooimeer. Note differences in scale.

Table 1: Composition of organic substrates used in the addition experiment

Substrate	C/N	% of OM*				
		Glucose	Xylose	Arabinose	Galactose	Mannose
Reed litter	80	29.4	19.8	3.5	1.4	0.2
Seston	6.2	8.6	0.7	nd	1.5	3.3
Cellulose	nd	79.6	2.2	nd	nd	0.5
Casein	3.6	0.3	nd	nd	1.9	0.2

*: Carbohydrate data were not corrected for possible losses during acid hydrolysis.
nd: not detected. No C/N ratio for cellulose is given because no N was detected.

Results

Seasonal variation was studied for β-glucosidase, exo-glucanase, endo-peptidase and esterase activities (Fig. 1). The highest enzyme activities were detected for the high organic matter site at 20 m inside the bed (Fig. 1A). At this site, all four enzyme activities varied consistently during both years, with higher activities in winter and lower in summer. The other two sites were followed only during the first year. Changes in activities at 10 m inside the bed occurred during two events (Fig. 1B). In July there was a sudden rise in all four activities and in winter the activities associated with cellulose decomposition were clearly higher, especially β-glucosidase. The sediment outside the bed showed low activities with little variation (Fig. 1C). Only esterase activity was higher in the first months of the study.

Activity of esterases was almost always higher than of other enzymes (Fig. 1), which was expected since esterase activity as measured with MUF-butyrate represents a non-specific hydrolase activity that includes other more specific activities analysed (Jones and Lock, 1989; Meyer-Reil and Köster, 1992). The second highest activity was different between sites. β-Glucosidase activity was second highest at both sites inside the bed, whereas endo-peptidase activity was second outside the bed.

To further elucidate this difference in activities between sites, several additional substrates were used on two dates (see Fig. 2) and relative activities were calculated by dividing the more specific activities by the general esterase activity. Patterns of relative activities were different between samples from 20 m inside the bed and outside the bed (Fig. 2). Inside the bed, activities associated with cellulose decomposition (β-glucosidase and exo-glucanase) and xylan decomposition (β-xylosidase), and mannosidase activity were relatively more important than outside the bed. Protein related activities (endo-peptidase) were more important outside the bed. The sediment sampled at 10 m inside the bed showed an intermediate pattern of relative activities. Relative activity of α-glucosidase was less effected by sampling site.

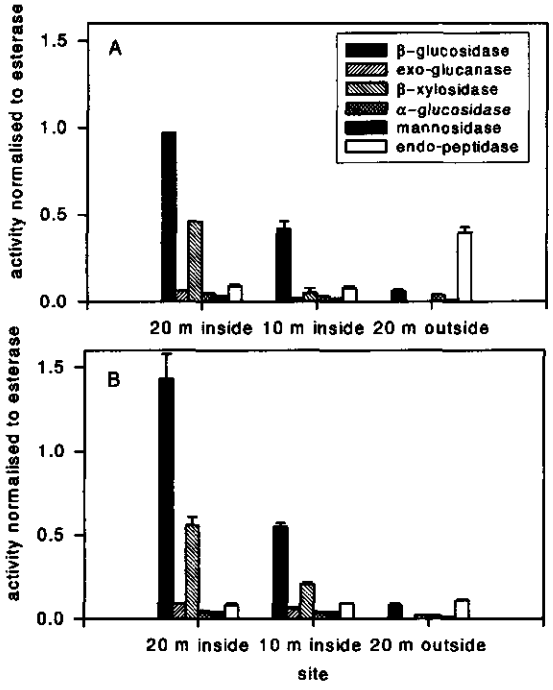

Figure 2. Enzyme activities relative to esterase (MUF-butyrate) at three sediment sites in the littoral of Lake Gooimeer sampled on 17 March 1992 (A) and on 11 November 1993 (B).

Addition of organic substrates with different compositions (Table 1) had a pronounced effect on enzyme activities in sediments sampled outside the reed bed. Absolute activities increased (data not shown) and patterns of relative activities were also affected depending on the addition (Fig. 3). Addition of reed litter or cellulose caused an increase particularly in relative exo-glucanase activities and to a lesser extent in relative β-glucosidase and β-xylosidase activities. These changes resulted in a shift to a pattern as for sediments sampled deep inside the bed. In the reed litter addition, the relative activities of exo-glucanases and β-xylosidases still increased between 10 and 25 days of incubation. Patterns of relative activities remained rather similar after the addition of seston, and the same applied for the control incubation. Addition of protein (casein) resulted in a decrease in polysaccharases and ,but only after 10 days of incubation, an increase in relative endo-peptidase activity. Organic matter contents at the end of the experiment suggested that almost all casein and approximately 50 % seston had been degraded, whereas most of the added reed litter and cellulose were not.

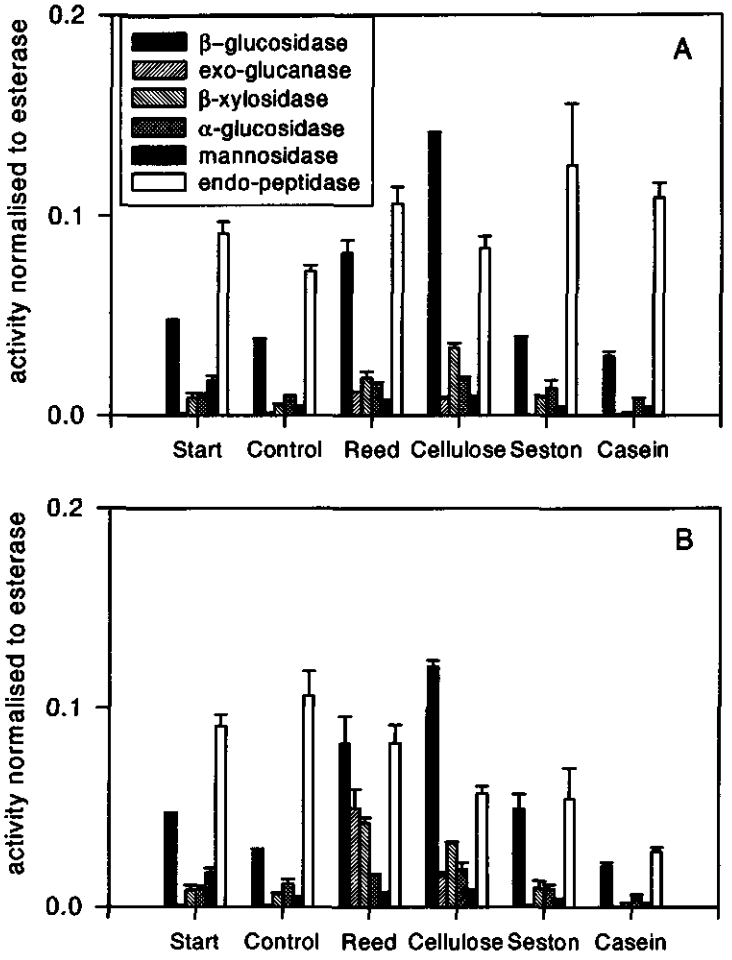

Figure 3. Effect of addition of different organic substrates on extracellular enzyme activities relative to esterase activity after 10 days (A) and 25 days (B) of incubation.

Discussion

There was a positive correlation between absolute enzyme activities and organic matter as activities were higher inside the reed bed than outside. Increased activities inside the bed during winter coincided with the input of fresh reed litter in autumn and its removal during storms in spring (Chapter 3). This relationship between enzymatic activities and organic matter contents or input, can be extended further as β-glucosidase activities have been studied in a wide range of sediments using different substrates and substrate concentrations. Although the methodological differences make a direct comparison in many cases difficult, β-

Table 2. Comparison of β-glucosidase activities in top-layers of aquatic sediments

Sediment type	Substrate (concentration)*	Activity µmol/g DM/h	Ref.
Freshwater littoral	MUF-gluc (S)		This study
20 m Inside bed		1 - 70	
10 m inside bed		0.1 - 0.8	
20 m outside bed		0.008 - 0.09	
Freshwater estuary (Hudson river)	PNP-gluc (S)		Sinsabaugh & Findlay (1995)
Typha marsh		7.5, 11.8	
Subtidal *Trapa* site		4.4	
Creek		1.3	
Shoal		0.2, 0.46	
Boreal River (benthic organic matter, mainly macrophyte litter)	PNP-gluc (S)	15 - 30	Sinsabaugh & Linkins (1990)
River (sand)	MUF-gluc (S)	0.02 - 0.05[1]	Marxsen & Fiebig (1993)
Tropical lagoon	PNP-gluc (S)	0.06 - 0.16	Hoppe et al. (1983)
Intertidal mud flat	MUF-gluc (NS)	0.03[2]	King (1986)
Shallow brackish (Kiel Bight)	MUF-gluc (NS)	0.01[2]	Meyer-Reil (1986a)
Continental slope (135 - 1680 m)	MUF-gluc (S)	0.0001- 0.0009[2]	Poremba & Hoppe (1995)
Deep sea (2900 - 4600 m)	MUF-gluc (S)	0.0001[2]	Poremba (1995)
Deep sea (4500 m depth)	MUF-gluc (S)	0.0001[2]	Boetius (1994)

*: MUF-gluc (methylumbelliferyl-β-glucose) or PNP-gluc (*p*-nitrophenyl-β-glucose) used at a saturating (S) or non-saturating (NS) concentration.

[1,2]: calculated from original data (on volume basis) using: 1) specific weight 2 g/ml and DM 80%, 2) specific weight 1.5 g/ml and DM 50%.

glucosidase activities (Table 2) show a positive correlation with expected organic matter sedimentation and mineralisation rates for the studied systems (Canfield 1993; Smith & Hollibaugh 1993; Middelburg et al. in press). Our activities inside the bed are among the highest reported, which is probably caused by a high primary production of cellulose rich litters in this system (Bacic et al. 1988; Wetzel 1992). Similar activities were only found in sediments that were dominated by the input of macrophyte derived litters as well (Table 2).

Besides a predominant effect of the amount of sedimentary organic matter loading on enzymatic activities, there was also an secondary effect caused by the biopolymer composition of the dominant carbon source for the sediments. An interesting finding of this study is that patterns of enzyme activities were very different between inside and outside the reed bed. This

difference was most likely caused by a change in dominant available carbon sources. In a recent study, we have shown that inside the bed macrophyte derived material dominated the sedimentary organic matter (Boschker et al. 1995b). Since macrophyte litter consists for a large part of polysaccharides, this would explain our high relative activities for enzymes involved in cellulose and xylan decomposition. Likewise, algal or cyanobacterial carbon sources have been found to dominate the sediment organic matter outside the bed (Boschker et al. 1995b), which explains the relatively high proteinase activities at this site since algae generally contain more protein than macrophyte litters (see C/N ratios in Table 1). This was confirmed by the experiment where organic substrates were added to sediment sampled outside the bed. Addition of reed litter or cellulose resulted in a shift in enzyme pattern to polysaccharide degrading activities, whereas addition of seston had little effect (Fig. 3).

These relationships between enzyme actvities and the polymeric composition of the organic matter sources, suggest that patterns of enzyme activities relative to esterases can be used to study sources of organic matter available in environmental samples. Most methods presently used to study sources of organic matter in ecosystems are based on chemical parameters like lipid biomarkers, carbohydrate compositions and lignin oxidation products (Cowie & Hedges 1984a; Meyers & Ishiwatara 1993). Also, stable isotope data have been widely used to discern carbon sources (Fry and Sherr (1984)). The chemical methods have the disadvantage that they show only absolute contributions. Patterns of relative enzyme activities most likely point to sources of organic matter that are available for micro-organisms in a system and might therefore give more relevant information on the biological functioning of the system. Relative exo-glucanase activities showed the largest differences between marcophyte and algal dominated sediments and should therefore be most useful in detecting sources of available carbon in ecosystems.

It is also interesting to notice that addition of cellulose caused an increase in relative β-xylosidase activity (Fig. 3), although the cellulose used contained only little xylose (Table 1). β-Xylosidases are a part of the enzyme system that is involved in the decomposition of xylan (Biely 1993) and this result therefore suggests that regulation of xylan and cellulose decomposition are closely linked in natural systems. This does make sense since cellulose and xylan mostly do occur together in litters of macrophytes (Bacic et al. 1988). It has been shown that in a number of bacterial isolates xylanase activity is induced by intermediates formed during cellulose decomposition or by cellulose itself (Forsberg et al. 1981; Biely 1993; Khanna & Gauri 1993).

In conclusion, our results suggest i) that absolute activities are mostly related to amounts of organic matter input and ii) that patterns of activities can be related to the composition of the dominant sources of organic matter. This last conclusion suggests that these patterns of enzymatic activities could be used to study sources of available organic matter in natural systems. It should be noticed though that in the system we studied mainly macrophyte and alga or cyanobacteria derived organic matter was found. Different sources of organic matter

may dominate in other systems and additional enzyme activities may have to be studied to differentiate between these sources.

Acknowledgements

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Chapter 6

An Inhibitor-Based Method to Measure Initial Decomposition of Naturally Occurring Polysaccharides in Sediments

H.T.S. Boschker, S.A. Bertilsson,* E.M.J. Dekkers, and T.E. Cappenberg

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Abstract. A method that can be used to measure the initial decomposition rates of polysaccharides in sediment samples was developed. It uses toluene to specifically inhibit microbial uptake of carbohydrates without affecting extracellular hydrolysis of polysaccharides. Accumulating carbohydrates were determined by high-performance liquid chromatography. Field-sampled litter from common reed (*Phragmites australis*), which contains cellulose and arabinoxylan as its main polysaccharides, was used as a model system. Toluene concentrations of between 1 and 10% resulted in the accumulation of similar amounts of monomeric carbohydrates, which was linear over time for most neutral sugars. Toluene (3%) did not have an effect on extracellular enzyme activities, and microbial sugar uptake was completely inhibited, as demonstrated with ^{14}C-labelled xylose and glucose. Experiments with enhancement cultures and fixed reed litter suggested that enzymatic hydrolysis of polysaccharides in reed litter was the main source of glucose, xylose, arabinose, and galactose accumulation. In contrast, the accumulation of high amounts of the alditols mannitol and glucitol was probably caused by lysis of the microbial population in toluene-treated reed litter. Glucose accumulated at rates of 1.3 and 0.10 μmol g·dry matter content^{-1}·h^{-1} under aerobic and anaerobic conditions, respectively, whereas xylose accumulation rates were only 10% of the glucose accumulation rates.

* Present address: Department of Water and Environmental Studies, Linköping University, S58183 Linköping, Sweden

Introduction

Polysaccharides, like cellulose and hemicelluloses, represent a major part of the organic material in aquatic environments (DeBlois & Wiegel 1990; Ljungdahl & Eriksson 1985). The initial step in the decomposition of these polymers to smaller intermediates is catalyzed by enzymes secreted by microorganisms and is generally regarded as the rate-limiting step in the microbial mineralization of organic matter (Benner et al. 1984a; Billen 1982; King 1986). Although large amounts of data exist on the processes and factors that govern the decomposition of bulk sediment organic matter, information on the decomposition of specific polymers under natural conditions is still lacking (Henrichs 1992; Webster & Benfield 1986).

Several approaches have been used to study the initial decomposition of polymeric material. The activities of extracellular enzymes in environmental samples are often assessed by adding an artificial substrate at a saturating concentration (Boschker & Cappenberg 1994; Burns 1978; King 1986; Sinsabaugh et al. 1991). The measured enzyme activity should be regarded as a potential value; the actual concentration of the natural substrate, its availability to the enzymes, or its interactions with other polymers in the sediment organic matter are not incorporated. Some of these problems can be circumvented by using specifically radiolabelled polymers (Benner et al. 1984a; Cunningham & Wetzel 1989; Schink & Zeikus 1982) or by following changes in the composition of the sedimentary matter itself (Benner et al. 1990; Wilson et al. 1986b). Another approach is the use of radiolabelled compounds to study the turnover of the free pool of intermediates that are formed during initial decomposition (Gocke et al. 1981; King & Klug 1982; Sawyer & King 1993). Problems with this last approach are that (i) not all intermediates will be readily available in radiolabelled form, and (ii) the free bioavailable concentration of the intermediates, which is also needed, might be hard to assess (Dawson & Liebezeit 1981; Gocke et al. 1981; Henrichs 1992).

In the study described here, a new approach to measuring the initial decomposition rates of naturally occurring polysaccharides in plant litter and sediment is presented. The method is based on the selective inhibition of microbial carbohydrate uptake by toluene, without affecting the extracellular hydrolysis of polysaccharides in the sample under study. The accumulation of hydrolysis products was followed over time by high-performance liquid chromatography (HPLC). This resulted in a sensitive method with a high degree of resolution for the products formed. Litter from common reed (*Phragmites australis* (Cav.) Trin. ex Steudel) was used as a model system to test the method. This material is the main carbon source for the sediment in the ecosystem studied (Boschker et al. 1995b) and could be collected year round, which facilitated testing. Like litter from

most grasses (Bacic et al. 1988), reed litter is mainly composed of cellulose, arabinoxylan, and lignin.

In this report, we show that (i) neutral sugars, which form the building blocks of the polysaccharides in reed litter, accumulated linearly over time, (ii) toluene effectively inhibited the uptake of glucose and xylose, (iii) the activities of the extracellular enzymes involved in the degradation of cellulose and xylan were not significantly affected by toluene, (iv) disturbance by leakage of neutral sugars from the microbial cytoplasm was negligible, and (v) the unexpected accumulation of alditols was probably due to lysis of the microorganisms in reed litter and not to the initial decomposition of polysaccharides. In a subsequent report (Chapter 7) we will describe the results of rate measurements on sediment cores, compare the carbohydrate accumulation rates with mineralization rates, and discuss the relative rates of accumulation of the different carbohydrates produced, including oligomers.

Materials and Methods

Reed litter sampling and incubations. The sampling site was situated in an extensive reed bed on the southern border of Lake Gooimeer, The Netherlands (Boschker et al. 1995b; De Haan et al. 1993). Litter, mainly derived from common reed, was collected by hand from the top centimeter of the sediment on several occasions during 1993. The material was immediately cut into 1-cm pieces and was stored at room temperature (20 to 23°C) under aerobic or anaerobic (100 % N_2) conditions. Litter for the anaerobic experiments was kept under water during sampling, and all further handling was conducted in an anaerobic glove box filled with N_2 and 1.5% H_2 (Coy Laboratory Products Inc.). The authors are aware that this litter sampling procedure might have influenced polysaccharide decomposition rates. The primary goal, however, was to test the method, which can be done best by using batches of similarly treated litter. Dry matter content (DM) was measured after drying the litter at 104 °C to a constant weight. Litter in both aerobic and anaerobic experiments contained 17% DM, on average, with little variation during the year.

Incubations were carried out at room temperature (20 to 23 °C) in slowly shaken 250- or 500-ml serum bottles. The bottles were sealed with butyl rubber stoppers fitted with a butyl rubber septum through which toluene additions (*Pro Analysi*, Merck) were made and samples for carbohydrate analysis (5 ml, see below) were taken. Between 3 and 10 g (wet weight) of litter was incubated in 50 or 100 ml of artificial lake water containing similar amounts of major ions as Lake Gooimeer water (2.1 mM HCO_3^-, 1.4 mM SO_4^{2-}, 3.7 mM Cl^-, 0.6 mM Mg^{2+}, 1.7 mM Ca^{2+}, 3.6 mM Na^+, 0.3 mM K^+). Aerobic incubation bottles were preincubated for 0.5 h and anaerobic bottles were preincubated for 1 week

before toluene addition. Bottles were intensively shaken directly after the addition of toluene (always given as % [vol/vol]) to accomplish an even distribution of the inhibitor.

Effects of toluene on microbial monomeric sugar uptake. The uptake of glucose and xylose under both aerobic and anaerobic conditions was studied with [U-^{14}C]glucose (10.8 GBq·mmol^{-1}, Amersham) and [U-^{14}C]xylose (2.74 GBq·mmol^{-1}, Amersham) in separate experiments. Toluene was added to the incubation bottles to final concentrations of 0 and 3%. The incubation bottles with 3% toluene were preincubated for either 15 or 90 min before tracer addition. A control incubation bottle was preincubated for 15 min with 3% toluene and 4% glutaraldehyde before tracer addition to determine the possible adsorption of the labelled sugar to reed litter.

Labelled sugars were added to shaking incubation bottles to a final activity of 2.2 kBq·cm^{-3}. Liquid samples of 1.5 ml were taken at distinct time intervals, immediately diluted in 1 ml of glutaraldehyde (final concentration, 4%) to stop all biological activity, and centrifuged (5,400 x *g*, 20 min, 19 °C) to pelletize the cells and particles. Two ml of the supernatant was drawn over an ion-exchange column containing 2 cm^3 of Dowex-1 anion exchange resin (0.2 to 0.4 mm) in the fluoride form to remove labelled CO_2, negatively charged or acidic end products, and intermediates (Mopper et al. 1992). The sample was then diluted with water purified in a Millipore-Q system (Milli-Q) to a volume of 5 ml, and the mixture was transferred to a scintillation vial. The activity in the sample was determined with a Packard Tri-Carb 4530 liquid scintillation counter with dual photomultipliers after the addition of 10 ml of Instagel II scintillation cocktail (Packard). The counts were corrected for background, losses in the ion exchange column, and quenching by the external standards, sample-channels ratio method.

Effects of toluene on extracellular enzyme activities. The effects of toluene on exoenzyme activities were studied under both aerobic and anaerobic conditions by comparing the activities with and without the addition of 3% toluene for the most important enzymes involved in the degradation of cellulose and xylan. Methylumbelliferyl-β-D-cellobiopyranoside (MUF-cel) was used to measure the combined activities of exoglucanases and most β-glucosidases (Boschker & Cappenberg 1994); β-xylosidases were assayed with methylumbelliferyl-β-xylopyranoside (MUF-xyl). After the addition of substrate to a final concentration of 0.4 mM, liquid samples (1 ml) were taken at distinct time intervals and were analyzed for methylumbelliferyl (MUF) content as described by Boschker and Cappenberg (1994). To correct for MUF adsorption on litter fragments, a separate recovery experiment was performed by adding 0.4 mM MUF to the incubation bottles.

Endoglucanase activity was measured as the rate of decrease in the viscosity of 1.5% carboxymethyl cellulose (CMC; medium viscosity, Sigma) (Almin & Eriksson 1967; Hulme 1988). Samples (5 ml) were centrifuged at 4,500 x *g* for 10 min to remove particles. The viscosity of the supernatant was determined by fall velocity in a small-bore

glass tube (diameter, 1.7 mm; length, 20 cm). Activity was calculated as the decrease in specific viscosity over time.

Endoxylanase activity was determined by using Remazol Brilliant Blue xylan (RBB-xylan), which was synthesized from oat-spelt xylan (Sigma) as described by Biely et al. (Biely et al. 1988). The incubation mixture contained 10 g of reed litter, 100 ml of 5 $g \cdot l^{-1}$ RBB-xylan and 1% Triton X-100. At distinct time intervals, 2 ml of the incubation mixture was combined with 4 ml of 96% ethanol to precipitate the unreacted RBB-xylan. Toluene (3%) was added together with the ethanol to samples from the control incubations, since toluene seemed to influence the precipitation of RBB-xylan. After standing at room temperature for 30 min, the precipitated RBB-xylan was removed by centrifugation (2,000 x *g*, 5 min, 20 °C). The concentration of RBB-dyed xylan fragments in the supernatant was measured spectrophotometrically at 595 nm. Endoxylanase activity was calculated as the amount of RBB produced over time.

Effects of toluene on microbial carbohydrate leakage. The possible release of carbohydrates from the cytoplasm of microorganisms caused by the toluene treatment was investigated in two experiments.

In the first experiment, enrichment cultures were prepared on a mixture of glucose and xylose under both aerobic and anaerobic conditions. A small reed litter fragment was used to inoculate 1 l of artificial lake water enriched with 5 mM (each) glucose and xylose and 0.01% yeast extract (Oxoid). Glucose and xylose were chosen as substrates, since they form the major building blocks of the polysaccharides found in reed litter (see Results). Both incubations were carried out under gentle shaking at 20 °C. After a 7-day incubation, cultures still had a low cell density. Therefore, extra glucose, xylose, and yeast extract were added to the final concentratons given above, and incubations were harvested 2 days later. At that time, the cells were microscopically found to be dividing. Liquid subsamples (200 ml) containing as little reed material as possible were taken and centrifuged at 6,000 x *g* for 20 min (4°C). The pellet was washed two times with 100 ml of artificial lake water. Microbial biomass was determined by first drying subsamples of the washed enrichment cultures at 104 °C to a constant weight and subsequently ashing them at 450 °C for 3 h.

The washed cultures were preincubated for 15 min before toluene (0 and 3%) was added. Samples were collected immediately before toluene addition for use as blanks and 1 h after toluene addition. Samples were analyzed for carbohydrates as described below.

To be able to compare sugar releases from enrichment cultures with releases from reed litter, the numbers of bacteria on reed litter were determined by fluorescent microscopy after staining with 4,6-diamidino-2-phenylindole (DAPI) (Starink et al. 1994). Bacterial numbers were converted to biomass by using a mean bacterial biovolume of 0.1 μm^3 (Starink et al. 1996), a carbon content of 2.2×10^{-13} g $C \cdot \mu m^{-3}$ (Bratbak & Dundas 1984), and the assumption that 50% of the organic matter in bacteria is carbon.

In the second experiment, the effects of enzymatic hydrolysis of polysaccharides and lysis of cells were separated by inhibiting enzyme activities with glutaraldehyde. Incubation bottles with reed litter were fixed with 2% glutaraldehyde and received two treatments: (i) addition of toluene (3%), after which sugar release was monitored over time, and (ii) mechanical disruption of the microorganisms in reed litter by sonication to determine the intracellular carbohydrate content. For the sonication treatment, 10 g of wet reed litter in 100 ml of artificial lake water was homogenized in a Waring blender for 30 s. A 10 ml subsample of the reed-water mixture was transferred to a scintillation vial (24 ml) and sonicated for 15 min in an MSE Soniprep 150 ultrasonic disintegrator (output power, 150 W) equipped with a miniprobe after the addition of sodium dodecyl sulfonate (SDS; final concentration, 0.01%) to facilitate disruption of the cells. The samples were cooled on ice during sonication. Samples were analyzed for carbohydrates as described below, except that filtration was carried out over Whatmann GF/F glass fiber filters because the normally used polycarbonate filters kept clogging. The efficiency of sonication in the disruption of microorganisms was tested by counting the numbers of bacteria before and after sonication (Starink et al. 1994). Sonication decreased the bacterial numbers by at least 96%.

Carbohydrate sample treatment and analysis. Carbohydrate samples were added to disposable centrifugation tubes, and the tubes were centrifuged at 3,000 x *g* for 10 min at 19 °C. The supernatants were filtered (0.2-μm-pore-size polycarbonate filter, standard surface, Poretics) under pressure and were washed under suction through ion-exchange columns to desalt the samples and remove humic substances (Mopper et al. 1992). The columns were filled with a mixture of 2 cm^3 of AG 50W-X8 (hydrogen form, 50-100 mesh, Bio-Rad) cation exchange resins and 2 cm^3 of AG 2W-X8 (carbonate form, 20-50 mesh, Bio-Rrad) anion exchange resins. All equipment that would be in direct contact with the samples was washed several times in Milli-Q and was dried overnight at 95 °C before use.

Carbohydrate concentrations were analyzed by using a Dionex 2000i/SP HPLC system (Dionex Corporation). Samples were injected into the system with a Marathon autosampler (Spark-Holland) with a loop size of 200 μl. Two analytical setups were used. First, monomeric neutral sugars were analyzed on a Carbopac PA1 (Dionex Corporation) anion-exchange column with Milli Q as the eluent at a flow rate of 1 $ml \cdot min^{-1}$. Every second day of analysis, the column was preconditioned for 1 h with 100 mM NaOH plus 25 mM sodium acetate in Milli-Q to shorten the retention times. To optimize detection, a postcolumn addition of 1.6 M NaOH in Milli-Q (0.3 ml min^{-1}) was made by using an AMMS II anion micromembrane suppressor (Haginaka et al. 1989). Second, alditols were separated on a CarboPac MA 1 analytical column (Dionex) with an eluent containing 480 mM NaOH in Milli-Q at a flow rate of 0.5 $ml \cdot min^{-1}$. The second analytical setup also gave a confirmation of the identities and concentrations of monomeric neutral sugars. Both

Table 1. Effects of toluene concentration on carbohydrate accumulation rate in reed litter

Condition	Toluene (%)	Activity[a] (μmol·g DM^{-1}·h^{-1})	
		Glucose	Xylose
Aerobic	0	ND[b]	ND
	1	1.21 ±0.18	0.060±0.004
	3	1.27 ±0.16	0.055±0.001
	10	1.33 ±0.14	0.066±0.004
Anaerobic	0	ND	ND
	1	0.049±0.004	0.022±0.001
	3	0.052±0.003	0.021±0.002
	10	0.052±0.008	0.025±0.002

[a] Values are means standaard ± deviations.

[b] ND, not detectable.

columns were used at a temperature of 35 °C. The detector was a Dionex PED in the integrated pulsed amperometric mode with waveforms optimized as described by La Course and Johnson (1991). External standards were used to calculate concentrations. Corrections were made for sugar recovery in the ion-exchange column step, for volume changes due to sampling, and for blank concentrations. The detection limits for the different carbohydrates ranged from 5 to 50 nM.

The carbohydrate composition of reed litter was determined after sulfuric acid hydrolysis as described by Cowie and Hedges (1984b).

Results

Carbohydrate composition of reed litter. Acid hydrolysates of reed litter contained 33% (wt/wt) glucose, 18% xylose, 3% arabinose, and 2% galactose, with minor amounts of mannose, rhamnose, and ribose. Under the assumption that, in grasses, glucose is mainly derived from cellulose and that xylose and arabinose are derived from arabinoxylan (Bacic et al. 1988), a cellulose-to-xylan ratio of approximately 1.6 was calculated. No alditols were detected, which might be due to their destruction under the hydrolysis conditions used.

Effect of toluene concentration. The accumulation rates of carbohydrates were similar for incubations with 1, 3, and 10% (vol/vol) toluene (Table 1). Therefore, 3% toluene was chosen for all further experiments. Without toluene, the carbohydrate

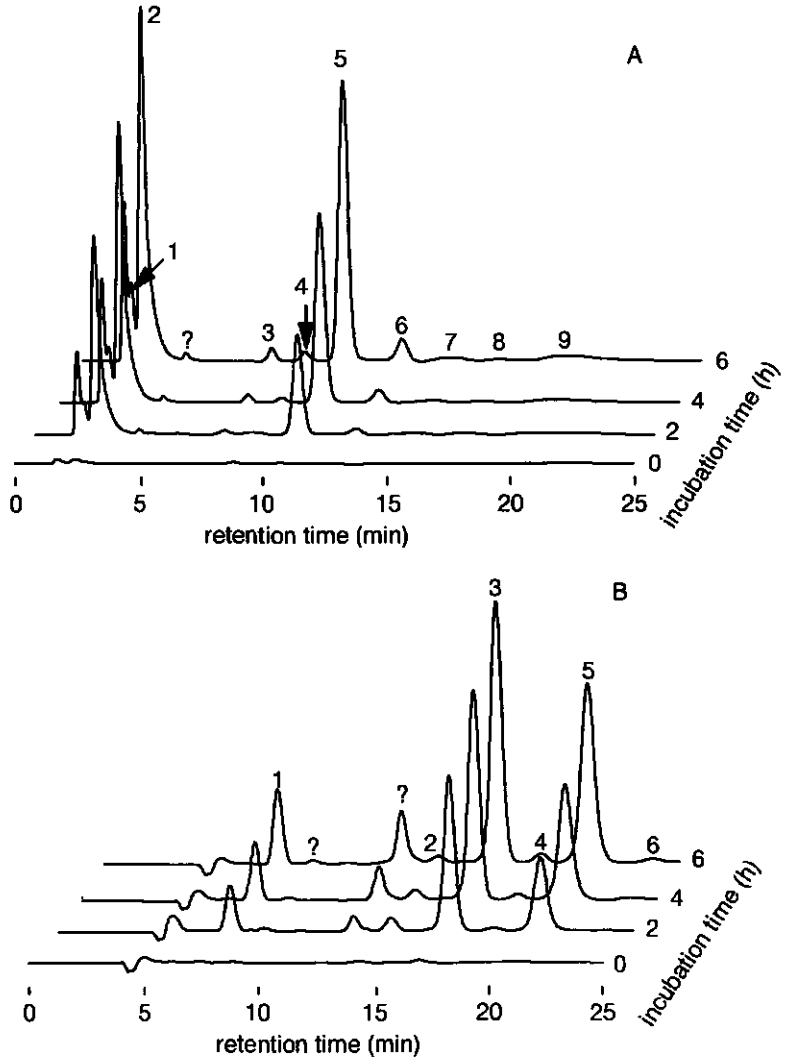

Figure 1. Example of carbohydrate accumulation over time in aerobically incubated reed litter in the presence of 3% toluene. Chromatograms of neutral sugar analysis on the CarboPac PA1 column (A) and alditol analysis on the CarboPac MA1 (B) are shown. Numbers show peak identification for the chromatograms after 6 h of incubation. Fig. A: 1 glycerol, 2 mannitol + glucitol, 3 arabinose, 4 galactose, 5 glucose, 6 xylose, 7 mannose, 8 fructose, 9 ribose. Fig. B: 1 glycerol, 2 glucitol, 3 mannitol, 4 arabinose, 5 glucose + xylose, 6 galactose. ? unidentified peak. The glucose and mannitol concentrations were 72 and 98 µM, respectively, after 6 h of incubation.

concentrations remained very low and showed no increase. Only glucose, xylose, and arabinose could be detected at maximum concentrations of 94, 84, and 6 nM, respectively, under aerobic conditions and at maximum concentrations of 32, 16, and 6 nM, respectively, under anaerobic conditions.

Accumulation of carbohydrates in the presence of 3% toluene. Example chromatograms of carbohydrate release from reed litter in the presence of 3% toluene under aerobic conditions are shown in Fig. 1. The neutral sugars glucose, xylose, arabinose, and galactose accumulated clearly over time (Fig. 1A). The amount of accumulation of mannose, fructose, and ribose was small. These chromatograms (Fig. 1A) also show an increase in several not-well-resolved peaks eluting shortly after the void volume of the column. On the basis of their retention times, these peaks might have been alditols or polyols. To investigate this possibility, we also analyzed the samples on a CarboPac MA1 analytical column, which is specifically designed for alditol separation (Fig. 1B). Mannitol, glycerol, and glucitol were identified as clearly accumulating compounds; in addition, their were two unidentified peaks. The neutral sugars, which were released from reed litter in the presence of toluene, were also the major carbohydrates in the reed litter hydrolysates. The high level of accumulation of glycerol, mannitol, and glucitol was not expected since they could not be detected in the sulfuric acid hydrolysates of reed litter. This suggests that alditols do not accumulate because of extracellular enzymatic hydrolysis of the polysaccharides in reed litter (see section below on the effect of toluene on microbial carbohydrate leakage).

The accumulation of neutral sugars was linear over time (Fig. 2). Glucose under anaerobic conditions (Fig. 2C) and xylose under aerobic conditions (Fig. 2D) showed a lag phase of about 1 h. Accumulation rates were calculated by regression analysis of the linear parts of the curves. The accumulation rate for glucose under aerobic conditions was 1.30±0.10 μmol $g \cdot DM^{-1} \cdot h^{-1}$, whereas it was 0.102±0.001 μmol·g $DM^{-1} \cdot h^{-1}$ under anaerobic conditions. The xylose accumulation rate under aerobic conditions was approximately 10% of the glucose accumulation rate, whereas both arabinose and galactose accumulation rates were 3.4 and 2.2% of this value. Accumulation rates for the three minor neutral sugars under anaerobic conditions (percentage of anaerobic glucose rate) were 9.4% for xylose, 5.6% for galactose, and 3.1% for arabinose.

Glycerol accumulated at a high rate during the first 2 h, after which the rate suddenly decreased (Fig. 2). Activities for glycerol based on the slow rate were 0.263±0.016 (aerobic) and 0.035±0.008 (anaerobic) μmol·g $DM^{-1} \cdot h^{-1}$. Mannitol was the dominant accumulation product under both aerobic and anaerobic conditions. The accumulation pattern of mannitol under aeroboc conditions was similar to that of glycerol. In other experiments under aerobic conditions, mannitol sometimes seemed to reach a plateau concentration or its accumulation rate decreased with time (e.g., see Fig. 4A). Under anaerobic conditions, mannitol showed a clear maximum plateau concentration after 4 h.

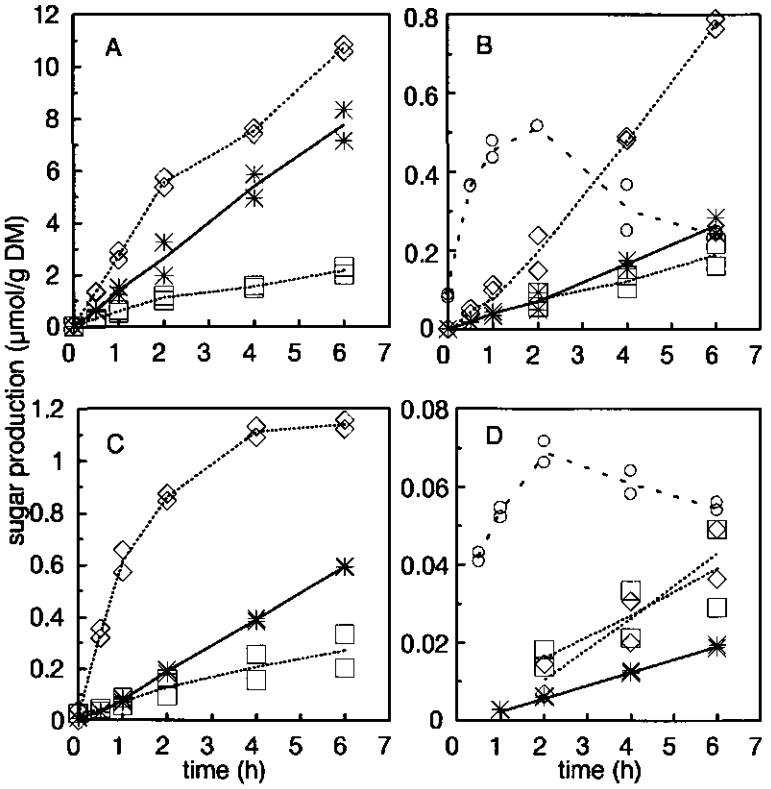

Figure 2. Carbohydrate accumulation in reed litter incubated under aerobic (A and B) and anaerobic conditions (C and D) in the presence of 3% toluene. Major compounds are shown in A and C (diamonds, mannitol; stars, glucose; squares, glycerol), and minor compounds are shown in B and D (circles, glucitol; stars, arabinose; squares, galactose; diamonds, xylose).

The behavior of the second unidentified peak (Fig. 1B) was similar to that of mannitol. Glucitol showed a maximum concentration after 2 h, after which the concentration decreased. This suggests that glucitol was not stable and reacted further to an unknown product or was metabolized by microorganisms not inhibited by toluene.

Effect of toluene on microbial monomeric sugar uptake. Toluene inhibited the uptake of radiolabelled glucose and xylose effectively under both aerobic and anaerobic conditions (Fig. 3). The inhibition of sugar uptake was similar for both the 15- and 90-min pretreatments with toluene, suggesting a rapid inhibition of microbial sugar uptake. Without toluene, glucose and xylose uptake was rapid and exponential during the first 10 min ($r^2 > -0.97$ for all experiments after linearization, $n = 8$). Glucose turnover was faster under aerobic conditions (turnover time, 2.7 min) than under anaerobic conditions (turnover time, 3.6 min). The same was found for xylose turnover times (5.3 min under

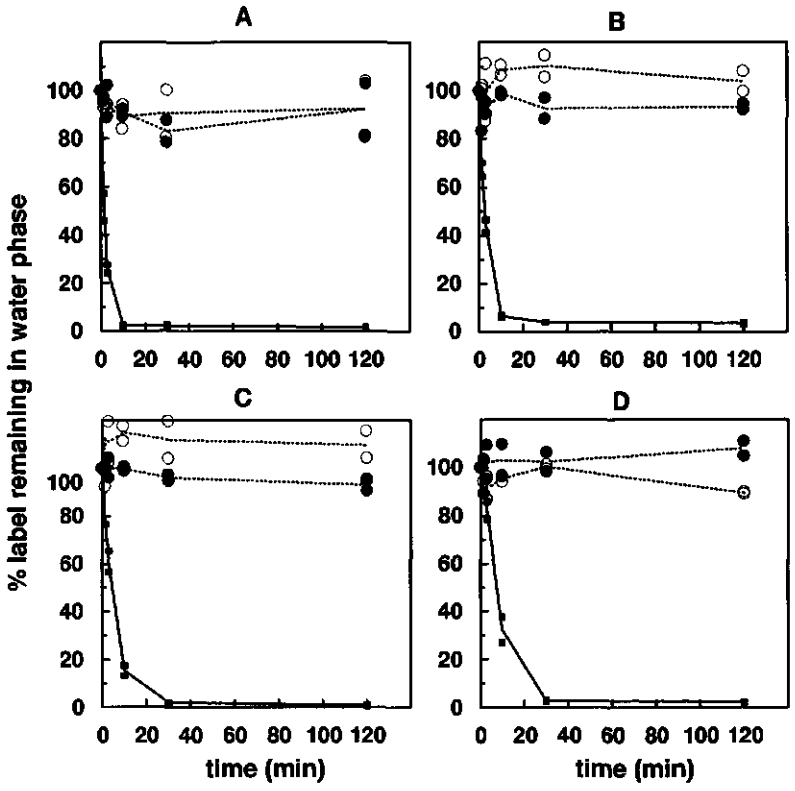

Figure 3. Effect of 3% toluene on aerobic (A) and anaerobic (B) glucose uptake and aerobic (C) and anaerobic (D) xylose uptake as determined with ^{14}C-labelled sugars. Shown are controls without toluene added (squares) and toluene additions with 15 (open cirlces) and 90 min (black circles) preincubation before labelled sugars were added.

aerobic conditions, 8.4 min under anaerobic conditions). No adsorption or uptake of labelled sugars could be detected in the reed litter incubations treated with 4% glutaraldehyde.

Effects of toluene on extracellular enzyme activities. Enzyme activities were similar in the incubations with and without toluene (Table 2). The effect of toluene was statistically significant for endoglucanases under aerobic conditions and for β-glucosidases plus exoglucanases and endoxylanases under anaerobic conditions. However, the relative effects were small, ranging from -5 to +20% between treatments.

Incubations with MUF-cel as the substrate and 3% toluene were also analyzed for sugar production. Glucose was mainly found. Cellobiose accumulation explained only 1.7% of the MUF production under aerobic conditions and 8% under anaerobic conditions, implying a rapid decomposition of the cellobiose unit of MUF-cel into two glucose molecules.

Table 2. Effect of 3% toluene on apparent activities in reed litter of extracellular enzymes involved in the decomposition of cellulose (glucan) and xylan

Substrate	Enzyme type	Condition	Activity ± SE	
			0% Toluene (activity[a])	3% Toluene (%[b])
MUF-cel	β-Glucosidase and exoglucanase	Aerobic	6.25±0.52	97.3± 9.0
		Anaerobic	8.95±0.25	95.1± 2.3*
CMC	Endoglucanase	Aerobic	3.17±0.18	120.7± 6.4*
		Anaerobic	3.43±0.23	100.0± 9.8
MUF-xyl	β-Xylosidase	Aerobic	7.45±0.34	95.5± 5.2
		Anaerobic	3.37±0.16	105.3± 5.6
RBB-xylan	Endoxylanase	Aerobic	1.44±0.05	104.9± 4.6
		Anaerobic	0.81±0.03	111.2± 4.8*

[a] Activities of the controls are reported as μmol MUF·g DM^{-1}·h^{-1} for the MUF-labelled substrates, as the decrease in specific viscosity·g DM^{-1}·h^{-1} for CMC, and as mg RBB formed·g DM^{-1}·h^{-1} for RBB-xylan.
[b] The activities of the different enzymes in the presence of 3% toluene are given as a percentage of that of the control (0% toluene).
* Significantly different from activity with 0% toluene (*t*-test; p=0.95).

Effect of toluene on microbial carbohydrate leakage. In the first experiment, enrichment cultures on glucose+xylose were prepared from reed litter fragments under both aerobic and anaerobic conditions. Toluene treatment released between 1.5 and 140 μmol·g microbial $biomass^{-1}$ of the different carbohydrates. Reed litter incubations under both aerobic and anaerobic conditions had an microbial biomass of 0.3 mg·g DM^{-1}. By using this biomass, calculations suggest that less than 4% of the carbohydrate released from toluene-treated reed litter could be explained by leakage or release from the bacterial population. This is true for all carbohydrates except glycerol under anaerobic conditions, for which 40% could be explained by the lysis of bacteria.

In the second experiment, the effects of leakage from the natural microbial population and the enzymatic decomposition of polysaccharides were separated by inhibiting enzymes with 2% glutaraldehyde. Unfortunately, glutaraldehyde severely interfered with the carbohydrate analysis. The detection limit increased dramatically to about 5 μM because of the high background signal and blanks. Only the dominant carbohydrates in the aerobic incubations could be analyzed accurately; the concentrations of the minor carbohydrates and all concentrations in the anaerobic incubations were too low. Also, the CarboPac PA1 column could not be used at all, since glutaraldehyde showed up in the region of the chromatogram where the carbohydrates normally appear. Because of this

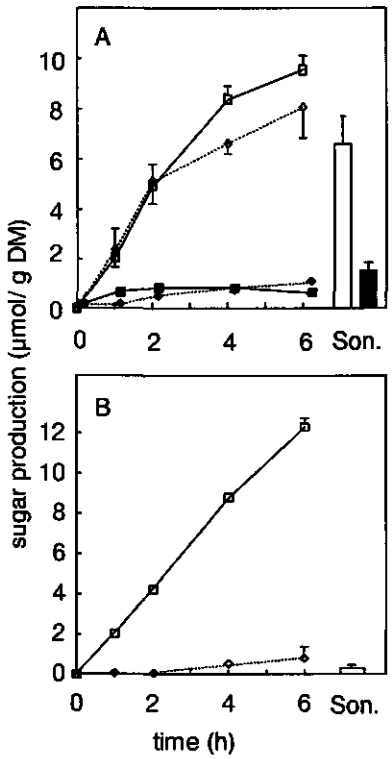

Figure 4. Accumulation of alditols (A) and glucose+xylose (B) in the presence of toluene in 2% glutaraldehyde fixed (dotted lines with diamonds) and non-fixed (solid lines with squares) aerobic incubations. Bars represent carbohydrate release after sonication. Alditols shown in A are mannitol (non-filled symbols and bar) and glucitol (filled symbols and bar). SD is shown by error bars (n=3). Anaerobic incubations are not shown (see text).

and because the CarboPac MA1 column does not separate glucose and xylose (Fig. 1B), data on these two sugars were not available separately.

The effect of 2% glutaraldehyde on carbohydrate accumulation under aerobic conditions in the presence of toluene is shown in Fig. 4. Also shown in Fig. 4 is the amount of carbohydrate released after disruption of the microbial cells by sonication, which gives an estimate of the intracellular amount of carbohydrates. The detected alditols (Fig. 4A) behaved differently from the combined glucose and xylose peak (Fig. 4B). The incubations fixed with glutaraldehyde accumulated a similar amount of alditols as the incubations not fixed with glutaraldehyde (Fig. 4A). In contrast, only 8% glucose+xylose accumulated in the fixed series compared with the amount accumulated in the nonfixed ones (Fig. 4B). For both alditols and glucose+xylose, release after sonication was similar to the accumulation with toluene after 6 h.

The sonication experiment was also conducted with nonfixed litter. The amounts of intracellular carbohydrate were overestimated in these experiments, since the activities of extracellular enzymes were not inhibited during sample handling. Under aerobic conditions, glucose, xylose, arabinose, galactose, and glycerol release by sonication could explain 39, 10, 26, 14, and 22% of the accumulation, respectively, after 6 h in the toluene treated litter. Under anaerobic conditions, sonication released higher amounts: 48, 28, 67, 36, and 35%, respectively. Mannitol and glucitol releases due to sonication were 65 and 100%, respectively, under anaerobic conditions.

Discussion

In the present study, 3% toluene was used to inhibit carbohydrate uptake by the microbial community in reed litter. Similar or higher concentrations of toluene have been used previously in enzyme assays in soils (Burns 1978; Kiss & Boara 1965) and sediments (Sinsabaugh et al. 1991) to prevent the microbial uptake of the dissolved product that is formed and microbial growth. We used toluene in a similar fashion, but we did not add an external substrate and followed the accumulation of a spectrum of products by a sensitive HPLC method. In this way, extracellular decomposition of natural polysaccharides occurring in situ could be followed.

We showed that toluene was an effective and fast-working inhibitor of microbial carbohydrate uptake. It probably disturbs the proper functioning of the cytoplasmic membrane by increasing its permeability (Felix 1982). As a result, transmembrane gradients like the proton gradient will collapse. This, in turn, decreases ATP formation and leads to the inhibition of the active uptake systems that depend directly or indirectly on ATP or the proton gradient as an energy source (Saier 1985). Carbohydrates will probably be taken up by active transport under natural conditions since the free extracellular pools of carbohydrates are very low (Dawson & Liebezeit 1981; Jørgensen & Jensen 1994; Mopper et al. 1992; Wicks et al. 1991; and this study). Nonactive uptake systems (Saier 1985) will also be affected by the toluene treatment. They depend on a concentration gradient over the semipermeable cytoplasmic membrane, which probably collapses in the presence of toluene. Although only the effective inhibition of glucose and xylose uptake was shown here, the nonspecific mode of action of toluene makes it unlikely that any other uptake systems are still functioning.

Many studies have been made on glucose turnover in sediments; some have reported turnover times of up to several hours (Krumböck & Conrad 1991; Toerien & Cavari 1982). The estimated turnover times for glucose in our reed litter incubations without toluene are comparable to those reported for intertidal marine sediments (Sawyer & King 1993) and about three times longer than those for sediments of eutrophic Wintergreen

Lake (King & Klug 1982). The short turnover times suggest that assimilation was efficient and that a highly active microflora was present. The xylose turnover times were of the same order of magnitude as the glucose turnover times. To the best of our knowledge, xylose turnover times have not been previously measured in sediments or in incubations of plant litter.

The enzyme assays showed that the activities of the major enzymes involved in the extracellular decomposition of cellulose and xylan were neither stimulated by a release of intracellular enzymes nor inhibited to a great extent by the toluene treatment. King (1986) reported that toluene inhibits β-glucosidase activity by 50% in a marine sediment. The lack of effect in our study could be due to a different type of β-glucosidase. In older studies, no inhibition was found for several types of hydrolytic enzymes in soils (Burns 1978; Kiss & Boara 1965). Sinsabaugh et al (1991) stated that toluene likely inhibits the activities of at least some exoenzymes, which puts forward the need for testing other types of enzymes. Possible candidates for the type of samples we worked on are so-called debranching enzymes (DeBlois & Wiegel 1990; Wong et al. 1988), which split the arabinose side chains of xylan, and the enzymes involved in the decomposition of lignin. It is known that the presence of lignin in macrophyte litter severely diminishes the decomposition rates of the associated polysaccharides (Benner et al. 1984a; Moran & Hodson 1989). Inhibition of one of the enzymes in the lignase system by toluene might therefore result in a decrease in polysaccharide decomposition.

Because toluene increases the permeability of the cell membrane, cytoplasmic compounds that interfere with the measurements might be released (Felix 1982). Except for glycerol, insignificant amounts of both neutral sugars and alditols were released from enrichment cultures by toluene. This suggests that leakage was not an important process. However, the enrichment technique is known to be highly selective with respect to the enriched species, and the types of intracellular carbohydrates might have been different from those in the natural population. Because of this, we also tried to show possible leakage from the natural population in reed litter using glutaraldehyde-fixed material. Toluene treatment of fixed material released only about 10% glucose+xylose during the same time period compared with that released from nonfixed material. Sonication released a percentage of glucose+xylose that was similar to that released from the toluene-treated fixed material. This suggests that the accumulation of glucose and xylose that we found when reed litter was incubated with toluene did not result from leakage of cytoplasmic compounds. High sugar concentrations in the cytoplasma are not to be expected, since most sugars are phosphorylated during their passage through the cell membrane or in the first metabolic step (Saier 1985). The situation was clearly different for the alditols. The release of mannitol and glucitol from toluene-treated and sonicated from experiments with fixed material was similar to that from nonfixed material used in controls. This suggests that alditols mainly accumulated because of leakage from the

cytoplasm and that the accumulation was not due to enzymatic activity. A decrease in the accumulation rate with time or a plateau concentration, which was found for mannitol in some experiments, is also in agreement with the fast release of a relatively small intracellular pool. It is well known that alditols are used as osmoregulatory agents in many heterotrophic microorganisms, especially fungi (Hellebust 1976; Yancey et al. 1982).

This new method should only be applied with short incubation times. Longer incubation times will probably result in a changed pool of exoenzymes caused by the slow rate of deterioration of extracellular enzymes, while de novo synthesis is inhibited by the absence of metabolism in permeabilized cells. With incubation times of 6 h or less used in the present study, no such effect could be detected since neutral sugars accumulated linearly over time. The observed lag phase of about 1 h for the accumulation of some sugars might be explained by the disturbance of interactions between enzymes and their substrates caused by the addition of toluene and/or the shaking. The lag phase would then be the time needed for the recovery from these interactions. Several of the extracellular enzyme assays performed also showed initial lag phases of similar lengths both with and without toluene (data not shown). The explanation that a certain amount of time is needed before toluene inhibits all microbial uptake seems less plausible since preincubations with toluene for both 15 and 90 min inhibited sugar uptake completely.

The rate of glucose accumulation was 10 times higher than rate of xylose accumulation under both aerobic and anaerobic conditions. The neutral sugar composition showed that the glucose/xylose ratio in reed litter was only about 1.8. Unless any other decomposition intermediate of xylan was accumulating, it can be concluded that cellulose was decomposed at a significantly higher rate than arabino-xylan. This is in contrast to experiments with litter bags in which the decomposition rates of xylan and cellulose in litter were similar (Moran et al. 1989; Wilson et al. 1986b). A high degree of interaction between the refractory lignin of organic materials and the more easily degradable structural polysaccharides of plant cell walls has been suggested as a limiting factor in the decomposition of organic material (Benner et al. 1984a; Moran & Hodson 1989; Valiela et al. 1984). A higher degree of association of lignins with xylan than with cellulose could explain the relatively low xylose production rates found in the present study.

The method described in this report provides a fast and sensitive way to quantify the initial decomposition of polysaccharides in macrophyte litter and sediment organic matter. It provides information on the decomposition rates of natural substrates. If the texture of the substrate remains unchanged and the conditions are not altered from the natural situation during the incubation with toluene, the method can be used to monitor the in situ decomposition rates of polysaccharides in sediments. Although we designed the method primarily for polysaccharides in aquatic environments, it might also be applicable, after

additional testing, to other systems like soils and the rumen and to other substrates like proteins or lipids.

Acknowledgments

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Chapter 7

Initial Decomposition of Polysaccharides in Plant Litter and Littoral Sediments

H.T.S Boschker, S.A. Bertilsson,* E.M.J. Dekkers, and T.E. Cappenberg

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Abstract. The microbial decomposition of cellulose and xylan was studied in macrophyte litter and in sediments from a reed bed in the littoral of Lake Gooimeer, The Netherlands. A recently developed method was used, which is based on the selective inhibition of microbial carbohydrate uptake by toluene without affecting extracellular hydrolyses of polysaccharides. Accumulation of hydrolysis products was followed in time using high performance liquid chromatography, and a similar pattern of accumulating carbohydrates for both litter and sediments was observed. Ratios between glucose and xylose accumulation rates suggested that xylan was degraded more slowly than cellulose, which was not in agreement with apparent long term removal rates of glucose and xylose from litter. Accumulation of oligomeric compounds besides xylose during the decomposition of xylan may explain this discrepancy. The turnover time of particulate glucose was estimated at 85 ± 14 d in the top centimetre of the sediment, and showed a three to four fold increase with depth. Comparison between glucose accumulation rates as a measure of cellulose decomposition and total carbon mineralization rates confirmed that cellulose decomposition was a major process in the mineralization of organic matter in littoral sediments.

*: Present address: Department of Water and Environmental Studies, Linköping University, S58183 Linköping, Sweden.

Introduction

Polysaccharides, like cellulose and hemicelluloses, represent a major part of the organic material in aquatic environments (Benner et al. 1984b; Ljungdahl and Eriksson, 1985). The initial step in the decomposition of these polymers to smaller intermediates is catalysed by enzymes secreted by micro-organisms and is generally regarded as the rate-limiting step in the microbial mineralization of organic matter (Benner et al. 1984a; Billen 1982; King, 1986). Although large amounts of data exist on processes and factors that govern the decomposition of bulk sediment organic matter, information on the decomposition of specific polymers under natural conditions is still lacking (Henrichs, 1992; Webster and Benfield, 1986).

Recently, we developed a method to measure momentary initial decomposition rates of naturally occurring polysaccharides in plant litters and sediments (Boschker et al, 1995a). The method is based on the inhibition of microbial carbohydrate uptake by toluene, which does not affect the extracellular hydrolyses of polysaccharides in the sample under study. The accumulation of hydrolysis products is followed in time using high performance liquid chromatography (HPLC), which results in a sensitive method with a high resolution for the products formed.

In the present article, we have used this method to study polysaccharide decomposition in macrophyte litter and in sediments from a reed bed in the littoral of Lake Gooimeer, The Netherlands. This reed bed is mainly covered with common reed (*Phragmites australis*). Like in other grasses, the main polysaccharides in reed litter are cellulose and arabino-xylan (Bacic, 1988). Recently it has been shown that reed litter is the major source of organic carbon matter for the sediments inside the reed bed (Boschker et al. 1995b). It is therefore likely that the initial decomposition of cellulose and arabino-xylan is an important process in the mineralization of organic matter in this system. We will discuss accumulation rates of the different carbohydrates produced, including oligomers, as measured with the toluene inhibition method in terms of decomposition of the two main polysaccharides in reed litter. In addition, carbohydrate accumulation rates will be compared with total carbon mineralization rates in plant litter and sediments. Conclusions will be drawn on the importance of polysaccharide decomposition in the sedimentary mineralization process.

Materials and methods

Initial decomposition rates of polysaccharides in sediments. Three to four sediment cores were collected by hand at a site 20 m inside an extensive reed bed on the

southern border of Lake Gooimeer, The Netherlands (Boschker et al, 1995b). Samples were taken on two occasions: i) 8 July 1993 when the sediment was covered by water (*in situ* temperature, 16 °C) and ii) 18 November 1993, when there was no overlying water inside the bed (*in situ* temperature, 5 °C). The November sampling was shortly after the first input of fresh litter to the sediment as a result of the senescence of common reed in autumn. Sediment cores were transported to the laboratory within 1 h and immediately sliced in an anaerobic glove box (Coy Laboratory Products Inc.; filled with N_2 and 1.5% H_2). Macrophyte roots were removed by hand from the sediment slices.

Between 5 and 14 g wet weight of each sediment slice was transferred anaerobically to a 250 ml serum bottle containing 50 ml of deoxygenated artificial lake water (Boschker et al. 1995a) and bottles were sealed using butyl-rubber stoppers fit with a butyl-rubber septum. Bottles were preincubated for 15 min before toluene was added to a final concentration of 3% (vol/vol, *Pro Analysi*, Merck). Aerobic incubations were bubbled with air during a 15 min preincubation period. Liquid samples (5 ml) were taken with syringes at three points between 45 and 180 minutes after addition of toluene and were treated and analysed for carbohydrates as described below. All incubations were made at *in situ* temperatures and were shaken slowly at about 30 rpm to increase contact between toluene and the sediment slurry.

On each occasion, subsamples from each slice of two replicate cores were dried at 104 °C, ground to a fine powder in an IKA sample mill, and analysed for carbohydrate composition as described below.

Initial decomposition rates of polysaccharides in reed litter. Standing dead reed litter was collected in December 1990, shortly after the senescence of reed plants. The material was cut into 1 cm pieces, air dried and stored until use at -20 °C. This reed litter was used in three experiments in which polysaccharide decomposition was studied and compared with total carbon degradation rates. Reed litter (0.5 or 1 g dry weight) was incubated at 15 °C in 500 ml Serum bottles, which further contained 100 ml of artificial lake water, 0.8 mg N added as nitrate for the aerobic or as ammonium for the anaerobic incubations, 130 µg PO4-P and a small inoculumn of fresh sampled sediment from the reed bed of Lake Gooimeer (less then 2 % additional organic carbon).

Short term aerobic and anaerobic incubations were preincubated for 10 days and were continuously shaken during this period (about 30 rpm). Total carbon mineralization was followed by measuring carbon dioxide and methane production as described below. At the end of the preincubation period, polysaccharide decomposition was measured by the toluene method, i.e. samples were incubated with toluene for 180 minutes and three aliquotes were taken in this period for carbohydrate analysis.

Initial decomposition rates of polysaccharides and carbon mineralization rates in reed litter were also determined after 150 days of preincubation under both aerobic and anaerobic conditions. Conditions were similar as in the short term incubations, with the

exception that in the aerobic incubations only 10 ml of artificial lake water was added to avoid the development of anoxic pockets in the incubation bottle. To determine if slurrying influenced the decomposition rate of reed litter, the incubation bottles were not shaken during the preincubation period when carbon dioxide and methane production was measured.

Similar reed litter incubations were made up to 1000 days primarily under aerobic conditions, and were analysed for organic matter losses and for carbohydrate composition in the remaining litter. The contents of the bottles was filtered over a Whatmann GF/F glass fiber filter. The filter was dried at 104 °C until stable weight and ground to a fine powder in an IKA sample mill. The remaining organic matter was determined from the loss of weight after combusting a subsample at 550 °C for 4 h. The carbohydrate composition of fresh and incubated reed litter was determined as described below.

Carbon mineralization in litter incubations. Mineralization rate of reed litter in the 10 and 150 day incubations was measured by following carbon dioxide, dissolved inorganic carbon and methane accumulation with time.

Carbon dioxide in the head space of incubation bottles was measured using an Infra Red Gas Absorbance analyser (IRGA) BINOS 1 (Leybold-Heraeus). A flow of nitrogen (1 $l.min^{-1}$) was first let through the reference cell of the IRGA and subsequently through the measuring cell. Between the two cells, gas samples (0.2 ml) could be injected. IRGA response was followed on a strip-chard recorder and peak height was used to calculate carbon dioxide concentrations. A 25 mM $BaCO_3$ suspension in Milli-Q was used for standardisation: 1 ml of this suspension was added to a 8 ml vial, the vial was immediately closed with a butyl-rubber septum and 0.2 ml 1 N HCl was injected through the septum. After an equilibration period, the headspace was sampled for carbon dioxide. Dissolved inorganic carbon in the water phase of the incubation bottle was determined in a 1 ml sample, which was acidified and sampled for carbon dioxide in the headspace similar to the $BaCO_3$ standard. Corrections were made for carbon dioxide partitioning between head space and water phase in the vials.

Methane was measured in the head space of anaerobic incubations using a Packard model 428 gas chromatograph equipped with a Chrompack Q column and a flame ionisation detector.

Carbohydrate sample treatment and analysis. Carbohydrate sample treatment and analysis were similar as described by Boschker et al. (1995a). In short, samples were centrifuged, filtered over 0.2-µm-pore-size polycarbonate filters, and put over a mixed ion-exchange column to remove ionic and humic substances that interfered with the analysis. Carbohydrate concentrations were determined by ion-chromatography with pulsed amperometric detection on a Dionex 2000i/SP HPLC system. Monomeric neutral sugars were analysed on a Carbopac PA1 (Dionex, 4x250 mm) anion exchange column with Milli-Q water as eluent and detection was optimised by a post column addition of 1.6

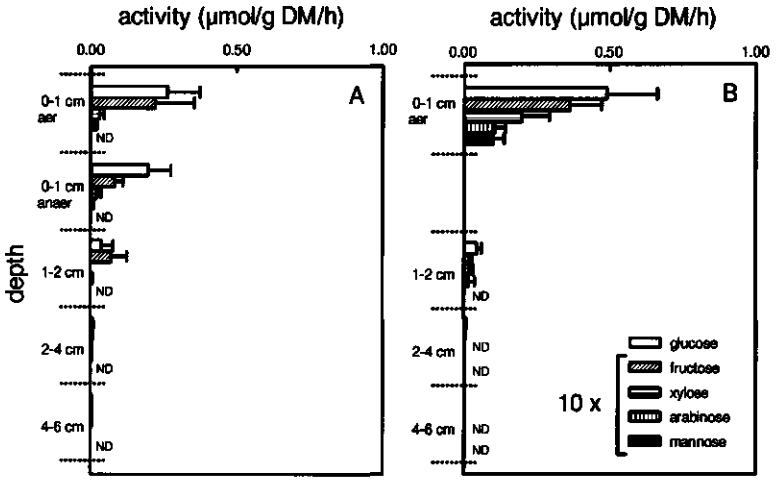

Figure 1. Accumulation rates of carbohydrates with depth in toluene treated, slurried sediments sampled in the reed bed of Lake Gooimeer on (A) 8 July 1993 (*in situ* temperature 18 °C) and (B) 18 November 1993 (5 °C). In November, plants had died down and first fresh reed litter had entered the sediment.

M NaOH using a AMMS II anion-micromembrane-suppressor (Dionex). Oligomeric neutral sugars were analysed on the same column with an eluent containing 100 mM NaOH in Milli-Q and between 25 to 100 mM Sodiumacetate (NaAc) depending on the compounds to be analysed. The detection limits for different carbohydrates ranged from 5 to 50 nM.

The following oligomeric compounds were available as references: i) cellodextrin mixture II (Merck) containing cello-oligomers with a degree of polymerisation (dp) from 2 to 8 and ii) xylo-oligomers (dp 2 to 4), which were a gift from P. Schijns, Department of Microbiology, Wageningen Agricultural University, The Netherlands.

The carbohydrate composition of reed litter and sediment samples was determined after sulphuric acid hydrolysis according to Cowie and Hedges (1984a).

Results

Initial decomposition rates of polysaccharides in sediment cores. In the littoral sediment of Lake Gooimeer almost all measured activity was found in the surface sediment (0-1 cm, Fig. 1). In deeper layers, only low accumulation rates (< 0.027 µmol·g $DM^{-1}·h^{-1}$ for glucose) could be detected. For one core sampled on 8 July 1993, sediment layers from below 6 cm depth were also incubated with toluene but showed no detectable activitie (detection limit, approximately 0.1 nmol·g $DM^{-1}·h^{-1}$). The top layers from cores

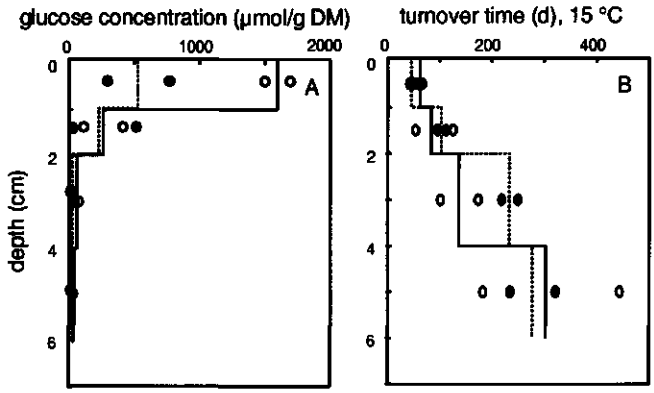

Figure 2. Total glucose content after acid hydrolysis of the sediment (A) and temperature corrected turnover times for glucose (B) in Lake Gooimeer sediments sampled on July (black circles) and November 1993 (open circles). Turnover times were calculated as the sediment glucose conctent divided by the glucose accumulation rate in the presence of toluene. A Q^{10} of 2 was used to correct accumulation rates at *in situ* temperatures to 15 °C.

sampled on 8 July 1993 were incubated under both aerobic and anaerobic conditions. Accumulation rates of glucose and fructose were slightly lower under anaerobic conditions, but differences were not statistically significant. Xylose rates were higher in the anaerobic incubations. Ratios between the glucose and xylose rates were rather high compared with their concentration ratios in reed litter (about 1.8; see further) and decreased between July and November from 80 to 25 in the top centimetre of the sediment. Accumulation of galactose could not be detected because of an incomplete separation from an unidentified compound, which did not accumulate significantly with time.

Decomposition rates were variable between cores from the littoral sediments of Lake Gooimeer, suggesting a high spatial heterogeneity. This was consistent with visual observations on sediment structure and occurrence of plant litter on the sediment. A depth integrated, glucose mineralization rate of 1.3 ± 0.5 g $C \cdot m^{-2} \cdot day^{-1}$ was calculated for the sediment on both dates. Although the *in situ* temperature was lower in November than in July, both dates gave similar activities, which is probably the result of the input of fresh reed litter in November.

Two replicate cores from every sampling date were analysed for their bulk carbohydrate compositions (Fig. 2A). The percentage of the organic matter explained by glucose in the top layer of the sediment was lower in July (20 %) than in November (34 %), and decreased to 13 and 10 %, respectively, in the deepest layer. Xylose to glucose ratios remained rather constant with depth (average ratio 1.70 ± 0.33), suggesting a similar turnover time for both carbohydrates, and were similar to ratios found in fresh reed litter (see further). Pool average, pseudo-first-order turnover times were calculated

Table 1. Rates of initial decomposition rate as measured with the toluene method and rates carbon mineralization in reed litter

	Incubation time 10 days		Incubation time 150 days	
	Aerobic	Anaerobic	Aerobic	Anaerobic
Activity (μmol.g DM^{-1}.h^{-1})				
Arabinose	0.020±0.002	0.013±0.001	0.010±0.000	0.003±0.000
Galactose	0.014±0.002	-	0.020±0.014	-
Glucose	0.94 ±0.25	0.21 ±0.03	1.92 ±0.54	0.61 ±0.11
Xylose	0.094±0.007	0.033±0.005	0.040±0.007	0.010±0.002
Total	1.11 ±0.26	0.25 ±0.04	1.99 ±0.57	0.62 ±0.11
Mineralization (mg C.gDM^{-1}.d^{-1})				
Carbohydrates	1.88 ±0.45	0.43 ±0.06	3.4 ±1	1.1 ±0.2
CO_2 and CH_4	1.89 ±0.21	0.71 ±0.01	1.12 ±0.15	0.56 ±0.05
Activity ratios				
Gluc/Xyl	10.1	6.3	48.4	58.0
Xyl/Arab	4.7	2.6	4.1	3.1

- , not detected

for glucose by dividing the glucose concentration (Fig. 2A) by the accumulation rate as measured with the toluene method (Fig. 1) and were corrected to a temperature of 15 °C using a Q^{10} of 2 to allow direct comparison between sampling dates (Chapter 3). Temperature corrected turnover times were similar on both sampling dates, with a turnover time of approximately 85 ± 14 d for the top centimetre of the sediment and a three to four fold increase in the 4 to 6 cm depth interval (Fig. 2B). In the 1 to 4 cm layer, there seems to be a shift in glucose turnover time with higher values for July than for November when there had been a recent input of fresh litter. Mainly data for glucose are reported here, because our results suggested that accumulation rates for other monosaccharides were too low to be correct, which resulted in unlikely long turnover times (see Discussion).

Initial decomposition of polysaccharides in reed litter. The measurements performed on decomposing reed litter are summarised in Table 1. Patterns of carbohydrates produced were similar to Lake Gooimeer sediment (Fig. 1), except that no fructose accumulation was found in litter incubations. Glucose production rates were

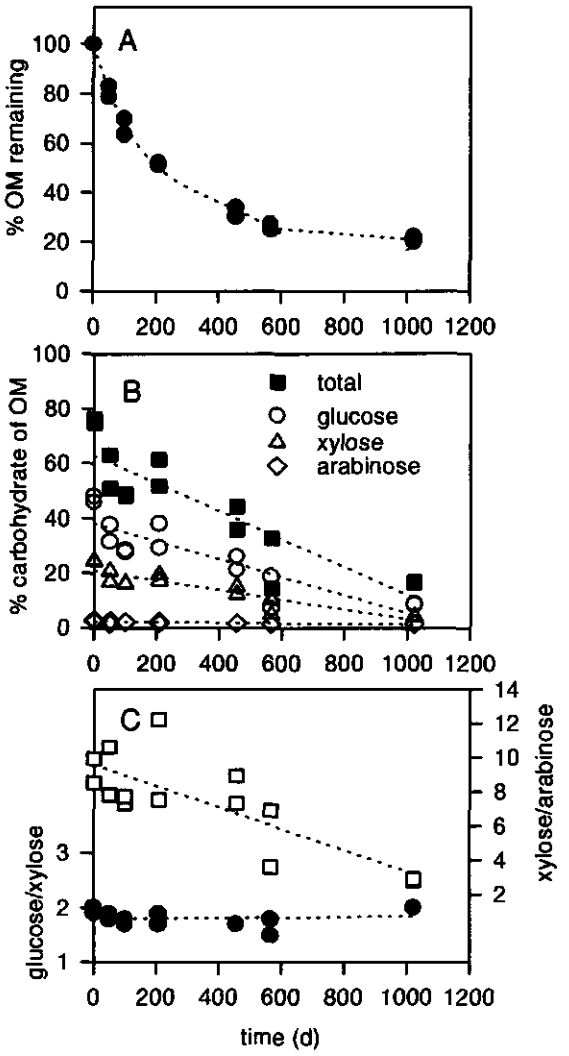

Figure 3. Changes in carbohydrate composition of reed litter during long term aerobic incubations at 15 °C. Amounts of particulate organic matter remaining in the incubations is shown in Fig. 3A. Carbohydrate composition of the remaining litter is shown in Fig. 3B and the ratios between the major carbohydrates in Fig. 3C (glucose/xylose, black circles; xylose/arabinose, open squares).

always the highest, followed by xylose and arabinose or galactose. Combined rates of glucose, xylose, arabinose and galactose were always higher for aerobic compared to anaerobic incubations. For the 10 day incubations, total carbohydrate production rates were in the same range as carbon mineralization data (Table 1). But after 150 days carbohydrate accumulation rates were 2 to 3 times higher than expected from carbon

mineralization. It should be noticed though that for the 10 day incubations both parameters were determined under shaking conditions, whereas for the 150 day incubations only carbohydrate accumulation measurements were shaken and carbon mineralization was measured under non-shaking conditions. For the long time incubations, pseudo-first-order turnover times for glucose were 50 ± 14 d under aerobic and 160 ± 30 d under anaerobic conditions.

Ratios between glucose and xylose hydrolysis rates were always much higher (Table 1) than the ratios between their concentration in reed litter itself (Fig. 3C). Especially reed litter that had been incubated for 150 days showed high ratios between glucose and xylose rates. Rates for xylose and arabinose, the two major monosaccharides in xylan, showed a relative constant ratio between experiments, which was lower than in the litter composition.

Carbohydrates were selectively degraded during a 2.5 year aerobic decomposition experiment, resulting in an relative accumulation of non-carbohydrate compounds (Fig. 3B). Ratios between glucose and xylose in the remaining litter were on average 1.78 ± 0.16 and did not change significantly with time (Fig. 3C), suggesting a similar removal rate for these sugars. In contrast, xylose to arabinose ratios decreased with time and therefore suggested a higher turnover rate for xylose then for arabinose. Anaerobic litter incubations were only run for 500 days, and showed an approximately twice as low organic matter loss but similar effects on carbohydrate composition as aerobic incubations. Aerobic pseudo-first-order glucose turnover times were calculated from the data in Fig. 3 and increased from 220 ± 50 d for the first 200 days of incubation to 450 ± 20 d from incubation day 450 onward.

Formation of oligomeric compounds. Three major compounds were detected in all incubations with longer retention times than monosaccharides, which suggested that they were oligomeric carbohydrates (Fig. 4). Non of the retention times of these compounds were comparable with the available cello-oligomers or the xylo-oligomers under both analytical conditions used. No compounds with higher retention times and therefore a higher degree of polymerisation were detected even during long runs with an eluent containing 100 mM NaAc in 100 mM NaOH. The three, unknown oligomeric compounds accumulated linearly in time.

Discussion

Cellulose and arabino-xylan are the principle polysaccharides in litter from grasses like common reed. Minor other polysaccharides are mixed-linked glucans, arabino-galactans and xyloglucans (Bacic et al., 1988). In the following discussion it is assumed

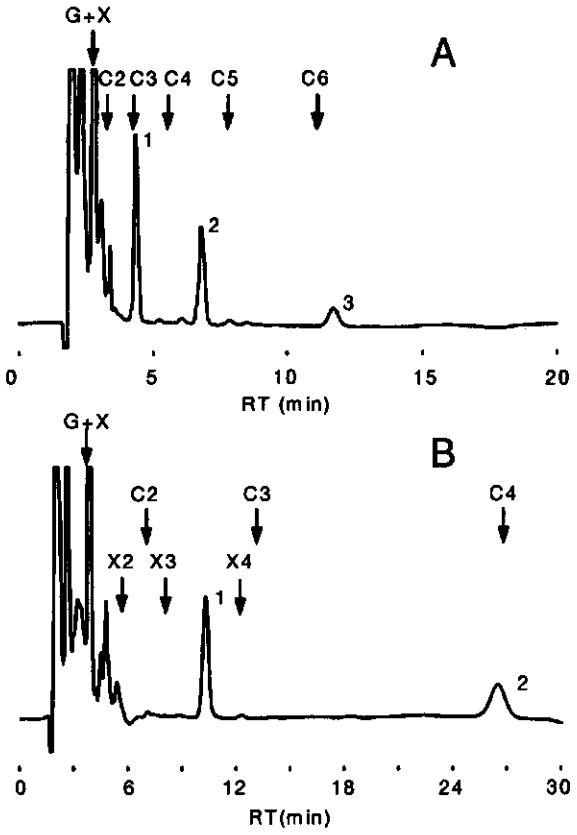

Figure 4. Chromatograms of oligomeric carbohydrates as found after 6 h in aerobic incubations of top layer sediment with toluene, November 1993. Variable amounts of the same oligomeric compounds were detected in other incubations with toluene. For A) 75 mM NaAc in 100 mM NaOH was used as eluent and for B) 25 mM NaAc in 100 mM NaOH. Numbers 1 to 3 refer to different oligomers detected. Retention times of reference cello-oligomers (G for Glucose and Cx for cello-oligomers with dp x) and xylo-oligomers (Xx for dp x) are indicated with arrows.

that glucose in reed litter is predominantly derived from cellulose, and that xylose and arabinose are from arabino-xylan (Wilson et al., 1986).

Initial decomposition rate measurements in reed litter and littoral sediment of lake Gooimeer resulted in accumulation of similar spectra of carbohydrates. This was expected, since reed litter derived material is the dominant carbon source in this sediment (Boschker et al., 1995b). Only fructose accumulated in sediment incubations whereas no accumulation was found with litter. Although living roots were removed as far as possible from the sediments, some remaining root sucrose, a glucose-fructose disaccharide, may be the source of this fructose (Barclay and Crawford, 1983; Best and Dassen, 1987). Glucose was the dominating accumulating product, suggesting that cellulose decomposition and a

subsequent utilisation of glucose was a major mineralization process in this sediment. Other major carbohydrates in reed litter polysaccharides like xylose and arabinose, accumulated at much lower rates than expected from litter composition. Assuming that both xylose and arabinose were the only products of arabino-xylan decomposition, this accumulation pattern would indicate that arabino-xylan was degraded with a much lower rate constant than cellulose as explained by glucose accumulation. The glucose/xylose production ratios increased with litter age both in litter incubations and in field studies, which suggested that xylan became less available with time. On the other hand, this lower rate of xylan decomposition based on xylose and arabinose accumulation rates is not in agreement with the apparent similar removal rate of different polysaccharide fractions or component monosaccharides in litters from grasses as shown by us (Fig. 3) and in other studies (Godschalk and Wetzel, 1978; Moran et al., 1989; Tanaka, 1991; Wilson et al.,1986).

There are several possible explanations for the apparent underestimation of xylan decomposition rates by the toluene method. An incomplete inhibition of carbohydrate uptake by toluene seems less likely since toluene has an unspecific mode of action and the effective inhibition of glucose and xylose uptake by toluene in reed litter incubations has been shown (Boschker et al., 1995a). An other explanation might be that an enzyme involved in the complete decomposition of xylan was inhibited by toluene. We recently showed that xylanases and β-xylosidase were not affected by 3% toluene (Boschker et al., 1995a), and other hydrolytic enzymes are also generally not inhibited (Burns, 1978; Kiss and Boara, 1965). However, no data are available on effects of toluene on debranching enzymes, which split the arabinose side chains of xylan (Biely, 1993), and on the enzymes involved in the decomposition of lignin or phenolic compounds that are associated with xylan (Kirk and Farrell, 1987). The inhibition of these enzymes might have affected xylan decomposition rates or the pattern of intermediates produced.

An alternative explanation for the apparently slow decomposition of xylan is that other compounds were accumulating in addition to the monosaccharides xylose and arabinose. We indeed detected accumulation of three oligomeric compounds, which were different from both linear cello-oligomers and xylo-oligomers. It is unlikely that the decomposition of cellulose would yield any oligomeric sugars other than linear cello-oligomers, since cellulose is made of a linear chain of (1-4)-β-linked glucose molecules (Ljungdahl and Eriksson, 1985). Arabino-xylan on the other hand is a heteropolymer, made of a linear xylan backbone with primarily arabinose side groups (Bacic et al., 1988). Decomposition of arabino-xylan could therefore yield xylo-oligomers with arabinose side groups besides xylose, arabinose and linear xylo-oligomers (Kormelink and Voragen, 1993; Puls and Schuseil, 1993). Unfortunately, no reference materials were available for these compounds and we therefore do not know their retention times and response factors under the analytical conditions used. If it can be shown that these oligomers are indeed

intermediates under natural conditions, this would have implications for carbon flow and the associated microbial populations. It is well known that certain types of polysaccharide degrading bacteria are able to or even preferentially take up oligomers like cello-oligomers and xylobiose (Helaszek and White, 1991; Hulcher and King, 1958; Hurtubise et al., 1995; Strobel et al., 1995), but as far as we know direct uptake of hetero-oligomers has not yet been shown. No cello-oligomers were accumulating in either litter or sediment incubations, which suggested that direct uptake of these compounds did not occur in cellulose decomposition. This is in contrast with the rumen, where cello-oligomers are thought to play an important role in cellulose decomposition and in the competition between cellulolytic and non-cellulolytic, cello-oligomer using micro-organisms (Russell, 1985; Shi and Weimer, 1996).

For the short term litter incubations, carbohydrate accumulation with toluene were in the same range as carbon mineralization rates (Tab. 1). Since reed litter is mainly made of polysaccharides, this suggested that carbohydrate accumulation rates were in the correct range. There is also lignin present in reed litter, but it is generally thought that lignin is less readily degraded (Benner et al, 1984a/b; Wilson, 1985) and it therefore should not be a major source of CO_2 production. A closer comparison between the two mineralization parameters is difficult, because i) our data suggest that xylan degradation was under estimated by xylose and arabinose accumulation and ii) the net growth yield of the microbial population on reed litter should be included in the comparison. For the long litter incubations, carbohydrate accumulation rates were 2 to 3 times higher than could be expected from carbon mineralization rates, which was probably the result of a difference in incubation conditions since carbohydrate accumulation rates were determined while shaken but bottles for mineralization rate mesurements were not shaken. Slurrying and mixing probably stimulates mineralization because it results in a fragmentation sediment and litter aggregates, and diminishes limitations caused by mass transfere resistance.

Carbon mineralization data were not available for Lake Gooimeer for the two dates in 1993, when carbohydrate initial decomposition rates were determined. In the previous two years, carbon mineralization rates, which were calculated from heat production rates of intact cores as determined by microcalorimetry according to Graf (1987) and Gustafsson (1987), were between 0.55 and 0.75 g $C \cdot m^{-2} \cdot day^{-1}$ for the same seasons when polysaccharide decomposition was measured (Chapter 3). This is again lower than found for the glucose hydrolysis rate, which was 1.3 ± 0.5 g $C \cdot m^{-2} \cdot day^{-1}$ on both dates. Under the assumption that slurrying affected carbohydrate accumulation rates in sediment to a similar extent as in reed litter, it was calculated that the actual *in situ* glucose accumulation rates were in the range of 0.4 to 0.7 g $C \cdot m^{-2} \cdot day^{-1}$ and therefore in the same range as the carbon mineralization. This suggests that decomposition of glucose containing polymers, probably mainly in the form of cellulose, is one of the main or even the main mineralization process in the littoral sediments of Lake Gooimeer.

Temperature corrected turnover times of the total sediment glucose pool showed a similar increase with depth on both sampling dates (Fig. 2). Since deeper sediment layers are older, this suggests that the reactivity of the residual glucose decreased with time and therefore with the degradation level of the reed litter. Similar increases in glucose turnover times were found in long term litter incubations, which also showed that carbohydrates were selectively faster degraded than non-carbohydrates. A decrease in polysaccharide decomposition rates with time has been found for many but not for all litter types (Godschalk and Wetzel, 1978; Moran et al., 1989; Tanaka, 1991; Wilson et al., 1986) and has been explained by the accumulation of more refractory non-carbohydrate materials like lignin that shield carbohydrates from further microbial decomposition (Wilson et al., 1986 a/b). Turnover times for glucose sediment top layers (85 d) were in the range as found for aerobic and anaerobic litter incubations, which showed turnover times of 50 and 160 d, respectively. Since the top centimetre of the sediment will be both partially aerobic and anaerobic, this suggests that relatively fresh reed litter was the dominant source for glucose in the top of the sediment. As discussed before, carbohydrate accumulation rates were probably overestimated resulting in turnover times that were too low. When carbohydrate removal rates were determined in long term litter incubations, we found indeed higher glucose turnover times of 200 d under aerobic conditions (Fig. 3). Others have found similar turnover times in plant litters (Tanaka, 1991; Wilson et al, 1986 a/b), but a much higher turnover time of 5.5 y was found for glucose in an intermitently anoxic, marine sediment (Hamilton and Hedges, 1988). Since organic carbon in this marine sediment was mostly derived from algae and since algae are generally much faster mineralized than macrophytes (Harvey et al., 1995; Otten et al., 1992; Webster and Benfield, 1986; Westrich and Berner, 1984), the longer glucose turnover time in this marine sediment may seem unexpected. It is, however, possible that most of the organic matter in this marine sediment was rather old or that decomposition rates were limited by anaerobic conditions. Recently though it has been shown that mineralization of organic matter in marine sediments with a relatively low organic matter content is mainly determined by sorption to the mineral phase of the sediment, which renders organic matter inaccessible to microbes (Keil et al., 1994). The similarity of turnover times of total glucose in reed litter and sediment suggests that sorption does not play an important role in littoral sediments of Lake Gooimeer. This is probably caused by the fact that amounts of organic matter were much higher in littoral sediment (between 20 and 90% of sediment dry weight) compared to marine sediments (<10%). In other words, there was simply not enough sorption surface available in littoral sediments and cellulose decomposition rates were probably biologically determined by a combination of the hydrolytic abilities of the microbial community and the properties of cellulose in reed litter.

In conclusion, we have shown that it is feasible to study the initial decomposition of polysaccharides in sediments with a recently developed method (Boschker et al., 1995a). At present, the method can only be used to study rates of cellulose decomposition. On the other hand, accumulation rates of xylose and arabinose were lower than could be expected from arabino-xylan decomposition rates in reed litter. This may be caused by the accumulation of oligomeric compounds during extracellular hydrolysis of arabino-xylan, which need further characterisation.

Acknowledgements

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Chapter 8

General Discussion

The reed bed as an ecosystem

Littoral systems of lakes are considered to be complex with respect to sedimentary, organic matter cycling, especially when compared with the pelagic, open water. This is mainly caused by the fact that the littoral is a transition zone between terrestrial and aquatic ecosystems, and it therefore resembles both systems in certain characteristics. The main source of organic matter for pelagic sediments is derived from sedimentation of seston. In littoral systems, a larger variety of organic matter sources are potentially important, ranging from again sedimentation of seston, production by benthic and epiphytic micro-algae, submerged macrophytes and finally emergent macrophytes at the land side of the littoral (Wetzel 1992). Since the subject of this thesis was the initial decomposition of biopolymers in littoral sediments, and the polymeric composition and degradability of these potential sources is rather different, it was important to know which carbon sources were dominant in our studied littoral.

In Lake Gooimeer, we could show that deep inside the reed bed, organic matter cycling was dominated by one source, namely macrophyte litter (Chapter 2). This greatly simplified the interpretation of the results in other chapters. It was, for example, possible to calculate a sedimentary carbon budget (Chapter 3) and to explain differences in enzyme activities between inside and outside the bed (Chapter 5). Also, it explained why the spectrum of carbohydrates produced during initial decomposition of polysaccharides were similar for reed litter and sediments from inside the bed (Chapter 7). Since plant litter is mainly made of lignocellulose, which has a rather simple and distinct polymeric composition, this makes the reed bed a good natural model system to study initial decomposition of biopolymers. Carbon sources gradually changed to an algal dominance going from inside the bed towards the lake (Chapter 2). This transition zone already begins inside the reed bed and no macrophyte derived material was found outside the bed, despite the high annual production of common reed.

Results of the budget study (Chapter 3) suggest that there are two major processes involved in the removal of sedimentary organic matter. Transport caused by erosion of the sediment during storms explains about 65% of the total carbon and only about 30% was removed by mineralization. Mineralization rates were mainly determined by temperature, changes in oxic and anoxic conditions, and by amounts of organic matter present (Chapter 3). Traditionally, littorals have been described as zones of sediment and organic matter accumulation, resulting in many cases in the formation of peat (Mitch & Gosselink 1993; Wetzel 1992). At the Gooimeer site, less than 5% of the annual production remained in the sediment over a period of 25 years. No short term accumulation could be detected during both years studied and results from 1992 even suggested a net removal of organic matter. This shows that sediment organic matter accumulation is variable and is governed by a delicate balance between input and outputs. Predicting sediment organic matter dynamics will be difficult in these types of systems, since it depends to a large extent on an episodic and variable sediment erosion during storms.

This thesis has been mainly directed to the aboveground produced reed litter. A major uncertainty in the functioning of the reed bed is the importance of root derived material for carbon mineralization. Estimating below-ground productivity is laborious in wetlands, and it is unknown how much and through which processes this production eventually becomes available to the microbial populations in the sediment (Schubauer & Hopkinson 1984). Below-ground standing stocks and productions can be high in similar systems (Hemminga et al. 1996; van der Linden 1980; Schierup 1978) and in rice fields a direct link between methane production and recently fixed carbon has been shown (Minoda & Kimura 1996). Although first sediment depth profiles of enzyme activities suggested that there was an underground input of organic matter (Chapter 4), no such effects were found in subsequent studies (data not shown in this thesis). Further studies are needed to clarify this matter, for instance by using the stable-carbon-isotope tracers methodologies as described by Hemminga et al. (1996) and Kimura & Minoda (1996).

The overall conclusion is that although at first the system looked complex both in structure and functioning, the results of this study suggest that carbon cycling in subsystems can be described in relative simple terms and functionalities.

Mechanism of initial decomposition of plant litter

Micro-organisms are generally not capable of direct uptake of particulate or polymeric organic matter. For bacteria, the limit for uptake lays around 600 daltons (Weiss et al. 1991). This means for instance that for cello-oligomers of 3 to 4 molecules

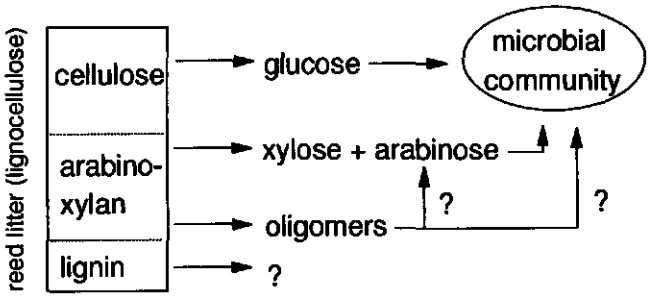

Figure 1. Conceptual scheme of enzymes and intermediates involved in the initial decomposition of biopolymers in reed litter. For the cellulase system, activities of endo-glucanases, exo-glucanases and β-glucosidases were detected. Endo-xylanase and β-xylosidase activities have been shown to play a role in xylan decomposition; activities of other enzymes, like debranching enzymes, have not been determined in this thesis.

length could be used directly, which has indeed been shown recently (Lou et al. 1996; Schlosser & Schrempf 1996; Strobel et al. 1995). It is therefore thought that the first step in the decomposition of biopolymers is an extracellular enzymatic hydrolysis. Figure 1 shows a conceptual model of enzymes and intermediates involved in the initial decomposition of the two major polysaccharides in reed litter, which is based on Chapters 4 to 7 of this thesis.

The major intermediate in the decomposition of cellulose seems to be its monomer glucose. Others have suggested that cello-oligomers, especially cellobiose, might be important intermediates (Russell 1985; Shi & Weimar 1996). For instance, many cellulolytic bacteria preferentially use cellobiose instead of glucose (Helaszek & White 1991; Hulcher & King 1958; Hurtubise et al 1995; Strobel et al 1996). No cello-oligomers were formed during sediment and litter incubations in the presence of toluene (Chapter 7), which suggests that these compounds are not important as intermediates in natural systems. This might be caused by to the high cello-oligomere-hydrolysing, β-glucosidase and exo-glucanase activities associated with reed litter (Chapters 4 and 5).

As shown in Chapter 7, several oligomeric carbohydrates accumulated in the presence of toluene, and although these compounds were not directly characterised results may suggest that they were intermediates in arabino-xylan decomposition. These compounds might be heterogenic xylo-oligomers with arabinose side groups, which would explain the low accumulation rates of xylose and arabinose. In view of the rather high β-xylosidase activity detected in reed litter (Chapter 6), it seems unexpected that these oligomeric compounds accumulated. It is possible though that the action of β-xylosidases is inhibited by the arabinose side-groups. Debranching enzymes, in this case arabinosidases, normally remove these side-groups. These debranching enzymes might have been inhibited by the toluene treatment, which would indeed result in an accumulation of arabino-xylo-

oligomers (Kormelink & Voragen 1993; Puls & Schuseil 1993). On the other hand, all tested hydrolytic enzyme activities were not or only to a small extent affected by the addition of toluene (Chapter 6). Several steps have to be taken to clarify these questions: i) the oligomeric products have to be characterised further so hydrolysis rates of arabino-xylan can be calculated, and ii) effects of toluene on arabinosidases and other debranching enzymes should be studied. If direct uptake of hetero-oligomers can be shown, it would have implications for carbon flow and the associated microbial populations. Uptake of these compounds has not been shown yet in micro-organisms.

In sediments, a diverse microbial community will be involved in the decomposition of polysaccharides and there probably will be a competition between these populations for intermediates. It has been suggested that bacteria involved in biopolymer decomposition can be divided into two groups: primary populations that actually hydrolysis the biopolymer and secondary populations that scavenge hydrolysis products and which have no capacity to degrade the native polymer. Their are probably also intermediate forms that can use partially degraded polymers like non-crystalline cellulose or long oligomers. In the rumen and in defined mixed cultures a substantial part of the carbon flow is directed towards the secondary populations (Chesson & Forsberg 1988; Ljungdahl & Eriksson 1985; Russell 1985). Whether these relations exist in sediments is unknown and will depend on the organisms present and their abilities to compete for substrates. At present, it is not known which organisms are active in biopolymer decomposition in natural systems. A wide variety of cellulolytic bacteria have been isolated from reed litter and other wetlands (Ljungdahl & Eriksson 1985; Mullings & Parish 1985; Tanaka 1993), but these might not reflect the actual populations active since isolation procedures are known to be highly selective for certain types of bacteria. Methods like DNA based techniques developed for the *in situ* identification of bacteria may be used to study organisms present in natural systems (Amann et al. 1995; Giovannoni et al. 1990; Muyzer et al. 1993). However, it will be difficult to identify specific functions like primary and secondary populations or population involved in cellulose or in xylan decomposition, since these traits are found in a large variety of unrelated groups and even within those genetic groups many of the possible traits can be found (Biely 1993; Ljungdahl & Eriksson 1985).

Competition for substrates between bacterial groups will also depend on the physical organisation of the decomposition of biopolymers. The most simple and widely used model is that intermediates produced through the action of extracellular enzymes are released into the sediment pore water, from which they are taken up by micro-organisms. From a substrate competition point of view this is a rather unlikely strategy for the primary populations since other, secondary organisms are also free to scavenge intermediates. A substantial fraction of the bacteria in sediments will be attached to the sediment matrix (Weisse & Rheinheimer 1978). A much more sensible strategy for these

bound microbes would be to release extracellular enzymes predominantly in the space between them and the sediment matrix. Intermediates formed during the decomposition of biopolymers would then also be released in this compartment and would be available to a greater extend for the primary organism. Many cellulolytic *Clostridia* seem to have such a strategy, since they excrete a kind of gel that not only binds them to the substrate but that also contains all the enzymes needed for the extracellular hydrolysis of cellulose (Ljundahl & Eriksson 1985). Several polymer hydrolysing strains in the rumen use a similar strategy in which vesicles with extracellular enzymes are released on the side of the bacterium that touches the polymer (Chesson & Forsberg 1988). If this strategy would indeed operate in sediments, pore water concentrations of intermediates might not be representative for the concentrations the competing bacteria are experiencing.

Rate determining/limiting step

Decomposition of organic matter is a stepwise chain of processes in which many discrete intermediates, and enzymatic and microbial processes can be distinguished (see Fig. 1, Chapter 1). In this thesis, a major assumption has been that the first process in this chain, the enzymatic extracellular hydrolysis of biopolymers, is the rate limiting step in the decomposition of organic matter in sediments and ecosystems in general. This assumption that has been stated by many authors (e.g. Billen 1982; King 1986; Meyer-Reil 1986), although not by all (Arnosti et al. 1994), and explains the attention that has been given to extracellular enzyme and their activities in natural systems (e.g. Burns 1978; Sinsabough et al. 1991). Although it will be apparent to most that a piece of paper (cellulose) will be much less susceptible to microbial decay than a similar amount of sugar (glucose), I would like to discuss some arguments for this assumption based on this thesis and other recent publications.

The main arguments arrive from a comparison of pools and their turnover times. Although the total carbon flux through the different pools of intermediates will be similar during the decomposition of a biopolymer in stable, undisturbed systems, the pool sizes and turnover times of individual compartments are different in sediments. As shown in this thesis, bulk glucose, which is mainly present as litter cellulose, has a pool size of around 1 mmol $C \cdot ml^{-1}$ sediment in the sediment top layer inside the reed bed of Lake Gooimeer and a turnover time of around 100 days (Chapter 7). The pool sizes of the intermediates formed during the decomposition of cellulose have a much lower pool size and are turned over much faster. Free concentrations of glucose the major hydrolysis product of cellulose were not higher than 0.4 nmol $C \cdot ml^{-1}$ under both aerobic and anaerobic conditions, and showed turnover times of about 3 min (Chapter 6). Similar ranges for glucose concentrations and turnover times were found by others (King & Klug

1982; Mopper et al. 1992; Sawyer & King 1993; Wicks et al 1991). Under anaerobic conditions this glucose will be first fermented to mainly acetate (Fig 1, Chapter 1). Although acetate pools and turnover rates were not determined in Lake Gooimeer sediments, literature data suggest that in eutrophic systems like Lake Gooimeer acetate concentrations will be in the range of 0.1 µmol $C \cdot ml^{-1}$ sediment and will have a turnover time of around 1 h (e.g. de Graaf et al 1996; Hordijk 1993; King 1991). These data for the different pools are in agreement with the idea that polymer hydrolysis is the rate determining step and that organic carbon once released from the polymer flows quickly through the rest of the decomposition chain. They also show that only the polymer accumulates to considerable amounts in sediments.

There are some exceptions to this general pattern. Several studies have found a transitional accumulation of acetate in sediments, especially during major shifts in environmental conditions like temperature and redox-conditions, or when organic loading suddenly changes or is extremely high (Alperin et al. 1994; Shannon & White 1996). It seems that during these events acetate consuming populations like methanogens or sulphate reducers are either inhibited or can not cope for some time with the increased production of acetate by fermenting bacteria, and that organic carbon mineralization to carbon dioxide is also limited by acetate consumption. No examples of short term accumulation of hydrolysis products like carbohydrates or amino acids are known so far from natural systems.

It has been shown that only a portion of the chemically-measured free concentrations of intermediates is available to micro-organisms; the remainder is probably adsorbed to some matrix (Gocke et al. 1981; King 1991; Wellsbury & Parkes 1995). Several authors have suggested that the existence of a non-bioavailable, adsorped pool limits degradation of the intermediates (see Henrichs 1992). Although the presence of a non-bioavailable pool will certainly have an effect on the turnover of the total intermediate pool as such, the question remains whether this will influence actual mineralization rates of bulk organic matter in sediments. It is more likely that carbon cycles through the bioavailable pool of the continuously produced intermediates and that the adsorbed pool does not participate in the turnover of the intermediate. In addition, non-bioavailable pools do not seem to increase with time or sediment depth, which should have been the case if the non-available pool was a net sink for organic matter. For instance, the non-bioavailable part of the total acetate concentration did not change much with depth in a marine sediment (Wellsbury & Parkes 1995).

If the initial enzymatic hydrolysis is indeed the rate limiting step in the decomposition, the question remains what intrinsic characteristics of polymeric organic matter do affect the rate of this step. Arnosti et al. (1994) recently showed that hydrolysis of several dissolved polysaccharides was faster than microbial uptake of hydrolysis products in sediments. Similar effects are sometimes found when polymer degrading

micro-organisms are grown on purified biopolymers (Ljundahl & Eriksson 1985). These observations suggest that the polymeric nature as such is not necessarily the determining factor in the degradability of biopolymers. An additional factor influencing degradation of polymers will be that biopolymers like lignocellulose are mainly found as particles. This does limit microbial degradation since the hydrolysable bonds in the centre of the particle will be shielded from the action of extracellular enzymes that can not penetrate the particle and hydrolysis is therefore limited to the exterior of the particle. Furthermore, biopolymers are seldom found in pure form in nature and as in lignocellulose are made of a composite of different molecules that might hamper each others decomposition. For macrophyte litter, it is generally found that a high initial lignin is negatively correlated to the decomposition rate (Taylor et al. 1989; Valiela et al. 1984). Moreover, the accumulation of more refractory compounds during litter decomposition does result in a decrease in decomposition rate with time (Wilson et al. 1986b).

In sediments and soils with a low organic matter content most organic matter will be found absorbed to the mineral matrix, especially to clays (Hedges & Keil 1995). Recently, Keil et al. (1994) showed that organic matter from 500 year old, marine sediments can be partially desorbed, and that ones desorbed it is degraded in a few days. This strongly suggests that organic matter mineralization is severely limited by absorption in these types of sediments and that desorption kinetics might determine decomposition rates. This might also explain why compound classes like carbohydrates, amino acids and lignin phenols which are thought to have different reactivities are apparently removed at similar rates in marine sediments (Cowie et al. 1992). It is less likely that sorption plays a role in sediments with relatively high organic matter content like the ones studied in this thesis or in other peat forming systems simply because there is not enough sorption surface available. Moreover, coarse reed litter will have to be first degraded or physically fractionated before it is small enough to absorb to clay particles. We indeed found that the turnover time for cellulose in Lake Gooimeer sediments was similar to cellulose turnover time in laboratory incubations with reed litter (Chapter 7).

Rates and methods

In this thesis, decomposition of organic matter has been studied by several methods both in sediments and in plant litter and results are summarised in Table 1. Turnover times in this table were calculated assuming that pseudo-first-order kinetics could be used. Pool average values are reported, which disregards that sub-pools with different reactivities will probably exist in sediments and reed litter.

Table 1. Comparison of results from methods used in this thesis to measure carbon mineralization rates and cellulose decomposition rates.

Process and methods[1]	Aerobic		Anaerobic	
	Rate[2]	Turnover time[3] (d)	Rate	Turn-over time (d)
(A) Carbon mineralization				
Microcalorimetry (sed., Ch 3)		910 ± 20		1700 ± 60
CO_2 production (litter, Ch 3)	2.7 ± 0.3	190 ± 20		
CO_2 production 10 d (lab., Ch 7)	1.9 ± 0.2	240 ± 25	0.71 ± 0.15	630 ± 130
CO_2 production 150 d (lab., Ch 7)	1.1 ± 0.2	400 ± 70	0.56 ± 0.05	800 ± 70
Litter decomposition (lab., Ch 3)		250 ± 10		500 ± 25
(B) Cellulose decomposition				
Toluene method July (sed., Ch 7)	0.24 ± 0.10	82 ± 14	0.20 ± 0.08	105 ± 20
Toluene method Nov. (sed., Ch 7)	0.94 ± 0.35	90 ± 12		
Toluene method (litter, Ch 6)	0.92 ± 0.12	75 ± 10	0.048 ± 0.003	1400 ± 90
Toluene method 10 d (lab., Ch 7)	0.94 ± 0.25	104 ± 28	0.21 ± 0.03	470 ± 70
Toluene method 150 d (lab., Ch 7)	1.92 ± 0.54	50 ± 14	0.61 ± 0.11	160 ± 30
Glucose removal (lab., Ch 7)		220 ± 50		
Glucose turnover (litter, Ch 6)[4]	0.18	390	0.046	1500
MUF-glu (sed., Ch 5)[5]	410, 960			
MUF-cel +GLN (sed., Ch 5)[5]	63, 132			

1 Sediment measurements (sed.) are for the top centimetre of the sediment 20 m inside the reed bed, except the microcalorimeter data which are for the top 3 to 4 centimetres. Litter measurements (litter) were done on recognisable pieces of litter collected from the top of the sediment. Laboratory measurements (lab.) were all done on standing litter collected in 1990 at the end of the growing season.

2 Carbon mineralization rates are in mg C·g DM^{-1}·d^{-1} and cellulose decomposition rates are in µmol glucose·g DM^{-1}·d^{-1}. All rates were normalised to a temperature of 15 °C using a Q^{10} of 2. Average ± SD.

3 Assuming first-order kinetics, turnover times were calculated either as the reciprocal value of the first order-rate constant or by dividing the pools size by the rate. Amounts of total, acid hydrolysable glucose were used as the cellulose pool size.

4 Glucose turnover rates were calculated dividing the maximum detected free glucose concentration by the turnover time of free glucose as determined using ^{14}C-labelled glucose (Chapter 6)

5 Data for enzyme activity measurements are other years but from similar months (July, November) as the sediment data using the toluene method.

Aerobic turnover times of organic carbon in reed litter were mostly around 200 d, with the exception of the 150 day incubations from Chapter 7 that showed a turnover time of 400 d. Turnover times for reed litter were faster than for sediment organic matter as determined by microcalorimetry, which suggested that part of the sediment pool was less reactive and probably existed of older, partially degraded reed litter. Degradation under anaerobic conditions was between 2 and 3 times more slowly than under aerobic conditions. In general, results of different methods for studying organic matter decomposition were in agreement with each other and showed consistent patterns.

Rates of aerobic cellulose decomposition as determined with the toluene method showed little variation and were between 50 and 100 d. In contrast with total organic matter, there was no difference in cellulose turnover time between sediment and reed litter. This suggested that mainly relatively fresh reed litter cellulose was degraded in Lake Gooimeer sediments. However, the decomposition rates as measured by toluene method were 2 to 4 times too high, when they are compared with glucose removal rates from reed litter. As discussed in Chapter 7, this discrepancy is most likely caused by the fact that toluene incubations were shaken, which generally stimulates microbial activities. It may be possible to inject toluene directly into sediment cores (de Graaf et al. 1996), which would give results closer to the *in situ* rates of polysaccharide decomposition. When sediment data for cellulose turnover are corrected for the 2 to 4 times overestimation caused by handling artefacts, results of the toluene method suggest that cellulose is selectively faster degraded than bulk sediment organic matter. Furthermore, comparing glucose hydrolysis rates with carbon mineralization data (Chapter 7) confirms that polysaccharide decomposition is one of the major mineralization processes inside the reed bed of Lake Gooimeer.

Anaerobic cellulose decomposition rates were rather variable but always lower than aerobic rates. It is likely that most of the variability in anaerobic rates was caused by differences in incubation techniques used. Surprisingly, the difference between anaerobic and aerobic rates was rather small for the sediment incubations in July. This is probably caused by the short preincubation period used (Chapter 7), which will not have been long enough for the enzymes and microbes to adapt to shifts in redox conditions. However, incubations with fallen litter always showed very large differences between anaerobic and aerobic rates. These incubations were made with field sampled fallen litter that was kept for some time in relatively large amounts. Under anaerobic conditions, it is possible that intermediates produced during this storage period, like volatile fatty acids, ammonia and sulphide, had an adverse effect on the microbial community and cellulose decomposition. Longer incubations that were made with relatively small amounts of dried standing litter showed ratios between anaerobic and aerobic rates of 3 to 4, which overlap with the ratios found for carbon mineralization.

Cellulose decomposition was also calculated from the turnover rates of dissolved glucose. Incubations were made with fallen litter and as discussed before the anaerobic rates derived from these incubations were probably adverse affected by accumulation of metabolic intermediates. The aerobic rates calculated from free glucose turnover rates are approximately 4.5 times too low when compared to data from the toluene method on similar litter incubations. There are two possible methodological reasons for this difference: either the turnover time as measured with ^{14}C-labelled glucose was too long, or the dissolved glucose concentration was underestimated. It should be noticed that the dissolved glucose concentrations used in the calculation were the highest in the range detected during litter incubations, and they are therefore already on the high side. It seems also less likely that the turnover time for free glucose was faster than measured since the methodology used results mostly in too short turnover times caused by sample handling times. An other possible explanation is that the model used to calculate glucose consumption rates is not correct. In this model it is assumed that all glucose that is hydrolysed from cellulose is released into the pore water. As discussed before, this is not a likely model from a substrate competition point of view, since non-cellulolytic microbes will be able to scavenge the released glucose. On the other hand, it is possible that only a part of the glucose is released into the pore water and the remainder is concentrated in a space between the cellulolytic microbe and the cellulose polymer to which it is attached. This will lead to an underestimation of the glucose concentration the microbes are experiencing, which would explain the low glucose turnover rates.

Enzyme activities as determined with MUF-labelled substrates were far higher than cellulose decomposition rates measured with the toluene method. This shows that enzyme activities measured with these artificial substrates do not reflect actual hydrolysis rates and should be regarded as potential values. It could be argued that lower concentrations of the artificial substrate should have been used than rate saturating amounts (Chapter 4), since these do not mimic the natural concentration. However, it is difficult to estimate the correct concentration, since one has to know the available pool size of the natural substrate and there is probably also a difference in reactivity between the natural and the added artificial substrates. Furthermore, MUF-labelled substrates were used at a concentration of 3 to 6 µmol glucose·ml^{-1} in this thesis, which is in fact much lower than the cellulose concentrations found in Lake Gooimeer sediments (around 200 µmol glucose·ml sediment^{-1}). Although enzyme activities do not give the correct *in situ* rates of biopolymer hydrolysis, the method can be used to compare activities at different sampling sites and to measure changes in activity with time. Also, the effects of various additions on the cellulolytic activities in sediment can be studied to characterise the enzymes present in sediments (Chapters 4 and 5).

In short, processes involved in the decomposition of organic matter and biopolymers in a littoral system have been studied in detail in this thesis. We showed that carbon cycling in a reed bed that covers a part of the littoral can be described in relative simple terms and functionalities. Decomposition of polysaccharides was one of the main processes fuelling the microbial community in the sediment of the reed bed that covered most of the land side of the littoral.

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Summary

This thesis deals with the microbial decomposition of organic matter in littoral sediments of lakes. Special attention was given to the initial step in the decomposition of polysaccharides that form a major component of macrophyte litter produced in these systems. This initial step, an extracellular enzymatic hydrolysis, is generally regarded as the rate limiting step in the decomposition of biopolymers in natural systems. The study site selected was an almost monospecific stand of common reed, *Phragmites australis*, that covers the upper littoral zone of Lake Gooimeer, The Netherlands.

The first two experimental chapters of this thesis deal with several general aspects of sedimentary carbon cycling, which are necessary for an implementation of a detailed study on the initial decomposition of biopolymers. In Lake Gooimeer, we could show that deep inside the reed bed, organic matter cycling was dominated by one source, namely macrophyte litter (Chapter 2). Plant litter is mainly made of lignocellulose, which has a rather simple and distinct polymeric composition. This makes the reed bed a good test system to study initial decomposition of biopolymers under natural conditions. Carbon sources gradually changed to an algal dominance going from inside the bed towards the lake (Chapter 2). This transition already begins inside the reed bed and no macrophyte derived material was found outside the bed, despite the high annual production of common reed.

Results of the budget study (Chapter 3) suggest that there are two major processes involved in the removal of sedimentary organic matter. Transport caused by erosion of the sediment during storms explains about 60% of the total carbon and only 30% was removed by mineralization. Mineralization rates were mainly determined by temperature, changes in oxic and anoxic conditions, and by amounts of organic matter present. Less than 5% of the annual production remained in the sediment over a period of 25 years. No short term accumulation could be detected during both years studied and results from 1992 even suggested a net removal of organic matter. This shows that sediment organic matter accumulation is variable and is governed by a delicate balance between input and outputs. Predicting sediment organic matter dynamics will be difficult in these type of ecosystems, since it depends to a large extent on episodic and variable sediment erosion during storms.

The remainder of the thesis deals with the initial decomposition of the two main reed-litter polysaccharides cellulose and arabino-xylan in sediments and the enzymes involved in this process. A method was developed to measure extracellular enzyme activities involved in cellulose decomposition by using artificial, fluorochrome labelled substrates (Chapter 4). Inhibition experiments with known substrates and inhibitors of cellulolytic enzymes were used to characterise the enzyme activities as measure in the assays. Results of the inhibition experiment suggested that the measured activity was of bacterial origin in the sediment used. This method was further extended to other enzymes and was used to

study temporal and spatial variability of enzyme activities during two years in the littoral sediments (Chapter 5). Absolute activities were mostly determined by organic matter input or content. β-Glucosidase activities were among the highest ever detected in sediments, which is probably the result of the high litter cellulose input to the sediment. Patterns of enzyme activities showed a distinct change in a gradient through the reed bed, which was consistent with the polymeric composition of dominant sedimentary carbon sources (Chapter 2). This suggests that patterns of enzyme activities may be used to study biologically available carbon sources in natural systems.

Although the enzyme assays are easy to use and allow information from a large number of samples to be gathered and compared, they give little information on the actual *in situ* hydrolysis rates of natural occurring polysaccharides in sediments. This is mainly caused by the use of an artificial substrate that may not be representative of the form and availability of the natural substrates. Therefore, a new method was developed to measure hydrolysis rates of naturally occurring polysaccharides in sediments. This new method is based on the selective inhibition of microbial uptake of hydrolysis products by 3% toluene without affecting the extracellular decomposition of polysaccharides. This approach was thoroughly tested (Chapter 6) and subsequently used to study initial decomposition of individual polysaccharides in reed litter and sediments (Chapter 7). The accumulation of hydrolysis products was followed over time by high-performance liquid chromatography, which resulted in a sensitive method with a high degree of resolution for the products formed.

A similar pattern of accumulating carbohydrates was found for litter and sediments (Chapter 7). Ratios between glucose and xylose accumulation rates suggested that arabino-xylan was degraded more slowly that cellulose, which was not in agreement with apparent rates of glucose and xylose removal from litter. Accumulation of heterogenic oligomeric compounds besides xylose during the decomposition of arabino-xylan may explain this discrepancy. If direct uptake of these hetero-oligomers can be shown, it would have implications for carbon flow and the associated microbial populations. The turnover time of particulate glucose was estimated at 85 ± 14 d in the top centimetre of the sediment, and showed a three to four fold increase with depth. Comparison between glucose accumulation rates as a measure of cellulose decomposition and total carbon mineralization rates suggested that cellulose decomposition was a major process in the mineralization of organic matter in littoral sediments.

Finally, implications of these studies are discussed in view of general functioning of littoral zones and the mechanisms of polymer decomposition in natural systems (Chapter 8).

Samenvatting

Afbraak van organisch materiaal in de litorale sedimenten van een meer.

H.T.S.Boschker

Het litoraal is de overgangszone van water naar land en combineert daardoor kenmerken van beide ecosystemen. Zo is het sediment in deze zone meestal met water verzadigd, waardoor zuurstof maar beperkt door kan dringen en het sediment grotendeels zuurstofloos is. Daarnaast is het sediment aan de landzijde begroeid met hogere planten, zoals riet (*Phragmites australis*) en lisdodde (*Typha* soorten). Rietkragen vormen een belangrijk onderdeel van het Nederlandse landschap, maar vertonen in grote delen van het land een achteruitgang. Deze vegetaties hebben een hoge produktiviteit hebben maar worden zelden direct begraasd door dieren. Het grootste deel van het plantenmateriaal zal daardoor in de herfst afsterven en uiteindelijk op het sediment terecht komen als rietstrooisel. Hier wordt het vervolgens afgebroken door micro-organismen, zoals bacteriën en schimmels. Één-cellige micro-organismen zijn echter niet in staat om grote stukken strooisel direct op te nemen. Ze scheiden daarom enzymen uit die de biologische polymeren in riet eerst afbreken tot eenvoudige verbindingen, om ze vervolgens op te nemen. Deze stap wordt de initiële afbraak van polymeren genoemd en is belangrijk omdat algemeen wordt aangenomen dat het de snelheidsbeperkende stap in het afbraakproces is.

Dit proefschrift behandelt de initiële afbraak van organisch materiaal in de litorale sedimenten van het Gooimeer, één van de randmeren ontstaan bij de inpoldering van het IJsselmeer. Bij de start van het onderzoek was er weinig bekend over de algemene dynamiek van het sedimentair organisch materiaal in deze litorale zone, terwijl dit soort informatie nodig was voor een gedetailleerde studie naar het initiële afbraakproces. In hoofdstuk 2 is bepaald of het organisch materiaal in het sediment alleen afkomstig is van rietstrooisel of dat er nog andere bronnen van belang zijn. In het litoraal komen namelijk ook diverse algen en ondergedoken waterplanten voor, die ook organisch materiaal produceren dat weer afgebroken wordt door micro-organismen. We hebben aannemelijk kunnen maken dat diep in de rietkraag alleen rietstrooisel van belang is als bron voor het sediment organisch materiaal. Meer naar de rand van de rietkraag worden algen steeds belangrijker, totdat rietstrooisel buiten de rietzone geen rol meer speelt en het organisch materiaal in het sediment geheel gedomineerd wordt door materiaal afkomstig van algen.

De resultaten van een koolstofbalansstudie in het tweede algemene hoofdstuk (Hoofdstuk 3) laten zien dat er twee belangrijke processen zijn die bijdragen aan het verwijderen van sediment organisch materiaal. Het was verrassend dat verreweg het grootste deel (ongeveer 60%) van het materiaal wegspoelt tijdens stormen en dat afbraak processen maar voor ongeveer 30% bijdragen aan de verdwijnen van sediment organisch

materiaal. Afbraakprocessen werden voornamelijk beïnvloed door de temperatuur, door verschillen in zuustofindringing van het sediment (veroorzaakt door waterpeil veranderingen) en door veranderingen in de hoeveelheid organisch materiaal. Minder dan 5% van de jaarlijkse rietproduktie werd voor langere tijd vastgelegd in het sediment. Gedurende de duur van het onderzoek kon geen opbouw van organisch materiaal worden gemeten, en resultaten van 1992 laten zien dat er zelfs netto materiaal verdween. Dit suggereert dat koolstofvastlegging in deze sedimenten het resultaat is van een delicate balans tussen aanvoer en afvoer. Het voorspellen van het verlandingsproces zal moeilijk zijn omdat het voornamelijk bepaald wordt door de intense en kortdurende erosie van het sediment tijdens stormen.

De hoofdstukken 4 to 7 zijn gewijd aan de initiële afbraak van biologische polymeren in rietstrooisel. De belangrijkste polymeren in riet zijn lignine en twee polysacchariden, cellulose en arabino-xylan. Het onderzoek heeft zich voornamelijk gericht op de afbraak van deze twee polysacchariden. In Hoofdstuk 4 is een methode beschreven om in sediment activiteiten te bepalen van extracellulaire enzymen die bij de afbraak van cellulose betrokken zijn. Uit de karakterisering van deze enzymactiviteiten blijkt dat deze in het sediment van de rietzone waarschijnlijk voornamelijk van bacteriën afkomstig zijn en niet van schimmels. Deze methode is vervolgens uitgebreid naar andere typen enzymen en gebruikt om de variatie in enzymactviteiten in plaats en tijd te onderzoeken in de litorale zone (Hoofdstuk 5). Absolute activiteiten werden voornamelijk bepaald door de veranderingen in hoeveelheid sediment organisch materiaal. Activiteiten van β-glucosidases, enzymen die bij de afbraak van cellulose betrokken zijn, waren hoog in vergelijking met waarden gevonden in de literatuur. Waarschijnlijk kan dit verklaart worden uit de hoge aanvoer van cellulose bevattend rietstrooisel. Het patroon van de verschillende enzymactiviteiten veranderde sterk in een gradiënt door de rietzone. Dit hing nauw samen met de polymeersamenstelling van de dominante bronnen van sediment organisch materiaal zoals beschreven in hoofdstuk 2. Patronen van enzymactiviteiten kunnen daarom gebruikt worden om biologisch beschikbare bronnen op te sporen in natuurlijke systemen.

Metingen van enzymactiviteiten zijn eenvoudig uit te voeren, waardoor informatie van een groot aantal monsters verzameld en vergeleken kan worden. Het probleem is echter dat de gemeten activiteiten weinig informatie geven over de snelheid waarmee biologische polymeren in het sediment worden afgebroken. De belangrijkste reden hiervoor is dat bij de enzymactiviteitsmetingen een kunstmatig substraat gebruikt wordt, dat meestal niet representatief is voor de vorm en de beschikbaarheid van het natuurlijke substraat. Daarom is een methode ontwikkeld waarmee afbraaksnelheden van de natuurlijke polysaccharide in rietstrooisel en sedimenten bepaald konden worden. De methode is gebaseerd op remming van de suikeropname door bacteriën met tolueen, zonder dat daarbij de extracellulaire afbraak van polysacchariden verstoord wordt. De accumulatie van afbraakprodukten (suikers) is vervolgens bepaald met hoge-druk vloeistof chromatografie, waarmee een heel scala aan afbraakproducten bepaald kan

worden. De tolueen methode is uitgebreid getest (Hoofdstuk 6) en vervolgens gebruikt om afbraaksnelheden van polysacchariden in rietstrooisel en in sediment te bepalen (Hoofdstuk 7). Het patroon van de geproduceerde suikers was vergelijkbaar voor rietstrooisel en sedimenten in de rietkraag. Glucose afkomstig van cellulose is het belangrijkste produkt dat gevormd werd. Hoge ratios tussen glucose en xylose produktie suggereerden dat cellulose veel sneller werd afgebroken dan arabino-xylan; de belangrijkste bron voor xylose. Dit was echter niet in overeenstemming met de snelheid waarmee glucose en xylose uit rietstrooisel verdwenen. Deze discrepantie werd mogelijk veroorzaakt door de produktie van oligomere suikers tijdens de afbraak van arabino-xylan. De omzettingstijd voor cellulose in sediment is geschat op 85 ± 14 d voor de toplaag van het sediment en neemt toe met een factor drie à vier in de diepere lagen. Wanneer de afbraaksnelheid van cellulose vergeleken wordt met de snelheid waarmee het totale sediment organisch materiaal wordt afgebroken, blijkt dat cellulose afbraak één van de belangrijkste processen in het sediment van de rietzone was.

De resultaten van deze studies worden tenslotte bediscussieerd in relatie tot het functioneren van rietkragen en het mechanisme van polymeer afbraak in natuurlijk systemen (Hoofdstuk 8).

Curriculum vitae

Op 7 mei 1962 werd ik geboren in het Brabantse Mierlo als Henricus Theodorus Siegmund Boschker, roepnaam Eric. Ik doorliep de HAVO op het Marianum te Groenlo, en vervolgens het VWO op het Maurick College te Vught. Na een jaar onder de wapenen, ben ik in 1983 begonnen aan mijn studie Milieuhygiëne aan de Landbouwuniversiteit Wageningen. In 1989, werd het ingenieurs diploma gehaald met als specialisaties microbiologie, aquatische ecologie, waterzuivering en ecotoxicologie/milieuchemie (stage RIZA, Lelystad). Ik werkte toen al een maand in wat toen nog werkgroep Mineralisatie van Organisch Materiaal van dr. Tom Cappenberg (Limnologisch Instituut Nieuwersluis) heette en de resultaten van het aldaar verrichte onderzoek zijn in dit proefschrift beschreven. Direct daarop in 1994, heb ik ruim een jaar binnen de werkgroep Microbiële Oecologie onder prof. dr. Riks Laanbroek gewerkt op hetzelfde instituut, dat ondertussen het Centrum voor Limnologie van het Nederlands Instituut voor Oecologisch Onderzoek was geworden. In het kader van een EEG-project heb ik onderzoek gedaan aan de identificatie van populaties van azijnzuur consumerende bacteriën in sedimenten. Op het ogenblik werk ik aan de koppeling tussen wortelproductie van schorplanten en microbiële processen in het sediment van deze schorren in de werkgroep Littorale Vegetaties onder dr. Martin Hemminga op het Centrum voor Estuariene en Mariene Oecologie van het Nederlands Instituut voor Oecologisch Onderzoek te Yerseke.