Carbon cycling in benthic diatom mats: Novel applications of LC/IRMS

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LC/IRMS analysis: A powerful technique to trace carbon flow in microphytobenthic communities in intertidal sediments

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Abstract

Microphytobenthic communities are important for primary production in intertidal marine sediments. Extracellular polymeric substances (EPS), comprising polysaccharides and proteins, play a key role in the structure and functioning of microphytobenthic biofilms and allow interactions between the benthic microalgae and the associated heterotrophic bacteria. The use of stable isotopes has provided major insights into the functioning of these microbial ecosystems. Until recently, gas chromatography - isotope ratio mass spectrometry (GC/IRMS) was the principal method for compound specific stable isotope analysis in these studies. Liquid chromatography linked to IRMS (LC/IRMS) is a more recently developed technique that broadens the range of compounds that can be targeted, in particular enabling the analysis of $^{13}$C in non-volatile, aqueous soluble organic compounds, such as carbohydrates and amino acids. In this paper we present an overview of the possibilities and limitations of the LC/IRMS technique to study metabolic processes in microphytobenthic biofilms consisting of mainly diatoms. With a preliminary in-situ labeling experiment, we show that the biosynthesis of carbohydrates and amino acids in EPS and total carbohydrate and amino acid pools can be determined by LC/IRMS. Water-extractable EPS were composed predominantly of carbohydrates, whereas amino acids played a minor role, both in terms of content and production. By using LC/IRMS, we will be able to quantify the biosynthesis of metabolites and, hence, to unravel in detail the metabolic pathways of the transfer of carbon from the diatoms via EPS to the bacteria.
Introduction

Coastal zones and estuaries play an important role in the global carbon cycle (Gattuso et al., 1998). Intertidal mudflats that are abundantly present in estuaries are amongst the most productive systems on earth providing feeding and nursery grounds for many species of birds, fish and shellfish. For a large part, these higher trophic level organisms depend on the primary production by microphytobenthos (MPB). During emersion, MPB form a temporary biofilm on the sediment surface and supply organic matter by photosynthesis (Underwood et al., 2005). It has been calculated that MPB may contribute up to 50 % of the total primary production within estuaries (Cahoon, 1999; Underwood & Kromkamp, 1999). A large number of studies endorse the complexity of the functioning of the microphytobenthic biofilm (Du et al., 2009; Hicks et al., 2011; Jesus et al., 2009; Kromkamp et al., 2006; Mouget et al., 2008; Paterson & Hagerthey, 2001; Stal, 2010; Vieira et al., 2011; Walters & Moriarty, 1993).

In temperate regions, MPB biofilms mainly consist of diatoms and associated bacteria, interacting with each other via extracellular polymeric substances (EPS) (Underwood et al., 2005). EPS are therefore important compounds in microphytobenthic biofilms and play a major role in the structure and functioning of the community (Stal, 2010). Recent studies have shown the importance of EPS exudation for the development of the biofilm (Bruckner et al., 2011). These authors showed that diatom growth and EPS production is influenced by the presence of certain heterotrophic bacteria. It has even been suggested that microphytobenthic biofilms are only formed in the presence of such bacteria (Bruckner et al., 2008).

EPS may occur in a range of different size classes (De Brouwer & Stal, 2001; Underwood and Paterson, 2003). Beside polysaccharides, EPS may also contain proteins, lipids and nucleic acids (Flemming & Wingender, 2010). Intracellular and extracellular carbohydrates (CHO) and amino acids (AA) play a central role in the functioning of MPB and their interaction with the bacterial community. During photosynthesis, glucose is produced and initially stored as an intracellular β-glucan, and subsequently further metabolized for the synthesis of amino acids and proteins and other structural cell materials of the diatoms. In addition, CHO and AA may both be exuded as EPS that then becomes available to the microbial community and finds its way further into the food web. EPS can be distinguished in two operationally defined fractions, water- and EDTA-extractable EPS (described in detail by de Brouwer & Stal (2001)). These fractions differ substantially in their composition and their
production seems to be under a different metabolic control. Water extractable EPS are probably intimately associated with the diatoms and can be readily used by heterotrophic bacteria, whereas EDTA-extractable EPS seem to be tightly bound to the sediment (Stal, 2003), and are more recalcitrant to microbial degradation (Domozych et al., 2005). Thus far, most studies focused on the measurement of the content of EPS fractions in the sediment but thereby neglecting production and turnover rates of these exudates.

At present, stable isotopes (SI) techniques are the state-of-the-art for the study of the carbon cycle in microphytobenthic biofilms. Stable isotope analysis can be used in two different ways for the study of MPB. First, at natural abundance level, the isotopic signature of an organism provides insight into the nature of the carbon sources that were used by comparing the isotopic signature of the organism with that of the available sources. For instance, Bouillion and Boschker (2006) collected natural abundance data of $\delta^{13}$C bacterial biomarker phospholipid-derived fatty acid (PLFA) to study bacterial carbon sources in a range of coastal sediments. A reanalysis of their data for bare intertidal areas showed that the bacteria present in intertidal marine sediments strongly depend on carbon derived from benthic diatoms (Fig. 5.1). Secondly, an isotopically enriched tracer compound can be added to the sediment surface and its fate can be subsequently followed in different carbon pools. This approach conveys important kinetic information of specific metabolic processes. The rate of incorporation of the isotopic label in a compound gives information on the synthesis. Similarly, the subsequent decay of the label provides information on the absorption and the rate of degradation of the same compound. By following the evolution of the label in a number of important pools, one can also deduce the pathway of carbon flow. A major benefit of the use of stable isotopes in environmental experiments is the ability to use them directly in the field, which yields more relevant in-situ information on the functioning of MPB. In order to understand the complex carbon flow in microphytobenthic biofilms it is important to be able to measure the stable isotopes in specific metabolic intermediates of the intracellular and extracellular pathways.
Fig. 5.1. Natural abundance $\delta^{13}C$ ratios of bacterial phospholipid-derived fatty acid (PLFA) in non-vegetated intertidal marine sediments. Results show that the sediment bacteria depend to a large extent on carbon derived from benthic diatoms as their enriched isotopic signal is more related to benthic diatoms than to phytoplankton derived material. Results are based on a reanalysis of the data presented in Bouillon and Boschker (2006).

Until recently, GC/IRMS was the principal technique to perform compound specific SI analysis (CSIA). The majority of SI studies published have dealt with various aspects of lipid biochemistry, such as the metabolism of free saturated and unsaturated fatty acids (Bellinger et al., 2009; Bouillon and Boschker, 2006; Evrard et al., 2012; Evrard et al., 2008; Oakes et al., 2012). GC/IRMS analysis of fatty acids is a simple and straightforward technique. Fatty acids are extracted prior to esterification to form fatty methyl esters (FAMEs) that makes them suitable for GC/IRMS analysis. Carbohydrates and amino acids can only be analyzed by GC/IRMS after heavy derivatisation, which makes them more volatile and less polar. A drawback of this technique is that some derivatives of CHO and AA are unstable and also substantial corrections of the measured stable isotopic composition are necessary because a large amount of extra carbon is added during derivatisation. Moreover, some protocols such as acetylation cause additional kinetic fractionation (Rieley, 1994). Fortunately, both carbon dilution and fractionation are reproducible and therefore recalculation to the original isotopic composition is possible. However, these corrections add uncertainties to the determination of the original isotopic ratios, decreasing accuracy and precision of the method. The need of derivatisation for CHO and AA analysis by GC/IRMS also substantially increases the complexity of the analytical procedures. This
combination of drawbacks and restrictions may be the reason that only a few studies have been published that directly traced carbon flow from diatoms to bacteria (Bellinger et al., 2009; Evrard et al., 2008).

Liquid chromatography (LC) eliminates many of the drawbacks of GC because compounds can be analyzed directly without the need for derivatisation. However, it was not until 2004 that an interface that coupled LC to IRMS became commercially available and the first applications of LC/IRMS were developed (Krummen et al., 2004). In the past years, a substantial number of applications to study metabolites have been published emphasizing the power and robustness of LC/IRMS for the analysis of amino acids, peptides, carbohydrates, fatty acids and nucleic acids (Boschker et al., 2008; Cabanero et al., 2010; Godin et al., 2008; Godin et al., 2005; Heuer et al., 2006; McCullagh et al., 2008; Schierbeek et al., 2007; Smith et al., 2009). Especially analysis of carbohydrates, amino acids and nucleic acids experience major benefits from the use of LC/IRMS because it is not necessary to make derivatives, avoiding laborious sample preparation. This all leads to more accurate results.

The aim of this paper was to provide an overview of the possibilities of the use of LC/IRMS for the study of metabolic processes in microphytobenthic biofilms. We also present a preliminary in-situ study on EPS dynamics in microphytobenthic biofilms by using LC/IRMS.
Analytical techniques

Isotope Ratio Mass Spectrometry

Currently, the most precise and accurate method for stable isotope analysis is isotope ratio mass spectrometry (IRMS). Due to this design, the precision of the isotope ratio is a few parts per thousand (0.0001 – 0.0003 %). Before a sample can be introduced into an IRMS it must be converted to a gas (N₂, CO₂, H₂, SO₂). This can be done by bulk combustion followed by separation of the produced gasses, or by chromatographic separation of the components of interest followed by online conversion of each single component to the required gasses, which is called compound specific stable isotope analysis (CSIA).

In the past, the measurement of the stable isotope ¹³C was performed with a manual procedure to convert the sample into CO₂ gas. Now it is possible to connect an IRMS to different preparation and separation instruments such as an elemental analyzer (EA) providing bulk data, and a gas chromatograph (GC) providing compound specific data. In 1988 the first commercially GC/IRMS system became available (Brand et al., 1989; Hayes et al., 1989). Middelburg et al. (2000) were the first to use in-situ ¹³C labeling to study the transfer of carbon from MPB to heterotrophic bacteria by using GC/IRMS analysis of biomarker PLFA.

LC/IRMS

Linking a GC directly to an IRMS system is possible because all compounds leave the separation column in the gaseous phase in an inert helium flow. The gas can be combusted and introduced into the IRMS after the removal of water. However, connecting an LC to an IRMS is more complicated because the compounds of interest need to be separated from the liquid eluent before being introduced in the IRMS. After several less successful attempts (Abramson et al., 2001; Brenna et al., 1997; Caimi & Brenna, 1993; McLean et al., 1996; Teffera et al., 1993), a wet oxidation interface linking LC to IRMS was developed by (Krummen et al., 2004). As illustrated in figure 5.2, the oxidation of the organic molecules into carbon dioxide (CO₂) gas is performed in a heated reactor where acid (phosphoric acid), oxidant (sodium peroxidisulfate) and LC eluent are mixed. Via chemical oxidation all eluting compounds are converted to CO₂, which is transferred from the eluent into a helium flow with a membrane separator. Finally, water vapor is removed and the purified CO₂ flow is carried into the ion source of the mass spectrometer. Currently, there are two available interfaces: the Isolink (Thermo Fisher,
Bremen, Germany) and the LiquiFace, a more recent development (Isoprime, Cheadle Hulme, UK (Morrison et al., 2010)).

The design of the LC/IRMS interface involves a number of analytical constraints. The current LC/IRMS systems are not compatible with organic and other carbon-containing eluents, preventing the use of many of the traditional LC methods. Organic solvents cannot be used because the continuous oxidation in the reactor unit would create an extremely high CO₂ background. The composition of the mobile phase is therefore restricted to inorganic acids, bases and buffers dissolved in high-quality Milli-Q water. Furthermore, the selection of the analytical column is important because column bleeding should be low, as the release of the bonded phase of the column during analysis also causes high and unstable background signals (Godin et al., 2007; McCullagh, 2010). These analytical constraints, together with the requirement of baseline separation of components in order to obtain accurate isotopic measurement of the compounds, are challenging the development of analytical methods. Fortunately there is an increase in the development of columns suitable for separating components in aqueous solutions such as mixed mode columns (McCullagh, 2010). Another limitation to take into account is that LC/IRMS is hitherto restricted to ¹³C analysis whereas GC/IRMS is able to measure multiple elements, such as ¹⁵N/¹⁴N, ¹⁸O/¹⁶O and ³H/²H, isotope ratios in addition to ¹³C/¹²C. Nevertheless, the introduction of the LC/IRMS technique has opened a new avenue for the study of a broad range of biological compounds.

The LC/IRMS system can also be used without column separation and via direct injection into the flow path of the system, bulk δ¹³C values can be determined. This feature is called flow injection analysis IRMS (FIA/IRMS) (McCullagh et al., 2011). The major advantage of FIA/IRMS over EA/IRMS is that it requires a lower amount of sample. Due to the more efficient sample transfer to the IRMS, typically 50-500 ng of carbon is required for FIA/IRMS compared to a few µg for EA/IRMS, to achieve a standard deviation lower than 0.3 ‰ (Boschker et al., 2008; Godin et al., 2005).
**Fig. 5.2.** Principles of the LC/IRMS interface. Compounds separated by LC are converted to CO$_2$ by wet-chemical oxidation. In the separation unit, this CO$_2$ is transferred from the liquid eluent into a helium flow. Before the CO$_2$ flow is carried into the isotopic mass spectrometer (IRMS) the water vapor is removed (Krummen et al., 2004).
Applications of LC/IRMS in metabolic studies

Carbohydrates
In organisms, polysaccharides have a primary function as structural components and as storage compounds. Because cellular and extracellular production of carbohydrates (CHO) plays a central role in the functioning of microphytobenthic biofilms, stable carbon isotope analyses of these compounds are important for the identification of carbon sources, their turnover rate in the sediment and tracing them into microbial biomass. Monomeric carbohydrates are found in many different forms including neutral carbohydrates, uronic acids and amino sugars. Neutral carbohydrates play an important role in the bulk CHO and in the MQ extracted EPS fraction, whereas uronic acids play an important role in the EDTA extractable EPS fraction (de Brouwer and Stal, 2001).

The carbohydrate polymers can be hydrolyzed to monomers (details see Material and Methods section). Cabanero et al. (2006) developed a method to analyze carbohydrates by LC/IRMS. They detected a limited number of sugars, i.e. sucrose, glucose and fructose. Boschker et al. (2008) extended the separation of carbohydrates by LC/IRMS to the most important monosaccharides and uronic acids that play a role in microphytobenthic biofilms. This method was applied to follow $^{13}$C labeling of carbohydrates in sediments by Oakes et al., 2010.

Amino acids
Similar to the case for carbohydrates, amino acids are equally important compounds in microphytobenthic biofilms. Amino acids are key building blocks of proteins but can also be present as free metabolites and in EPS. Godin et al. (2005) developed an LC/IRMS method to analyze amino acids. This method was improved by McCullagh et al. (2006) and finally optimized by Smith et al. (2009), who were able to separate all biological amino acids such as those found in microphytobenthic biofilms.

Short chain organic acids
Short chain organic acids (SCOA) such as acetic acid play an important role in the central metabolism of MPB mats. Acetate is a key metabolite in anaerobic metabolism and in the cycling of organic carbon in marine sediments. Acetate is produced by fermentation of organic matter and also by reduction of CO$_2$ by acetogenic bacteria. Concentrations in the pore water of surface sediments tend to be low, around 10 μM, because of rapid turnover (Heuer et al., 2009). In addition, various SCOA are important intermediates in the central metabolism of all organisms and are known to
be important as exudates of plant roots (Carvalhais et al., 2011). Acetate and other SCOA are important substrates for heterotrophic bacteria (Sundh, 1992). Therefore, the measurements of these compounds could provide valuable information on the diatom-bacteria interaction. By sampling the pore water of the sediment, 10 different SCOA such as citrate, malate, succinate, acetate and lactate can be directly determined by using LC/IRMS (Heuer et al., 2006).

Applications of FIA/IRMS
As mentioned above, the LC/IRMS interface can also be used to measure bulk carbon isotope ratio determination by injecting off-line extracted compounds directly into a continuous flow of the mobile phase. An example is the extraction of specific 16S ribosomal RNA from marine sediment by magnetic bead capture hybridization (Miyatake et al., 2009). 16S rRNA, a component of the prokaryotic ribosome, is an excellent phylogenetic marker and the analysis of $^{13}$C in this molecule is an attractive approach to identify the groups of bacteria that are involved in the coupling of carbon flow between benthic diatoms and heterotrophic bacteria.

The FIA mode of the LC/IRMS can also be used to analyze $\delta^{13}$C of dissolved organic carbon (DOC) in aqueous solutions (Alberic, 2011). Thus far, this analysis is restricted to samples with salinities lower than 1‰ because at higher salinity the samples are incompletely oxidized by the interface.
Materials and Methods

Field site
In February 2011, an in-situ $^{13}$C-labeling experiment was performed at the Zandkreek mudflat situated along the southern shore of the Oosterschelde estuary in the South-West of the Netherlands (51°32′41″N, 3°53′22″E). The sampling site was located 0.15 m below the mean tidal level and the exposed period was approximately 6 h per tidal cycle. Salinity and sediment temperature were 28.5 and 4.5 °C, respectively. During the experiment a diatom biofilm was present on the surface, varying in density depending on the time of the day, probably because of migration of the diatoms.

$^{13}$C labeling and sampling
The experiment was started shortly after the immersion period. Two 50 × 50 cm stainless steel frames were pushed into the sediment to a depth of 8 cm in order to define the labeling and the sampling area. The two frames were treated as duplicates. Initially, unlabeled control samples were taken just outside the frames as described below. The in-situ labeling experiment was started by spraying the surface of the sediment within each frame with 200 mL of $[^{13}$C] sodium bicarbonate (99 % $^{13}$C; Cambridge Isotope Laboratories, Andover, USA) with ambient salinity to obtain a final concentration of 1 g $^{13}$C m$^{-2}$ (Middelburg et al., 2000).

Label incorporation was measured in samples taken 4 h after the label was sprayed on the sediment. The top 1.5 cm of the sediment was sampled by pushing a core liner (inside diameter 10 cm) into the sediment and sampling the top of the sediment with a spatula. Samples were collected and mixed from two randomly chosen positions within the sampling grid of each frame. Sediment samples were divided in portions of approximately 10 g wet weight each. Samples for TOC, bulk carbohydrate and bulk amino acid analysis were directly frozen in liquid N$_2$ and after lyophilization stored at -20 °C prior to analysis. Sediment samples for EPS carbohydrate extraction and EPS amino acid extraction were immediately transferred to the laboratory and within 30 minutes after sampling processed as described by de Brouwer and Stal (2001). Two operationally defined EPS fractions were distinguished: EPS MQ and EPS EDTA, and were analyzed for both carbohydrates and amino acids.

Analytical procedures
Carbon content and isotopic composition of TOC were analyzed by using an elemental analyzer/isotope ratio mass spectrometer (EA/IRMS)
LC/IRMS to trace carbon flow

(Boschker et al., 1999). Carbon content and isotopic composition of carbohydrates and amino acids in bulk sediment, EPS MQ and EPS EDTA fractions were analyzed by LC/IRMS (Boschker et al., 2008; McCullagh et al., 2006). For carbohydrates, freeze dried sediment (500 mg, for bulk analysis) and MQ and EDTA EPS extracts (4 mL) were hydrolyzed to monosaccharides under acidic conditions using a modified method according to Cowie and Hedges (1984). Instead of neutralizing the hydrolysates with barium carbonate, the samples were neutralized with strontium carbonate, which resulted in an increase yield of the extract. EDTA was removed from the EDTA EPS hydrolysate with an onGuard IIA cartridge (Dionex, Breda, the Netherlands) brought into the chloride form. After applying 1 mL of sample, the column was washed with 20 mL MQ. Both volumes were combined, freeze-dried and finally dissolved in 750 μL of MQ before injection into the LC/IRMS. For amino acids, freeze dried sediment (700 mg, for bulk analysis) and MQ and EDTA EPS extracts (4 mL) were hydrolyzed with 6 M HCl for 20 h at 110 °C and were purified by cation exchange chromatography (Veuger et al., 2005) before they were analyzed by LC/IRMS.

Liquid chromatography was carried out using a Surveyor liquid chromatograph connected to an LC Isolink interface and a Delta V Advantage IRMS (all from Thermo Fisher, Bremen, Germany). Isodat 3.0 software was used to control the LC/IRMS system. Samples were injected with an autosampler using partial loop injection. Carbohydrate concentrations and isotope ratios were analyzed by LC/IRMS equipped with a CarboPac PA20 column (Dionex, Breda, the Netherlands). The eluent was 1 mM sodium hydroxide at a flow rate of 0.3 mL min⁻¹. The carbohydrate method has been described in detail by Boschker et al. (2008). Amino acid separation was done on a Primseep A column (4.6 mm × 250 mm, particle size 5 μm (SIELC technologies, Prospect Heights, IL, USA). Elution was by a linear gradient containing two mobile phases: MQ and 0.2 % sulfuric acid at a flow rate of 700 μL min⁻¹. The amino acid method is described in detail by McCullagh et al. (2006).

Calculations

The isotopic abundance of a sample was calculated relative to a reference. At the level of the natural abundance the variation in the isotopic ratio is so small that it is convenient to express the variation of isotopic ratio, the δ¹³C value, in per mill (‰). The practical advantage of using the δ¹³C (‰) notation instead of the ¹³C/¹²C ratio notation is that small variations of the digits after the decimal point are easier to handle.
The delta ‰ notation is defined as
\[
\delta^{13}\text{C}_{\text{sample}} \text{ (‰)} = [(R_s / R_{st}) - 1] \times 1000
\]

where \( R_s \) is the ratio of \(^{13}\text{C}/^{12}\text{C}\) in the sample and \( R_{st} \) is the ratio of the international standard used (for carbon \( R_{st} = 0.0111802 \pm 0.0000009 \)). For metabolic studies it is more convenient to calculate the absolute amount of \(^{13}\text{C}\) incorporated into different carbon pools over the background. This value is expressed as excess \(^{13}\text{C}\) and is calculated from \( \delta^{13}\text{C}_{\text{sample}} \) as:

\[
\text{Excess }^{13}\text{C} \text{ (mol }^{13}\text{C g}^{-1} \text{ DW}) = \frac{\left( \frac{\delta^{13}\text{C}_{\text{sample}}}{1000+1} \times R_{st} \right) - \left( \frac{\delta^{13}\text{C}_{\text{background}}}{1000+1} \times R_{st} \right)}{\left( \frac{\delta^{13}\text{C}_{\text{sample}}}{1000+1} \times R_{st} + 1 \right)} \times C_{\text{sample}}
\]

where \( \delta^{13}\text{C}_{\text{background}} \) is the \( \delta^{13}\text{C} \) value of the unlabeled sample and \( C_{\text{sample}} \) is the pool size in mol of carbon per gram of dry weight sediment (mol C g\(^{-1}\) DW).
Results and Discussion

We successfully applied the available methods analyzing $^{13}$C in carbohydrates and amino acids to study carbon flows in microphytobenthic mats. The chromatograms demonstrated a satisfactory separation of CHO and AA in the bulk, MQ extractable EPS and EDTA extractable EPS (Fig. 5.3 A, B). Although peak areas of EPS MQ and EPS EDTA were low, they were sufficient to determine $^{13}$C labeling (Godin et al., 2005). Peak areas for bulk CHO and bulk AA were considerably higher. In general the $\Delta \delta$ values after 4 h of labeling were on average not higher than 500 ‰.

All carbohydrates were baseline separated except fucose, which elutes at the end of the slope of a large peak in the beginning of the chromatogram originating from mainly amino acids. In order to improve the baseline separation of fucose, the intensity of the AA peak can be decreased by cation exchange purification as described in the materials and methods section. Glucose, the isotopic most enriched carbohydrate in the bulk fraction, is a key component of intra- and extracellular polysaccharides produced by MPB. Glucose is also the most important component of the EPS MQ fraction in terms of content and production. The EPS EDTA fraction was more diverse in terms of both carbohydrate content and production (Table 5.1). These findings are in line with other published data (Bellinger et al., 2009; de Brouwer & Stal, 2001; Underwood & Paterson, 2003).

For amino acids, 8 of the 14 eluting components were baseline separated. Optionally up to 21 components could be baseline separated utilizing the chromatographic conditions described by Smith et al. (2009), but this would more than double the runtime to 4 hours. Proline was the most important component in the EPS MQ fraction both in terms of content and production. In the EPS EDTA fraction methionine dominated in terms of content followed by threonine, which dominated in terms of production.
Fig. 5.3. Examples of LC/IRMS chromatograms demonstrating the separation of extracellular carbohydrates (A) and amino acids (B) in the MQ extractable EPS fraction obtained from an enriched intertidal mudflat sediment. Label incorporation for each component is shown in excess $^{13}$C (nmol $^{13}$C g$^{-1}$ DW) for samples taken 4 h after the label was sprayed on the sediment. Shown is the analysis of 6 different carbohydrates and 14 amino acids (i.e. alanine (ala), arginine (arg), asparic acid (asp), glycine (gly), histine (his), isoleucine (ile), leucine (leu), lysine (lys), phenylalanine (phe), proline (pro), serine (ser), tyrosine (tyr), valine (val), hydroxyproline (hyp)), *=unknown, RP=reference pulses.
Table 5.1. Carbohydrate (CHO) and amino acid (AA) content and production in MQ and EDTA extractable EPS extracted from an intertidal marine sediment after 4h in-situ $^{13}$C labeling. LC/IRMS data is given of 6 different carbohydrates (i.e. fucose (fuc), rhamnose (rha), galactose (gal), glucose (glc), xylose (xyl), and mannose (man)) and 14 amino acids (i.e. alanine (ala), arginine (arg), asparic acid (asp), glycine (gly), histidine (his), hydroxyproline (hyp), isoleucine (ile), leucine (leu), lysine (lys), methionine (met), phenylalanine (phe), proline (pro), serine (ser), threonine (thr), tyrosine (tyr), valine (val)), nd = not detectable.

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In order to reconstruct the fate of carbon, we analyzed concentrations and $^{13}$C label incorporation in bulk sediment (TOC), bulk CHO and bulk AA (Fig. 5.4). CHO and AA explained only 9% and 5% of the TOC content, respectively. However, CHO were much more important for the carbon processing as they explained 42% of the total carbon fixation. The absolute amount and incorporation of C in the different pools varied between the duplicate frames due to the spatial heterogeneity of MPB. However, the relative distribution between the different pools of carbohydrate was more or less equal.

Both EPS fractions consisted mainly of CHO, while AA were only present in low amounts (Fig. 5.5 A, B). More than 80% of the EPS production could be explained by MQ extractable CHO and this fraction therefore presented the most important and dynamic pool within the EPS. AA were only produced in small quantities. These results show that production and turnover rates yield further insight into EPS dynamics and provide a broader view than concentrations alone.

In this study, label incorporation was measured 4 h after the label was sprayed on the sediment. It would be interesting to follow the label for a longer period of time providing kinetic information on label distribution in the various metabolic pools. Further study of lipid $^{13}$C biomarkers will lead to the unraveling of the transfer of carbon from MPB to the heterotrophic communities in intertidal mudflat sediments.

Previous studies found rapid transfer of carbon from MPB into the heterotrophic community (Evrard et al., 2008; Middelburg et al., 2000). Others found that EPS-derived carbohydrates were major intermediates in the transfer of carbon between the MPB and bacteria (Bellinger et al., 2009). The fate of organic matter in the benthic food web (including meio- and macro fauna) was studied by Evrard et al. (2012) and Oakes et al. (2012).
Fig. 5.4. Amount and production of bulk Total Organic Carbon (TOC), Carbohydrates (CHO) and amino acids (AA) in an intertidal marine sediment after 4 h \textit{in-situ} $^{13}$C labeling incorporation. Amount is expressed in $\mu$mol C g$^{-1}$ DW and production is expressed in nmol $^{13}$C g$^{-1}$ dry weight (DW).

Fig. 5.5. Carbohydrate (CHO) and amino acid (AA) content (A) and production (B) in MQ and EDTA extractable EPS extracted from an intertidal marine sediment after 4 h \textit{in-situ} $^{13}$C labeling. Amount is expressed in $\mu$mol C g$^{-1}$ DW, whereas production is given in nmol $^{13}$C g$^{-1}$ dry weight (DW).
Concluding remarks

In this study, we show that MQ extractable EPS is the major component produced by MPB and that it mainly consists of glucose with minor contributions from other carbohydrates and amino acids.

The introduction of LC/IRMS was a major step towards the unraveling of metabolic processes in the coupling of the transfer of carbon from the diatoms via EPS to heterotrophic bacteria. This innovation allows direct measurement of carbon isotopes in a wide range of low molecular weight compounds and macromolecules both for natural abundance and isotopic enrichment studies. The strength of this method lies in the straightforward analysis of compounds without the need for derivatisation. As LC/IRMS can be used to quantify the biosynthesis of metabolites and shows the direct distribution of the applied $^{13}$C tracer in various carbon pools it is also a valuable tool for biology in general.

A major innovation in LC/IRMS would be the capability of measuring nitrogen isotopes, which would open up new avenues to study the nitrogen cycle. The development of new stationary phases such as mixed mode phases and the emergent application of high temperature chromatography may also provide new opportunities (Godin et al., 2008). Improvements in the sensitivity and robustness of another mass spectrometry technique, LC/MS/MS, have opened new possibilities for studying macromolecules (Zhang et al., 2007). However, the precision of the LC/MS/MS technique for the determination of low levels of enrichments at or close to natural abundance is insufficient and this hampers its use in in-situ labeling studies,

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