

Biomarker and carbon isotopic constraints on bacterial and algal community structure and functioning in a turbid, tidal estuary

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Abstract

We studied planktonic community structure and isotopic composition using compound-specific ¹³C analysis of phospholipid-derived fatty acids (PLFA) along the Scheldt estuary during a spring bloom. A comprehensive set of other carbon cycle parameters was also determined. Based on dissolved carbon dioxide and oxygen concentrations and primary and bacterial secondary production, the trophic status of the estuary changed from strongly net heterotrophic in the upper to net autotrophic in the lower estuary. Concentrations of algal PLFA and pigments and microscopic identifications of dominant phytoplankton revealed the same trend: a mixed community of green algae and diatoms dominated the freshwater phytoplankton and there was a major diatom bloom at intermediate salinities. Bacterial biomass (also based on PLFA) was much lower for the diatom bloom in the lower estuary than in the net heterotrophic upper estuary. Carbon isotopic ratios of the main diatom marker PLFA, 20:5ω3, were mainly related to changes in δ¹³C values of dissolved inorganic carbon and showed a residual variation of ~5‰, which is probably related to changes in growth conditions along the estuary. The green algal marker 18:3ω3 was much more depleted in ¹³C than the diatom markers, suggesting that these two main phytoplankton groups use a different inorganic carbon source or carbon dioxide fixation mechanism. Isotopic ratios of the different bacterial PLFA detected were very similar and ranged between -30‰ and -21‰. They closely tracked δ¹³C values in particulate organic carbon (POC), indicating that POC or material with the same ¹³C signature was the main bacterial carbon substrate. In the lower, marine side of the estuary, isotope ratios of bacterial and algal PLFA were similar, suggesting a coupling between primary production and bacterial consumption of organic matter. However, δ¹³C values of bacterial PLFA in the upper estuary were enriched compared with algal PLFA (between 6‰ and 15‰), indicating an uncoupled algal–bacterial system with allochthonous subsidies, such as terrestrial C3 organic matter or sewage supporting bacterial growth.

Estuaries form the transition zone between rivers and the sea and receive large amounts of organic matter and nutrients. Organic matter imported by rivers is efficiently processed, with the result that upper parts of estuaries are often net heterotrophic systems, where respiration exceeds local primary production (Smith and Hollibaugh 1993; Heip et al. 1995). Low oxygen saturation and high carbon dioxide partial pressures therefore often characterize estuaries (Frankignoulle et al. 1998). Nutrients released by mineralization are transported to the more marine parts of the estuary, where primary production is stimulated and large algal blooms may develop because of better light conditions.

As estuaries are often net heterotrophic, this indicates that local primary production is not the only carbon source driving microbial mineralization processes and that other sources of organic material are important as well. Several approaches have been used to detect and quantify this uncoupling between primary and bacterial secondary productivity. A number of studies have shown that primary and bacterial sec-

ondary productivity cannot be related directly to each other both in magnitude and in timing of peak productivities (e.g., Findlay et al. 1991; Goosen et al. 1997). Goosen et al. (1997) showed, for instance, that estimates of annual bacterial secondary production were high in different sections of the Scheldt estuary (200–2,600 g C m⁻² yr⁻¹) and could not be supported by the much lower net primary production (40–280 g C m⁻² yr⁻¹). This was especially true for the upper, freshwater part of the estuary, where bacterial production was about 10 times higher than primary production. Stable isotope analysis provides a useful, alternative approach to study estuarine carbon cycling. The rationale is that isotopic signatures of heterotrophic organisms contain information on their substrates. However, application of this approach to planktonic microalgae and bacteria is difficult because their biomass is masked by other particulate organic materials and physical separation of algae and bacteria from other seston components is not possible in turbid estuaries.

Analysis of specific biomarkers, however, offers a possibility to specifically study the isotopic composition of major components of the plankton. Coffin et al. (1990) isolated estuarine DNA that was shown to be primarily of bacterial origin and used it as an isotopic marker to indicate that bacteria used a variety of carbon sources, including local phytoplankton production and terrestrial material. Lipids derived from the biological membranes, such as phospholipid-derived fatty acids (PLFA), offer interesting opportunities as specific markers are found in both bacteria and eukaryotic algae (Tunlid and White 1992; Canuel et al. 1995; Boschker and Middelburg 2002). PLFA have been widely used as bio-

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Acknowledgments

We thank the crew of *RV Luctor* for assistance during sampling; Nico Goosen and Monique de Bie for measuring bacterial production rates; and Joop Nieuwenhuize, Jan Peene, and Jan Sinke for technical and analytical support. Two anonymous reviewers are thanked for their helpful comments. This research was partly funded by the European Union (EUROTROPH: EVK3-CT-2000-00040) and the Netherlands Organization for Scientific Research (Dutch–Flemish Cooperation). This is publication number 3350 of the Netherlands Institute of Ecology (NIOO-KNAW).

markers to study microbial communities in sediments and soils. However, there are few studies on pelagic systems in general and there is only one report for estuarine planktonic communities (Canuel et al. 1995). PLFA analysis offers an advantage over the more widely studied total fatty-acid pool, as the former is mainly associated with biomass (Tunlid and White 1992; Boschker and Middelburg 2002), whereas detritus may contribute significant quantities to the total fatty-acid pool. This total pool is consequently used to trace sources and processing of organic matter in estuaries (e.g., Mannino and Harvey 1999; Canuel 2001). We and others have recently shown that carbon isotope analysis of PLFA offers the possibility of studying carbon sources used by sedimentary bacteria (Boschker et al. 1999; Cifuentes and Salata 2001) and to determine the link between benthic primary producers and heterotrophic bacteria in labeling studies (Middelburg et al. 2000).

In the present study, PLFA concentrations and stable carbon isotope ratios were determined in the Scheldt estuary during a spring bloom in the lower estuary. During this period, there was a strong contrast between a highly heterotrophic upper estuary and an autotrophic lower estuary. PLFA results will be used to describe algal and bacterial communities along the estuarine gradient and will be compared with algal pigment data and microscopical observations. Stable isotope ratios of algal biomarker PLFA will be used to quantify isotopic fractionation between inorganic carbon and algae during photosynthesis. They will also be used to reveal the importance of phytoplankton-derived carbon as a bacterial energy source and will be compared with bacterial PLFA to assess whether local primary production or external carbon inputs support bacterial growth in different parts of the estuary.

Methods

Description of the estuary and sampling—The Scheldt River (Fig. 1) drains an estimated 21,000 km² of Northern France, Belgium, and the Netherlands, an area with approximately 10 million inhabitants. Its estuary is tidally dominated and in general vertically well mixed, with an average residence time for freshwater of 2 months. Turbidity is high, especially in the upper estuary, where suspended matter concentrations range from 25 to 200 mg L⁻¹. The Scheldt estuary receives large amounts of nutrients and untreated sewage, and on an annual basis, it is net heterotrophic over the whole salinity gradient (Heip et al. 1995; Frankignoulle et al. 1998). Improved wastewater treatment has led to a gradual increase in water quality since the 1980s. Based on data from the long-term monthly monitoring program of the Netherlands Institute of Ecology, oxygen saturation in the upper part varies between 0% and 50% and is usually around 90% in the lower estuary except during spring blooms, when 120% or more can be reached.

On 28 and 29 April 1997, surface water (upper 1 m) was sampled at 15 stations in the estuary with a 20-liter Niskin bottle from the *RV Luctor*. Sampling was approximately during peak biomass of the spring bloom in the lower estuary, which gave a maximum contrast between a strongly hetero-

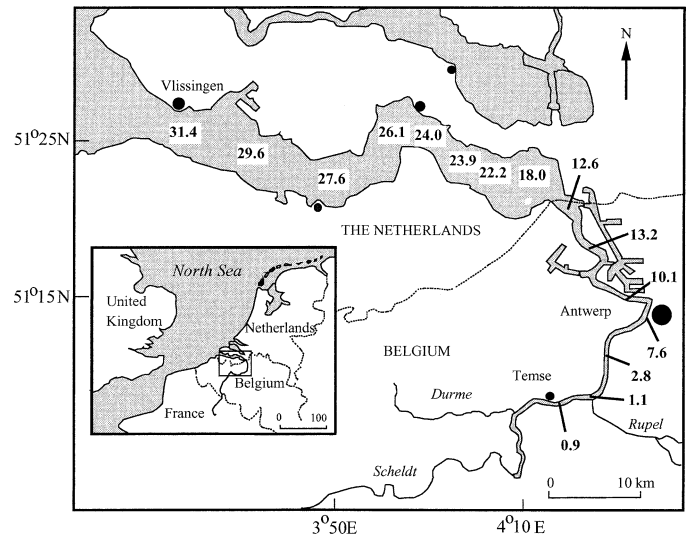


Fig. 1. Map of the Scheldt estuary with sampling sites and salinities.

trophic upper and an autotrophic lower estuary (Fig. 2A). Sampling locations covered most of the salinity gradient in the estuary, ranging from 0.9 at Temse to 31.4 at Vlissingen (Fig. 1). Duplicate samples were taken at several stations and samples were subsampled and processed for different analyses within 20 min. At the time of sampling, salinity, temperature, pH, and oxygen concentrations were measured with a CTD probe (H2O Hydrolab). Dissolved carbon dioxide partial pressure (pCO₂) was recorded continuously with an equilibrator coupled directly to a photo-acoustic infrared detector, and results presented are the average of 10 measurements (over a 20-min period) while sampling.

PLFA concentrations and $\delta^{13}\text{C}$ values—Suspended material from 1.2–2 liters of water was collected on precombusted GF/F glass-fiber filters (Whatman), which were directly added to the PLFA extraction mixture and stored at -20°C . PLFA on the filters were extracted and analyzed as in Boschker et al. (1999). In short, lipids were extracted in chloroform–methanol–water using a modified Bligh and Dyer method and fractionated on silicic acid into different polarity classes. The most polar fraction containing the PLFA was derivatized by mild methanolysis to yield fatty-acid methyl esters (FAME). Concentrations were determined by gas chromatograph–flame ionization detection (GC-FID). Identification is based on comparison of retention times with reference materials on two analytical columns with different polarities and by gas chromatography–mass spectrometry (GC-MS). Carbon isotopic composition of individual FAME was determined with a gas-chromatograph combustion–interface isotope-ratio mass spectrometer (GC-c-IRMS); a HP G1530 GC (Hewlett Packard) connected to Delta-plus IRMS via a type-III combustion interface from Thermo Finnigan (Bremen). Internal FAME standards (12:0 and 19:0) and external FAME and alkane references mixtures were used to check the accuracy of the isotopic ratios determined by the GC-c-IRMS. Stable carbon isotope ratios for individual PLFA were calculated from FAME data by correcting for

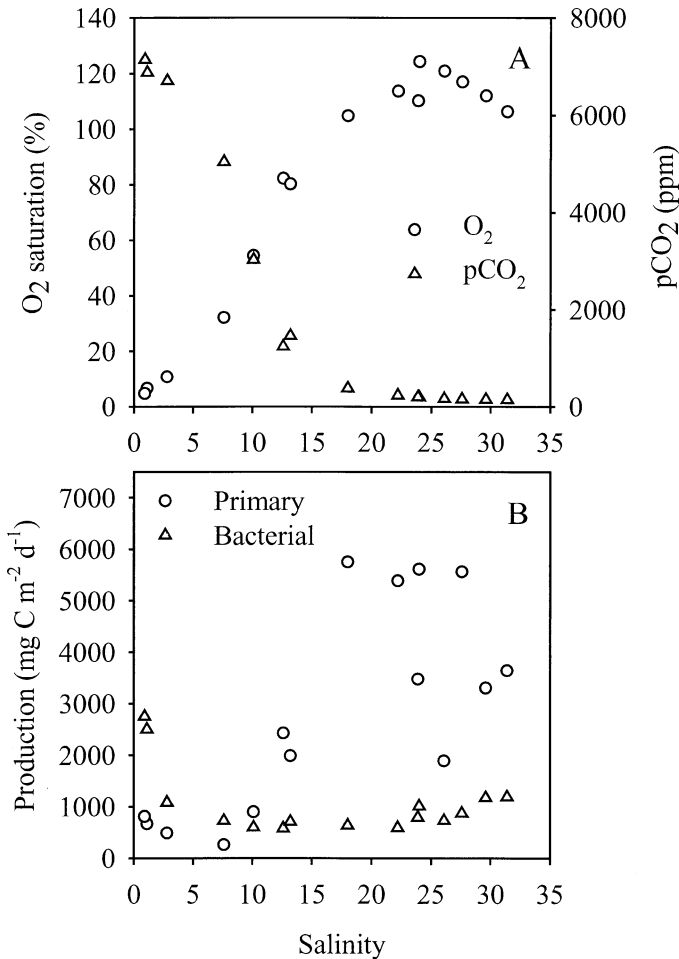


Fig. 2. Background data showing (A) pCO₂ and oxygen saturation and (B) primary and bacterial secondary production.

the one carbon atom in the methyl group that was added during derivatization. Stable carbon isotope data are expressed in the delta notation relative to Vienna Pee Dee Belemnite (PDB).

Additional analysis—Primary production was measured with the ¹⁴C-incorporation method using an incubation period of 2 h (Goosen et al. 1997). The thymidine incorporation method was used to estimate bacterial secondary productivity (Goosen et al. 1997). Phytoplankton was identified microscopically in 1% formaldehyde-fixed samples. Samples for pigment analysis were collected on GF/F glass-fiber filters and stored at -80°C before analysis by high-performance liquid chromatography. Light attenuation was measured with a Licor LI-192 SB cosine light sensor.

Nutrients were determined by standard methods on a SKALAR segmented flow autoanalyzer. Suspended matter was collected on precombusted GF/F glass fiber filters, which were freeze dried and weighed. These filters were also used to analyze particulate organic carbon (POC) content and total nitrogen content with an elemental analyzer (EA), and stable isotopes ratios of POC by EA-IRMS (Boschker

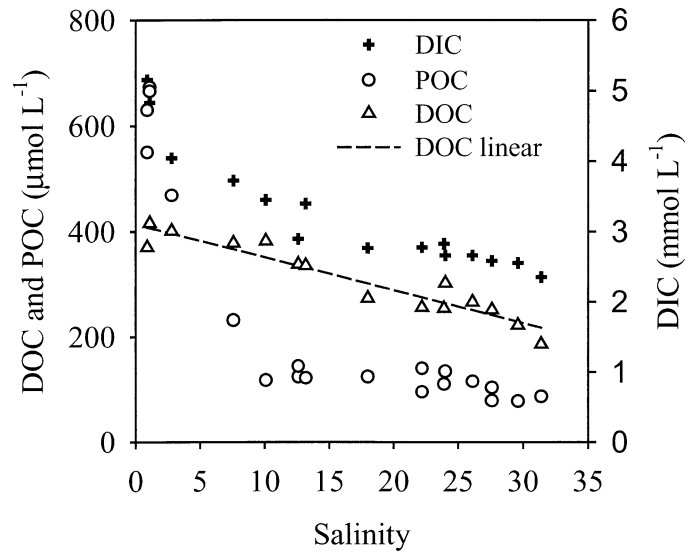


Fig. 3. Distribution of DIC, POC, and DOC along the estuary.

et al. 1999). Samples for DOC were filtered through precombusted GF/F filters, stored frozen, and analyzed using a combined ultraviolet-wet oxidation technique. Dissolved inorganic carbon concentrations (DIC) were determined by headspace analysis after acidification.

Results

Background data—At the time of sampling, the upper and lower sections of the Scheldt estuary differed strongly in biogeochemical characteristics (Fig. 2). In the upper part of the estuary, oxygen was undersaturated and pCO₂ was highly supersaturated compared with the atmosphere, suggesting a strongly net heterotrophic system (Fig. 2A). The pCO₂ decreased with increasing salinity, probably due to carbon fixation, outgassing, and mixing with North Sea water. In the lower estuary, oxygen was supersaturated and pCO₂ undersaturated (about 150 parts per million) compared with the atmosphere and therefore indicated a net autotrophic system during this time of the year. The distribution of primary production and bacteria secondary production over the estuary showed the same transition between net heterotrophy and autotrophy at a salinity of about 10, with bacterial production far exceeding primary production in the upper estuary and the reverse situation in the lower estuary (Fig. 2B). The ratio between bacterial production and primary production was 0.24 ± 0.11 (average \pm SD) above salinity 10.

The estuary was turbid, with suspended particulate matter (SPM) concentrations decreasing sharply from 120 mg L⁻¹ at the freshwater end member to around 10 mg L⁻¹ at salinity 13, whereafter SPM remained almost constant (data not shown). POC (Fig. 3) showed a similar profile as SPM, but dissolved organic carbon (DOC) concentrations decreased almost linearly with salinity ($R^2 = 0.91$, Fig. 3) and therefore appeared to behave conservatively. POC concentrations in the upper estuary were higher than DOC, whereas DOC dominated in the algal bloom in the lower estuary. POC-to-

Table 1. PLFAs detected in this study and their interpretations for the Scheldt estuary. Some algal biomarkers may also be found in other groups of algae that are, however, not abundant in the Scheldt estuary (see *Discussion*).

PLFA	Group
i14:0, i15:0, a15:0, i16:0	Bacteria, mainly <i>Cytophaga/Flavobacteria</i> and Gram positive*
18:1 ω 7c	Bacteria, mainly Gram-negative Proteobacteria*
18:2 ω 6, 18:3 ω 3, 18:4 ω 3	Green algae (Chlorophyceae)†
20:5 ω 3, 22:5 ω 3	Diatoms (Bacillariophyceae)†
16:2, 16:3, 16:4 ω 1	Some green algae and diatoms†
14:0, 15:0, 16:0, 16:1 ω 7c, 18:0, 18:1 ω 9c	General PLFA, found both in algae and bacteria
Sum of all PLFA	Community biomass
Sum of all polyunsaturated PLFA	Algal biomass, minor contribution from other eukaryotes
Sum of bacterial PLFA detected	Bacterial biomass

* Ratledge and Wilkinson (1988), Tunlid and White (1992).

† Volkman et al. (1989), Ahlgren et al. (1992), Dunstan et al. (1994).

chlorophyll *a* ratios were high (around 250 mg C : mg Chl*a*) in the freshwater part, suggesting a major contribution of detritus, but decreased to values between 30 and 70 mg C : mg Chl*a* in the lower estuary, i.e., within the range reported for phytoplankton (Wetsteyn and Kromkamp 1994). This indicates that phytoplankton biomass dominated the POC pool. Molar C : N ratios of SPM varied between 6.4 and 10.3, with higher values in the upper estuary (salinity < 13, C : N = 8.9 ± 0.6) than in the lower estuary (salinity > 13, C : N = 6.7 ± 0.2).

Nutrient concentrations were very high in the freshwater part of the estuary, with $580 \mu\text{mol L}^{-1}$ dissolved inorganic nitrogen (DIN), $8 \mu\text{mol L}^{-1}$ PO_4 , and $220 \mu\text{mol L}^{-1}$ H_4SiO_4 , and decreased sharply with increasing salinity. At salinities above 20, nutrient concentrations were relatively low ($0.3\text{--}1.6 \mu\text{mol L}^{-1}$ PO_4 and $0.2\text{--}2.8 \mu\text{mol L}^{-1}$ H_4SiO_4), with the exception of DIN, which remained high ($45\text{--}125 \mu\text{mol L}^{-1}$). Light extinction coefficients were around 6 m^{-1} in the upper estuary, suggesting that light limitation was important for

phytoplankton (Heip et al. 1995) and decreased to around 1 m^{-1} from salinity 15 onward.

PLFA and pigment concentrations—Microscopic observations revealed that phytoplankton in the upper estuary consisted of a mixture of small centric diatoms (predominantly *Cyclotella* sp.) and green algae (mainly small Chlorophyceae, like *Scenedesmus* and *Euglena* spp.). The bloom in the lower estuary was dominated by large centric diatoms (*Skeletonema*, *Thalassiosira*, *Odontella*, *Coscinodiscus*, and *Rhizosolenia* spp.).

Table 1 lists the PLFA that were used in this study to indicate different planktonic taxa in the Scheldt estuary. Some of the algal biomarker PLFA used in this study may be specific for the Scheldt estuary only (see *Discussion*). The sum of the concentrations of all PLFA, which is a measure of total eukaryote, predominantly algal, and bacterial biomass (Table 1), showed a bimodal distribution, with high concentrations in the freshwater part that decreased quickly to a minimum at a salinity of about 10 (Fig. 4). A second peak in sum of all PLFA was detected in the lower estuary between salinity 15 and 25, where chlorophyll *a* data revealed an algal bloom (Figs. 4, 5C). Algal PLFA (sum of all polyunsaturated PLFA) and chlorophyll *a* showed a similar distribution as the sum of all PLFA. However, algal PLFA contributed less to the total PLFA in the upper estuary than in the lower estuary. Bacterial PLFA peaked in the upper estuary and were only slightly elevated in the diatom bloom of the lower estuary (Fig. 4). Algal PLFA and bacterial PLFA do not add up to the total PLFA, as nonspecific PLFA, like 16:0 (Table 1), were also detected. Individual bacterial PLFA showed similar trends as the combined bacterial PLFA data, suggesting that no major changes in bacterial community structure occurred as can be detected within the resolution of these biomarkers. The ratio between branched PLFA (i14:0, i15:0, a15:0 and i16:0) and the monounsaturated PLFA (18:1 ω 7c) varied only between 0.62 ± 0.10 in the upper estuary to 0.86 ± 0.07 in the lower estuary. Bacterial production showed a similar distribution as bacterial biomass (compare Figs. 2B, 4), implying a rather uniform growth rate.

Diatoms and green algae, the dominant phytoplankton groups in the Scheldt, can be differentiated well by both

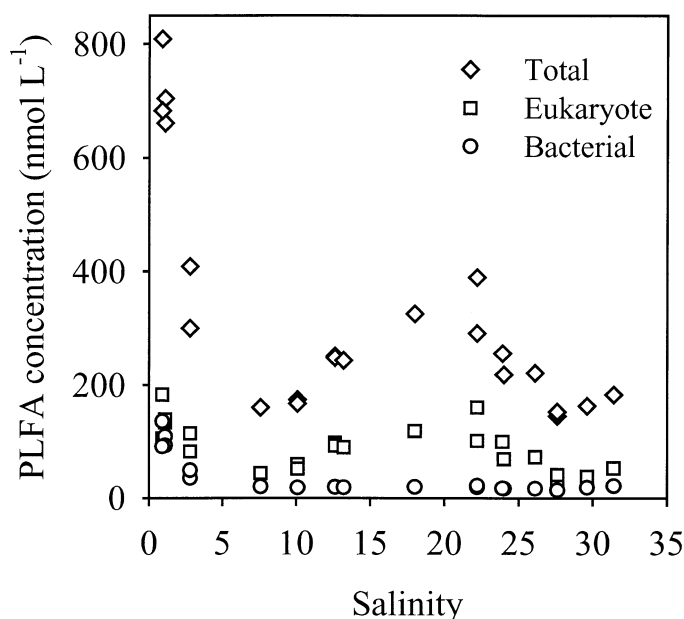


Fig. 4. Concentration of combined PLFA concentrations showing the sum of all PLFA, bacterial PLFA, and eukaryotic PLFA.

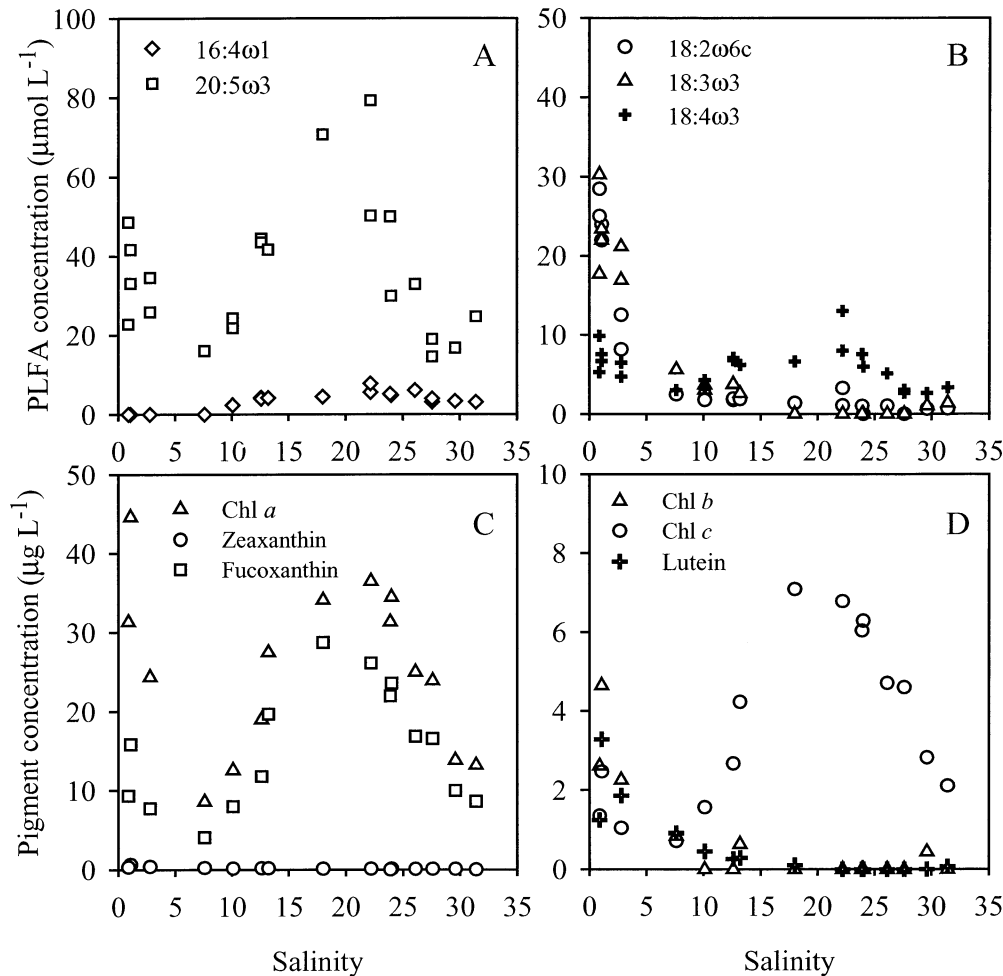


Fig. 5. PLFA and pigment concentrations along the salinity gradient of the Scheldt estuary: (A) diatom-derived PLFA, (B) green algal PLFA and another 18 polyunsaturated PLFAs, (C and D) concentrations of phytoplankton pigments.

PLFA (Table 1) and pigments. The marker PLFA for diatoms, 20 : 5 ω 3 and 22 : 6 ω 3 (Fig. 5A, latter not shown) dominated algal-specific PLFA over the whole of the estuary and showed the same distribution as total algal PLFA. Diatom-derived pigments, like chlorophyll *c*, fucoxanthin, and diadinoxanthin (last not shown), the dominant auxiliary pigments in diatoms and the Scheldt estuary, followed the same bimodal distribution as the PLFA markers for diatoms (Fig. 5C,D). The green algal markers 18 : 2 ω 6 and 18 : 3 ω 3 were highest in the upper estuary, which is in agreement with the presence of chlorophyll *b* and lutein, two auxiliary pigments in green algae. Both types of green algal biomarkers dropped rapidly when salinity increased (Fig. 5). The PLFA 16 : 2 and 16 : 3, which are both found in diatoms and green algae, also showed a bimodal distribution (data not shown). Although green algae were not a major component of the plankton in the lower part of the estuary, small amounts of 18 : 2 ω 6, 18 : 3 ω 3 (only in the two most marine stations), and especially 18 : 4 ω 3 were detected in the lower estuary (Fig. 5B). The PLFA 16 : 4 ω 1 had a divergent distribution because it was absent from the upper estuary (Fig. 5A). Duplicate samples analyzed at several stations generally gave similar concen-

trations for the various PLFA. Zeaxanthin was detected in small amounts in the upper estuary (Fig. 5C), which may have been derived from a small cyanobacterial community or from the abundant green algae that also may contain minor amounts of this auxiliary pigment (Jeffrey et al. 1997). The pigments echinenone, dinoxanthin, peridinin, violaxanthin, and 19-hexanoyloxyfucoxanthin were not detected. Concentrations of pheophytines, biomarkers for algal detritus, were higher in the upper estuary (up to 2.6 $\mu\text{g L}^{-1}$) than in the diatom bloom of the lower estuary (about 0.5 $\mu\text{g L}^{-1}$).

Isotopic composition of PLFA—Duplicate samples analyzed at several stations generally gave similar stable isotope ratios for the various PLFA. Algal PLFA that were sufficiently abundant and separated from other compounds for isotope analysis by GC-c-IRMS were 18 : 3 ω 3, 20 : 5 ω 3, and 22 : 6 ω 3. Only the isotope ratios for 18 : 3 ω 3 and 20 : 5 ω 3 are presented in Fig. 6A, as they are the most abundant polyunsaturated PLFA in the two dominant phytoplankton taxa, namely green algae and diatoms, respectively. The other diatom-specific PLFA, 22 : 6 ω 3, had similar isotope ratios as 20 : 5 ω 3. The 18 : 3 ω 3 green algal PLFA could only be de-

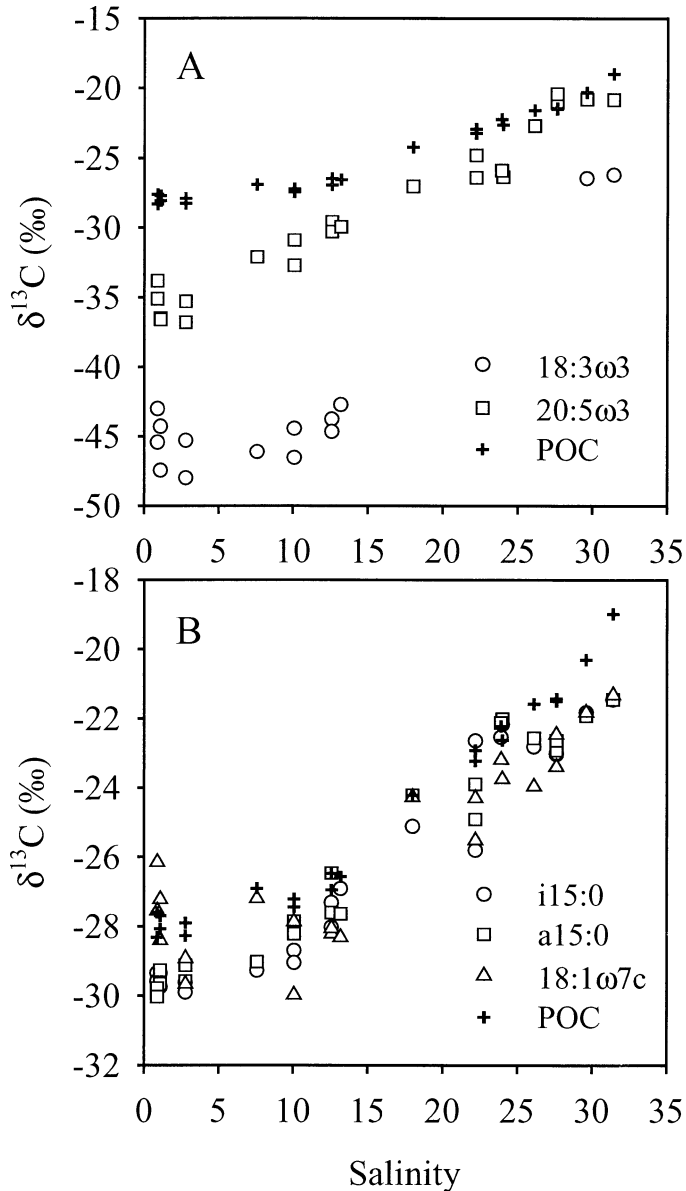


Fig. 6. (A) Stable isotope ratios of algal and (B) bacterial PLFA in relation to POC ratios. Notice differences in y-scale between both figures.

ected in the upper estuary and in the two most marine stations.

Stable isotope ratios of POC were approximately -28% in the upper estuary and increased to typical marine phytoplankton, end-member ratios of around -20% at the highest salinity (Fig. 6). At the freshwater side of the estuary, differences in isotopic characteristics between algal PLFA and POC were substantial and ranged between 7% and 16% , depending on the algal marker (Fig. 6A). The green algal marker 18:3 ω 3 had exceptional negative values of -42 to -48% in the upper estuary, and its ratio showed little change, with increasing salinities until its concentration became too low for isotopic analysis by GC-c-IRMS. The diatom marker 20:5 ω 3, however, became more positive going

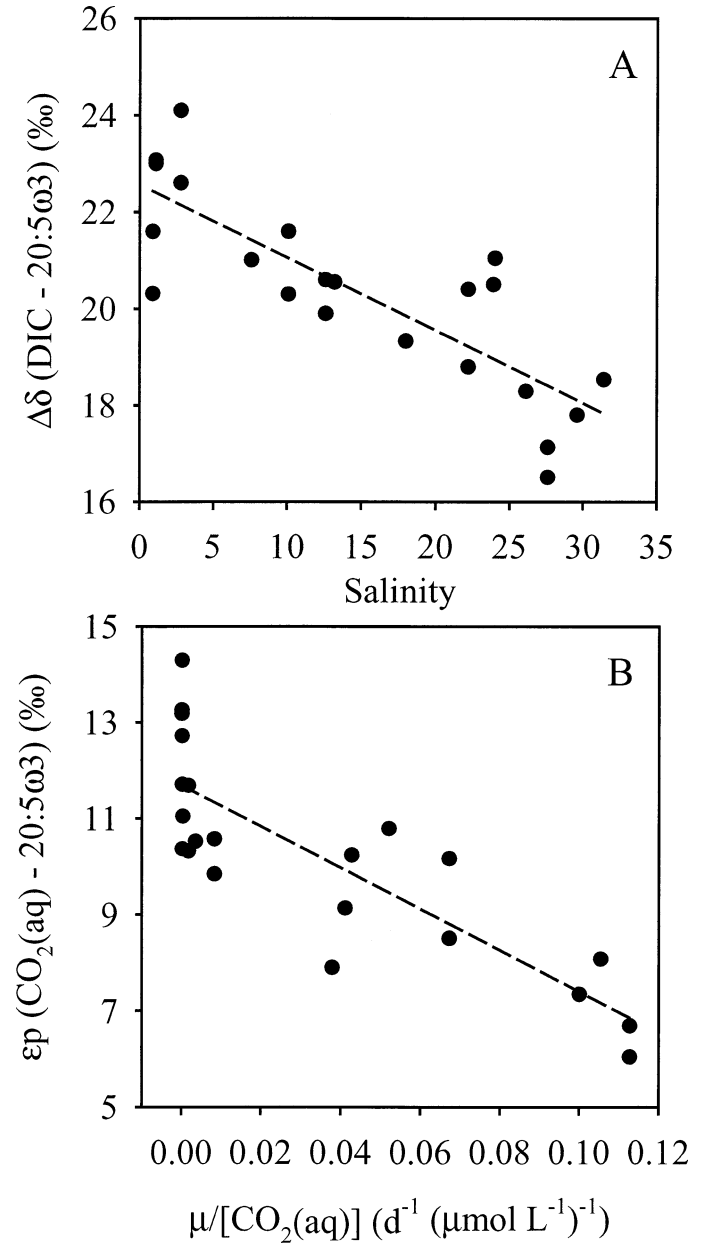


Fig. 7. (A) Isotopic fractionation between DIC and the diatom PLFA 20:5 ω 3 in relation to salinity and (B) between $\text{CO}_2(\text{aq})$ and the diatom PLFA in relation to growth rate over the $\text{CO}_2(\text{aq})$ concentration. DIC data are from Hellings et al. (2001). Lines show results of linear regression analysis: (B) $\epsilon p_{\text{CO}_2-20:5\omega 3} = 11.7 \pm 0.3 - 43 \pm 6 \times \mu/[\text{CO}_2(\text{aq})]$ ($R^2 = 0.69$).

downstream and seemed to track changes in $\delta^{13}\text{C}$ -DIC (as presented in Hellings et al. [2001]). At the marine side of the estuary, all algal PLFA had similar or slightly depleted ratios compared with bulk POC (Fig. 6A).

Approximately 70% of the carbon isotope gradient in the diatom marker PLFA 20:5 ω 3 could be explained by changes in $\delta^{13}\text{C}$ -DIC ratios along the Scheldt estuary. The residual variation in the isotopic fractionation between DIC and diatom PLFA ($\Delta\delta^{13}\text{C}_{\text{DIC-PLFA}}$) decreased approximately linearly with salinity ($R^2 = 0.67$; Fig. 7A). This indicates that iso-

topic fractionation during photosynthesis was not constant in diatoms but was affected by changes in the growth conditions, physiology, and species composition of the diatom community along the estuary.

Carbon isotope ratios of individual bacterial PLFA are shown in Fig. 6B. The bacterial PLFAs i15:0 and a15:0 were sufficiently abundant and well separated from other PLFAs to yield reliable isotope ratios. The peak of the most abundant bacterial PLFA, 18:1 ω 7c, however, showed some overlap with a less abundant monounsaturated PLFA (18:1 ω 9c) in the GC-c-IRMS chromatograms and its $\delta^{13}\text{C}$ values may therefore be biased. Despite this potential problem, the data for 18:1 ω 7c are presented because these may be indicative for groups of bacteria other than the methyl-branched i15:0 and a15:0 PLFA (see *Discussion*). All three bacterial-specific PLFAs show the same trend in isotopic ratio along the estuary (Fig. 6B), suggesting that any inaccuracies in the $\delta^{13}\text{C}$ results of 18:1 ω 7c were rather small.

The isotopic composition of all three bacterial PLFAs closely tracked POC $\delta^{13}\text{C}$ values over the whole estuary (Fig. 6B). Bacterial PLFA ratios were on average 1.2‰ depleted compared with POC, with no significant differences among the three PLFA. At the marine end member, algal PLFA was similar (20:5 ω 3) or somewhat depleted (18:3 ω 3, a minor compound in this part of the estuary) in $\delta^{13}\text{C}$ compared with bacterial PLFA (Fig. 6). While going upstream, bacterial PLFA became gradually more enriched compared with algal PLFA, until they were between 6‰ and 15‰ more positive.

All data from this study are available as Web Appendix 1 at http://www.aslo.org/lo/toc/vol_50/issue_1/0070a1.pdf

Discussion

PLFA as biomarkers for estuarine community composition—The distribution of polyunsaturated, algal-derived PLFA was generally in agreement with algal distribution patterns based on pigment data and microscopic observation. All data showed that there was a mixed community of green algae and small diatoms at the freshwater stations, which rapidly declined at increasing salinity. Large diatoms dominated the major bloom in the lower estuary, leading to a second peak in algal biomass. The diatom marker 20:5 ω 3 (Fig. 5) was found in the highest concentrations of the algal PLFA over the whole of the estuary. Other PLFA commonly found in diatoms, 16:2, 16:3, and 22:6 ω 3, showed the same bimodal distribution as 20:5 ω 3. The green algal markers 18:2 ω 6 and 18:3 ω 3 were most abundant in the upper estuary and quickly decreased at higher salinity. This is in agreement with Muylaert et al. (2000), who reported that freshwater green algae were not able to survive at the increased and variable salinities as found in the upper estuary. In addition, stable isotope ratios of the green algal PLFA marker 18:3 ω 3 showed little variation in the upper estuary despite (Fig. 6) changes in the isotopic composition of the DIC pool (Hellings et al. 2001), which also suggests that green algae were not growing in the upper estuary and decayed.

Although green algae were not a major component of the plankton in the lower estuary, small amounts of 18:2 ω 6,

18:3 ω 3 (only in the two most marine stations), and especially 18:4 ω 3 were detected in the lower estuary. The latter compound can also be found in some green algae but could also be derived from diatoms in which it is a relatively minor component (Volkman et al. 1989; Ahlgren et al. 1992; Dunstan et al. 1994). *Phaeocystis* blooms, which develop in the North Sea during early spring (Lancelot and Mathot 1987), are regularly washed into the estuary and cell counts can be high. *Phaeocystis* contains abundant highly unsaturated 18 fatty acids like 18:4 ω 3 and especially 18:5 ω 3 (Virtue et al. 1993). However, the 18:5 ω 3 marker was not detected in our samples, which is in agreement with the absence of 19-hexanoyloxyfucoxanthin, an auxiliary pigment generally found in Prymnesiophytes (Jeffrey et al. 1997). *Phaeocystis* colonies were also not seen. The exact origin of the minor polyunsaturated 18-carbon PLFA in the brackish and marine parts of the estuary therefore remains unknown. The only PLFA that was absent in the upper but present in the lower estuary was 16:4 ω 1. This fatty acid is found in some diatom species, like *Skeletonema*, *Coscinodiscus*, and *Rhizosolenia* (Dunstan et al. 1994), which are prominent features in the lower estuary but are lacking in the upper estuary.

Several of the identifications in Table 1 may be specific for the Scheldt estuary and should be used with care in other systems, although the phytoplankton composition based on pigment analysis in many turbid, temperate estuaries shows similar features (Lemaire et al. 2002). For instance, the diatom markers 20:5 ω 3 or 22:6 ω 3 are also found in other groups of algae, such as the Chrysophyceae, Dinophyceae, and Cryptophyceae, but these groups are only a minor component of the phytoplankton, and 20:5 ω 3 and 22:6 ω 3 can therefore be attributed primarily to diatoms. Similar arguments can be used for 18:3 ω 3, which is generally the most abundant polyunsaturated fatty acid in riverine green algae that are washed into the upper estuary, but it can also be found in cyanobacteria (Ahlgren et al. 1992). However, the absence of specific pigment echinenone and the presence of only small amounts of zeaxanthin (Jeffrey et al. 1997) indicate that cyanobacteria are a minor component of the phytoplankton in the upper estuary.

An advantage of using PLFA is that bacteria-specific compounds are detected as well (Table 1). Bacterial PLFA concentrations were highest in the upper estuary and only slightly elevated in the diatom bloom in the lower estuary (Fig. 5). This limited increase in concentrations of bacterial PLFA and bacterial secondary production (Fig. 2) in the lower estuary indicate that the diatom bloom supported less bacterial biomass and growth than the allochthonous organic matter delivered by the river into the upper estuary. Goosen et al. (1997) reported a similar distribution of bacterial numbers and bacterial secondary production in the Scheldt estuary during spring of 1991. Coupling between algae and bacteria during bloom periods depends strongly on the physiological condition of the algae, and bacteria usually lag behind the development of the algal biomass and only increase when the bloom collapses and the algal-derived material becomes available in a dissolved form (Billen and Fontigny 1987). The status of the bloom in the lower estuary is not known, but the high algal biomass and primary production and low quantities of pheophytines suggest that it was not yet in a

state of collapse. Primary production in the bloom was, however, sufficient to fuel the observed bacterial secondary production in the lower estuary. The ratio between bacterial and algal production in the bloom (0.24) was well within the range of reported growth yields for bacteria (Del Giorgio and Cole 1998).

Individual bacterial PLFA detected showed similar trends as the combined bacterial PLFA data. This suggests that there was no major shift in bacterial community structure, which may be due to the relatively low taxonomic resolution of the PLFA analysis. Several studies using molecular techniques have shown that the estuarine bacterial community is dominated by various bacteria belonging to the Proteobacteria and the Cytophaga–Flavobacteria, although a substantial variation may occur within these groups along the estuarine gradient (Crump et al. 1999; Selje and Simon 2003). Given this composition, the Cytophaga–Flavobacteria are most likely the dominant source of the methyl-branched PLFA, like i15:0 and a15:0, with possible contributions from Gram-positive bacteria, and the monounsaturated bacterial marker PLFA 18:1 ω 7c is probably mostly derived from the Proteobacteria (Ratledge and Wilkinson 1988).

Overall, the results of the algal PLFA were in good agreement with pigment concentrations and microscopical counts, which strengthens the identifications of the algal PLFA markers as used in this study. The advantages of PLFA over other biomass markers are that bacterial biomarkers are also detected and that PLFA are amenable to compound-specific isotope analysis by GC-c-IRMS.

The isotopic composition of phytoplankton—Diatoms were found over the whole estuary and the diatom biomarker 20:5 ω 3 showed a gradual change in isotopic ratio along the estuary from approximately -35‰ in the river Scheldt to -20‰ at the mouth of the estuary (Fig. 6). This variation was primarily (by 70%) caused by a gradient in DIC isotopic ratios (Hellings et al. 2001). However, there remained a residual variation in the isotopic fractionation between DIC and 20:5 ω 3 of about 5‰ (Fig. 7A), indicating that isotopic fractionation by diatoms was not constant. Factors that influence isotopic fractionation by phytoplankton have been intensively studied in recent years, both in the field and in laboratory cultures, as it has been suggested that isotopic signatures can be used as a proxy for paleo-pCO₂ (Rau 1994, and references therein). Our study is the first to investigate algal isotopic fractionation in an estuary under a wide range of dissolved CO₂ concentrations.

Isotopic fractionation in diatoms against dissolved carbon dioxide was analyzed using the diffusion limitation model (Laws et al. 1995). This model involves algal growth rates, algal volume: surface ratios (i.e., cell size) and pCO₂ as major factors, and implies that plots of isotopic fractionation versus growth rate over CO₂(aq) concentration follow a linear function. We used the following, simplified equation to analyze our diatom biomarker data:

$$\epsilon p_{\text{CO}_2-20:5\omega_3} = \epsilon_{\text{pmax}} - a \times \mu / [\text{CO}_2(\text{aq})] \quad (1)$$

where $\epsilon p_{\text{CO}_2-20:5\omega_3}$ is the isotope fractionation between dissolved carbon dioxide (CO₂(aq)) and the diatom marker PLFA 20:5 ω 3, ϵ_{pmax} is the maximum isotope fractionation

factor, μ is the community growth rate, and a is an empirical factor. The marker PLFA 20:5 ω 3 is used as a representative of the diatom biomass and it should be noted that lipids like PLFA are generally somewhat depleted compared with the total biomass (Schouten et al. 1998; Hayes 2001). The dissolved carbon dioxide $\delta^{13}\text{C}$ values were calculated according to Mook et al. (1974) using $\delta^{13}\text{C}$ values of DIC for the spring bloom in 1998 (Hellings et al. 2001). The maximum fractionation during photosynthesis (ϵ_{pmax}) is primarily a result of carbon fixation by rubisco and β -carboxylations. Dissolved carbon dioxide concentrations ([CO₂(aq)]) were calculated from partial pressures as measured with an equilibrator (pCO₂; Fig. 2). Diatom growth rates (μ) were estimated from the community production and chlorophyll *a* (Chl*a*) concentrations assuming a factor of 30 mg C:mg Chl*a* between carbon and chlorophyll *a* content (Wetsteyn and Kromkamp 1994). Diatoms dominated phytoplankton biomass in much of the estuary, except in the freshwater section, where green algae were also important although our results suggest that green algae were not actively growing. Consequently, the use of whole community growth rates in Eq. 1 may have underestimated diatom growth rates at the upper freshwater stations. Deviations from this simple model have been reported and have been attributed to nutrient or light limitations of growth or to bicarbonate uptake instead of CO₂(aq) (Burkhardt et al. 1999; Riebesell et al. 2000; Rau et al. 2001).

The residual variation in carbon isotope fractionation between diatoms and CO₂(aq) could be described well by Eq. 1 (Fig. 7B), implying that the diffusion limitation model applies (Goericke et al. 1994; Laws et al. 1995). The relationship presented in Fig. 7B ($\epsilon p_{\text{CO}_2-20:5\omega_3} = 11.7 \pm 0.3 - 43 \pm 6 \times \mu$; $R^2 = 0.69$) does not change significantly when the freshwater stations, with their mixed phytoplankton community, are excluded (salinity <8) and only samples are considered where diatoms dominate the phytoplankton (salinity >8: $\epsilon p_{\text{CO}_2-20:5\omega_3} = 10.9 \pm 0.4 - 33 \pm 6 \times \mu$; $R^2 = 0.71$). Growth rates, as estimated from chlorophyll *a* concentration and primary production, were very low in the upper part of the estuary ($0.08 \pm 0.02 \text{ d}^{-1}$, between salinity 0.9 to 7.6), probably because the algae were light limited in this turbid part of the estuary. However, estimated growth rates for the bloom in the lower estuary were high ($0.6 \pm 0.2 \text{ d}^{-1}$, between salinity 10.6 to 31.4). In addition, CO₂(aq) concentrations were very high in the upper estuary ($350 \mu\text{mol L}^{-1}$) and decreased strongly to below saturation at higher salinities ($6 \mu\text{mol L}^{-1}$). The decrease in CO₂(aq) concentrations and the concomitant increase in algal growth rates with increasing salinity could therefore explain the gradient in isotopic fractionation, especially as the size of the diatoms also increased downstream.

Although the data follow the diffusion model, the parameters obtained deviate from the traditional interpretation. One, the intercept, which gives the maximum fractionation (10.9–11.7), was much lower than for eukaryote rubisco and β -carboxylation reactions (25–28‰ against CO₂(aq); Goericke et al. 1994). The fatty acids that we studied are in general somewhat depleted compared with the total biomass (Schouten et al. 1998; Hayes 2001), which would decrease the maximum isotope fractionation even further. Limited iso-

tope fractionation for diatoms has been reported before in a variety of systems and has been attributed to growth conditions during blooms (Fry 1996). Two, the slope of the regression line (-33 to -43 d^{-1} ($\mu\text{mol L}^{-1}$) $^{-1}$) was lower than expected given the large gradients in environmental conditions that influence isotopic fractionation. Especially for the community of relatively fast growing, large-bodied diatoms in the bloom, a larger decrease in isotopic fractionation would be expected at higher μ [$\text{CO}_2(\text{aq})$] $^{-1}$ ratios (Popp et al. 1998). However, Pancost et al. (1997) reported a similar maximum fractionation and slope for diatom-derived sterols in the Peru upwelling region, especially for larger diatoms in the >20 μm fraction ($\epsilon_{\text{pmax}} = 11.7\text{‰}$, slope = -54 d^{-1} ($\mu\text{mol L}^{-1}$) $^{-1}$). It is possible that the diatoms in the bloom were nutrient limited, as concentrations of silicate and phosphorus were low and this may influence isotopic fractionation in algae (Riebesell et al. 2000). Moreover, the partial uptake of bicarbonate instead of $\text{CO}_2(\text{aq})$ by the diatoms using a carbon-concentrating mechanism could explain the lower fractionation (Kaplan and Reinhold 1999). This would be remarkable given the very high $\text{CO}_2(\text{aq})$ concentrations found in the upper Scheldt estuary. Recently, preference of bicarbonate by coastal phytoplankton has been shown (Pancost et al. 1997; Rau et al. 2001; Tortell and Morel 2002).

The stable isotope ratios of the green algal marker PLFA (18:3 ω 3) in the upper estuary were much more negative than the diatom PLFA and seem to comply with the use of $\text{CO}_2(\text{aq})$ and the diffusion model. These very negative isotope ratios of around -45‰ also suggest that there was little contribution from macrophytes, which contain similar PLFA as green algae but with isotopic ratios of around -30‰ . Although our results suggest that these green algae were washed into the estuary from the Scheldt river and decayed, the growth conditions more upstream from our sampling sites were very similar with very high $\text{CO}_2(\text{aq})$ concentrations and a strongly depleted $\delta^{13}\text{C}$ -DIC pool of about -14‰ in spring (Hellings et al. 2001). The high $\text{CO}_2(\text{aq})$ concentrations in combination with low growth rates and a small cell size imply that isotopic fractionation was probably close to maximum if the CO_2 diffusion model applies ($\epsilon_{\text{pmax}} =$ about 26‰ ; Goericke et al. 1994). Calculating the isotopic composition from these data would give $\delta^{13}\text{C}$ for green-algal PLFA of about -51‰ ($= -14\text{‰}$ ($\delta^{13}\text{C}_{\text{DIC}}$) + -8‰ (DIC to $\text{CO}_2(\text{aq})$) + -26‰ (carbon fixation) + -3‰ (biomass to PLFA)), which is only somewhat depleted compared with $\delta^{13}\text{C}$ values of 18:3 ω 3 in Fig. 6A, given the uncertainties in the calculations. The large difference in $\delta^{13}\text{C}$ of the diatom and green algal biomarkers therefore may suggest that these algal groups were using a different inorganic carbon source or carbon-fixation metabolism.

Bacterial carbon sources in relation to the metabolic state of the estuary—The isotopic composition of all three bacterial PLFA closely tracked POC $\delta^{13}\text{C}$ values over the whole estuary (Fig. 6B). This implies that POC or a carbon pool with the same isotopic composition was the main carbon source for bacterial growth. This other pool may be DOC, and our results then imply that DOC will have the same isotope distribution as POC along the estuary. POC and DOC concentrations were in the same range in the Scheldt

estuary. In DOC-dominated rivers, POC and DOC isotopic ratios are often not the same along the estuary, and DOC isotopic mixing curves suggest a strong cycling (Cifuentes and Eldridge 1998). Bacterial PLFA ratios were on average 1.2‰ depleted compared with POC with no significant differences between individual PLFA. Lipids tend to be depleted in ^{13}C compared with the bacterial biomass and food source usually by around 3‰ , although a wider range of values has been reported (Abraham et al. 1998; Boschker et al. 1999; Hayes 2001). The difference in isotopic ratios between POC and bacterial PLFA is therefore somewhat on the low side, which may suggest that bacteria predominantly used an ^{13}C -enriched fraction of the available POC such as carbohydrates or protein. It has also been reported that bacteria are, on average, somewhat heavier than their substrate (Coffin et al. 1990; Hullar et al. 1996).

At the marine end of the estuary, algal PLFAs were similar (20:5 ω 3, major compound) or only somewhat depleted (18:3 ω 3, minor compound) in $\delta^{13}\text{C}$ compared with bacterial PLFA and POC, indicating that local production by phytoplankton may be an important source for bacterial growth in this part of the estuary. Other data, like Chl *a*:POC ratios (30–70) and C:N (6.7 ± 0.2) ratios confirm that the POC in this part of the estuary is mainly phytoplankton with little detritus. At lower salinities, however, algal PLFA became gradually more depleted compared with bacterial PLFA and POC, and they were between 6‰ and 15‰ more negative at the freshwater end member, suggesting that other carbon sources than phytoplankton were important and that bacteria used this allochthonous subsidy preferentially. Isotopic signatures of bacterial PLFA suggest that the dominant substrate supporting bacterial production had an isotopic ratio of around -26‰ , if one considers an isotopic fractionation between bacterial PLFA and biomass of approximately -3‰ (Boschker et al. 1999; Hayes 2001). This isotopic signature suggests that the bacterial carbon substrate was primarily of terrestrial or anthropogenic sewage origin (Hellings et al. 1999). Isotope ratios of POC in the freshwater end of the estuary are also in agreement with a major contribution from C3 terrestrial or sewage material. Hellings et al. (1999) calculated that only approximately 20% of the POC pool was algal derived at our most upstream stations. Our chlorophyll *a*:POC ratios also imply a major contribution from detritus to the POC pool. Consistent with our study, it appears that terrestrial or other external inputs are often, but not always, important as bacterial substrates in the upper reaches of estuaries (Coffin et al. 1989, 1990; Cifuentes et al. 1996), whereas local phytoplankton production is mostly indicated as the main bacterial substrate at the marine end of estuaries (Coffin et al. 1990). For the upper Scheldt estuary, our study is in agreement with the strongly net heterotrophic nature (Frankignoulle et al. [1998] and Fig. 2A, this study) and the uncoupling of primary and bacterial secondary productivity (Goosen et al. [1997] and Fig. 2B this study) and provides the first direct evidence that bacteria are indeed primarily using nonlocally produced material in the upper Scheldt estuary.

Carbon cycling and community structure in tidal estuaries—The Scheldt estuary is one of the best characterized

estuaries and is often used as a model system for temperate tidal estuaries (Heip et al. 1995; Frankignoulle et al. 1998). Tidal estuaries are characterized by intensive mixing, high suspended-matter concentrations, turbid waters, and poor light conditions (Heip et al. 1995). Rivers entering estuaries deliver riverine algal and bacterial communities as well as riverine and terrestrial organic matter. In upper estuaries, these allochthonous communities and organic matter sources are mixed with autochthonous, endemic communities and estuarine organic matter pools. We have shown that PLFA can be used to delineate algal and bacterial material from other organic matter in estuaries, consistent with the results of Canuel et al. (1995) for San Francisco Bay, USA. Moreover, stable isotope analysis of algal PLFA made it possible to trace the differential dynamics and isotope fractionation of green algae and diatoms along the estuarine gradient. Fractionation by diatoms was not constant and depended on changes in growth conditions, species composition, and/or cell size along the Scheldt estuary. Furthermore, green algae and diatoms showed different fractionation factors. Phytoplankton in other estuaries may show similar features, as the upper reaches of estuaries are generally characterized by high CO₂(aq) concentrations and depleted DIC isotope ratios. The detected low fractionation factors in diatoms also imply that, in estuaries with less depleted DIC isotopic ratios, diatoms may become isotopically indistinguishable from terrestrial C3 material, which will complicate estuarine food-web reconstructions based on carbon isotopes only.

In the upper estuary, bacterial biomass and POC $\delta^{13}\text{C}$ signatures are similar, indicating that bacteria primarily rely on abundant terrestrial, C3-derived organic matter rather than on local algal material. This is consistent with the well-known weak coupling and unbalance between bacterial and primary production in turbid estuaries (Findlay et al. 1991; Heip et al. 1995; Goosen et al. 1997). In less turbid estuaries, light conditions are more favorable for phytoplankton growth and local algal production may be a more important carbon source. Our observations in the less turbid, lower Scheldt estuary clearly show that bacteria here mainly depend on algal-derived organic matter and do not rely on allochthonous subsidies, consistent with observations in coastal waters (Billen and Fontigny 1987).

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Received: 18 December 2003

Accepted: 12 July 2004

Amended: 4 August 2004