

Seasonal and spatial variation in the organic carbon and nitrogen concentration and their stable isotopic composition in *Zostera marina* (Denmark)

S. Papadimitriou,¹ H. Kennedy, and D. P. Kennedy

School of Ocean Sciences, University of Wales–Bangor, Menai Bridge, Anglesey LL59 5AB, United Kingdom

J. Borum

Freshwater Biological Laboratory, University of Copenhagen, Helsingørsgade 51, DK-3400 Hillerød, Denmark

Abstract

The spatial and seasonal variation in the concentration and stable isotopic composition of carbon and nitrogen was studied in *Zostera marina* (eelgrass) meadows in Roskilde Fjord and Øresund, Denmark. Organic carbon (C_{org}) and nitrogen (N_{org}) concentrations in new tissues (leaf, rhizome, roots) ranged from 11 to 40 mmol g⁻¹ C and 0.4 to 3.6 mmol g⁻¹ N, whereas their stable isotopic composition in new leaf ranged from -22‰ to -8‰ ($\delta^{13}C_{leaf}$) and +7‰ to +20‰ ($\delta^{15}N_{leaf}$). The $\delta^{13}C_{leaf}$ correlated weakly ($r^2 \sim 0.3$) with the $\delta^{13}C$ of total dissolved inorganic carbon (DIC) in the surface waters ($\delta^{13}C_{DIC}$ range: -5.4‰ to -0.3‰). The temporal change was more pronounced than the spatial change for N_{org} , the atomic C:N ratio, and $\delta^{13}C_{leaf}$ but was much smaller for C_{org} and $\delta^{15}N_{leaf}$. Eelgrass plants had higher N_{org} concentrations, lower C:N ratios, and more depleted $\delta^{13}C_{leaf}$ in winter than summer, reflecting the temporal imbalance between inorganic nutrient supply and plant demand imposed by seasonality in the growth rate. The apparent carbon isotope enrichment factor of new leaf relative to external DIC ($\epsilon_{eelgrass-DIC}$ range: -12.1‰ to -5.3‰) indicated that eelgrass must be reliant on direct HCO_3^- uptake, especially during the period of high growth rate (May to September). The steep spatial gradient of $\delta^{15}N_{leaf}$ reflected changes in the isotopic composition of the source of assimilated inorganic nitrogen. The strongly positive $\delta^{15}N_{leaf}$ values measured inside the fjord indicated an anthropogenic origin for inorganic nitrogen, which comes from riverine input (riverine $\delta^{15}N - NO_3^- = +11.8‰ \pm 1.1‰$).

Sea grasses (aquatic angiosperms) derive nutrient elements for growth from the ambient pool of dissolved inorganic ions in surface waters by uptake through their leaves but are known also to exploit the pool of remineralized nutrients in the pore waters of the underlying sediments by uptake through their extensive root and rhizome system (Thursby and Harlin 1982; Short and McRoy 1984; Hemminga et al. 1994). The availability of nutrient elements in the surface waters in subtropical and temperate ecosystems can become seasonally limiting as a result of enhanced biological productivity during spring and summer. As a result, the elemental composition of sea grass biomass has been found to exhibit strong seasonal enrichment and depletion patterns, with nitrogen concentration and its molar ratio to carbon being a characteristic example of this pattern (Harrison and Mann 1975; Pedersen and Borum 1993; Van Lent and Verschuure 1994).

The stable isotopic composition of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) in the biomass of aquatic primary producers reflects primarily the kinetic isotope fractionation that results from slower biological assimilation of the heavy isotope, ^{13}C

and ^{15}N respectively, as well as the isotopic composition of their source in the dissolved inorganic pool of these elements (Fogel and Cifuentes 1993; Waser et al. 1998; Burkhardt et al. 1999). Sea grasses are known to rely primarily on the C_3 cycle of carbon assimilation, yet exhibit isotopically enriched $\delta^{13}C$ values relative to typical C_3 plants (Andrews and Abel 1979; Hemminga and Mateo 1996). In the C_3 cycle, the active inorganic carbon substrate that is assimilated into biomass is CO_2 , which can be supplied to the site of enzymatic assimilation by passive diffusion across the cell membrane from the naturally occurring pool of $CO_2(aq)$ in the surrounding water, by extracellular conversion of HCO_3^- to CO_2 and diffusive CO_2 entry into the cell, and by active uptake of HCO_3^- and intracellular conversion to CO_2 . The latter is a well-established pathway of inorganic carbon acquisition in many sea grass species (Sand-Jensen and Gordon 1984; Beer et al. 2002). Sea grasses assimilate nitrogen in the form of dissolved ammonium and nitrate, with preference for the former ion (Short and McRoy 1984; Hemminga et al. 1994). Theoretically, isotope fractionation is less when demand exceeds supply, and the isotopic composition of the biomass will be close to that of the source of assimilated inorganic substrate, and vice versa (Fogel and Cifuentes 1993; Waser et al. 1998). The $\delta^{13}C$ and $\delta^{15}N$ of sea grass tissues can be expected to be influenced by a number of often interacting factors, such as light availability, nutrient concentrations, and shoot density, as well as species-specific physiological factors. In addition, translocation of old and newly assimilated photosynthetic material from aged to actively growing young tissues has been established as an important mechanism of the nutrient cycle in sea grasses (Ped-

¹ Corresponding author (s.papadimitriou@bangor.ac.uk).

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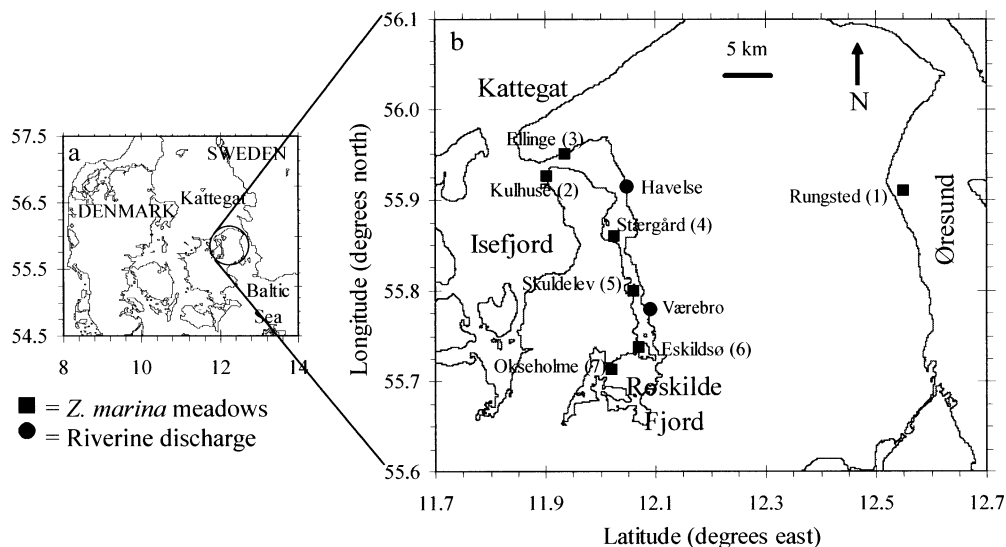


Fig. 1. Study area in Zealand, Denmark.

ersen and Borum 1992; Marbá et al. 2002). The above can be expected to contribute to temporal variability in the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of sea grass tissues on seasonal timescales. Seasonal variability in sea grass $\delta^{13}\text{C}$ has been documented (Stephenson et al. 1984; Anderson and Fourqurean 2003) but has not been a distinct feature of sea grass $\delta^{15}\text{N}$ (e.g., Fourqurean et al. 1997; Anderson and Fourqurean 2003), which has been found to reflect mostly the isotopic composition of the source inorganic nitrogen (Udy and Dennison 1997; Fourqurean et al. 1997; McClelland and Valiela 1998). The $\delta^{15}\text{N}$ of sea grasses can mirror that of the nitrogen source to sea grass habitats because the supply of inorganic nitrogen can be limiting during periods of high growth rates of aquatic plants due to rapid exhaustion of its low standing stock in the aquatic environment (generally $<30 \mu\text{mol L}^{-1}$), leading to minimal isotopic difference between plant and source nitrogen.

Sea grasses form diverse ecosystems in environmental conditions that range from tropical to polar and from oligotrophic to eutrophic (Hemminga and Mateo 1996). The eelgrass *Zostera marina* is the most widespread sea grass species in the Northern Hemisphere (Den Hartog 1970). Signs of decline of sea grass habitats worldwide (Duarte 2002) and their role in the global cycles of carbon and nitrogen through their prolific productivity (e.g., Capone and Carpenter 1982; Duarte and Chiscano 1999) have both been at the center of increased scientific interest in these shallow-water macrophytes in the past two decades. Extensive analysis of the carbon and nitrogen elemental and isotopic composition of primary producers in undisturbed and perturbed sea grass meadows showed the sensitivity of sea grasses to environmental conditions; this and their sessile habit offer the potential for using sea grass tissue chemistry as an environmental indicator, a useful tool in an effective coastal ecosystem management (Fourqurean et al. 1997; McClelland and Valiela 1998). As part of a wide-scope program investigating the response of European sea grasses to changing environmental conditions, we obtained measurements per-

taining to the concentration and isotopic composition of carbon and nitrogen in young, actively growing tissues of *Z. marina* in the well-studied and closely monitored estuarine ecosystems in Roskilde Fjord and Øresund, Denmark. We present seasonal and spatial patterns of these measurements in a number of meadows against the background of prolonged nutrient enrichment in the area. Our aims were (1) to examine whether the nitrogen content of eelgrass is a reflection of spatial and temporal changes in the inorganic nitrogen load of the surface coastal waters, (2) to assess to what extent the isotopic composition of carbon in eelgrass reflects that of the external dissolved inorganic carbon (DIC), and (3) to evaluate whether the long-term nutrient enrichment in the area is reflected in the isotopic composition of nitrogen in eelgrass.

Methods

Study sites—Eelgrass tissues and water samples were collected from *Z. marina* meadows located at one station in the straits of Øresund and six stations along Roskilde Fjord, in the northern coast of Zealand, Denmark (Fig. 1). The stations are located along a eutrophication gradient, with increasing inorganic nutrient concentration in the surface waters from Øresund to the innermost part of Roskilde Fjord (see below). There were no systematic changes in eelgrass shoot density and size in the shallow meadows along the nutrient gradient, but the lower depth limit of the meadows decreased from 6 m in Øresund to 2.3 m in the innermost part of the fjord (unpubl. data). All samples were collected at approximately 1.5 m water depth, except at three locations where the meadows were also sampled close to their lower depth limit at approximately 5 m water depth at station 1 (Rungsted, Øresund), 4 m at station 3 (Ellinge, Roskilde Fjord), and 2 m at station 7 (Okseholme, Roskilde Fjord). All meadows were sampled in August 2001, whereas the shallow and deep meadow at station 1, the shallow meadow at station 3, and

Table 1. Range of concentrations of dissolved inorganic nitrate (including nitrite) (NO_3^-) and ammonium (NH_4^+), both in $\mu\text{mol L}^{-1}$, as well as temperature (θ , in $^\circ\text{C}$) and salinity (S) ranges in the surface waters of Øresund (Rungsted, station 1) and Roskilde Fjord (fjord entrance: Kulhuse, station 2; fjord apex: Okseholme, station 7). Detailed data are available at <http://mads-en.dmu.dk> (the national database for marine data, National Environmental Research Institute, Denmark).

	Rungsted (station 1)			Kulhuse (station 2)			Okseholme (station 7)		
	2001	2002	2003	2001	2002	2003	2001	2002	2003
NO_3^-	0–6.5	0–8.3	0–6.8	0–34	0–46	0–32	0–60	0–102	0–72
NH_4^+	0–2.1	0–3.7	0–2.1	0–8.6	0–6.4	0–7.9	0–7.9	0–8.8	0–12.5
θ		0.1–21.4			0.2–22.6			0.0–24.1	
S		7.6–25.7			14.0–23.7			9.7–14.1	

the shallow meadow at station 5 (Skuldelev, Roskilde Fjord) were also sampled in June, July, September, and October 2002, as well as in February, March, and May 2003. Water samples were also taken from the two main rivers entering

Roskilde Fjord, Værebros and Havelse (Fig. 1). The rivers have a mean annual discharge of 2.5×10^7 and 1.9×10^7 m^3 based on a 10-yr period, accounting for approximately 50% of the total freshwater input to the fjord.

Roskilde Fjord is a 50-km-long narrow estuary, with an average water depth of 3 m, a surface area of 122.3 km^2 , seasonally fluctuating temperature, and variable, low salinity (Table 1). The strait of Øresund is one of the links of the low-salinity Baltic Sea to the high-salinity North Sea, and its surface waters have low dissolved inorganic nitrogen concentration (Table 1). Roskilde Fjord has been receiving large inputs of nitrogen and phosphorus via freshwater and direct discharge of treated municipal waste, as well as via atmospheric deposition for several decades (Kamp-Nielsen 1992; Middelboe and Sand-Jensen 2000). Dissolved nitrate plus nitrite concentrations (hereafter, dissolved nitrate) as high as 60 and 185 $\mu\text{mol L}^{-1}$, dissolved ammonium concentrations up to 40 and 30 $\mu\text{mol L}^{-1}$, and ~ 6 mmol L^{-1} total DIC were measured in October 2003 (unpubl. data) in the rivers Værebros and Havelse, respectively. As a result, the estuarine waters are highly enriched in dissolved inorganic nitrogen throughout the fjord, with seasonally fluctuating concentrations (e.g., Fig. 2). At its maximum in February to early March, the concentration of dissolved nitrate and ammonium in the waters of the fjord in the past 3 yr reached 30–100 $\mu\text{mol L}^{-1}$ and 6–12 $\mu\text{mol L}^{-1}$, respectively, depending on location (Fig. 2; Table 1). Overall, the seasonal fluctuation of the concentration of the dissolved inorganic nitrogen species in the surface waters was dampened outside the fjord (e.g., Øresund) and was amplified in the innermost reaches of the fjord (e.g., Okseholme).

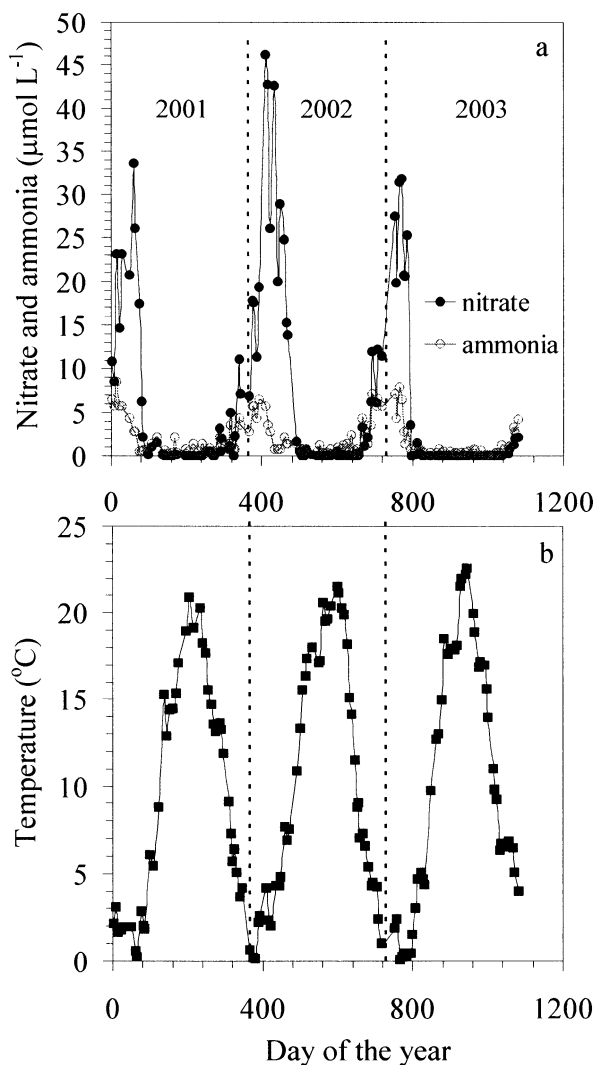


Fig. 2. (a) Dissolved nitrate and ammonium concentration and (b) temperature versus day of the year in the surface waters in Kulhuse (station 2) at the entrance of Roskilde Fjord, Denmark (data from the National Environmental Research Institute, Denmark, <http://mads-en.dmu.dk>).

Sample collection and analysis—New, actively growing leaf, rhizome, and roots of the sea grass *Z. marina* (for definition, see Pedersen and Borum 1993) were collected and prepared for elemental and isotopic analyses as described in Kennedy et al. (2004). Surface water samples for the measurement of the isotopic composition of total DIC ($\delta^{13}\text{C}_{\text{DIC}}$) were collected and processed as described by Papadimitriou et al. (2004). The isotopic composition of dissolved nitrate ($\delta^{15}\text{N} - \text{NO}_3^-$) in riverine water samples was determined with the diffusion method of Sigman et al. (1997). The organic carbon (C_{org}) and nitrogen (N_{org}) concentrations of sea grass tissues were determined on a EUROPA Scientific ROBOPREP C:N Analyzer. Isotopic measurements of carbon and nitrogen were conducted on a PDZ-EUROPA GEO 20/

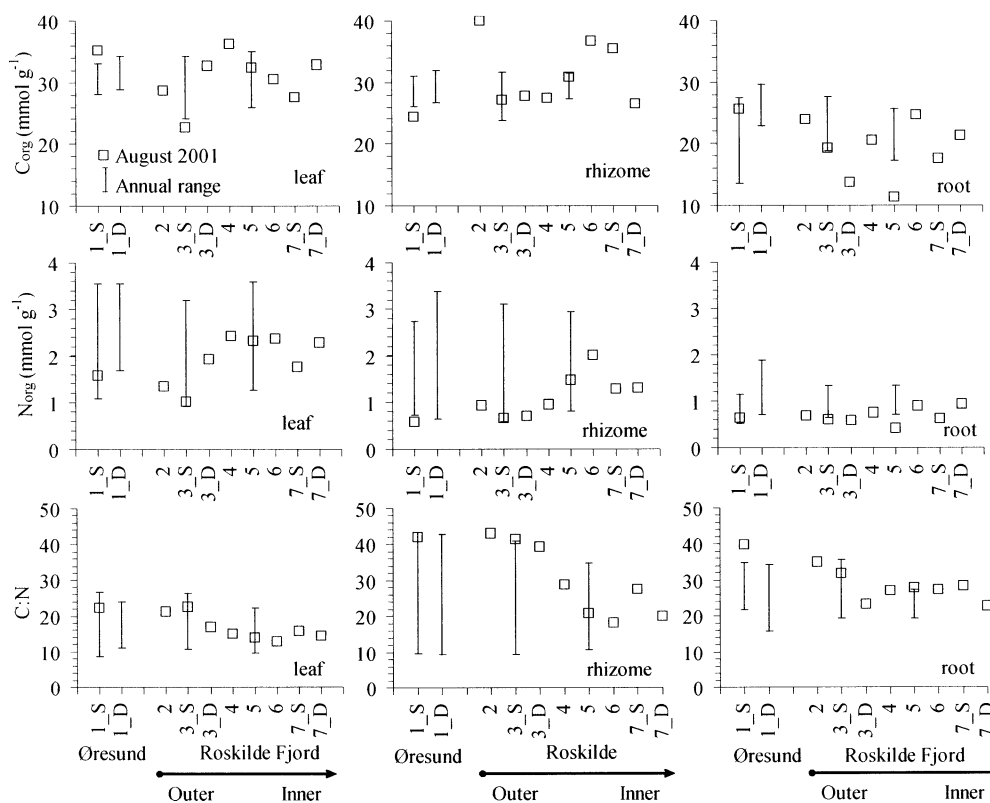


Fig. 3. The concentration of organic carbon (C_{org}) and nitrogen (N_{org}) and their molar ratio in new tissues (leaf, rhizome, and roots) of *Zostera marina* versus station number (station numbers are as in Fig. 1). For stations 1, 3, and 7, S denotes shallow meadows and D denotes deep meadows. The annual range of values between June 2002 and May 2003 is shown in detail in Fig. 4.

20 and a SIRA VG II mass spectrometer, respectively. They are reported in the δ notation relative to standards Vienna Pee Dee Belleminite for carbon and air for nitrogen, i.e., $\delta_{sample} = 1,000[(R_{sample}/R_{standard}) - 1]$, where $R = {}^{13}\text{C}/{}^{12}\text{C}$, or $R = {}^{15}\text{N}/{}^{14}\text{N}$. Elemental analyses were conducted in triplicate and isotopic analyses in single samples, except when testing the precision of the determination (i.e., analytical and sample variability) on triplicate samples of new leaf from the three sites of the annual study. The triplicate samples represent material from three different shoots within a meadow and indicated a precision better than 10% on the basis of 1σ relative to the mean values of C_{org} and N_{org} , and better than 0.02‰ and 0.08‰ for their respective isotopic composition. The precision of $\delta^{15}\text{N} - \text{NO}_3^-$ measurements (1σ of five replicates) was $\leq 0.7\text{‰}$.

Results

Organic carbon and nitrogen concentration in *Z. marina* tissues—The C_{org} concentration of eelgrass tissues exhibited considerable spatial variability without a coherent pattern with respect to location (Fig. 3), as well as a moderate seasonal increase by a factor of 1.2 to 2.0 from minimum values in June–July to a maximum in February–March irrespective of location in the study area or plant tissue (Fig. 4). The seasonal range of C_{org} concentration in the three plant tissues was comparable to or smaller than its spatial range of values

(Fig. 3). New leaves and rhizomes were richer in carbon than roots at all times, with an annual average concentration ($\pm 1\sigma$) of 30.1 ± 3.2 , 28.4 ± 2.4 , and $23.1 \pm 3.7 \text{ mmol g}^{-1}$, respectively (equivalent to $36.1\% \pm 3.9\%$, $34.1\% \pm 2.9\%$, and $27.8\% \pm 4.5\%$, respectively, by dry weight), on the basis of all available measurements ($n = 24$).

A spatial gradient of increasing N_{org} and decreasing molar C:N ratio toward the apex of the fjord was observed in new leaf and rhizome tissue (Fig. 3). The detailed seasonal measurements showed a considerable rise in N_{org} and concurrent lowering of the C:N ratio in above- and belowground eelgrass tissues by a factor of 2–5, with an apparent onset of the seasonal increase in September–October toward a maximum in February–March, when C:N ratios as low as 9–10 were measured in new leaves and rhizomes regardless of location (Fig. 4). The seasonal range of N_{org} concentrations and C:N ratios in all plant tissues was comparable to, or larger than, their spatial range of values (Fig. 3). Leaf and rhizome tissue consistently had higher nitrogen concentrations than root tissue, with annual average concentrations of ($\pm 1\sigma$, $n = 24$) 2.0 ± 0.8 , 1.5 ± 0.9 , and $0.9 \pm 0.3 \text{ mmol g}^{-1}$, respectively (equivalent to $2.9\% \pm 1.1\%$, $2.1\% \pm 1.2\%$, and $1.3\% \pm 0.4\%$, respectively, by dry weight). The corresponding annual average ($\pm 1\sigma$, $n = 24$) molar C:N ratio was 16.7 ± 5.5 , 25.3 ± 11.9 , and 26.1 ± 5.2 for new leaf, rhizome, and root, respectively.

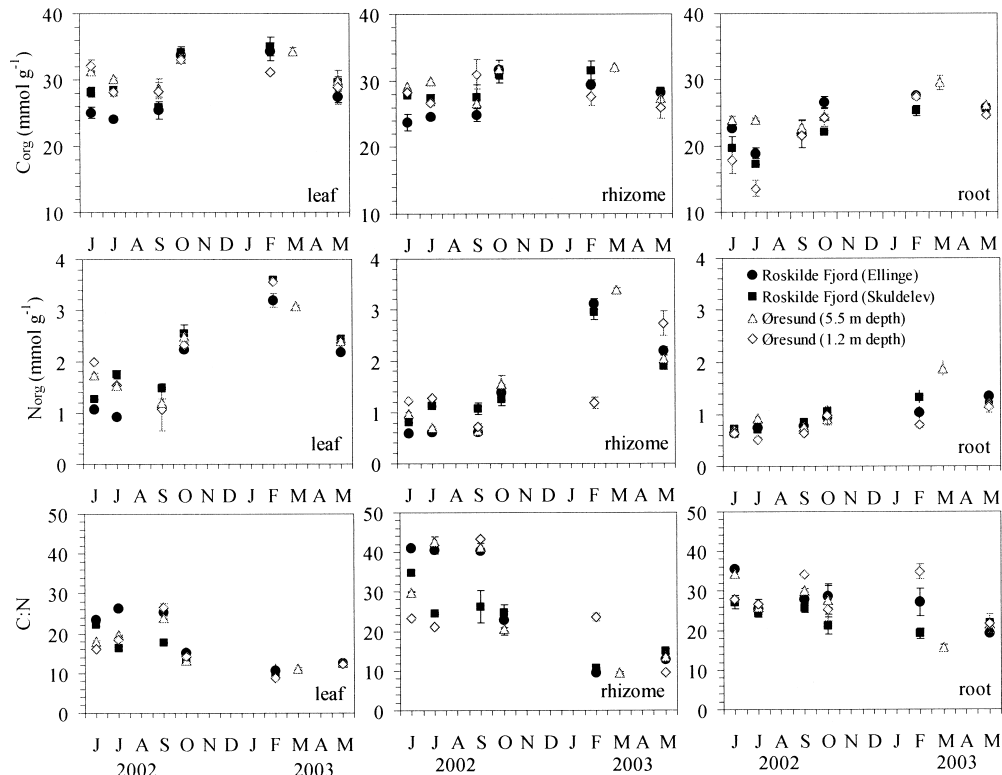


Fig. 4. The concentration of organic carbon (C_{org}) and nitrogen (N_{org}) and their molar ratio in new tissues (leaf, rhizome, and roots) of *Zostera marina* versus calendar month. Error bars indicate 1σ of triplicate measurements, which is smaller than symbol size when not shown.

Isotopic composition of carbon and nitrogen in Z. marina tissues—The stable isotopic composition of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) measured along the spatial gradient indicated that young rhizome and root tissue (data not shown) were isotopically similar to new leaf tissue. Regression analysis on pairs of plant tissues ($n = 9$, $r = 0.91$ – 0.98 , $p \leq 0.001$) yielded slopes close to unity (range from 0.8 ± 0.3 to 1.0 ± 0.3) and intercepts that included zero (range from -1.7 ± 1.8 to 1.0 ± 3.9). Although this correspondence of isotopic composition among the different eelgrass tissues may not be representative over large temporal scales, seasonal isotopic measurements are presented and discussed on the basis of the isotopic data from new leaf only ($\delta^{13}C_{leaf}$ and $\delta^{15}N_{leaf}$).

A considerable spatial range of $\delta^{13}C_{leaf}$ values was measured in the study area, which extended over at least 5‰ (Fig. 5a) and up to 10‰ in February 2003 (Fig. 5b). At all locations, the spatial distribution was marked by a tendency for isotopic depletion in the innermost part of the fjord (i.e., $\delta^{13}C_{leaf} < -12$ ‰; stations 5–7) relative to the outer part of the fjord and the open water meadow in Øresund (i.e., $\delta^{13}C_{leaf} > -12$ ‰; stations 1–4). Annually, the $\delta^{13}C_{leaf}$ range extended over 4–5‰ in the meadow in Øresund and 7–9‰ in the meadows within the fjord (Fig. 5a), which was comparable with the spatial range. The $\delta^{13}C_{leaf}$ exhibited a seasonal cyclical pattern regardless of location (Fig. 5b). Isotopic values were most enriched during the summer months and were at a minimum in February, when $\delta^{13}C_{leaf}$ values of -13 ‰, -16 ‰, and -22 ‰ were measured in the meadows in Øre-

sund, Ellinge, and Skuldelev, respectively. On the basis of all available observations, the $\delta^{13}C_{leaf}$ ranged over approximately 14‰, extending from -22 ‰ to -8 ‰, with an overall mean ($\pm 1\sigma$, $n = 31$) of -11.6 ‰ ± 2.8 ‰.

A steep spatial gradient of $\delta^{15}N_{leaf}$ was observed in the study area, which extended over 13‰, from $+7$ ‰ in the meadow in Øresund (station 1) to $+20$ ‰ in the innermost part of the fjord (Okseholme, station 7) (Fig. 5c). In comparison, the annual range of $\delta^{15}N_{leaf}$ values (2 – 4 ‰) at each station was much smaller. There was no clear seasonal pattern in the $\delta^{15}N_{leaf}$ in any of the meadows, and the observed temporal trends varied between locations. The available data exhibit a general tendency for lower $\delta^{15}N_{leaf}$ values in 2003 than in 2002 by 2–3‰ within the fjord (Fig. 5d, filled symbols). A similar trend was seen in the shallow meadow in Øresund (Fig. 5d, open symbols), albeit to a lesser extent (i.e., 1‰ approximate annual isotopic difference). In the deep meadow in Øresund (Fig. 5d, open symbols), the reverse trend was observed in the $\delta^{15}N_{leaf}$, i.e., isotopic enrichment by up to 2.5‰ in May 2003 relative to the values measured in June 2002. The overall mean $\delta^{15}N_{leaf}$ was $+10.2$ ‰ ± 3.9 ‰ ($\pm 1\sigma$, $n = 31$).

Isotopic composition of total DIC in Z. marina meadows—The isotopic composition of total DIC ($\delta^{13}C_{DIC}$) in the surface waters exhibited a distinct spatial gradient, from -0.3 ‰ in the open water meadow in Øresund (station 1, Rungsted) to -5.4 ‰ in the inner fjord (Fig. 6a). The $\delta^{13}C_{DIC}$ values measured seasonally between June 2002 and May

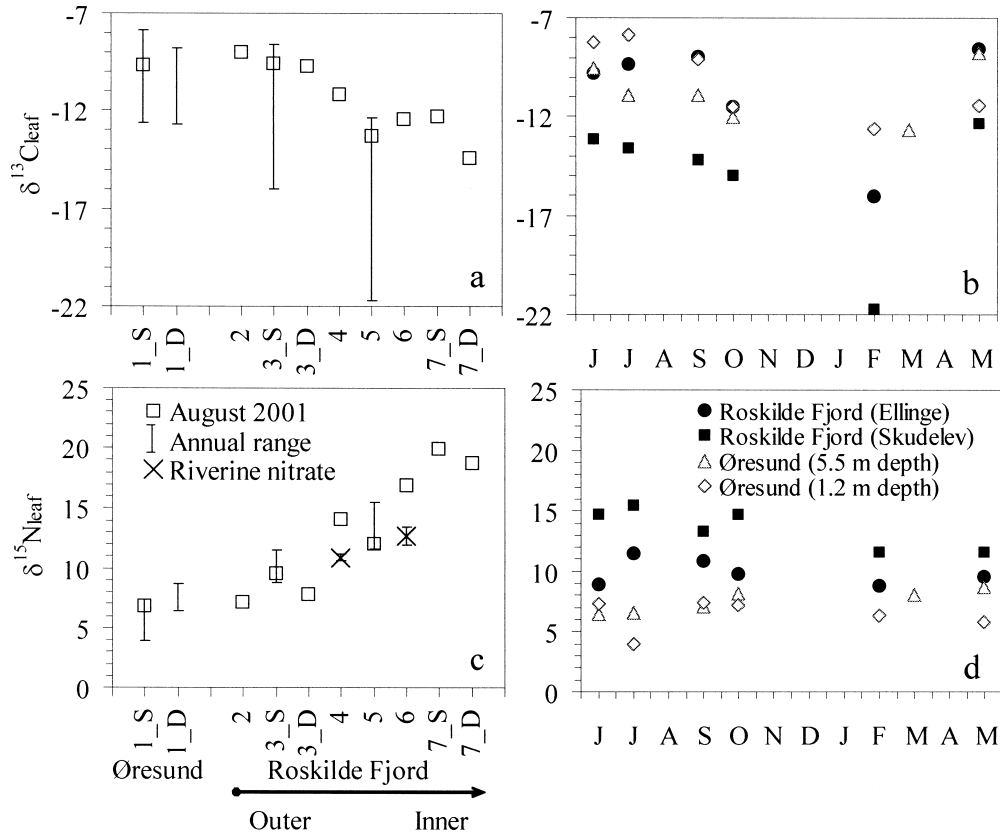


Fig. 5. Isotopic measurements in new leaf from *Zostera marina* plants: (a) $\delta^{13}\text{C}_{\text{leaf}}$ versus station, (b) $\delta^{13}\text{C}_{\text{leaf}}$ versus calendar month, (c) $\delta^{15}\text{N}_{\text{leaf}}$ versus station, and (d) $\delta^{15}\text{N}_{\text{leaf}}$ versus calendar month. Station identity in (a) and (c) is as in Fig. 1.

2003 were generally more depleted than those measured in August 2001 in all meadows (Fig. 6a) but exhibited a similar spatial gradient of approximately 3–4‰ between Øresund and the interior of the fjord at any one sampling period (Fig. 6b). The temporal data indicated a tendency for more depleted $\delta^{13}\text{C}_{\text{DIC}}$ in surface waters in February–March than in

June–July, resulting in an annual range of approximately 1–2‰ at any one of the four meadows (Fig. 6a). Specifically, the within-meadow temporal $\delta^{13}\text{C}_{\text{DIC}}$ measurements ranged from -0.3‰ to -2.9‰ in Øresund (Rungsted), from -2.2‰ to -4.0‰ in the outer fjord (Ellinge), and from -3.7‰ to -5.4‰ in the inner fjord (Skuldelev).

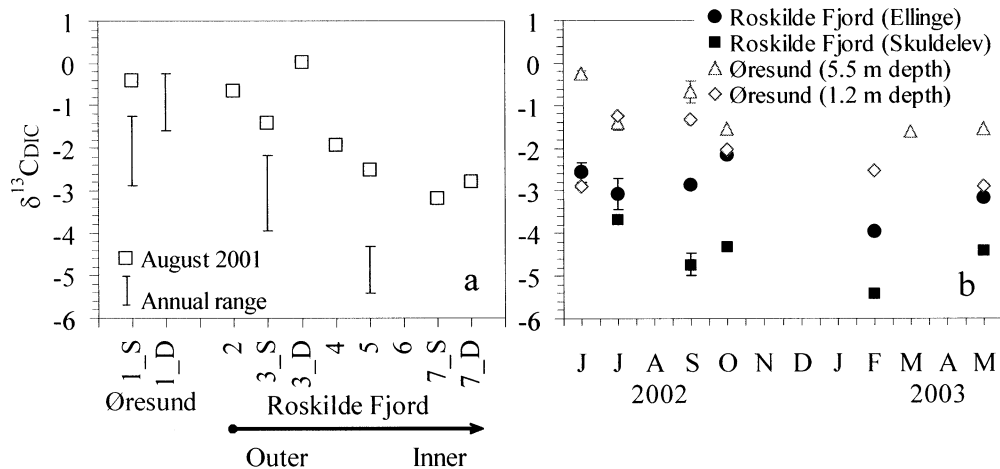


Fig. 6. The isotopic composition of total dissolved inorganic carbon ($\delta^{13}\text{C}_{\text{DIC}}$) in the surface waters overlying *Zostera marina* meadows: (a) $\delta^{13}\text{C}_{\text{DIC}}$ versus station, with station identity as in Fig. 1, and (b) $\delta^{13}\text{C}_{\text{DIC}}$ versus calendar month.

Isotopic composition of total DIC and dissolved nitrate in the riverine waters—The $\delta^{13}\text{C}_{\text{DIC}}$ and $\delta^{15}\text{N} - \text{NO}_3^-$ in the freshwater discharged into the fjord by the rivers Værebro and Havelse were measured on one occasion in October 2003. The freshwater $\delta^{13}\text{C}_{\text{DIC}}$ was considerably more depleted than in the estuarine water, averaging $-13.8\text{‰} \pm 0.6\text{‰}$ ($n = 4$). The freshwater $\delta^{15}\text{N} - \text{NO}_3^-$ was $+12.6\text{‰} \pm 0.7\text{‰}$ ($n = 5$) and $+10.9\text{‰} \pm 0.3\text{‰}$ ($n = 5$) in Værebro and Havelse, respectively.

Discussion

*Seasonal changes in the concentration of nitrogen and the C:N ratio in *Z. marina* tissues*—There was considerable spatial and temporal variability on seasonal scale in the carbon and nitrogen concentration, as well as in their molar ratio in young above- and belowground tissues of *Z. marina* in Øresund and Roskilde Fjord (Figs. 3, 4). The values measured during the summer–autumn 2002 and the winter–spring 2003 are within previously reported spatial and temporal ranges in *Z. marina* tissues (Harrison and Mann 1975; Pedersen and Borum 1993; van Lent and Verschuure 1994). A tendency for higher concentrations of C_{org} and N_{org} was apparent in the young *Z. marina* tissues formed in winter compared with those formed in summer (Fig. 4). The most pronounced seasonal change was seen in the nitrogen content and C:N ratio by a factor of about 2–5, a feature that has been previously recorded in temperate *Z. marina* meadows (Harrison and Mann 1975; van Lent and Verschuure 1994; Fourqurean et al. 1997) and agrees with earlier measurements in Øresund (Pedersen and Borum 1993). Furthermore, it mirrored the seasonal dynamics of the pool of total dissolved inorganic nitrogen in the surface waters (Fig. 2), with minimum and maximum concentrations of N_{org} in young eelgrass tissues coincident with nutrient-impooverished and nutrient-replete ambient conditions, respectively. This is consistent with the mechanism of nitrogen uptake, assimilation, and storage during the relatively short period of maximum nutrient availability in the water column to support growth by internal mobilization when external nutrient concentrations remain low (Pedersen and Borum 1992; Udy and Dennison 1997; Marbá et al. 2002).

After seasonal reestablishment of nutrient-replete conditions in the water column between December and February–March (depending on location and year), the concentration of total dissolved inorganic nitrogen was consistently higher within the fjord than in the open water location in Øresund (Table 1). This may be expected to lead to a corresponding spatial nitrogen enrichment of eelgrass tissues. A spatial gradient was previously recorded in *Z. marina* leaves along a temperate estuary and was attributed to a corresponding gradient in nutrient availability (Fourqurean et al. 1997). A rise in N_{org} concentrations coupled with C:N decline toward the inner fjord characterized the young eelgrass tissues in August 2001 (Fig. 3). This distinct but nonmonotonic spatial gradient was not evident in the seasonal data obtained a year later (Fig. 4). The available data, therefore, cannot confirm a systematic spatial correspondence between the nitrogen concentration of *Z. marina* tissues and its load in the water

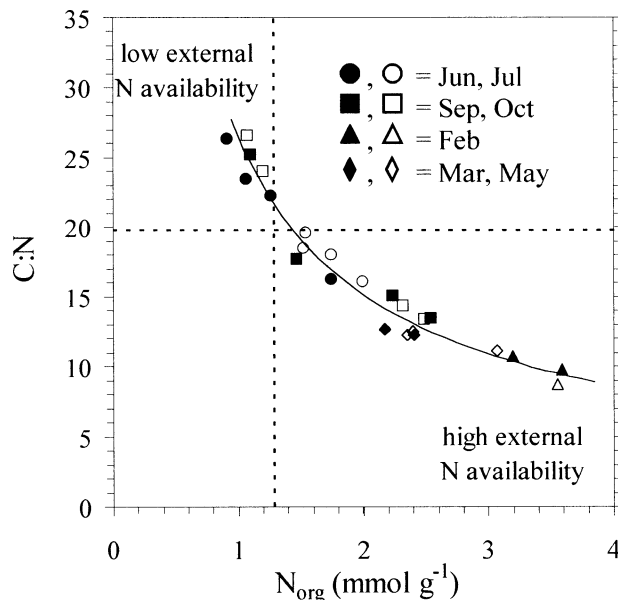


Fig. 7. Molar organic carbon to nitrogen ratio (C:N) versus nitrogen concentration (N_{org}) in new leaf of *Zostera marina* between June 2002 and May 2003. Open and closed symbols indicate observations from Øresund and Roskilde Fjord, respectively. Dashed lines indicate the threshold values for N_{org} and C:N ratio in sea grasses related to the balance between external nitrogen supply and internal demand by the plants (from Duarte 1990).

column in this particular coastal area. The discrepancy may be expected because measurements of the concentration of total dissolved inorganic nitrogen in surface waters are instantaneous values, whereas those in eelgrass tissues integrate also contributions from reclamation of older tissue nitrogen (Pedersen and Borum 1992) and nitrogen uptake from the ammonium pool of the pore waters in the underlying sediments (Thursby and Harlin 1982; Short and McRoy 1984; Hemminga et al. 1994).

The present N_{org} and C:N values covaried ($r = -0.868$, $n = 71$) as a result of the dependence of the C:N ratio primarily on the nitrogen concentration of plant tissue (Duarte 1992). Their functional relationship in the new leaves can be described by the power function, $\text{C:N} = 26.6(\pm 1.4)[\text{N}_{\text{org}}]^{-0.81(\pm 0.07)}$ ($r = -0.981$, $n = 24$; Fig. 7), while the observations from young rhizomes and roots followed a very similar trend. The fitted coefficient of attenuation of the C:N ratio as N_{org} increases is in excellent agreement with that reported for a compilation of values from marine plants, including sea grass leaves (Duarte 1992). On the basis of the global median for sea grasses, the N_{org} concentration and C:N ratio suggested to represent conditions of nitrogen demand by the plants exceeding external supply are $<1.3 \text{ mmol g}^{-1}$ and >20 , respectively (Duarte 1990). In comparison, the current N_{org} and C:N in new leaves suggest that such conditions may occur during the period of maximum growth (Fig. 7) and low dissolved inorganic nitrogen concentrations in the surface waters (Fig. 2) (Pedersen and Borum 1993). The N_{org} and C:N of new leaves gradually passed this threshold during the period of low growth toward N_{org} concentrations as high as 3.6 mmol g^{-1} (equivalent to 5% N_{org} by dry weight) and C:N values as low as 9–10 in February

(Fig. 7). Actively growing young leaves of *Z. marina* have been demonstrated to rely entirely on nitrogen reclamation and translocation of newly acquired nitrogen from older eelgrass tissues (Pedersen and Borum 1992). It would appear that neither nitrogen resources within the plant nor its capacity for nitrogen uptake from the pore water of the underlying sediments (Thursby and Harlin 1982; Short and McRoy 1984; Hemminga et al. 1994) is sufficient to sustain during the period of high growth rates the elevated N_{org} and low C:N ratio measured outside this period (i.e., winter) in these *Z. marina* meadows.

Seasonal changes in the stable isotopic composition of nitrogen and carbon of Z. marina tissues—The isotopic composition of carbon and nitrogen in the new leaf of *Z. marina* exhibited a wide range of values. Trends in space and time similar to those measured here have been described previously in temperate eelgrass (Stephenson et al. 1984; Fourqurean et al. 1997) and subtropical *Thalassia testudinum* meadows (Anderson and Fourqurean 2003). The overall mean $\delta^{13}C$ and $\delta^{15}N$ values in Øresund and Roskilde Fjord are not disparate from those previously reported for *Z. marina* (Stephenson et al. 1984; Hemminga and Mateo 1996; Fourqurean et al. 1997), but their ranges are much wider, extending over 13–14‰.

Many factors influence the isotopic composition of carbon and nitrogen in the tissues of marine plants, often in concert. These factors include: the isotopic composition of the assimilated inorganic substrate, fractionation resulting from kinetically slower assimilation of the heavy isotope during enzymatic fixation of inorganic substrates into biomass, seasonal storage and translocation of biochemical compounds isotopically distinct from whole plant tissue, and seasonally fluctuating availability of inorganic substrates in concert with demand imposed by natural plant growth patterns (Fogel and Cifuentes 1993; Hemminga and Mateo 1996). Below, the isotopic measurements of eelgrass carbon and nitrogen are examined separately against this background.

Stable isotopic composition of carbon—The isotopic composition of substrate CO_2 and, by extension, of its source in the ambient (i.e., external) bulk DIC pool can be of primary importance in determining the isotopic composition of sea grass carbon (e.g., Hemminga and Mateo 1996). This is pertinent to the present study area, where there was a temporally persistent, strong spatial $\delta^{13}C_{DIC}$ gradient. The $\delta^{13}C_{DIC}$ values were enriched by 3–4‰ in the open water meadows in Øresund relative to those in the inner part of Roskilde Fjord (Fig. 6). This trend is maintained by the mixing of water from outside the fjord with isotopically depleted DIC from the main freshwater discharge into the inner fjord (see sections 2 and 4.4) and through the addition of respiratory CO_2 via in situ heterotrophic activity. The $\delta^{13}C_{leaf}$ was positively correlated with the contemporaneous $\delta^{13}C_{DIC}$ ($r = 0.566$, $p = 0.001$, $n = 30$; Fig. 8), inferring that changes in bulk $\delta^{13}C_{DIC}$ are reflected in the eelgrass tissues (Hemminga and Mateo 1996; Fourqurean et al. 1997). However, this relationship explained only 30% (i.e., $r^2 = 0.296$) of the spatial and temporal variability in $\delta^{13}C_{leaf}$, whereas the most nega-

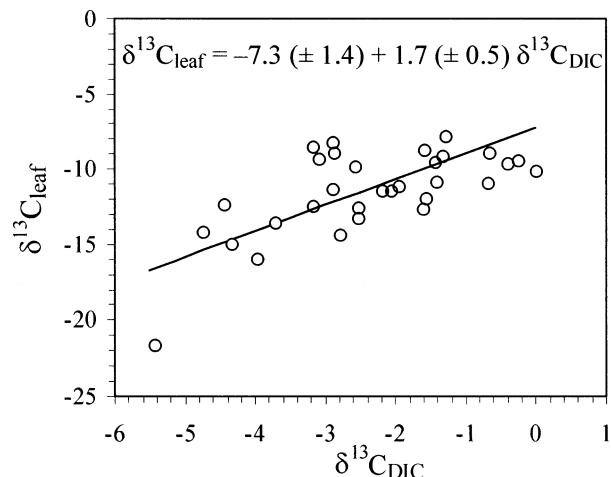


Fig. 8. The relationship between the stable isotopic composition of new leaf carbon ($\delta^{13}C_{leaf}$) and the stable isotopic composition of external DIC ($\delta^{13}C_{DIC}$) in *Zostera marina* meadows.

tive $\delta^{13}C_{leaf}$, which is not included in the linear correlation, was offset to the main trend.

The low degree of correlation between $\delta^{13}C_{DIC}$ and $\delta^{13}C_{leaf}$ may be due to the disparity between the timescales that the two carbon reservoirs represent. Bulk $\delta^{13}C_{DIC}$ measurements represent instantaneous values, which can be subject to diurnal variation, whereas the $\delta^{13}C_{leaf}$ measurements represent an integrated value of a number of processes operating on different timescales. Assimilation of carbon derived via root uptake from the DIC reservoir of the pore water in the underlying sediment (e.g., Hemminga and Mateo 1996) is also a possible interfering factor, although seasonal measurements of pore water $\delta^{13}C_{DIC}$ from the upper 6 cm of the local sediments (ranging from $-1.6‰$ to $-7.5‰$; unpubl. data) did not correlate with the contemporaneous $\delta^{13}C_{leaf}$. Further complexity is added by several physiological and chemical factors affecting the kinetics of carbon isotope uptake and assimilation by marine plants, which intervene between the bulk source $\delta^{13}C$ and final biomass $\delta^{13}C$ (Fogel and Cifuentes 1993; Burkhardt et al. 1999).

A salient characteristic of the temporal measurements of $\delta^{13}C_{leaf}$ was its distinct cyclic seasonal pattern from minimum values in February–March to maximum values in May–July, which was reproducible in all the studied meadows (Fig. 5b). The cyclic seasonality of $\delta^{13}C_{leaf}$ has been previously reported (Stephenson et al. 1984) and appears to be in concert with the seasonal fluctuation of the growth pattern of temperate sea grasses (e.g., Pedersen and Borum 1992; Lent and Verschuere 1994), following the increase in incident irradiance and temperature in spring and summer. This line of evidence provides a link between the dynamics of uptake and assimilation of carbon isotopes by eelgrass and the seasonality in their carbon demand imposed by their growth rate.

Theoretical models and empirical relationships from field and laboratory observations have often shown the importance to the $\delta^{13}C$ of aquatic algae of fluctuations in the balance between the supply of and intracellular demand for external inorganic carbon (Hollander and McKenzie 1991;

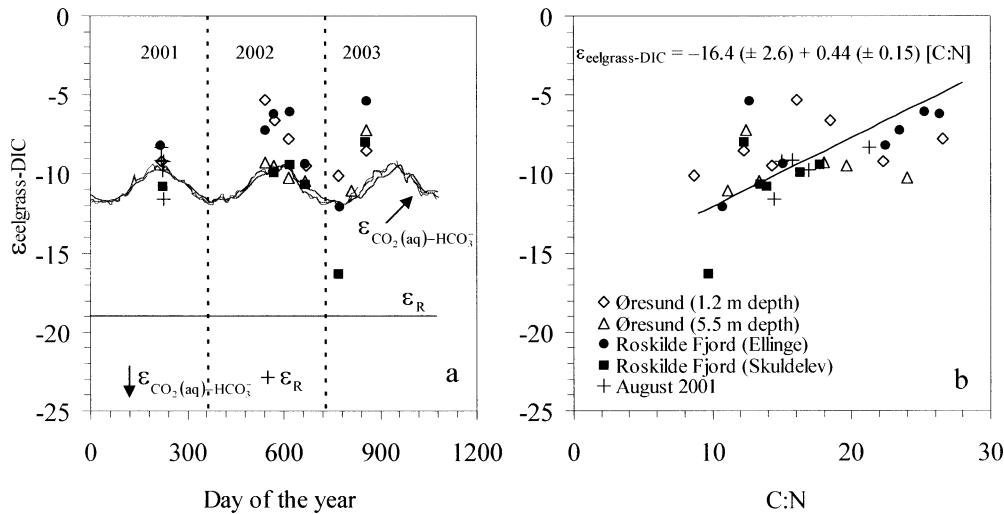


Fig. 9. Apparent carbon isotope enrichment factor ($\epsilon_{\text{eelgrass-DIC}} = \delta^{13}\text{C}_{\text{leaf}} - \delta^{13}\text{C}_{\text{DIC}}$) in new leaf of *Zostera marina*: (a) $\epsilon_{\text{eelgrass-DIC}}$ versus day of the year from 1 January 2001 to 31 December 2003, and (b) $\epsilon_{\text{eelgrass-DIC}}$ versus molar C:N ratio of new leaf. The curves and horizontal solid line in (a) indicate theoretical limits for $\epsilon_{\text{eelgrass-DIC}}$ (see text for details).

Fogel and Cifuentes 1993; Burkhardt et al. 1999). The systematics of carbon isotope uptake and assimilation by marine plants during photosynthesis can be described by a net isotope enrichment factor, $\epsilon_p \cong \delta^{13}\text{C}_p - \delta^{13}\text{C}_{\text{substrate}}$, where $\delta^{13}\text{C}_p$ = isotopic composition of the product of photosynthesis and $\delta^{13}\text{C}_{\text{substrate}}$ = isotopic composition of the inorganic substrate assimilated, i.e., CO_2 (Fogel and Cifuentes 1993). On the basis of the conceptual model of Burkhardt et al. (1999), the apparent ϵ_p of eelgrass relative to external DIC can be expressed as follows:

$$\begin{aligned} \epsilon_{\text{eelgrass-DIC}} &\cong \delta^{13}\text{C}_{\text{leaf}} - \delta^{13}\text{C}_{\text{DIC}} \\ &= \epsilon_{\text{CO}_2(\text{aq})-\text{HCO}_3^-} \frac{F_a}{F_a + F_b} + \epsilon_R \frac{F_{-a}}{F_a + F_b} \quad (1) \end{aligned}$$

where $\delta^{13}\text{C}_{\text{leaf}} \cong \delta^{13}\text{C}_p$, with $\delta^{13}\text{C}_p$ as before, ϵ_R = kinetic isotope enrichment factor by the enzyme ribulose 1,5-bisphosphate carboxylase oxygenase (RUBISCO), F_{-a} = flux of CO_2 out of the cell, and F_a, F_b = flux into the cell of the source of substrate CO_2 , i.e., $\text{CO}_2(\text{aq})$ and HCO_3^- , respectively, from the bulk external DIC reservoir.

Equation 1 was arrived at by considering that, at the pH range of natural coastal waters, the majority of the DIC pool is in the form of HCO_3^- , hence $\delta^{13}\text{C}_{\text{DIC}} \approx \delta^{13}\text{C}_{\text{HCO}_3^-}$, and the isotopic difference between substrate (CO_2) and bulk external source (DIC) can be approximated by the isotope enrichment factor between CO_2 and HCO_3^- , i.e., $\delta^{13}\text{C}_{\text{CO}_2(\text{aq})} - \delta^{13}\text{C}_{\text{DIC}} \approx \epsilon_{\text{CO}_2(\text{aq})-\text{HCO}_3^-}$. Also, isotopic fractionation during inorganic carbon flux in and out of the cell was ignored as comparatively minimal (Burkhardt et al. 1999). The following limits can be set on $\epsilon_{\text{eelgrass-DIC}}$ in relation to growth patterns. When diffusive loss of CO_2 from the cell is minimal ($F_{-a} \sim 0$), ϵ_R is not expressed, and $\epsilon_{\text{eelgrass-DIC}} \rightarrow \epsilon_{\text{CO}_2(\text{aq})-\text{HCO}_3^-}$ or $\epsilon_{\text{eelgrass-DIC}} \rightarrow 0$ when the plants are entirely reliant on external $\text{CO}_2(\text{aq})$ uptake ($F_b \approx 0$) or direct uptake of external HCO_3^- ($F_a = 0$), respectively. This situation can be expected to occur during the period of fast growth rates, when ele-

vated inorganic carbon demand by the plant can closely match the external supply, allowing minimal CO_2 escape from the cell. Furthermore, isotopically enriched values in the range of $\epsilon_{\text{CO}_2(\text{aq})-\text{HCO}_3^-} < \epsilon_{\text{eelgrass-DIC}} \leq 0$ can occur only when eelgrass plants are entirely reliant on direct uptake of external HCO_3^- and its intracellular conversion to substrate CO_2 . Elevated cell leakage of CO_2 increases the contribution of ϵ_R to $\epsilon_{\text{eelgrass-DIC}}$, which is maximized when $[F_{-a}/(F_a + F_b)] \rightarrow 1$, whereupon $\epsilon_{\text{eelgrass-DIC}} \rightarrow \epsilon_{\text{CO}_2(\text{aq})-\text{HCO}_3^-} + \epsilon_r$ or $\epsilon_{\text{eelgrass-DIC}} \rightarrow \epsilon_R$, depending on the DIC species utilized as source of substrate CO_2 . This situation could characterize the seasonal phase of slow growth rate, when the carbon demand by the plant is relaxed, external supply is in comparative excess, and more substrate CO_2 can escape the cell.

The $\epsilon_{\text{eelgrass-DIC}}$ for *Z. marina* from the available $\delta^{13}\text{C}_{\text{leaf}}$ and $\delta^{13}\text{C}_{\text{DIC}}$ observations can be examined relative to the theoretical values set by the above limits. The theoretical values were computed using an $\epsilon_R = -19\text{‰}$ as a conservative estimate of the extent of enzymatic carbon isotope fractionation in sea grasses (ϵ_R range: -30‰ to -19‰ ; Guy et al. 1993), the equilibrium $\epsilon_{\text{CO}_2(\text{aq})-\text{HCO}_3^-}$ based on the temperature function in Zhang et al. (1995), and the temperature data available for Øresund and Roskilde Fjord at Kulhuse (fjord entrance) and Okseholme (fjord apex) (Fig. 2). The observed $\epsilon_{\text{eelgrass-DIC}}$ (Fig. 9a) maintains the distinct seasonality seen in $\delta^{13}\text{C}_{\text{leaf}}$ (Fig. 5b), with more negative values during the period of low biomass and growth rate (February) and less negative values during the period (May to July) when biomass and growth rate are high (Pedersen and Borum 1993). Further support for the influence of growth rate on $\epsilon_{\text{eelgrass-DIC}}$ is provided by its significant linear correlation with the corresponding leaf C:N ratio (Fig. 9b; $r = 0.565$, $p = 0.001$, $n = 31$), which is used here as a proxy for growth rate on account of its growth-related seasonality (Figs. 4, 7). The observed $\epsilon_{\text{eelgrass-DIC}}$ varied mostly between -12.1‰ and -5.3‰ , with an overall average of $-9.1\text{‰} \pm 2.2\text{‰}$ ($n =$

31), and was generally close to, or less negative than, $\epsilon_{\text{CO}_2(\text{aq})-\text{HCO}_3^-}$, the upper limit expected under conditions of reliance on external $\text{CO}_2(\text{aq})$ uptake closely matched by plant demand. The excursion of $\epsilon_{\text{eelgrass-DIC}}$ beyond that expected for external $\text{CO}_2(\text{aq})$ uptake was evident on many occasions during the period of fast growth rate from May to September in all meadows, when $\epsilon_{\text{eelgrass-DIC}} > -9\text{‰}$ (Fig. 9a), with an average of $-7.1\text{‰} \pm 1.1\text{‰}$ ($n = 12$). By comparison with the theoretical limits set by the model for the utilization of the different external DIC species, this range of values (i.e., $\epsilon_{\text{CO}_2(\text{aq})-\text{HCO}_3^-} < \epsilon_{\text{eelgrass-DIC}} \leq 0$) suggests reliance on direct uptake of external HCO_3^- . This mechanism of inorganic carbon acquisition is known for *Z. marina* (Beer et al. 2002) and is clearly indicated by the present data set at least during the period of fast rates of primary production within the limitations associated with the current measurements of external $\delta^{13}\text{C}_{\text{DIC}}$ (instantaneous value) and the $\delta^{13}\text{C}$ in bulk new leaf biomass (several days).

Stable isotopic composition of nitrogen—The uptake and assimilation of the stable isotopes of nitrogen are subject to the same constraints of enzymatic kinetic fractionation, with the expression of the fractionation being subject to the external inorganic nitrogen availability relative to plant demand (Fogel and Cifuentes 1993; Waser et al. 1998). Dissolved inorganic nitrogen in the surface waters of Øresund and Roskilde Fjord consisted of both nitrate and ammonium, with a systematic seasonal exhaustion and replenishment pattern (Fig. 2). Sea grasses have the capacity to utilize both forms of dissolved inorganic nitrogen in surface waters through leaf uptake (Short and McRoy 1984). Of the two forms, nitrate exceeded ammonium concentrations by a factor of 10 throughout the study area during the brief period of maximum concentrations in winter (Fig. 2; Table 1). Assuming a constant $\delta^{15}\text{N}$ of the inorganic nitrogen substrate assimilated by the plants, a seasonal pattern of $\delta^{15}\text{N}_{\text{leaf}}$ may be expected on the basis of plant metabolism of stable isotopes in relation to substrate availability in the water column but was not evident in the current measurements (Fig. 5d). This is not unexpected, considering that aquatic grasses also have access to the pool of microbially regenerated inorganic nitrogen in the pore waters of the underlying sediments through root uptake (Short and McRoy 1984; Dennison et al. 1987). In addition to this, young sea grass tissues rely heavily on reclamation and translocation of nitrogenous compounds from older plant tissues (Borum et al. 1989; Pedersen and Borum 1992). The $\delta^{15}\text{N}_{\text{leaf}}$, therefore, can be expected to integrate the $\delta^{15}\text{N}$ of current and past assimilated inorganic nitrogen from both the surface water and the underlying sedimentary pore water. These processes can dampen considerably any seasonality in the isotopic signal that might be anticipated on the basis of the strong seasonality in the availability of dissolved inorganic nitrogen in the surface waters.

The most marked aspect of the current $\delta^{15}\text{N}_{\text{leaf}}$ measurements was their progressive isotopic enrichment with proximity to the apex of Roskilde Fjord to values as high as $+20\text{‰}$ in the innermost station (Fig. 10). This strong spatial gradient apparently persisted in the 3-yr course of this study (Fig. 5c) and suggests influence on the $\delta^{15}\text{N}_{\text{leaf}}$ of the isotopic

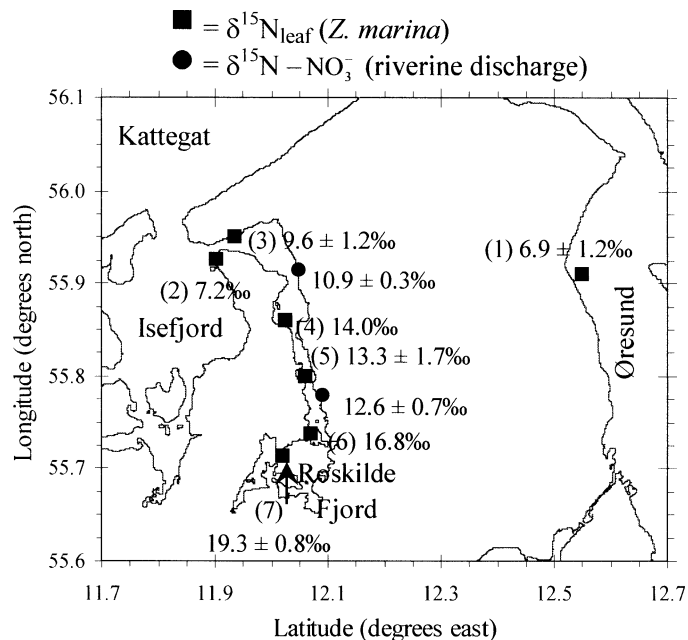


Fig. 10. Spatial distribution of the $\delta^{15}\text{N}$ of new leaf of *Zostera marina* and of riverine NO_3^- in Øresund and Roskilde Fjord, Denmark. The values are means $\pm 1\sigma$ where $n \geq 2$, whereas the numbers in parentheses indicate station identity as in Fig. 1b.

composition of the source of assimilated inorganic nitrogen rather than its concentration and, hence, availability (Fourqurean et al. 1997; McClelland and Valiela 1998). Roskilde Fjord has been a heavily eutrophic ecosystem for several decades because of large anthropogenic nutrient loads delivered to the system (Kamp-Nielsen 1992; Middelboe and Sand-Jensen 2000). The measured riverine $\delta^{15}\text{N} - \text{NO}_3^-$ (Figs. 5c, 10; overall average: $+11.8\text{‰} \pm 1.1\text{‰}$) is within the range of values ($+10\text{‰}$ to $+20\text{‰}$) reported for the $\delta^{15}\text{N}$ of freshwater and estuarine pools of dissolved inorganic nitrogen derived from anthropogenic waste (Heaton 1986; Cifuentes et al. 1989; McClelland and Valiela 1998). For comparison, the $\delta^{15}\text{N} - \text{NO}_3^-$ in pristine riverine waters was found to be approximately $+4\text{‰}$ (Brandes and Devol 2002). Given the long-term quality of sea grass $\delta^{15}\text{N}$ as an integrator of the isotopic composition of source inorganic nitrogen (e.g., McClelland and Valiela 1998), the measured spatial gradient of $\delta^{15}\text{N}_{\text{leaf}}$ (Fig. 5c) can be perceived as a simple dilution line, reflecting physical mixing of anthropogenically derived and open water inorganic nitrogen, the latter equivalent to the $\delta^{15}\text{N}_{\text{leaf}}$ measured in Øresund ($+6.9\text{‰} \pm 1.2\text{‰}$, $n = 14$).

The range of $\delta^{15}\text{N}_{\text{leaf}}$ values overlaps with the measured riverine $\delta^{15}\text{N} - \text{NO}_3^-$ in the meadows closest to the major riverine discharge (i.e., stations 4 and 5; Figs. 5c, 10) but indicates an isotopic enrichment relative to this source of inorganic nitrogen by as much as 7–8‰ in the innermost part of the fjord. This tentative comparison is based on spot measurements of the riverine $\delta^{15}\text{N} - \text{NO}_3^-$ entering the fjord, which might not be representative of the annual average riverine input. An additional factor that can affect the $\delta^{15}\text{N}_{\text{leaf}}$ is the assimilation of ammonium from surface and sediment

pore waters, which can have a different nitrogen isotopic composition from the riverine NO_3^- . Microbial nitrification and denitrification can enrich strongly in ^{15}N the dissolved inorganic nitrogen reservoir of surface waters (Cifuentes et al. 1989; De Brabandere et al. 2002; Sebilo et al. 2003) with their large isotopic fractionations (Cline and Kaplan 1975; Mariotti et al. 1981) and important contribution to the cycling of nitrogen in estuarine environments in general (Cifuentes et al. 1989; De Brabandere et al. 2002) and in Roskilde Fjord in particular (Kamp-Nielsen 1992). The observed enrichment of $\delta^{15}\text{N}_{\text{leaf}}$ in the innermost part of the fjord relative to the instantaneous local riverine $\delta^{15}\text{N} - \text{NO}_3^-$ value (Fig. 5c) can be the result of assimilation of the residual reservoir of similarly affected inorganic nitrogen in the surface waters. A more detailed approach that covers all the above processes and different dissolved inorganic nitrogen pools is clearly required to resolve the cycling of nitrogen isotopes in this ecosystem and their signature in local eelgrass tissues.

In summary, the N_{org} , molar C:N ratio, and $\delta^{13}\text{C}_{\text{leaf}}$ of the new leaf of eelgrass plants (*Z. marina*) from a number of meadows in the estuarine waters of Roskilde Fjord and Øresund, Denmark exhibited large temporal fluctuations in concert with the imbalance between external supply of inorganic nutrients and internal demand imposed by seasonality in the growth rate. Compared with theoretical values, the apparent carbon isotope enrichment factor of new leaf relative to external DIC ($\epsilon_{\text{eelgrass-DIC}}$ range from -12.1‰ to -5.3‰) indicated predominantly partial expression of enzymatic carbon isotope fractionation, especially during the fast growth season (spring–summer), when carbon assimilation must occur under conditions of closely matching external supply and plant demand. During this period, furthermore, eelgrass plants must be entirely reliant on direct uptake of external HCO_3^- leading to the observed isotopically enriched $\delta^{13}\text{C}_{\text{leaf}}$. The link to growth rate lends some degree of temporal predictability to the $\delta^{13}\text{C}_{\text{leaf}}$, but the ensuing annual range of values can be large (here extending over 14‰), which must be taken into consideration in environmental monitoring and food web studies that use $\delta^{13}\text{C}$ as a tracer. The $\delta^{15}\text{N}_{\text{leaf}}$ in contrast, was characterized by a steep spatial gradient of increasing isotopic enrichment within the inner reaches of the fjord, extending over 13‰ . Because eelgrass plants appear to grow mostly during the period of low external inorganic nitrogen availability in spring and summer, minimal nitrogen isotope fractionation can be expected during assimilation, resulting in a strong signal of the source of the assimilated inorganic nitrogen in eelgrass tissues. The strongly positive $\delta^{15}\text{N}_{\text{leaf}}$ and riverine $\delta^{15}\text{N} - \text{NO}_3^-$ values measured well inside the fjord suggest an anthropogenic origin of the assimilated inorganic nitrogen, which is introduced mainly via riverine input. With its potential for identification of the origin of inorganic nitrogen that enters coastal ecosystems, the $\delta^{15}\text{N}$ of sea grasses can be an informative environmental monitoring parameter.

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