Biochemical characterisation and fatty acid profiles of 25 benthic marine diatoms isolated from the Solthörn tidal flat (southern North Sea)

Bettina Scholz · Gerd Liebezeit

Abstract Twenty-five intertidal diatom species were isolated from the Solthörn tidal flat (Lower Saxony, southern North Sea) and grown in semi-continuous cultures under standardised conditions, in order to observe differences in their biochemical gross compositions (e.g. protein, lipid, carbohydrate and ash contents). Composition, expressed as % dry weight, indicated that the majority of species (52 %) contained only <15 % protein but had nearly twice the total amount of carbohydrate and two to three times higher ash content. In addition, most species contained a relatively constant percentage of lipids (19.4 to 25.6 %), whereas extraordinary high lipid contents (>30 %) were found for *Amphora exigua*, *Gyrosigma spenceri*, *Pleurosigma angulatum* and *Gyrosigma littorale*. Glucose, galactose, mannose and ribose constituted the majority of the sugars detected, although the levels of these varied between species. Lipid class composition showed high concentrations of phospholipids and galactolipids as major constituents (19–22 % and 40–43 % of total lipids). The major fatty acids in most species were 14:0, 16:0, 16:1(n-7) and 20:5(n-3). Significant differences in biochemical gross compositions were found in the temperature (10, 30 °C) and salinity tests (20, 35 PSU), suggesting special intracellular acclimatisation processes that provide possible explanations for the adaptability of the species to environmental variations and the distinct differences in the diatom assemblages.

Keywords Benthic marine diatoms · Biochemical composition · Carbohydrates · Cell volumes · Chlorophyll *a* · Fatty acids · Lipid classes · Proteins

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Introduction

Diatoms are the major group of photoautotrophic organisms inhabiting marine intertidal fine sediments (Admiraal 1984; Underwood 1994). Diatom-dominated biofilms are important in the ecology of mudflats as they exhibit high rates of primary production (Pinckney and Zingmark 1991), influence erosion and deposition of sediment (Underwood and Paterson 1993; Paterson 1994) and affect sediment water nutrient fluxes (Sundbäck and Granéli 1988; Rysgaard et al. 1995).

Intertidal mudflats constitute a highly unstable environment due to continuously changing water levels and currents as well as temporary exposure to air and higher solar irradiation and temperatures (Reineck 1983). Most marine benthic diatom species, isolated from intertidal environments, showed only slight differences in growth responses to temperature and salinity fluctuations in laboratory trials (e.g. Admiraal 1977; Scholz and Liebezeit 2012a). Salinity influences diatom physiology directly by exerting an osmotic stress. Upon this stress, algae exhibit a wide range of adaptations at the molecular, cellular and organism level (Hare and Cress 1997; Bohnert et al. 2001). Besides free amino acids (e.g. proline), several other organic osmolytes have been detected in marine and estuarine diatoms such as cyclohexanetetrol (Fujii et al. 1995; Garza-Sánchez et al. 2009), glycerol (Dickson and Kirst 1987) and mannose (Paul 1979). In addition to their function in the alleviation of osmotic stress, some organic osmolytes also have shown cryoprotective properties during freezing (e.g. Helliot and MortainBertrand 1999; Takagi et al. 2000). These include the protection of membranes and proteins by sugars (Uemura and Steponkus 2003) and the scavenging of reactive oxygen species by proline (Hare and Cress 1997). The main lipid classes of the thylakoid membrane of diatoms are galactolipids (Harwood 1998). For polar diatoms, it has

been shown that both lipid classes contained high concentrations of certain polyunsaturated fatty acids (PUFAs, Mock and Kroon 2002) such as stearidonic (18:4 (n-3)) or eicosapentaenoic acid (20:5 (n-3)). In particular, 6,9,12,15-hexadecatetraenic acid, which is a typical constituent of diatom lipids, has so far only been found in cold-acclimated cells of other algal groups (Thompson et al. 1992) and is assumed to be responsible for increase of membrane fluidity at low temperatures (Jüttner 2001).

Species dominance in intertidal flats is probably related to small intraspecific differences in, e.g. salinity and temperature tolerance (Underwood 1994). Therefore, the main objective of the present investigation is to find first indications of acclimatisation to fluctuating salinities and temperatures on the level of biochemical gross compositions. Thus, laboratory trials were conducted to determine their protein, lipid, carbohydrate and ash contents under standardised growth conditions in semi-continuous cultures as well as in relation to different salinities and temperatures. Furthermore, lipid classes as well as fatty acid and carbohydrate compositions were also considered under standardised growth conditions. To our knowledge, most of these Solthörn tidal flat isolates have so far not been considered in studies describing the biochemical compositions.

Material and methods

Diatoms were isolated by dilution series of samples from microbial biofilms on sediments in the intertidal zone of the Solthörn tidal flat, located in the eastern part of the Inner Jade near the village of Tossens in Lower Saxony, Germany (53° 34′ 2.03″ N 8° 13′ 54.66″ E, Scholz and Liebezeit 2012b, c). Samples were taken at low tide from sediments characterised by mixed sands at biweekly intervals from June 9 to August 22, 2008. In a first step, subsamples of approximately 2–3 g were evenly distributed on glass Petri dishes, diluted with 10 mL sterile filtered sea water from the sampling area (spreading plating method, Hasle and Fryxell 1970) and stored until isolation at 18 ± 2 °C and an irradiance of 400 µmol photons m^{-2} s^{-1} (Phillips, Germany, Master TL-D 18 W/840). PAR irradiance (400–700 nm) was measured with an underwater quantum sensor LI-192 (Q 17079) connected to a Licor Data Logger LI-1000. In the second step, both epipsammic and epipelic species were collected with shortened glass pipettes under the microscope and distributed over agar plates, using f/2 medium (Guillard 1975). Artificial seawater salt (Tropic Marin®, GmbH Aquarientechnik, Germany) dissolved in de-ionised water was used with a salinity of 30 PSU and pH of 8.0. Diatom identification was conducted as described in Scholz and Liebezeit (2012b).

Determination of growth rates measurement of cell sizes

The counting of samples was performed microscopically, every second day, using a Neubauer Improved counting chamber with 0.1 mm depth (LO Laboptik GmbH, Germany). Only cells which exhibited red fluorescence under UV light were counted. At least 1,000 cells were counted in each sample at ×400 magnification. The specific growth rate (μ) was calculated according to $\mu = \ln_{c1} - \ln_{c0}/t_1 - t_0$ where c_1 and c_0 denote cell counts at times t_1 and t_0, respectively, within the exponential phase. Data are presented in Scholz and Liebezeit (2012a).

In addition, the diatoms were embedded in Naphrax (refractive index 1.74, Northern Biological Supplies Ltd., UK), and dimensions were measured under a light microscope at ×1,000 magnification. Cell volumes were calculated according to the method described by Snoeijs et al. (2002).

Cultivation of monocultures prior to experiments

Non-axenic cultures of individual species were grown in 2 L Erlenmeyer flasks (approximately 600 mL culture volume) using f/2 medium as a base medium (pH 8.04, salinity=30 PSU). To reflect Solthörn tidal flat conditions (Scholz and Liebezeit 2012c), the medium was modified: (1) we used two nitrogen sources, 500 µM $NaNO_3$ and 150 µM NH_4Cl; (2) the N/P ratio was changed to 17:1 and (3) $NaHCO_3$ was added after autoclaving to a final concentration of 2 mMol/L^{-1}. The irradiance was 600 µmol photons m^{-2} s^{-1} (Phillips, Germany, Master TL-D 18 W/840) at a light/dark regime of 16:8 h; cultures were maintained at 18 ± 2 °C. Glass beads (Sartorius, Ø 300–700 µm) were placed on the bottom of culture vessels with a layer of approximately 3 mm thickness and used as substratum. Salinity, pH and conductivity were measured using handheld probes (YK-31SA, YK-2001PH SI Model 33, Engineered Systems and Designs-Model 600, Philips W9424). Cultures were transferred to new medium every 7 days to maintain optimal growth. After biofilm development (21 days after inoculation), the culture broths were removed with a sterile sleeve until only a thin liquid film remained over the biofilms. There was only minimal loss of cells (0.5–0.8 %) during the removal of the culture broth. Subsequently, the experiments were started by addition of fresh culture medium.

Experimental setup

Besides the standardised conditions described above, two different test series (one-factor-at-one-time) were conducted in addition, with variations in salinities (20, 35 PSU) and temperatures (10, 30 °C). Different salinities were obtained by dissolving defined quantities of artificial sea salt in de-

ionised water, whereas temperature experiments were carried out in an incubator (Certomat T plus, Sartorius, Germany). Once per day, 60 mL of culture suspension was replaced by fresh medium (semi-continuous culture) which gave a dilution rate of 0.6 d^{-1}. The experiments were started with eight replicate flasks per treatment and species. The position of replicate flasks was randomly changed every other day to eliminate any location effects due to minor changes in external conditions.

Harvesting and sample treatment

Generally, biofilms were allowed to grow without physical disturbance on the glass beads, from which diatoms could be easily removed for sampling by shaking on a rotating shaking device (350 rpm), thus dislodging the biofilm from the substrate and homogenising the culture. Microscopic observation of the beads after shaking confirmed the complete release of the cells. Biomass was harvested by centrifugation at 680 rpm for 10 min. The eight replicates with the diatom biomass were subdivided each into nine aliquots and used for different extraction procedures as described below.

Analyses

A spectrophotometric method adapted from Parsons et al. (1984) using Lorenzen's equations (Lorenzen 1967) was used to determine chlorophyll a (chl a) contents in the samples. Measurements were conducted following the method described by Brito et al. (2009). Tests were performed in triplicate. Chl a concentrations were calculated in $\mu g \times \mu m^{-3} \times 10^{-10}$.

Total protein, lipid, carbohydrate and ash contents in the diatom species were determined at exponential phase (16–26 days after inoculation, for details see Scholz and Liebezeit 2012a), growing under standardised (e.g. 20 °C, 30 PSU) and different salinity (20, 35 PSU) and temperature (10, 30 °C) conditions. The compositions of lipid classes, fatty acids and carbohydrates were only determined for the standardised assays. All tests were performed in triplicate. To rupture diatom cells, all samples were homogenised with a mortar and pestle prior to assay.

Protein was determined spectrophotometrically using a Pierce test kit (BCA 23225) based on the procedure of Lowry et al. (1951) and standardised bovine serum albumin. In brief, filtered diatoms were prepared for analysis in four steps, with cooling intervals in an ice bath. Firstly, the filters were suspended in 4 mL 0.1 M NaOH in a 15-mL polypropylene test tube. Then the filter was ground in a tissue homogeniser for 1 min and sonicated for 20 s. Samples were diluted to 8 mL with 0.1 M NaOH, heated to 60 °C, sonicated for 10 s and centrifuged (4 °C, 800×g, 10 min). A total of 10 μL of each supernatant was transferred to each

triplicate well in a 96-well microplate; 200 μL of BCA reagent was added to each well and incubated for 30 min at 37 °C. Finally, the microplate was analysed at 526 nm with a microplate reader.

Total soluble carbohydrates were determined using the phenol sulphuric acid method of Kochert (1978) and Ben-Amotz et al. (1985) incorporating the modifications of Mercz (1994) and Buttery (2000). The samples were homogenised in 1 M H$_2$SO$_4$, and after heating at 100 °C for 60 min, 0.3 mL of the supernatant was transferred into a fresh vial and made up to 2 mL with deionized water. Sets of glucose standards were prepared and 1 mL of 5 % (w/v) phenol solution was added to all samples, after which 5 mL concentrated H$_2$SO$_4$ was added. Absorbance was read at 485 nm. For carbohydrate composition, the extraction followed the method of Karsten et al. (1991), using 70 % EtOH. Saccharides were identified and quantified by thin layer chromatography (TLC Silica Gel 60 F254; Merck) according to the method described by Zhang et al. (2009). As standards arabinose, galactose, glucose, fucose, mannose, rhamnose, ribose and xylose (Merck) were used.

Lipids

The total lipid determination was based on the method of Bligh and Dyer (1959) as modified by Kates and Volcani (1966) and adapted by Mercz (1994) and Buttery (2000) using methanol/chloroform/de-ionised water (2:1:0.8, $v/v/v$). The extracts were dried under a stream of ultrapure N$_2$ gas and placed in a vacuum desiccator over silica gel overnight and then weighed. The lipids were resuspended in 100 μL 2:1 (v/v) chloroform/methanol, sealed, flushed with N$_2$ and then stored at −80 °C until lipid classes analysis. Lipid class were quantified by thin layer chromatography–flame ionisation detection using an Iactroscan Analyzer with methods described by Parrish (1987) and modified by Kreeger et al. (1997). Free fatty acids (FA), diglycerides (DG), hydrocarbons, triglycerides (TG), sterols, galactolipids and phospholipids were quantified using standards of nonedecane, triolein, oleic acid, dipalmitin, cholesterol, digalactocyl diglyceride and phosphatidyl choline, respectively.

Fatty acid and PUFA compositions

Lipid fractions were transesterified by methanolysis (1 % H$_2$SO$_4$ in methanol) for 3 h at 70 °C (Blank et al. 2002). After cooling, the resulting fatty acid methyl esters (FAMEs) were extracted with n-heptane and dried over anhydrous sodium sulphate. The FAMEs were analysed with argentation chromatography according to the method of Wilson and Sargent (1992). TLC plates were sprayed with a solution of 5 % silver nitrate in acetonitrile and activated by heating at 110 °C for 30 min. Plates were

developed in a TLC chamber saturated with toluene/aceto-nitrile (96:4, v/v). C14:0, C16:0, C16:1, C16:2ω4, C16:3, C16:4, C18:0, C18:4ω3, C20:5ω3, C24:0 and C22:6ω3 markers were used as standards. Samples and standards were visualised by spraying the plates with a solution of 3 % Cu(C$_2$H$_2$O$_2$)$_2$H$_2$O and 8 % H$_3$PO$_4$, followed by heating at 180 °C for 20 min. Plates were scanned from the origin to the solvent front, using a Shimadzu CS-9 10 scanning densitometer, and the integrated areas were computed by a Shimadzu CRl A data processor (Shimadzu Scientific Instruments). Plates were scanned at 350 nm (tungsten source) in the reflectance mode. The beam dimensions were 4.0×0.2 mm and the scan speed was 24 mm/min^{-1}. Each lane was scanned three times and the average values for the three scans were determined. The ratio of the density (pV-sec) of each marker to the density (pV-sec) of the internal standard was calculated for both the standard and sample lanes on each plate. The density ratios for the different standard amounts (1.0, 3.0, 5.0 and 10.0 pg) were used to obtain standard curves for each marker. The ratios of the density of each marker in the sample lane to the density of the internal standard in the same lane were used to determine the absolute amounts of each marker from their respective standard curves.

Total ash weights were determined by following the methods of Mercz (1994) and Buttery (2000). The glass fibre filters were placed within crucibles before combustion at 475 °C for 24 h. After combustion, the samples were weighed to five decimal places and total ash was calculated by subtracting the initial weight of the filter.

Statistical analyses

To assess variability of abundances and chemical compositions, analyses of variance (ANOVA) were performed on logarithmically transformed data to adhere to normality. In particular, abiotic parameters (temperatures, salinities) were used as source of the variance, while biochemical compositions of lipids, proteins, carbohydrates and ash contents were defined as dependent variables. All tests were performed with the programme XLSTAT 2011, Version 2011.2.08 Addinsoft.

Results

Cell volumes and chl a contents

The mean cell volume varied between 72 μm^3 for *Navicula perminuta* and 15.6×10^3 μm^3 for *Pleurosigma angulatum* (Table 1). Of the 25 isolates, 36 % had a cell volume <500 μm^3 (classified as small diatoms) and 32 % had a cell volume >5,000 μm^3 (classified as large diatoms). In addition, 28 % showed cell volumes >1,000 μm^3 and were

classified as medium-sized diatoms in the following. Only 4 % of the diatom species were found in the cutoff between the groupings <500 and >1,000 μm^3 (*Pinnularia ambigua* [23]). The chl a contents varied as a function of cell volume, being the highest for *P. angulatum* [24].

Biochemical compositions under standardised conditions

The gross compositions of each species (protein, lipid, carbohydrate and ash contents) expressed in % of dry weight (DW) showed partly significant differences between the individual species (Table 1). In detail, 24 % of the 25 species were rich in protein, ranging from 25.9 % (*Navicula gregaria* [17]) to 31.2 % (*N. salinicola* [20]) of DW and contained 7.5 to 9.6 % carbohydrate and 9.1 to 18.7 % ash. The majority of species (52 %) contained only <15 % protein but had nearly twice the total carbohydrate (20.3 [1] to 31.5 % [10]) and two to three times the ash (21.3 [5] to 32.5 % [9]) of the other species. In addition, 24 % of the isolated species showed protein contents between 15 and 25 %, with nearly twice the total carbohydrate (20.8 to 37.1 %) but significantly lower ash contents (8.7 to 18.6 %, ANOVA $F_{1,24}$=24.8, $P<0.0001$) as compared to the majority. Similar to the dry weight, the comparison of the ash-free dry weight (AFDW) showed a high level of differences between the taxa analysed. Here, protein ranged from 40.7 to 61.8 %, whereas carbohydrates varied between 21.3 and 47.0 % (Table 1). Glucose, galactose, mannose and ribose constituted the majority of the sugars detected, although the levels of these varied between species (Fig. 1). From this glucose showed the highest proportions in each species, varying between 0.4 and 1.8 μg μm^3×10^{-8}. In much lower concentrations, the isolates contained arabinose, xylose, rhamnose, fucose and ribose in addition to the major neutral sugars detected in all cultures.

Most species had similar relative lipid content (19.4–25.6 % DW; 11.2–19.7 % AFDW). Extraordinary high lipid contents (>30 % DW) were found for *Amphora exigua* [6], *Gyrosigma spenceri* [13], *P. angulatum* [24] and *Gyrosigma littorale* [12]. Lipid class composition showed high contents of phospholipids and galactolipids as major constituents in the diatom species (19–22 % and 40–43 % of the total lipids, respectively, Fig. 2). Within the individual species, fatty acids varied between 6 and 12 % of the total lipids, being the highest for *Achnanthes brevipes* [1]. The major fatty acids in most species were 14:0, 16:0, 16:1(n-7) and 20:5(n-3) (Fig. 3). The polyunsaturated fatty acids 16:2(n-4), 16:3(n-4), 16:4(n-1), 18:4(n-3) and 22:6 (n-3) also comprised a significant proportion (>4 % each) of the total fatty acids in the isolates. Seven species contained significant proportions of arachidonic acid, 20:4(n-6) (3.9–6 %, [3, 7, 9, 13, 14, 18, 23]), but most species examined contained very low proportions of (n-6) polyunsaturated fatty acids.

Table 1 Cell volumes, chlorophyll a concentrations in $\mu g\ \mu m^{-3} \times 10^{-10}$ and biochemical gross composition of 25 diatom species as percent dry weight (DW) and ash-free dry weight (AFDW), obtained from mono cultures grown under standardised growth conditions (400 μmol photons $m^{-2}\ s^{-1}$, 18 °C, 30 PSU, pH 8.0, nitrate as main nitrogen source (500 μM $NaNO_3$ and 150 μM NH_4Cl), N/P ratio 1:17)

| No.	Species (author)	Cell volume (μm³)	chl a (μg μm⁻³)×10⁻¹⁰	Protein (% DW)	Protein (% AFDW)	Lipid (% DW)	Lipid (% AFDW)	Carbohydrate (% DW)	Carbohydrate (% AFDW)	Ash (% DW)
1	Achnanthes brevipes (C. A. Agardh)	12,136±249.8	529.5±10.7	11.9±1.4	54.2±0.2	19.6±1.2	18.1±0.5	23.5±2.0	26.0±1.0	27.8±0.6
2	Achnanthes delicatula hauckiana (Grunow) Lange-Bertalot	465±32.9	20.3±1.4	13.2±1.5	43.0±1.2	29.8±3.1	18.7±0.6	27.2±2.6	36.1±0.4	28.6±0.9
3	Achnanthes exigua (Grunow)	81±3.6	3.5±0.2	12.8±2.3	59.1±1.4	25.6±2.8	18.4±0.3	20.3±1.9	22.5±1.1	28.0±0.4
4	Amphora angusta (Gregory)	5,727±153.4	235.0±6.6	9.6±0.8	50.1±2.0	21.4±4.6	12.6±0.4	26.5±2.8	36.3±1.0	28.3±1.1
5	Amphora arenaria (Donkin)	1,147±86.3	49.5±3.7	10.4±1.6	46.3±0.9	24.7±2.9	15.1±0.3	29.4±3.3	37.3±1.4	21.3±2.4
6	Amphora exigua (Gregory)	1,216±92.5	47.3±3.9	12.1±2.0	52.9±0.6	32.1±4.5	24.3±0.5	30.5±3.9	22.8±1.1	26.5±3.3
7	Amphora graeffii (Cleve)	3,335±102.7	144.2±4.4	11.5±1.1	53.6±1.1	24.4±2.8	19.2±0.8	25.4±1.6	27.2±0.8	29.5±1.6
8	Cocconeis peltoides (Hustedt)	192±12.4	8.7±0.5	14.6±0.9	46.7±3.4	20.9±1.0	16.1±0.2	27.3±2.7	34.2±1.4	18.4±4.5
9	Cylindrotheca closterium (Ehrenberg) Reiman & Lewin	153±11.9	7.2±0.6	10.7±0.6	56.2±1.4	20.3±2.1	18.1±1.1	26.1±1.9	23.7±1.0	32.5±6.1
10	Diploneis didyma (Ehrenberg) Cleve	9,889±192.6	431.4±8.3	12.5±1.3	43.7±0.7	25.3±1.8	19.1±0.5	31.5±2.1	35.2±2.5	20.7±3.0
11	Diploneis littoralis (Donkin) Cleve	7,620±164.7	329.2±7.1	11.9±0.7	54.1±0.6	22.9±1.5	18.7±0.9	20.4±1.5	27.2±1.1	9.6±0.7
12	Gyrosigma littorale (W. Smith) Griffith & Henfrey	288±31.3	12.4±1.3	19.8±2.4	35.1±1.1	30.1±4.3	26.9±0.6	36.3±4.0	38.0±1.6	14.3±2.9
13	Gyrosigma spenceri (Bailey ex Queckett) Griffith & Henfrey	9,844±186.2	467.7±8.1	20.7±3.1	49.7±0.9	34.5±2.4	22.3±1.1	37.1±4.6	29.0±0.8	18.6±3.8
14	Gyrosigma peisoneis (Grunow) Hustedt	1,696±103.8	73.3±4.5	18.3±1.6	51.1±2.4	22.4±0.9	14.4±0.7	20.8±1.7	34.5±1.3	8.7±0.3
15	Navicula digito-radiata (Gregory) Ralfs	5,929±139.4	256.1±6.0	29.3±0.5	53.8±2.0	18.9±1.4	16.1±0.9	9.5±0.7	30.1±1.0	11.2±1.0
16	Navicula forcipata (Greville)	2,373±116.5	102.5±5.2	17.2±0.8	48.3±1.6	19.7±2.9	11.2±0.6	21.3±1.1	40.5±2.4	11.1±0.3
17	Navicula gregaria (Donkin)	1,264±83.7	49.1±3.8	25.9±0.3	61.8±1.2	17.3±1.4	14.9±1.0	9.1±0.9	23.4±1.6	9.9±1.0
18	Navicula perminuta (Grunow)	72±8.3	3.1±0.4	26.5±1.9	58.2±0.9	23.8±1.6	18.3±1.2	7.5±2.7	23.5±2.2	9.1±0.7
19	Navicula phyllepta (Kützing)	201±11.9	9.6±0.5	27.3±4.6	55.1±1.4	23.1±4.9	20.4±2.5	8.0±3.8	24.5±1.5	9.6±5.1
20	Navicula salinicola Hustedt	44±9.7	2.2±0.4	31.2±3.1	59.5±2.3	25.5±2.7	19.2±0.7	9.6±2.9	21.3±1.3	9.3±2.3
21	Nitzschia constricta (Gregory) Grunow in Cleve & Grunow	483±13.5	21.9±0.6	18.5±1.7	48.1±1.5	29.7±4.2	19.7±0.4	26.5±3.1	32.2±1.7	17.3±2.6
22	Nitzschia epithemoides (Grunow) in Cleve & Grunow	1,864±109.4	84.5±4.7	16.9±0.7	44.3±1.0	22.3±2.5	20.7±1.1	20.8±4.6	35.0±2.0	14.7±1.7
23	Pinnularia ambigua (Cleve) (=Biremis ambigua (Cleve) D.G.Mann)	829±26.8	34.0±1.3	12.3±1.1	40.7±1.4	19.4±1.5	12.3±0.6	22.3±1.0	47.0±1.8	8.4±0.3
24	Pleurosigma angulatum (Queckett) W. Smith	15,632±263.7	675.2±12.5	26.9±3.8	52.8±0.9	33.5±4.9	26.7±1.3	8.1±5.3	20.5±1.1	18.7±1.6
25	Tryblionella navicularis (Brébisson ex Kützing) Ralfs in Pritchard	5,738±158.6	247.9±6.9	12.5±1.3	46.3±0.7	24.2±1.7	18.3±0.8	25.7±2.0	35.4±2.6	9.3±0.9

Values are means (± SE, $n=24$)
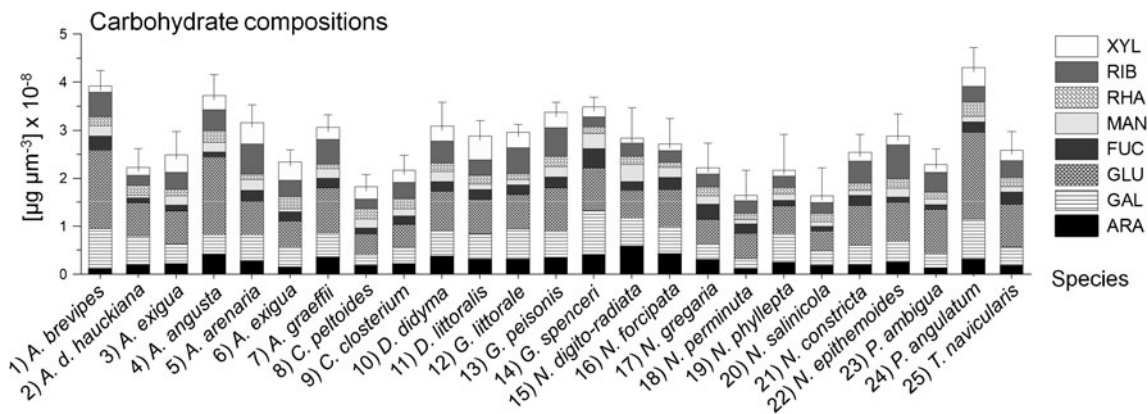
Fig. 1 Carbohydrate compositions of 25 diatom species, grown under standardised conditions, in µg µm$^{-3} \times 10^{-8}$, including means ± SD ($n=24$). Abbreviations: *ARA* arabinose, *GAL* galactose, *GLU* glucose, *FUC* fucose, *MAN* mannose, *RHA* rhamnose, *RIB* ribose, *XYL* xylose

Influence of salinity and temperature variations on the biochemical gross composition

The effect of variations in salinity and temperature on the 25 isolates is shown in Figs. 4 and 5. In comparison to values obtained from the standardised treatments (Table 1), relative differences in chl *a* concentrations of the 25 isolates varied only in a narrow range in the salinity and temperature variations (−0.5 to 1.2 %, Figs. 4a and 5a). Significantly higher levels of protein and lipids were found in most of the species tested in the high salinity experiment (35 PSU, up to 10.9 %, Fig. 5b, c; ANOVA $F_{1,48}=29.1$, $P<0.0001$) and an increase in carbohydrates at both low and high salinities (Fig. 5d; ANOVA $F_{2,23}=25.7$, $P<0.0001$). On the contrary, significantly lower levels of protein, lipids and carbohydrates were found in most of the tested species in the high temperature treatment (30 °C, Fig. 4b–d; ANOVA $F_{1,48}=34.5$, $P<0.0001$). The ash content of all species increased with lower salinity, with the greatest increase in *Amphora graeffii* [7] (4.5 % dry weight, Fig. 5e, ANOVA $F_{1,24}=24.8$, $P<0.0001$). In contrast, the ash

content in the high salinity test series varied only in a narrow range (−0.8 to 2.0 % dry weight, Fig. 5e, ANOVA $F_{1,24}=3.0$, $P=0.076$), similar to the high temperature test series (Fig. 4e). Moreover, similarities between lipid and carbohydrate levels in the low salinity and low temperature test series are noteworthy (Figs. 4c, d and 5c, d).

Discussion

Biochemical compositions and fatty acid profiles under standardised conditions

Proteins, carbohydrates and lipids are the major constituents of algal cells, and their relative portions vary with algal species (Brown et al. 1997). According to Parsons et al. (1961), the biochemical composition of diatoms is similar to that of other microalgae. Rapidly growing cells during exponential growth phase contain 30 to 60 % protein, 10 to 50 % carbohydrate and 5 to 20 % lipid (ash-free dry weight)
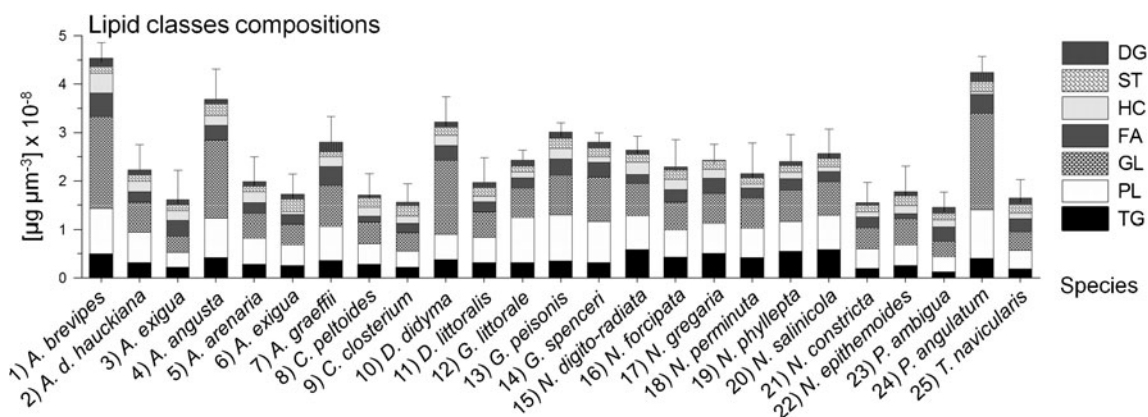

Fig. 2 Lipid classes compositions of 25 diatom species, grown under standardised conditions, in µg µm$^{-3} \times 10^{-8}$, including means ± SD ($n=24$). Abbreviations: *FA* free fatty acids, *DG* diglycerides, *HC* hydrocarbons, *TG* triglycerides, *ST* sterols, *GL* galactolipids, *PL* phospholipids

Fig. 3 Relative fatty acid compositions of 25 diatom species in % ($n=24$), grown under standardised conditions. Values expressed as percent of total fatty acids detected for each species
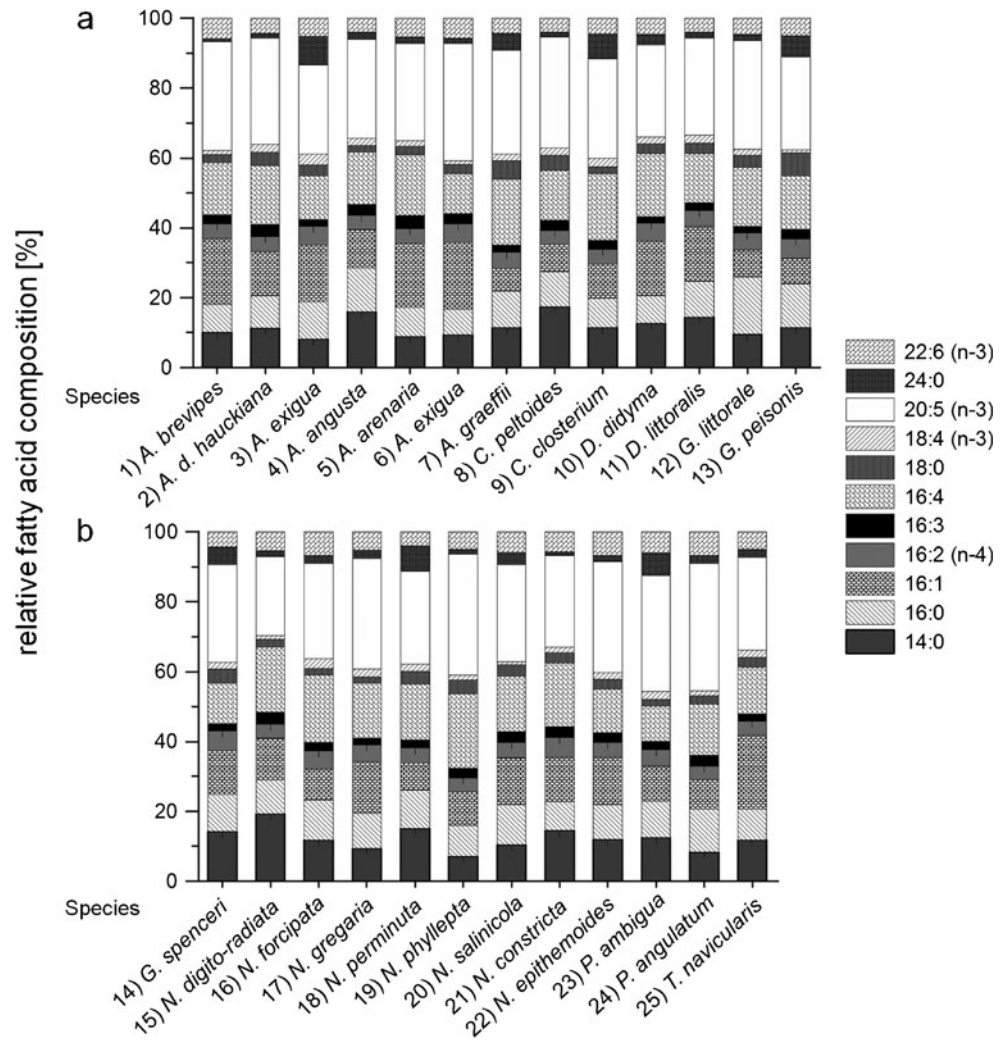
(Brown et al. 1997). In particular, planktonic diatoms contain 1.9–6.92 μg μm^{-3}×10^{-8} protein, 3.5–9.02 μg μm^{-3}×10^{-8} carbohydrate and 3.0–15.6 μg μm^{-3}×10^{-8} lipid (e.g. Lynn et al. 2000). In general, the results in the present study are within this range (Table 1, Figs. 1 and 2), although three classes of combinations in protein, carbohydrates and ash contents could be distinguished under standardised growth conditions, whereas, with the exception of four species, lipid contents did not vary considerably. The major lipid class in the isolates was polar lipids (48–66 %), although in some species, TG (0.3–25 %) and free fatty acids (0.6–15 %) were also abundant in the exponential phase cultures. In addition, Fig. 2 shows that the percentage of TG in general is lower compared to glycolipids and phospholipids. TGs have been reported to increase from 5 % in unstressed cultures to 70 % of total cell volume in nitrogen-stressed cultures of the planktonic diatom *Chaetoceros gracilis* Schütt (= *Chaetoceros neogracile* S.L.VanLandingham) (Lombardi and Wangersky 1995). Although the occurrence and the extent to which TG is produced appear to be species/strain-specific and are ultimately controlled by the genetic design of

individual organisms, oleaginous algae produce only small quantities of TG under optimal growth or favourable environmental conditions (Hu 2004). Synthesis and accumulation of large amounts of TG accompanied by considerable alterations in lipid and fatty acid composition occur in the cell when species are placed under stress conditions imposed by chemical or physical environmental stimuli, either acting individually or in combination. The major chemical stimuli are nutrient starvation, salinity and growth medium pH. The major physical stimuli are temperature and light intensity (Hu et al. 2008). In the present investigation, the initial nitrogen concentrations as well as the semi-continuous cultivation were sufficient to avoid nutrient-limiting circumstances and thus, nitrogen assimilation was not controlled during the course of the experiments. However, differences in the quality of the compounds accumulated during the cultivation processes, such as high TG concentrations or carbohydrates, support the assumption of nutrient limitation in some of the isolates.

The fatty acid profiles of the five diatom taxa were characteristic of most Bacillariophyceae: high proportions

Fig. 4 Differences in chlorophyll *a* contents (**a**) and biochemical gross compositions (protein (**b**), lipids (**c**), carbohydrates (**d**) and ash contents (**e**)) of 25 diatom species as percent of dry weight (*n*=8). The differences were calculated for each species, based on the data in Table 1, for low (10 °C) and high temperatures (30 °C)
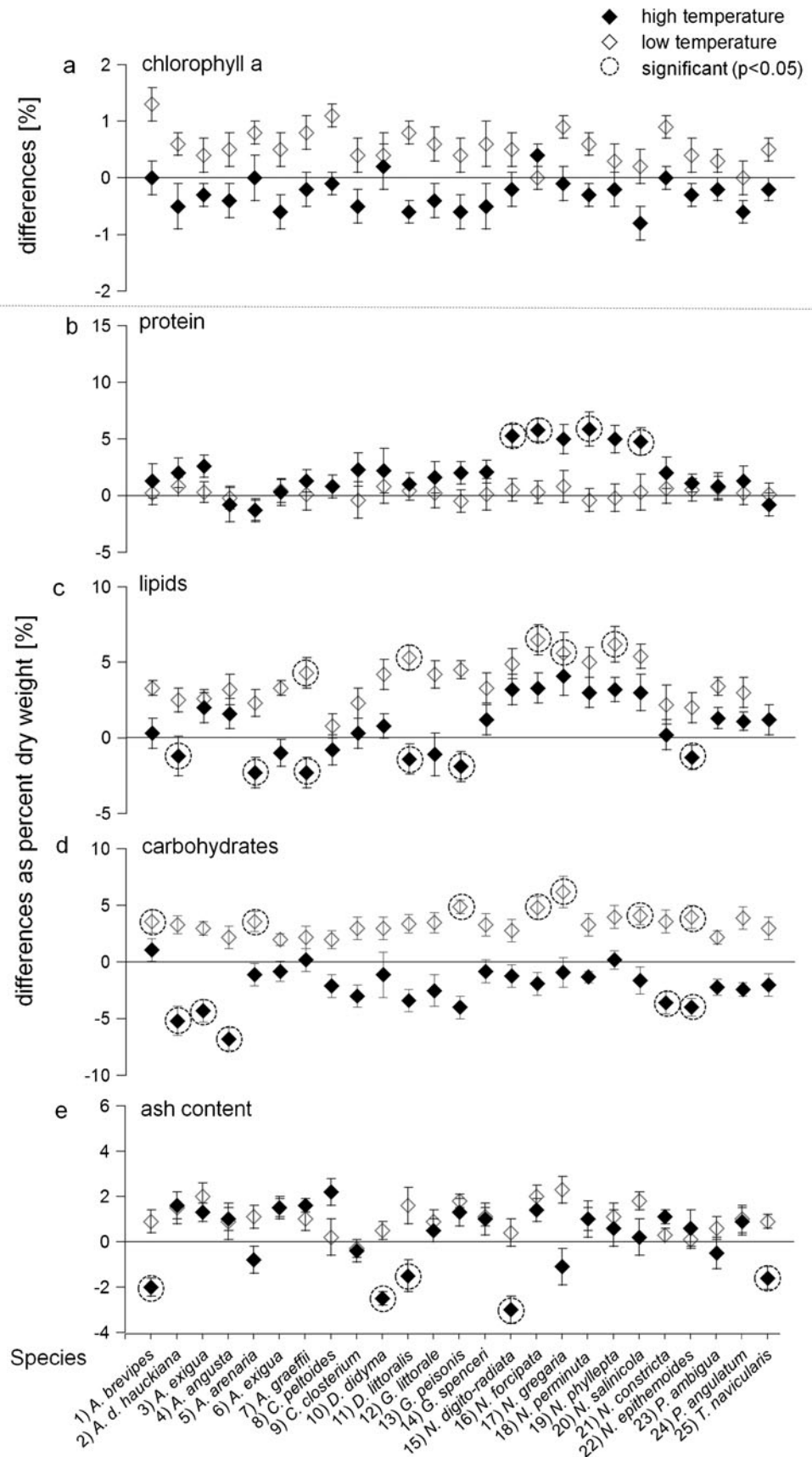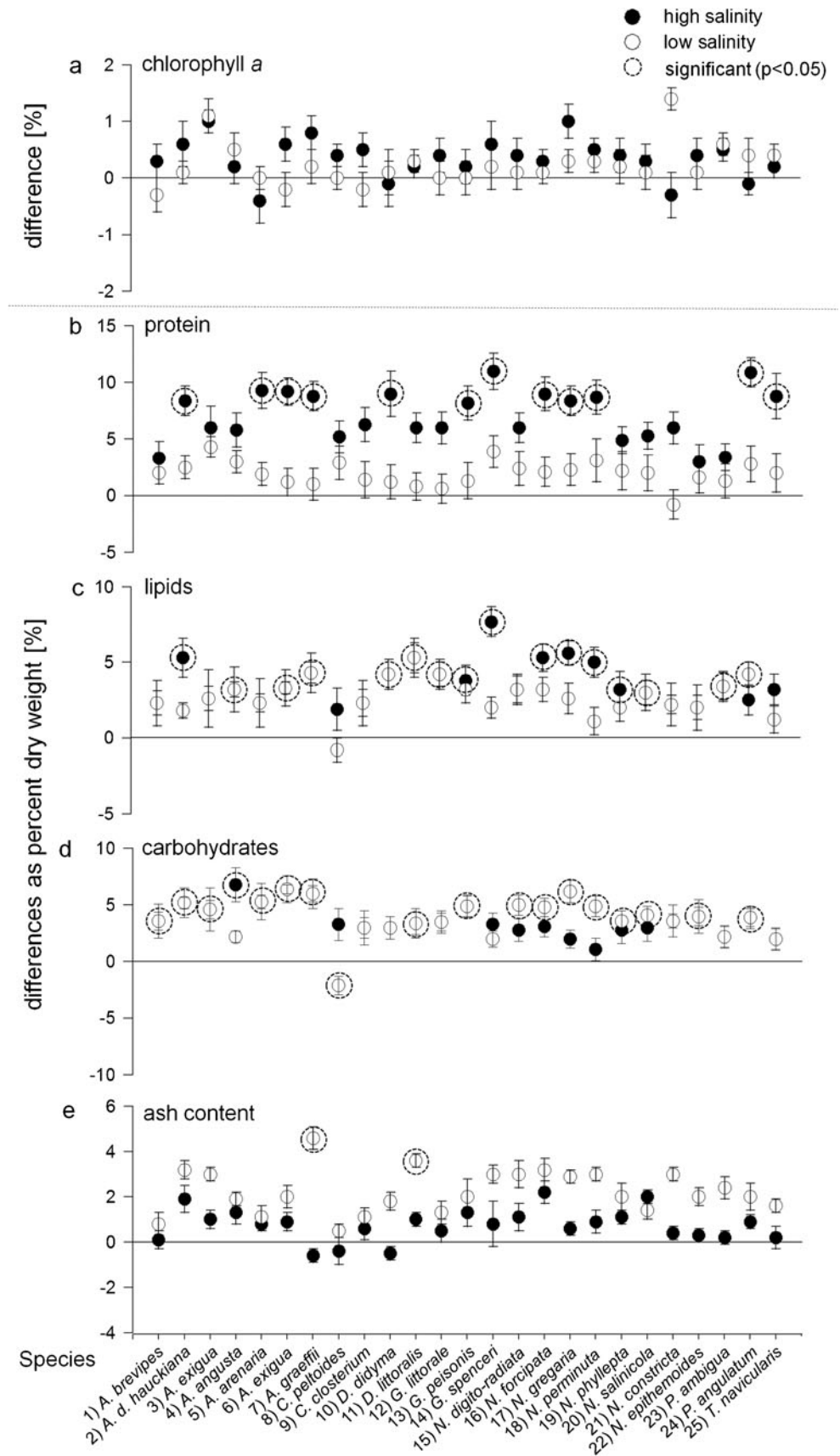
Fig. 5 Differences in
chlorophyll *a* contents (**a**) and
biochemical gross compositions
(protein (**b**), lipids (**c**),
carbohydrates (**d**) and ash
contents (**e**)) of 25 diatom
species as percent of dry weight
(*n*=8). The differences were
calculated for each species,
based on the data in Table 1, for
low (20 PSU) and high
salinities (35 PSU)

of 16:1(n-7) and 16:0 with variable, but usually high, proportions of 20:5(n-3) (Kates and Volcani 1966; Ackman et al. 1968; Dunstan et al. 1994; Renaud et al. 1994; Brown et al. 1997). In contrast, most species in the present study contained unusually high proportions of myristic acid (14:0, up to 13.9 %). For instance, values of 14:0 of up to 7.8 % of total fatty acids has been reported in *Gomphonema parvulum* (Kützing) Kützing (Jüttner 2001) and 8.8 % in the polar diatom *Fragilariopsis cylindrus* (Grunow in Cleve & Möller) W. Krieger in Helmcke & Krieger (Krell 2006) obtained by GC-MS analysis. In the present study, argentation TLC was applied, which can separate fatty acids based on the total number of ethylenic bonds. Although GC remains the most valuable and most efficient method of resolution and quantisation of lipids, including the non-polar higher molecular mass components, argentation TLC has been extensively utilised for analytical and semi-preparative resolution of methyl and glyceryl esters of saturated and unsaturated fatty acids (Arab and Akbar 2002). According to Özcimder and Hammer (1980), the recovery of highly unsaturated esters (n-5, n-6) was about 80 % and concluded that the decrease was a result of oxidation. Due to this observation, it is high likely that some of the unsaturated FA esters were underestimated and therefore the relative proportions were shifted for the benefit of the short-chain FA esters.

Finally, although glucose has been referred to be the major monosaccharide by most investigations including the present data, other monosaccharides (e.g. fucose) are dominant in some planktonic diatom cultures (Myklestad 1995). It may be concluded that the biochemical composition of the exponential phase cells of different species of marine benthic diatoms grown under identical conditions differs markedly. Similar observations were made by Myklestad (1974) for several planktonic species.

Effects of temperature and salinity on the biochemical gross composition

The specific growth rates of the 25 benthic marine diatoms in a preceding investigation did not vary considerably in a broad salinity (15–35 PSU) and temperature range (10–35 °C; Scholz and Liebezeit 2012a), whereas in the present study, significant differences in biochemical compositions were found in the temperature (10, 30 °C) and salinity tests (20, 35 PSU). In general, protein is the major cellular component in rapidly growing cells, while carbohydrate is the dominating substance among the extracellular products, especially polysaccharides (Myklestad 1995). Little is known about the ecological significance of the increased production of extracellular carbohydrates for the enhancement of the growth and survival of the producing species, especially under fluctuating physical conditions. The composition of extracellular polysaccharides has

been observed to be different than that of the intracellular reserve (Allan et al. 1972; Myklestad et al. 1972) and more similar to that of the mucilaginous sheath surrounding the diatom (Allan et al. 1972). Myklestad (1974) observed marked distinctions between different planktonic diatom taxa in the accumulation of the storage product glucan and extracellular polysaccharide. The author recorded for stationary phase cultures (12 days) that the ratio of extracellular polysaccharide to cellular carbohydrate varied from 0.01 in *Skeletonema costatum* (Greville) Cleve to 1.25 in *Chaetoceros curvisetus* Cleve and the content of glucan was 81.0 and 24.8 % of the organic dry matter, respectively (Myklestad 1974). Although the present study did not differentiate between extracellular products and intracellular pools, an increase in relative carbohydrate contents at both low and high salinities was recorded in the present investigation, suggesting a direct relation in the acclimatisation processes. In contrast, lower levels of carbohydrates were found in most of the tested taxa in the high temperature treatment, which is similar to data presented by Renaud et al. (2002). The authors observed that carbohydrate contents were significantly higher between 25 and 30 °C decreasing at lower and higher temperatures. Here, carbohydrates seemed not to be involved in the extreme temperature acclimatisation process.

Several authors reported changes in cellular lipid characteristics in planktonic species from variations in salinity (e.g. Renaud and Parry 1994; Chelf 1990) and temperatures (James et al. 1989). In the present study, higher levels of relative protein and lipid contents were found in most benthic taxa in the high salinity assays. In contrast, Araújo and Garcia (2005) found for the planktonic diatom *Chaetoceros wighamii* Brightwell lowered protein content at a salinity of 35 PSU, while other constituents increased. In addition, Fabregas et al. (1985) observed a decrease in protein content with a salinity increase. Since lipids are a heterogeneous group of compounds of varying structures and functions, it is likely that the measurement of total lipids may not be sufficient to indicate different trends occurring among the individual lipid classes. For example, temperature has a major effect on the types of fatty acids produced by microalgae (Ackman et al. 1968; Sato and Murata 1980; Thompson et al. 1992). Many microalgal species respond to decreased growth temperatures by increasing the ratio of unsaturated to saturated fatty acids (Ackman et al. 1968; Mortensen et al. 1988; James et al. 1989; Thompson et al. 1992; Renaud et al. 1995). Thus, further detailed investigations are needed in which the amounts of individual fatty acids and the composition of carbohydrates in relation to different salinities and temperatures have to be analysed.

Applications in applied phycology

The biochemical composition of diatoms is of considerable interest and has been dealt with in applied phycology studies

in areas such as aquaculture, the nutritional quality of foods fed to cultured animals (e.g. Wikfors et al. 1984) and biofuel research (Griffiths and Harrison 2009). Planktonic diatoms, in particular, are regarded as useful neutral lipid sources, both as liquid fuel precursors and as foods for marine culture of zooplankters (Ahlgren et al. 1990), larval and postlarval shrimp (Chu 1989), copepods (Bourdier and Amblard 1989) and juvenile oysters (Tsitsa-Tzardis et al. 1993). In contrast, benthic diatoms are very unlikely to have high potential for aquaculture or biodiesel production as they tend to settle in cultures. Furthermore, as the fatty acid profiles showed, the lipid contents of most benthic species in the present investigation were rather low, ranging from 19.4 to 25.6 % DW. On the other hand, the results of the acclimatisation potential gave evidence of the ability to manipulate diatom lipid quantity, which might be necessary for an effective use of the species as fuel or food resource (McGinnis et al. 1997). Finally, mutual changes among lipids, polysaccharides and proteins can help explain basic adaptations in algal physiological functions and are important for the food quality in aquatic food webs with subsequent trophic level effects (Volkman et al. 1989; Lynn et al. 2000).

Conclusions

The biochemical compositions of the 25 benthic diatoms, isolated from an intertidal environment, showed partial pronounced species-specific characteristics. Furthermore, all the taxa in the current study seemed to alter their relative protein, carbohydrate and lipid contents in response to variations in temperatures and salinities, but not all responses were equal. Generally, the intracellular concentrations of these compounds depend on environmental conditions and on the physiological potential of each diatom species. In summary, some benthic diatoms are able to react in a more efficient way, e.g. producing more thermo- and osmoprotectants, and are therefore able to grow over wider environmental ranges. These different acclimation potentials indicate niche separation in intertidal flats.

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