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Lipid class and fatty acid composition of *Pseudo-nitzschia multiseries* and *Pseudo-nitzschia pungens* and effects of lipolytic enzyme deactivation

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Abstract

Lipid class and fatty acid compositions of stationary phase cultures of the toxic diatoms *Pseudo-nitzschia multiseries* and *P. pungens* were determined. The lipid class compositions of both diatoms were similar, with phospholipids, triacy1glycerols and acetone-mobile polar lipids as the major classes. Both pennate diatoms also displayed similar fatty acid compositions that were typical of diatoms, with high levels of 14:0, 16:1n-7, 16:2n-4 and 20:5n-3. In addition, elevated levels (>7%) of the fatty acid 16:4n-1 were found in both species and this fatty acid has potential as a signature compound in differentiating *Pseudo-nitzschia* species from other diatoms. The taxonomic similarities of the diatoms were apparent in the very similar lipid class and fatty acid compositions. Treatment of cells with boiling water was effective in deactivating lipolytic enzymes and resulted in a significant decrease ($P < 0.05$) in free fatty acids, a breakdown indicator and a general increase in polyunsaturated fatty acids in all samples. Thus, the routine use of boiling water to deactivate lipolytic enzymes in diatom samples is recommended.

Keywords: *Pseudo-nitzschia multiseries*; *P. pungens*; Lipid class; Fatty acid; Lipolysis

1. Introduction

Lipid analyses of toxic algae can reveal signature compounds that may be useful in screening water or seafood samples. These signature compounds are often molecules with unusual structures that can be used to establish the presence of the alga or of organisms that have consumed the alga. The pennate diatom, *Pseudo-nitzschia multiseries*, is a domoic acid producer which was responsible for an outbreak of amnesic shellfish poisoning in Canada (Addison & Stewart, 1989). Its morphology is very similar to that of its relative, *Pseudo-nitzschia pungens*, which was thought to be nontoxic (Bates, Worms & Smith, 1993); however, recent data have shown this organism to also be a domoic acid producer (Rhodes, White, Syhre & Atkinson, 1996). The two organisms have overlapping measurements of length, width and number of costae per unit length (Hasle, Lange & Syvertsen, 1996) and the only differentiating feature is the number of rows of poroid areolae or striae between costae. Chemotaxonomic differences, particularly regarding lipids, may be useful in differentiating toxic *Pseudo-nitzschia* species from other diatoms. For example, the fatty acid, 16:4n-1, has been suggested as a signature compound of *P. multiseries* (Parrish, deFreitas, Bodennec, Macpherson & Ackman, 1991) and it is known that proportions of this acid fluctuate with scale of culture (Whyte, Ginther & Townsend, 1995). This study was undertaken to evaluate the usefulness of 16:4n-1 and other fatty acids as biomarkers for *P. multiseries*.

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Table 1
Lipid class composition and concentration of early stationary phase cultures of *P. pungens* and *P. multiseries*. Lipid class compositions are reported as percent of total lipids detected. Lipid concentrations are in pg cell^{-1} (mean ± S.D., $n = 3$). – = not detected

	P. multiseries		*P. pungens*	
	treated	untreated	treated	untreated
Total lipids (pg cell^{-1})	2.7 ± 0.4	3.6 ± 0.8	6.5 ± 0.7	6.3 ± 0.5
Lipid class				
Hydrocarbons	15.0 ± 11.8	3.9 ± 2.9	9.1 ± 2.3	1.1 ± 1.3
Steryl/wax Esters	0.2 ± 0.2	0.8 ± 0.4	1.1 ± 0.1	2.3 ± 2.4
Methyl esters	2.2 ± 2.0	–	–	–
Ethyl ketones	4.1 ± 0.6	3.8 ± 0.7	2.9 ± 0.4	3.0 ± 0.1
Triacylglycerols	21.4 ± 3.1	23.6 ± 0.6	29.0 ± 6.9	24.4 ± 3.4
Free fatty acids	5.7 ± 0.4	18.0 ± 4.4	6.1 ± 0.6	13.8 ± 2.8
Alcohols	2.1 ± 3.7	–	–	3.4 ± 1.6
Sterols	6.9 ± 1.4	5.6 ± 0.5	2.7 ± 2.3	3.1 ± 2.0
Diacylglycerols	1.1 ± 0.5	–	–	–
Acetone-mobile polar lipids	17.4 ± 3.3	30.9 ± 5.0	19.9 ± 2.3	28.5 ± 1.6
Phospholipids	23.8 ± 5.7	13.4 ± 0.3	29.4 ± 4.8	20.3 ± 4.6
Total	100%	100%	100%	100%

A second objective was to determine the extent of degradation of acyl lipids by lipolytic enzymes. The effects of in vitro hydrolysis on the lipid composition of the diatom, *Skeletonema costatum*, were recently examined (Berge, Gouygou, Dubacq & Durand, 1995) and were found to be responsible for reduced amounts of glycolipids and excesses of free fatty acids (FFA). However, the addition of boiling water to the sample after centrifugation effectively deactivated lipolytic enzymes. A similar approach, using boiling water treatment, was applied here to *P. multiseries* and *P. pungens* cultures to observe changes in lipid composition due to lipase activity. Diatoms also contain lipoxygenases (Gerwick, 1994) which are responsible for polyunsaturated fatty acid (PUFA) degradation and act only on FFA (Eriksson, 1987). Boiling water treatment was also expected to affect these enzymes and result in altered fatty acid compositions.

2. Results and discussion

2.1. Cultures

Batch cultures of *P. multiseries* and *P. pungens* were grown for 37 days until they reached early stationary phase with growth rates of 0.01 divisions d^{-1} and 0.04 divisions d^{-1}, respectively. At this time, the cultures had concentrations of 1.95×10^6 cells ml^{-1} and 1.08×10^6 cells ml^{-1}, respectively. Approx. 3/4 of the cultures were harvested and each portion was divided into 6 or 8 equal fractions, half of which were treated with boiling water after filtration. After a further 26 days growth, the remaining portion of the cultures was harvested and treated in the same manner. It was not possible to count the cells at this time as many of the *P. multiseries* cells were clumped, while many of the *P. pungens* cells were in chains. There was also a significant amount of detritus in both cultures. These microscopic observations plus evidence of sporulation indicate that the cultures were senescent. All stationary phase and senescent cultures were silica limited with silicate levels of only 20% of those of the original medium, while nitrate and phosphate concentrations were greater than 80% of the original.

Table 2
Lipid class composition of senescent cultures of *P. pungens* and *P. multiseries*. Results are reported as percent of total lipids detected (mean ± S.D., $n = 34$). – = not detected

Lipid class	*P. multiseries*		*P. pungens*	
	treated	untreated	treated	untreated
Hydrocarbons	7.7 ± 4.5	9.8 ± 3.5	6.9 ± 5.5	8.5 ± 5.0
Steryl/wax esters	1.2 ± 2.1	0.6 ± 0.1	0.8 ± 0.3	0.3 ± 0.4
Methyl esters	–	–	0.1 ± 0.1	–
Ethyl ketones	1.0 ± 1.0	0.8 ± 1.5	0.6 ± 0.9	0.2 ± 0.1
Methyl ketones	–	0.3 ± 0.5	–	–
Triacylglycerols	7.0 ± 5.6	1.0 ± 1.2	12.2 ± 1.6	0.6 ± 0.9
Free fatty acids	9.4 ± 3.2	19.7 ± 1.4	12.1 ± 2.5	26.7 ± 6.0
Alcohols	–	1.1 ± 0.5	1.4 ± 1.2	0.6 ± 0.5
Sterols	5.9 ± 1.6	8.4 ± 2.0	5.6 ± 2.1	7.5 ± 4.7
Diacylglycerols	1.8 ± 2.3	5.4 ± 4.0	2.0 ± 0.4	6.7 ± 3.5
Acetone-mobile polar lipids	26.5 ± 2.8	26.4 ± 3.0	22.4 ± 1.2	25.6 ± 5.0
Phospholipids	39.4 ± 1.7	26.6 ± 1.7	36.0 ± 3.1	23.3 ± 1.1
Total	100%	100%	100%	100%

In a secondary study, another strain of *P. multiseries* was cultured for comparison of strain differences. Portions of this culture were harvested in a similar manner in both stationary and senescent phases and half of these samples were treated with boiling water. Nutrient analyses indicated that these cultures were also silicate limited.

2.2. Lipolytic enzyme deactivation

Tables 1 and 2 display the lipid class composition of all cultures with and without boiling water treatment prior to extraction. Free fatty acid (FFA) proportions in all treated cultures were less than half those found in the corresponding untreated samples. Absolute amounts of FFA per cell were also significantly lower ($P < 0.05$) in the treated cultures. This indicates that boiling water treatment did deactivate lipolytic enzymes. In both cultures harvested in early stationary phase (Table 1), the untreated samples contained higher levels of FFA and acetone-mobile polar lipids (AMPL), which include the breakdown indicator monoacy1glycerol (MAG) and lower levels of phospholipids (PL) than treated samples. With boiling

water treatment, however, proportions of FFA and AMPL were substantially decreased with corresponding increases in PL. Obviously, PL, rather than triacy1glycerols (TAG), is the source of the FFA and MAG. This is in agreement with the results from *Skeletonema costatum* (Berge et al., 1995) and indicates that the high levels of FFA reported in other studies (Dunstan, Volkman, Barrett, Leroi & Jeffrey, 1994; Parrish et al., 1991; Volkman, Jeffrey, Nichols, Rogers & Garland, 1989) may be artefactual.

The senescent cultures (Table 2) harvested a month later displayed a different pattern among treated and untreated samples. A decrease in FFA proportions and an almost equivalent increase in PL with boiling water treatment was still apparent, but these samples did not contain a significantly different proportion of AMPL. In addition, a substantial increase in TAG was observed in the treated samples. These senescent cultures were unhealthy with sporulation starting to occur and significant levels of bacteria were present with cultures containing 25–30% detritus. Presumably the boiling water deactivated any lipases associated with intact diatoms, but, in unhealthy cultures such as these, autolysis and the resulting bacterial growth would be expected to generate large amounts of FFA. It is likely that bacteria made a significant contribution to the lipid data, partially explaining the differences in composition in the stationary phase and senescent cultures. The difference in growth phase may also be a factor.

A FFA content of 25% has previously been suggested as a reasonable value to use as a maximum proportion normally produced by cells in seawater (Parrish, 1988). It was also recommended that higher values be taken as an indicator of in situ degradation. However, fatty acids tend not to exist naturally as free carboxylic acids because they may interfere with enzyme function (Gurr & Harwood, 1991), so reports of high levels of FFA are likely artefacts due to cell damage. In this study, precautions were taken to deactivate lipases, but a FFA level of approximately 6% was still obtained in the healthy stationary phase cultures. Perhaps levels of FFA above 10% of total lipids would be a more appropriate maximal value to accept in lipid results. Values above this level would indicate poor sample handling techniques unless significant degradation due to bacteria was a possibility. Obtaining FFA percentages near zero may not be a reasonable expectation in routine lipid analyses and, in degraded algal detritus, proportions as high as 15% may be occurring naturally (Table 2).

2.3. Lipid class results

Treated early stationary phase *P. multiseries* samples (Table 1) contained significantly different ($P < 0.05$) amounts of TAG, AMPL and PL than the treated senescent culture (Table 2), while the treated early stationary phase *P. pungens* culture contained significantly different ($P < 0.05$) amounts of TAG, diacylglycerols and FFA than the treated senescent culture. Senescent cultures are particularly susceptible to autolysis and the resulting cell exudates commonly support extensive bacterial growth (Parsons, Masayuki & Hargrave, 1984). In this case, it is likely that bacteria were involved in lipid breakdown, as well as making a contribution to the biomass. Because of this bacterial growth in the senescent cultures, the focus will be on lipid class and fatty acid composition of cells harvested in early stationary phase. Boiling water treatment was effective in deactivating lipases and, in light of this, only early stationary phase samples treated with boiling water will be discussed in terms of lipid class composition.

The amount of total lipid per cell varied significantly ($P < 0.05$) between *P. multiseries* and *P. pungens* with *P. pungens* containing approximately three times the amount of lipid per cell. The concentrations of 2.7 ± 0.4 and 6.5 ± 0.7 pg cell^{-1} in *P. multiseries* and *P. pungens*, respectively, are at the low end of the range of 2.6 to 67 pg cell^{-1} reported in the literature for pennate diatoms (Dunstan et al., 1994; Parrish et al., 1991; Parrish & Wangersky, 1987; Renaud, Zhou, Parry, Thinh & Woo, 1995). Qualitatively, the lipid class proportions were quite similar in *P. multiseries* and *P. pungens* after treatment with boiling water, but some differences are notable. For example, proportions of steryl/wax esters, sterols and PL were significantly different ($P < 0.05$) in the two cultures and, generally, *P. pungens* contained larger percentages of TAG, AMPL and PL. The similarities in lipid class proportions reflect the taxonomic similarities of the two organisms, while differences in amounts of lipids per cell may indicate that conditions were not identical in the two culture mediums.

The lipid class composition of stationary phase *P. multiseries* has previously been reported, but very different results were obtained (Parrish et al., 1991). Most noticeably, FFA were found to comprise 40% of total lipids, with TAG and PL comprising only 2.1 and 6.7%, respectively. Such high levels of FFA suggest extensive degradation of acyl lipids which is also the likely cause of the small proportions of TAG and PL found in that study. Attempts to deactivate lipolytic enzymes were made in that study by treating the cells with isopropyl alcohol (Christie, 1989) and by fast freezing in liquid nitrogen, but high levels of FFA persisted. Since boiling water treatment was not employed at that time, comparisons between those results (Parrish et al., 1991) and the current study may be difficult.

Table 3
Fatty acid composition of early stationary phase cultures of *P. pungens* and *P. multiseries*. Results are reported as percent of total fatty acids (mean ± s.d., n = 3). – = not detected

Fatty acid	*P. multiseries*		*P. pungens*	
	treated	untreated	treated	untreated
Branched				
i-15:0	0.18 ± 0.05	0.12 ± 0.11	0.46 ± 0.03	0.48 ± 0.02
ai-15:0	–	–	0.36 ± 0.04	0.59 ± 0.33
i-17:0	0.75 ± 0.10	0.77 ± 0.03	0.52 ± 0.09	0.39 ± 0.11
Subtotal	0.93 ± 0.14	0.89 ± 0.09	1.35 ± 0.14	1.46 ± 0.22
Saturates				
14:0	12.20 ± 0.49	13.73 ± 0.40	13.3 ± 1.0	12.64 ± 0.27
15:0	0.59 ± 0.08	0.52 ± 0.02	0.41 ± 0.03	0.33 ± 0.02
16:0	5.05 ± 0.41	5.15 ± 0.34	2.99 ± 0.09	2.71 ± 0.57
18:0	0.06 ± 0.10	–	–	0.38 ± 0.22
22:0	–	–	0.05 ± 0.08	–
24:0	1.70 ± 0.17	1.61 ± 0.05	3.70 ± 0.34	3.4 ± 1.0
Subtotal	19.60 ± 0.57	21.00 ± 0.27	20.4 ± 1.3	19.5 ± 2.1
Monoenes				
16:1n-9	–	–	0.26 ± 0.44	0.21 ± 0.37
16:1n-7	23.25 ± 0.82	25.3 ± 1.0	22.80 ± 0.45	25.10 ± 0.36
16:1n-5	1.43 ± 0.03	1.58 ± 0.03	1.90 ± 0.03	2.00 ± 0.02
18:1n-9	1.30 ± 0.23	1.13 ± 0.16	0.80 ± 0.23	0.29 ± 0.13
18:1n-7	3.16 ± 0.24	2.84 ± 0.19	1.21 ± 0.10	1.58 ± 0.48
18:1n-5	0.43 ± 0.07	0.39 ± 0.03	0.40 ± 0.02	0.44 ± 0.08
20:1n-11	0.21 ± 0.36	0.07 ± 0.12	0.24 ± 0.11	0.08 ± 0.08
20:1n-9	–	–	0.08 ± 0.07	0.07 ± 0.06
Subtotal	29.78 ± 0.28	31.27 ± 0.99	27.68 ± 0.17	29.78 ± 0.67
Polyunsaturates				
16:2n-7	3.64 ± 0.18	3.97 ± 0.07	3.49 ± 0.19	3.86 ± 0.09
16:2n-4	4.84 ± 0.28	4.93 ± 0.13	4.20 ± 0.16	3.99 ± 0.07
16:3n-4	2.35 ± 0.12	2.55 ± 0.08	1.79 ± 0.09	1.53 ± 0.06
16:4n-1	6.87 ± 0.33	7.41 ± 0.20	7.69 ± 0.31	6.04 ± 0.42
18:2n-6	0.37 ± 0.04	0.39 ± 0.03	0.16 ± 0.01	0.17 ± 0.02
18:3n-6	0.27 ± 0.07	0.37 ± 0.02	0.25 ± 0.02	0.30 ± 0.02
18:4n-3	0.65 ± 0.04	0.73 ± 0.00	0.13 ± 0.00	0.15 ± 0.02
20:2n-6	0.08 ± 0.13	–	–	–
20:3n-6	–	–	0.04 ± 0.06	–
20:4n-6	0.29 ± 0.02	0.33 ± 0.03	0.59 ± 0.02	0.61 ± 0.06
20:4n-3	0.08 ± 0.07	0.09 ± 0.08	–	–
20:5n-3	27.3 ± 1.8	24.3 ± 1.0	30.79 ± 0.87	31.5 ± 2.2
Subtotal	46.7 ± 2.6	45.07 ± 0.54	49.1 ± 1.6	48.1 ± 2.7
Total	97.1 ± 2.5	98.24 ± 0.37	98.55 ± 0.19	98.82 ± 0.26
16:1/16:0	4.91 ± 0.46	5.24 ± 0.52	8.34 ± 0.37	10.3 ± 2.0
ΣC16/ΣC18	7.66 ± 0.75	8.69 ± 0.23	15.4 ± 1.8	14.5 ± 3.9

Table 4
Fatty acid composition of senescent cultures of *P. pungens* and *P. multiseries*. Results are reported as percent of total fatty acids (mean ± S.D., $n = 34$). – = not detected

Fatty acid	*P. multiseries*		*P. pungens*	
	treated	untreated	treated	untreated
Branched				
i-15:0	0.17 ± 0.16	0.19 ± 0.04	1.38 ± 0.22	1.46 ± 0.11
ai-15:0	–	–	1.25 ± 0.24	1.37 ± 0.12
i-17:0	0.77 ± 0.04	0.65 ± 0.02	0.74 ± 0.10	0.58 ± 0.07
Subtotal	0.94 ± 0.18	0.84 ± 0.05	3.37 ± 0.55	3.40 ± 0.26
Saturates				
14:0	11.81 ± 0.62	13.80 ± 0.38	13.6 ± 1.4	14.48 ± 0.38
15:0	0.46 ± 0.03	0.54 ± 0.05	0.40 ± 0.04	0.40 ± 0.02
16:0	7.46 ± 0.13	8.13 ± 0.40	3.76 ± 0.34	3.43 ± 0.25
18:0	1.03 ± 0.06	0.92 ± 0.18	0.57 ± 0.16	0.51 ± 0.05
24:0	2.11 ± 0.04	1.88 ± 0.13	4.87 ± 0.72	4.47 ± 0.27
Subtotal	22.86 ± 0.71	25.27 ± 0.70	23.2 ± 1.7	23.29 ± 0.17
Monoenes				
16:1n-9	1.05 ± 0.14	0.89 ± 0.28	0.73 ± 0.49	0.92 ± 0.10
16:1n-7	19.07 ± 0.81	25.9 ± 1.5	19.97 ± 0.60	23.77 ± 0.87
16:1n-5	1.18 ± 0.02	1.39 ± 0.03	1.88 ± 0.10	2.14 ± 0.07
18:1n-9	2.42 ± 0.23	2.37 ± 0.10	1.07 ± 0.53	0.81 ± 0.06
18:1n-7	19.4 ± 1.5	16.66 ± 0.89	4.99 ± 0.69	5.23 ± 0.43
18:1n-5	1.52 ± 0.12	1.26 ± 0.10	0.60 ± 0.10	0.64 ± 0.03
20:1n-11	–	–	0.09 ± 0.18	0.05 ± 0.11
20:1n-9	–	–	0.04 ± 0.08	–
Subtotal	44.6 ± 1.2	48.5 ± 1.1	29.4 ± 1.5	33.56 ± 0.82
Polyunsaturates				
16:2n-7	1.32 ± 0.09	1.74 ± 0.12	2.32 ± 0.19	2.61 ± 0.13
16:2n-4	2.79 ± 0.12	3.29 ± 0.16	3.02 ± 0.18	3.02 ± 0.09
16:3n-4	0.98 ± 0.10	1.23 ± 0.10	1.20 ± 0.13	0.88 ± 0.05
16:4n-1	4.63 ± 0.45	5.57 ± 0.33	7.22 ± 0.35	4.63 ± 0.33
18:2n-6	0.48 ± 0.08	0.52 ± 0.11	0.32 ± 0.12	0.30 ± 0.04
18:2n-4	0.43 ± 0.13	0.35 ± 0.11	0.21 ± 0.16	0.15 ± 0.04
18:3n-6	0.21 ± 0.02	0.23 ± 0.03	0.28 ± 0.04	0.39 ± 0.02
18:4n-3	0.41 ± 0.02	0.46 ± 0.03	0.13 ± 0.09	0.16 ± 0.01
20:4n-6	0.43 ± 0.01	0.34 ± 0.02	0.62 ± 0.03	0.66 ± 0.01
20:4n-3	0.17 ± 0.01	0.20 ± 0.04	–	–
20:5n3	18.3 ± 1.0	9.9 ± 1.6	26.2 ± 3.3	24.68 ± 0.57
Subtotal	30.1 ± 1.7	23.9 ± 1.1	41.5 ± 3.6	37.48 ± 0.81
Total	98.53 ± 0.23	98.42 ± 0.42	97.40 ± 0.27	97.72 ± 0.23
16:1/16:0	2.86 ± 0.15	3.47 ± 0.24	6.04 ± 0.55	7.87 ± 0.76
ΣC16/ΣC18	1.50 ± 0.16	2.12 ± 0.16	5.02 ± 0.91	5.08 ± 0.38

2.4. Fatty acid composition

The fatty acid compositions (Tables 3 and 4) of all four samples were typical of diatoms (Dunstan et al., 1994; Viso & Marty, 1993; Volkman et al., 1989) with 14:0, 16:1n-7, 16:4n-1 and 20:5n-3 among the most abundant fatty acids. High ratios of 16:1/16:0 and ΣC16/ΣC18 are also characteristic of diatoms and elevated levels, as compared to other microalgal classes, were obtained for these cultures (Tables 3 and 4). Several reports of fatty acid compositions of the related *Nitzschia* species are available in the literature (Dunstan et al., 1994; Nichols, Palmisano, Smith & White, 1986; Parrish et al., 1991; Viso & Marty, 1993) and are generally similar to the results obtained here with the fatty acids mentioned above predominating. The senescent cultures contained high levels of 18:1n-7 (> 5% of total fatty acids). Such levels are not expected in diatoms and also serve to indicate substantial bacterial growth in these senescent cultures, as this fatty acid is commonly employed as a bacterial indicator (Perry, Volkman & John, 1979; Wakeham & Breier, 1991).

The fatty acid composition of these treated cultures was also examined to identify any fatty acid or fatty acid ratio that could be employed as a signature compound for toxic *P. multiseries*. The fatty acid 16:4n-1

Table 5
Variation in the proportions of major fatty acids in two strains of *P. multiseries* in stationary and senescent growth phases. Both strains were treated with boiling water prior to extraction

	KP105			CCMP 1660		
	stationary	senescent	% change	stationary	senescent	% change
14:0	18.0	16.0	−10.7	12.2	11.8	−3.2
16:0	4.5	6.9	+53.1	5.1	7.5	+47.7
16:1n-7	28.9	15.5	−46.3	23.3	19.1	−18.0
16:2n-4	5.1	2.4	−52.0	4.8	2.8	−42.4
16:3n-4	5.2	3.3	−37.4	2.4	1.0	−58.3
16:4n-1	4.7	4.3	−7.9	6.9	4.6	−32.6
20:5n-3	21.8	14.8	−32.0	27.3	18.3	−33.0

is commonly found in diatoms at levels of 1–2% of total fatty acids but was present in this study at proportions up to about 7.5% in both *P. pungens* and *P. multiseries*. This fatty acid has been proposed as a marker for toxic *P. multiseries* (Parrish et al., 1991), but clearly, with similar levels produced by *P. pungens*, it would not be possible to distinguish these two species. However, with elevated amounts of that acid present in both cultures, it may be possible to use 16:4n-1 to differentiate *Pseudo-nitzschia* species from other diatoms. In addition, the general diatom marker, ΣC16/ΣC18, did show consistent variation among cultures of *P. multiseries* and *P. pungens*. This ratio was significantly higher ($P < 0.05$) by a factor of approximately two in cultures of *P. pungens* due to the lower proportions of 18:1n-9 and 18:1n-7 in that culture. In the laboratory, this ratio could be applied as a biomarker to differentiate cultures of the two species. However, fatty acid compositions of phytoplankton are known to vary with culture conditions (Chu, Phang & Goh, 1996; Harrison, Thompson & Calderwood, 1990; Reitan, Rainuzzo & Olsen, 1994; Renaud et al., 1995) and ratios of fatty acids should be applied cautiously as biomarkers. There were also significant differences ($P < 0.05$) in the proportions of other fatty acids, including 16:0, 16:3n-4, 18:1n-7 and 24:0, in the two cultures, but these acids are commonly found in many classes of microalgae and would not be useful in detecting *P. multiseries* in a field situation. Application of the ratio ΣC16/ΣC18 as a biomarker in the field would also be problematic, as elevated levels of this ratio are characteristic of almost all diatoms. In natural samples, perhaps some other class of compounds, such as bacillariolides (Wang, Maranda, Hargraves & Shimizu, 1993), would be more useful as biomarkers.

In a secondary study (Table 5), the fatty acid composition of another strain of *P. multiseries*, KP105, was compared to that of the strain discussed above, CCMP 1660. The proportions of several major acids fluctuated widely among the two strains. Similar variability among clones grown under identical culture conditions has been observed in the fatty acids of *Isochrysis galbana* (Alonso, Grima, Pérez, Sánchez & Camacho, 1992). These fluctuations in proportions also raise some doubt concerning the usefulness of fatty acid biomarkers for the identification of specific species. However, proportions of major fatty acids in stationary and senescent phases of both strains did vary consistently with decreased proportions of all fatty acids in Table 5, with the exception of 16:0, in the senescent cultures. This suggests that, despite the extensive bacterial growth, there is consistency in the biochemical alterations of fatty acid proportions in response to stress in the two strains.

As with the lipid class composition, the fatty acid compositions were also different in cultures with and without boiling water treatment. In general, boiling water treatment resulted in a larger proportion of PUFA and a smaller proportion of monounsaturated fatty acids. This suggests that, in addition to the deactivation of lipolytic enzymes, some mechanism of PUFA degradation was also inhibited. It is possible that labile PUFA were more prone to exposure to oxygen or light as FFA, leading to autoxidation, rather than when esterified in acyl lipids. Boiling water treatment resulted in a higher proportion of esterified fatty acids that were protected from degradation by incorporation in tissues and membranes. A second explanation involves the lipoxygenase enzyme commonly found in diatoms. In untreated samples, lipases may have produced a larger proportion of FFA on which lipoxygenases could act, resulting in less PUFA than in treated samples. It is also possible that boiling water treatment is capable of deactivating lipoxygenases as well as lipases. Whatever the explanation, PUFA levels were higher in treated samples, suggesting that, in addition to questionable reports of high FFA proportions in the literature, PUFA levels in diatoms may also be higher than currently thought. As previously indicated (Berge et al., 1995), treatment of all phytoplankton samples, particularly diatoms, with boiling water to deactivate any lipolytic enzymes would seem to be a good practice.

3. Experimental

Cultures of *Pseudo-nitzschia multiseries* (CCMP 1660) and *P. pungens* (CCMP 1572) were obtained from the Bigelow Lab, ME, USA. They were grown in f/2 medium (Guillard & Ryther, 1962) enriched with Se (0.01 μmol l^{-1}) and during a 37 day growth period, they were transferred to new medium in a larger flask and enriched with nutrients 3 times. Illumination was provided on a 14:10 light: dark cycle at 15°C. About

300 ml samples were taken from these early stationary phase cultures for lipid analyses and cell counts using a Coulter Multisizer II (Carl Zeiss, Inc). The remaining 100 ml of each culture were then transferred to new medium with half the silica concentration. The cultures were grown for a further 26 days without transfer or nutrient replenishment. Cultures of diatoms were also obtained from the National Research Council of Canada, Halifax, NS, for use in the secondary study. *P. multiseries* (KP 105) was grown in L1 medium (Guillard & Hargraves, 1993) enriched with Si and portions were harvested in both stationary and senescent phases.

Cells were filtered on precombusted GF/C glass fibre filters. Immediately after filtration, 10 ml of boiling distilled H_2O was poured over half of the filtered cells and an instant colour change from greenish-brown to bright yellow was observed. Lipids were then extracted by grinding and sonication with $CHCl_3$:MeOH 2:1 following a modified Folch, Lees & Sloane-Stanley (1957) method (Parrish, 1999). Extracts were concentrated to 0.5 ml and up to 30 μl of each was applied to Chromarods-SIll for sepn of lipid classes by TLC. A short development in Me_2CO was used to concentrate each sample into a narrow band on the silica gel surface. After drying and conditioning the rods, lipid classes were sepd in a step-wise procedure using developing systems of increasing polarity. Each rod was scanned in an latroscan MK V. Lipid classes were measured by FID. Most TLC-FID analyses were completed within a week of sampling.

Fatty acids were determined as methyl esters by GC. The methyl esters were prepared using BF_3 in MeOH and the resulting FAME were analysed on a 30 m fused silica column (0.32 mm i.d.) coated with Omegawax. Hydrogen (flow rate 2 ml min^{-1}) was used as the carrier gas and gas lines were equipped with an oxygen scrubber. The following temperature ramp was employed: 65°C for 0.5 min, hold at 195°C for 15 min after ramping at 40°C min^{-1} and hold at 220°C for 0.75 min after ramping at 2°C min^{-1}. Helium (make-up gas) and air (combustion) had flow rates of 30 ml min^{-1} and 300 ml min^{-1}, respectively. Both the injector and detector were isothermal at 260°C. Autoinjection, with a sample size of 1 μl and a solvent plug size of 0.8 μl, was used. Detection was by FID. FAME were identified by comparison of R_ts with known standards (PUFA1 and PUFA2, Supelco). The identity of several PUFA was confirmed by GC-MS.

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