Pergamon

PII: S0031–9422(98)00095–8

Phytochemistry, Vol. 49, No. 5, pp. 1241–1247, 1998

Printed in Great Britain
0031–9422/98/$—see front matter

EFFECTS OF ENVIRONMENTAL FACTORS AND METALS ON *SELENASTRUM CAPRICORNUTUM* LIPIDS

CHRISTIAN J. MCLARNON-RICHES,* CAROLE E. ROLPH, DAVID L. A. GREENWAY and PETER K. ROBINSON

Department of Applied Biology, University of Central Lancashire, Preston PR1 2HE, U.K.

(*Received in revised form* 13 *December* 1997)

Key Word Index—*Selenastrum capricornutum*; *Kirchneriella*; Chlorophyceae; acyl lipid biosynthesis; sterol biosynthesis; illumination; temperature; heavy metals.

Abstract—Studies of the effects of illumination, temperature and heavy metal exposure (Cu^{2+}, Zn^{2+}, Cd^{2+}) on *Selenastrum capricornutum* have shown that these factors alter both the total fatty acid and free sterol composition of cells in batch culture. Dark treatment resulted in a decrease in the relative proportions of oleate (18:1) and an increase linoleate (18:2), together with an increase in the relative proportion of the sterol 24-ethyl-5α-cholest-7-en-3β-ol (Δ^7-chondrillastenol) and a decrease in 24-ethyl-5α-cholesta-7,22-dien-3β-ol (chondrillasterol). A shift in temperature from 25° to 10° led to an increase in the relative proportion of oleate and a decrease in linoleate and parinate (18:4), together with a significant increase in the relative proportion of 24-methyl-5α-cholest-7-en-3β-ol (ergostenol). Generally, exposure to heavy metal ions led to an increase in oleate (with all three metals) and altered the relative proportions of linoleate and parinate (changes being metal specific). Metal (ion) treatment also significantly increase Δ^{22} desaturation of the 24-ethyl sterol components. The changes in the composition of many of the individual lipid components in response to heavy metal ion exposure occurred at concentrations which did not significantly affect the organism's specific growth rate. For example, the relative proportion of oleate was affected with only 1 μM Cu^{2+} in solution ($P < 0.10$).

INTRODUCTION

Selenastrum capricornutum Printz (CCAP 278/4) is a freshwater unicellular green microalga which is used worldwide as a chemical toxicity bioassay organism in protocols recommended by the EEC, ISO, OECD and others. Such protocols generally involve batch culture studies of the organism with a range of concentrations of toxicant, and periodic evaluation of cell number to determine the EC-50 value for the toxicant in question. The organism itself was originally isolated from the River Nitelva, Norway in 1959. Unfortunately the organism is not a true *Selenastrum* at all but is, in fact, a species of the closely related *Kirchneriella* [1]. Perhaps surprisingly, considering the widespread use of *S. capricornutum*, there have been no detailed studies of the lipid composition of this alga, though there have been some studies of the fatty acid [2] and sterol [3] composition of other (true) members of the genus *Selenastrum*. Described herein are the results of studies of both the total fatty acid composition and the free sterol composition of *S. capricornutum* (CCAP 278/4). The effects of varying environmental conditions (illumination and temperature) on lipid metabolism have been determined, as have the effects of exposure to the heavy metals copper, zinc and cadmium. As this organism is so widely used within bioassays, in which cell growth is used to evaluate toxic effect, particular attention has been given to the reproducibility, speed and sensitivity of the lipid responses to heavy metal stress.

*Author to whom correspondence should be addressed. Tel: +44 (0)1772 893511; fax:00 44 (0)1772 892903; E-mail:c.j.riches@uclan.ac.uk

RESULTS AND DISCUSSION

Typical total fatty acid and free sterol profiles for *S. capricornutum* (CCAP 278/4) grown under routine growth conditions are shown in Fig. 1. *S. capricornutum* was found to contain predominantly C_{16} and C_{18} fatty acids with palmitate (16:0) and oleate (18:1) accounting for about 50% of the total fatty acids. Such a composition is fairly typical of freshwater green microalgae [4–6] and is in broad agreement with the results of Hyne *et al.* [2] who studied an unidentified species of *Selenastrum*. Our studies, however, do

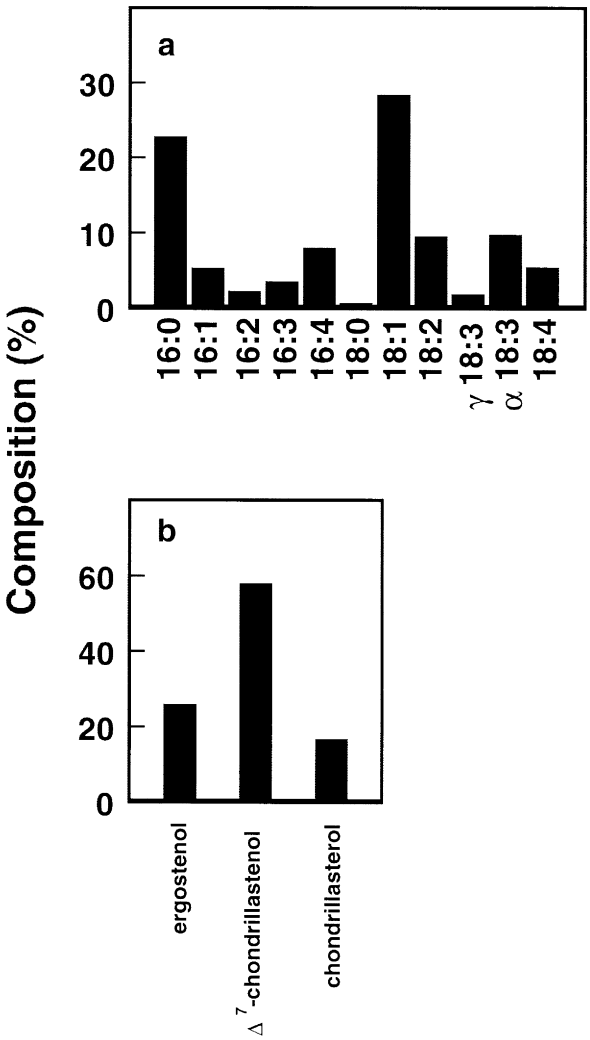

Fig. 1. Fatty acid (a) and sterol (b) composition of *S. capricornutum* (CCAP 278/4). Results presented as mean ($n = 3$).

reveal *S. capricornutum* to have a 3-fold greater proportion of oleate and a 3-fold lower proportion of linolenate (18:3) than found by Hyne *et al.* [2].

Analysis of the free sterol composition by GC and GC-MS revealed that *S. capricornutum* contains three major sterols, identified as 24-methyl-5α-cholest-7-en-3β-ol (ergostenol), 24-ethyl-5α-cholest-7-en-3β-ol (Δ^7-chondrillastenol) and 24-ethyl-5α-cholesta-7,22-dien-3β-ol (chondrillasterol) with trace levels of three other sterol components (<5%) which we were unable to identify using GC-MS. These results are in accordance with the suggestion that a particular algal sterol profile will contain only two or three dominant sterols [7]. The relative proportions of the three sterols are shown in Fig. 1. The occurrence and the relative abundance of these 24-methyl and 24-ethyl sterols in *S. capricornutum* is in good agreement with results from studies of *S. gracile* [3]. Indeed, ergostenol, Δ^7-chondrillastenol and chondrillasterol are known to be widely distributed in members of the Chlorophyceae, though their relative compositions vary considerably within the group [3].

The ability of algae to modify their lipid metabolism in response to changing environmental conditions is essential for their growth and survival [8]. However, these adaptations are often species specific, and generalised responses are rarely applicable to all members of the group. With this in mind, results from our investigation will be discussed.

When cells were subjected to periods of darkness, few significant or reproducible effects were observed amongst the C_{16} fatty acid components. Consequently, discussion is restricted to the C_{18} fatty acid components. Fatty acid composition was little affected by one day of darkness with only the relative proportion of oleate changing significantly (Table 1) whilst cells subjected to dark treatment for seven days showed a further decrease in the relative proportion of oleate and an increase in linoleate (18:2). Similar results have been reported with marine algae during periods of low light intensity or darkness [9–12]. However, these results are very different from those reported by Hitchcock and Nichols [13] in which dark grown *Chlorella vulgaris* accumulated oleate and linoleate, at the expense of linolenate. Other authors have also found that illumination can affect the relative compositions of algal C_{16} fatty acids, palmitate and palmitoleate (16:1) [10, 14, 15] though evidence for such changes was not found in any of our studies ($P > 0.05$).

The alterations in the cellular content of individual fatty acids are considered to be governed to a large extent by changes in the abundance of complex lipids such as phospholipids, galactolipids and triacylglycerols (TAGs) [12]. The phospholipids and galactolipids are usually polyunsaturated and are used as structural lipids, whilst the TAGs are used as storage molecules and contain predominantly saturated and monounsaturated fatty acids [9, 16]. Typically, during periods of high light intensity, when energy input is greater than energy utilisation, TAGs are synthesised to store the excess energy, thus accounting for the increase in levels of saturated and monounsaturated fatty acids. During darkness cells utilise the TAG stores to produce structural galactolipids for the production of thylakoid membranes and provide energy to maintain cellular processes [8]. These processes may account for the decrease in saturated and monounsaturated fatty acids and increases in the levels of polyunsaturated fatty acids during the period of darkness. Certainly the total cellular fatty acid content of *S. capricornutum* varied significantly with dark treatment, with cells exposed to darkness for 7 days having only 50% of the total fatty acid content (μg mg^{-1} freeze dried cells) of cells grown under continuous illumination.

As with the fatty acid composition, the sterol composition of *S. capricornutum* was not greatly affected by one day exposure to darkness (Table 1) though

Table 1. Effect of decreased illumination on total fatty acid and free sterols composition

	7-day-old cells	+ dark for 1 day	+ dark for 7 days
Fatty acid (% composition)			
18:0	0.9 ± 0.1	0.7 ± 0.1NS	0.9 ± 0.1
18:1	26.9 ± 0.3	23.6 ± 0.8*	21.9 ± 0.2‡
18:2	8.9 ± 0.2	10.1 ± 0.8NS	11.5 ± 0.4†
α-18:3	14.5 ± 0.5	15.4 ± 0.5NS	15.8 ± 0.3NS
18:4	5.4 ± 0.2	6.2 ± 0.1NS	5.8 ± 0.6NS
Sterol (% composition)			
Ergostenol	30.2 ± 1.5	28.4 ± 0.2NS	25.9 ± 0.5 PS
Δ^7-Chondrillastenol	51.2 ± 0.7	54.3 ± 0.1†	58.4 ± 0.8†
Chondrillasterol	18.6 ± 0.9	17.2 ± 0.3NS	15.6 ± 0.4*

Results presented as mean ± SE ($n = 3$). NS = $P > 0.10$, PS = $P < 0.10$, * = $P < 0.05$, † = $P < 0.01$, ‡ = $P < 0.001$.

Table 2. Effect of temperature downshift on total fatty acid and free sterol composition

	7-day-old cells	+ downshift to 10° for 1 day	+ downshift to 10° for 7 days
Fatty acid (% composition)			
18:0	1.4 ± 0.1	0.8 ± 0.1†	1.3 ± 0.1NS
18:1	30.5 ± 0.9	30.4 ± 1.2NS	46.1 ± 1.5‡
18:2	9.4 ± 0.1	5.9 ± 0.1‡	1.6 ± 0.2‡
α-18:3	13.5 ± 0.4	19.0 ± 0.9†	14.7 ± 0.7NS
18:4	5.4 ± 0.1	4.8 ± 0.4NS	3.1 ± 0.4†
Sterol (% composition)			
Ergostenol	33.2 ± 0.7	33.8 ± 0.6NS	48.9 ± 0.3‡
Δ^7-Chondrillastenol	47.3 ± 2.6	49.4 ± 0.2NS	39.8 ± 0.4*
Chondrillasterol	19.5 ± 2.3	16.8 ± 0.7NS	11.3 ± 0.3*

Results presented as mean ± SE ($n = 3$). NS = $P > 0.10$, PS = $P < 0.10$, * = $P < 0.05$, † = $P < 0.01$, ‡ = $P < 0.001$.

there was a significant increase in the relative proportion of Δ^7-chondrillastenol. Longer term exposure resulted in a further increase in Δ^7-chondrillastenol and decreases in the relative proportions of chondrillasterol and ergostenol. Published literature dealing with the effect of illumination on the sterol metabolism of algae is scarce. In a recent investigation of the diatom *Phaeodactylum tricornutum*, Veron *et al.* [17] found that the distribution of sterols between free and conjugated forms, as well as the sterol profile, was strongly dependent on light quality. Unfortunately the compositions of these conjugates were not analysed in our work. However, the modifications of free sterol composition reported herein are certainly in contrast to those from a study of the freshwater green macroalga *Cladophora* where the sterol composition remained relatively constant when irradiance was varied [12]. Studies of higher plants have shown that light can affect both the sterol composition and the overall amount of sterol in plant tissues [18–20]. The actual mechanism whereby light modifies the rate and pattern of sterol biosynthesis is poorly understood, though it has been suggested that it may be a phytochrome-mediated process [21].

Cells responded to a seven day period of reduced temperature (Table 2) with dramatic changes in their fatty acid composition. The results indicate a major and significant increase in the relative proportions of oleate with a concomitant decrease in the relative proportions of linoleate and parinate (18:4) in *S. capricornutum*. This led to a comparatively small increase in the overall degree of fatty acid unsaturation (as determined by the unsaturation index). These results differ from many other studies of microalgae and higher plants, where there is a general trend to increase the degree of fatty acid unsaturation with a decrease in temperature, usually associated with an increase in the levels of C_{18} polyunsaturated fatty acids [22–24]. Such effects are thought to be a response to maintain membrane fluidity, and hence function, at low temperatures [25] and recent investigations have suggested that this crucial desaturation of fatty acids is associated with protecting the photosynthetic machinery from photoinhibition under cold conditions [26]. However, Thompson *et al.* [27] point out that the total cellular fatty acid composition of cells exposed to low temperature is, in fact, a balance between an increase in membrane fatty acid unsaturation (an adaptation to produce increased membrane fluidity, as described previously) and chlor-

osis leading to accumulation of TAGs containing short chain saturated fatty acids. The balance between these two processes may well vary between algal species thus leading to a characteristic fatty acid profile for each species in response to environmental stress. Our results for *S. capricornutum* do agree closely with those from a study by Sukenik *et al.* [10] in which *Nannochloropsis* (Eustigmatophyceae) was subjected to a temperature shift from 25° to 18°. Cells responded to this lower temperature with increased levels of oleate and decreased levels of stearate and linoleate, similar to results presented herein.

The effect of temperature on sterol biosynthesis has been investigated in relatively few higher plants [28–30] and even fewer algae. Published results are conflicting and again responses appear to be highly species specific. In our investigation of *S. capricornutum*, low temperature affected the sterol profile, leading to a large and significant increase in the 24-methyl sterol composition and a significant decrease in the 24-ethyl sterol composition after 7 days (Table 2). Modifications of sterol structures affect cellular plasticity and membrane permeability [31] and may represent a cellular response to altered membrane fluidity at lower temperatures. Nabil and Cosson [32] have shown that there is a seasonal variation in sterol composition of the red alga *Delesseria sanguinea* in which temperature must have an influence. Our results are also generally similar to those from a recent study of sterols in the diatom *Phaeodactylum tricornutum* [17] in which 24-methylcholesterol accumulated at low temperatures. It was suggested that this accumulation was a consequence of the slowing down of sterol biosynthesis.

Preliminary experiments revealed that exposure of cells to 124 μM Cu^{2+}, 20 μM Zn^{2+} or 5.7 μM Cd^{2+}, in batch culture, reduced the specific growth rate to 50% of the control (metal-free) rate. Experiments similar to those described above showed that one day or seven day treatment of cells with these concentrations of metal ions had no significant effect upon either the total fatty acid or sterol compositions ($P < 0.05$—data not included). Experiments to assess the long term effect of these metal ions thus involved growth of the cells for 10 days (control cells) or 20 days (metal-treated cells) in batch culture, after which time cells were in early stationary phase. With such treatment, significant changes in the sterol and fatty acid compositions were observed (Table 3).

The relative proportions of a number of the individual C_{18} fatty acids showed some significant changes upon metal exposure. Most apparent was the significant increase in oleate in Cu^{2+}-, Zn^{2+}- and Cd^{2+}-treated algal cells in comparison with the control cells ($P < 0.05$). Such results are consistent with previous studies of marine algae [33]. Smith *et al.* [34] suggested that this increase in the relative proportions of oleate may arise from a decrease in acyl-ACP transacylase activity, which would slow fatty acid transfer into complex lipids, thus enhancing chain lengthening of palmitate to stearate and subsequent desaturation to oleate. The relative proportions of linoleate and parinate were also significantly altered by metal exposure. However Zn^{2+}-treatment increased the relative proportion of linoleate and decreased parinate whereas Cu^{2+}- and Cd^{2+}-treatment reduced the relative proportion linoleate and increased parinate. Therefore fatty acid biosynthesis in *S. capricornutum* is clearly inhibited in different ways by the three metal ions under investigation.

The relative proportions of ergostenol remained fairly constant upon metal exposure and only showed a small (though significant) difference from the control with Cd^{2+} treatment (Table 3). A more general response to all three metals was the significant increase in the relative composition of chondrillasterol ($P < 0.05$) and a concomitant decrease in Δ^7-chondrillastenol ($P < 0.05$) when compared with control cells. Whilst this increase in Δ^{22} desaturation occurred with all three metals, it was most prevalent with Cu^{2+} exposure. Similar changes in the 24-ethyl sterol composition, upon heavy metal exposure, have also been observed in the marine diatom *Asterionella* (with the

Table 3. Effect of metal exposure on total fatty acid and free sterol composition Cells treated with 124 μM Cu^{2+}, 20 μM Zn^{2+} or 5.7 μM Cd^{2+}

	Control	Cu^{2+}	Zn^{2+}	Cd^{2+}
Fatty acid (% composition)				
18:0	0.5±0.0	0.6±0.1NS	0.7±0.1NS	0.6±0.1NS
18:1	28.3±0.4	31.7±0.3†	30.2±0.2*	32.4±0.7†
18:2	9.5±0.2	8.7±0.1*	11.3±0.5*	8.3±0.1†
α-18:3	9.6±0.8	10.7±0.3*	9.8±0.6NS	8.6±0.4 PS
18:4	5.3±0.2	5.7±0.1NS	3.6±0.1†	6.3±0.1*
Sterol (% composition)				
Ergostenol	25.8±0.1	25.9±0.2NS	25.5±0.1NS	24.2±0.2†
Δ^7-Chondrillastenol	57.7±0.4	52.3±0.1†	55.2±0.7*	55.9±0.4*
Chondrillasterol	16.5±0.4	21.9±0.3†	19.3±0.6*	19.9±0.5†

Results presented as mean ± SE ($n = 3$), NS = $P > 0.10$, PS = $P < 0.10$, * = $P < 0.05$, † = $P < 0.01$, ‡ = $P < 0.001$.

sterols 24-ethylcholest-5-en-3β-ol and 24-ethylcholesta-5,24(28)*Z*-dien-3β-ol) [35]. Such modifications may represent an attempt by the cell to increase the plasma membrane fluidity by the introduction of such desaturated sterols into the membrane, since heavy metals such as Cd^{2+} are known to decrease membrane fluidity [36]. Alternatively, Jones *et al.* [35] have shown that key enzymes in sterol biosynthesis (e.g. S-adenosylmethionine methyl transferase, which methylates C-24 of the sterol side chain), are susceptible to heavy metal inhibition, which may directly lead to modifications of the sterol profile as described herein.

The physiological response of algae to heavy metals depends not only on the metal species used but also on the concentration and duration of exposure [37]. To determine the sensitivity of lipid response to metals, *S. capricornutum* was cultured in media containing 1, 12 and 124 μM Cu^{2+}. To our knowledge this is the only study to date to investigate the sensitivity of microalgal lipid profile response to heavy metal exposure. These Cu^{2+} concentrations are broadly within the range reported in polluted freshwater environments. Patterson and Miner, for example, described Cu^{2+} concentrations as high as 1.4 mM in untreated industrial effluents, with 0.33 mM remaining in the effluent even after treatment [38]. Cu^{2+} concentrations found in domestic sewage are much lower, typically 2.4 μM [39]. Dojlido and Best suggest that natural surface waters typically contain 0.03 μM Cu^{2+}, though waters in the vicinity of mineworkings may have Cu^{2+} concentrations as high as 32 μM [39].

The results reveal that whilst the specific growth rate was not significantly affected at 1 and 12 μM Cu^{2+} (data not included), there were significant changes in lipid profiles even at these low concentrations. Kent and Caux [40] similarly found that when microalgae were exposed to the insecticide fenitrothion, a shift in fatty acid profile (from one dominated by linolenate to one dominated by oleate) occurred before there was any reduction in growth rate. The changes in fatty acid and sterol profile observed with Cu^{2+} exposure were generally similar to those described in Table 3. The dose-response relationships of the individual sterol and fatty acid components (Figs 2 and 3) reveal that significant changes in the composition of Δ^7-chondrillastenol and also linoleate were only apparent at 124 μM Cu^{2+} ($P < 0.05$). Significant changes in the composition of chondrillasterol and parinate were noticeable at the 12 μM Cu^{2+} concentration ($P < 0.05$) and only 1 μM Cu^{2+} was necessary to cause changes in the oleate composition of the cells ($P < 0.10$). Thus it appears that the fatty acid and sterol profiles are sensitive indicators of Cu stress in *S. capricornutum*, showing noticeable alterations long before whole cell responses are apparent.

In conclusion, *S. capricornutum* has a fatty acid profile typical of freshwater green microalgae, and contains only three major sterols. Alterations of environmental factors (illumination and temperature) lead to significant changes in the fatty acid and free sterol composition after seven days, whereas shorter (one day) exposure to these conditions produced little significant response in these lipids. In contrast, short term incubations with metals over one day or seven days produced no significant changes in lipid metabolism. However, long-term incubations over 20 days resulted in significant alterations in both the fatty acid and sterol profiles. The fatty acid composition of algal cells was a particularly sensitive indicator of metal exposure, with concentrations of Cu^{2+} as low as 1 μM having an effect on algal lipid metabolism, yet showing no observable effect on specific growth rate. An increase in the ratio of the sterols chondrillasterol:Δ^7-chondrillastenol was also characteristic of heavy metal exposure. Indeed, it is possible that such lipid profile modifications may serve as a useful indicator of chronic heavy metal pollution in freshwater environments.

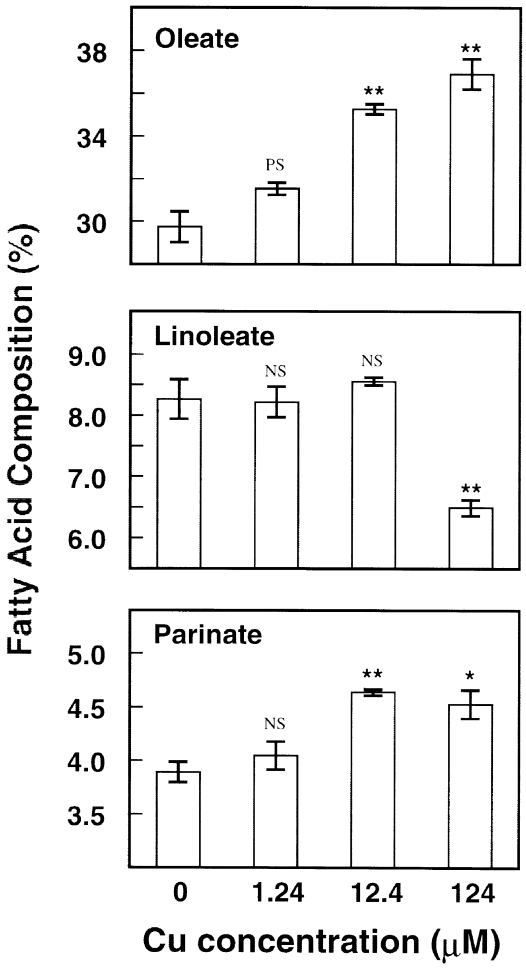

Fig. 2. Changes in fatty acid composition with copper exposure. Results presented as mean ± SE ($n = 3$). In comparison with control values—NS = $P > 0.10$, PS = $P < 0.10$, * = $P < 0.05$, ** = $P < 0.01$. ‡ = $P < 0.001$.

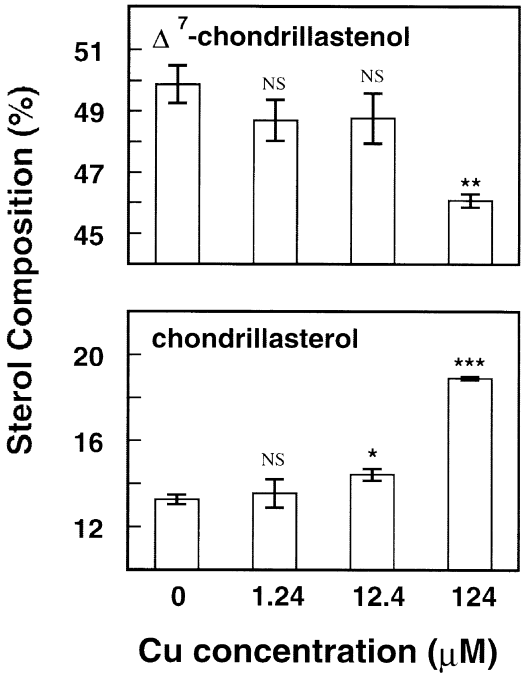

Fig. 3. Changes in sterol composition with copper exposure. Results presented as mean ± SE ($n = 3$). In comparison with control values—NS = $P > 0.10$, PS = $P < 0.10$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

EXPERIMENTAL

Cultivation

Selenastrum capricornutum Printz (CCAP 278/4) was obtained from the Culture Collection of Algae and Protozoa, Windermere, U.K. Cells were grown in batch culture in 100 ml EG:JM medium [41] in glass 250 ml Erlenmeyer flasks. Cultures were incubated in a Gallenkamp cooled orbital incubator at 100 rpm with continuous illumination (70 μmol m^{-2} s^{-1} Photosynthetically Active Radiation) and maintained at a temp. of 25°, unless otherwise stated. To determine the effects of illumination and temp. cells were grown to mid-exponential phase under a light and temp. regime as described above, then subjected either to a dark period or a decrease in temp. from 25° to 10° for up to one week. To determine the effect of metals on lipid composition, cells in mid-exponential phase growth (day 7) were inoculated into shake flasks and grown to late-exponential phase in the presence or absence of $CuSO_4$, $ZnSO_4$ or $CdSO_4$. Cell number was determined using a Coulter Multisizer. Cells were harvested by centrifugation (10,000 *g* for 10 min); the resultant cell pellet was then washed thoroughly and resuspended in deionised water prior to snap freezing in liquid N_2 and freeze drying.

Lipid extraction and GC analysis

Thirty milligrams of freeze-dried cells were continuously stirred in $CHCl_3$–MeOH (2:1) and the extracts were filtered through Whatman No. 1 filter paper into 5% (w/v) Na_2SO_4. The lower phase was removed after 24 h and evapd under N_2 before preparing fatty acid Me esters by acid catalysed transmethylation using 2.5% H_2SO_4 in dry MeOH at 70° for 2 h, as described previously [42]. Free sterol components were sepd from other lipids by TLC, 5α-cholestanol was used as int. standard and sterol acetates were prepared according to ref. [43].

Fatty acid compositions were analysed using a 30 m x 0.25 mm (0.25 μm film thickness) Omegawax capillary column. Samples (1 μl) were injected in a splitless injection mode with N_2 as carrier gas and the injector at 250°. Detection was by FID at 260° and involved a temp. programme, holding the column at 55° for 1 min, then raising the column temp. to 205° at 15° min^{-1}. Identification of fatty acids was made by comparison with authentic fatty acid standards.

Free sterol compositions were analysed using a 30 m x 0.25 mm (0.25 μm film thickness) SAC-5™ (bonded phase poly(5% diphenyl/95% dimethyl siloxane)) fused silica capillary column. Samples (1 μl) were injected in a splitless injection mode with N_2 as carrier gas and the injector at 300°. Detection was by FID at 300° and involved a temp. programme, raising column temp. from 50° to 200° at 50° min^{-1}, holding for 30 s, then raising column temp. to 265° at 5° min^{-1}. Sterol identity was confirmed by GC-MS with a 25 m x 0.22 mm HP1 (0.25 μm film thickness) quartz capillary column plumbed directly into the ion source. Helium was used at the carrier gas and a temp. programme involved raising column temp. from 50° to 300° at 12° min^{-1}.

Statistical analysis

Data were subjected to Analysis of Variance and Student's *t*-test.

Acknowledgements—C.J.M.-R. gratefully acknowledges funding from the University of Central Lancashire for this project. The authors would like to thank Dr John Goad, University of Liverpool, for assistance with GC-MS.

REFERENCES

1. Canter-Lund, H. and Lund, J. W. G., *Freshwater Algae Their Microscopic World Explored*. Biopress Ltd, Bristol, 1975.
2. Hyne, R. V., Podovan, A., Parry, D. L. and Renaud, S. M., *Australian Journal of Marine Freshwater Research*, 1993, **44,** 389.
3. Paoletti, C., Pushparaji, B., Florenzano, G., Capella, P. and Lercker, G., *Lipids*, 1976, **11,** 266.
4. Volkman, J. K., Jeffrey, S. W., Nichols, P. D., Rogers, G. J. and Garland, C. D., *Journal of Experimental Marine Biology and Ecolology*, 1989, **128,** 219.

5. Cranwell, P. A., Jaworski, G. H. M. and Biddey, H. M., *Phytochemistry*, 1990, **29,** 145.
6. Renaud, S. M., Parry, D. L. and Thinh, L., *Journal of Applied Phycology*, 1994, **6,** 337.
7. Rzama, A., Dufourc, E. J. and Arreguy, B., *Phytochemistry*, 1994, **37,** 1625.
8. Roessler, P. G., *Journal of Phycology*, 1990, **26,** 393.
9. Sukenik, A. and Carmeli, Y., *Journal of Phycology*, 1990, **26,** 463.
10. Sukenik, A., Zmora, O. and Carmeli, Y., *Aquaculture*, 1993, **117,** 313.
11. Koskimies-Soininen, K. and Nyberg, H., *Phytochemistry*, 1991, **30,** 2529.
12. Napolitano, G. E., *Journal of Phycology*, 1994, **30,** 943.
13. Hitchcock, C. and Nichols, B. W., *Plant Lipid Biochemistry*. Academic Press, London, 1971.
14. Renaud, S. M., Parry, D. L., Thinh, L., Kuo, C., Padovan, A. and Sammy, N., *Journal of Applied Phycology*, 1991, **3,** 43.
15. Floreto, E. A. T., Hirata, H., Ando, S. and Yamasaki, S., *Botanica Marina*, 1993, **36,** 149.
16. Sukenik, A., Carmeli, Y. and Berner, T., *Journal of Phycology*, 1989, **25,** 686.
17. Veron, B., Dauguet, J. and Billard, C., *European Journal of Phycology*, 1996, **31,** 211.
18. Bae, M. and Mercer, E. J., *Phytochemistry*, 1970, **9,** 63.
19. Grunwald, C., *Plant Physiology*, 1978, **61,** 76.
20. Goad, J. L., *Biochemistry Society Transactions*, 1983, **11,** 548.
21. Hartmann, M. A., Beneviste, P. and Durst, F., *Phytochemistry*, 1972, **11,** 3003.
22. Patterson, G. W., *Lipids*, 1970, **5,** 597.
23. Pohl, P. and Zurheide, C., in *Marine Algae in Pharmaceutical Science*, ed. H. A. Hoppe, T. Levring and Y. Tanaka. Walter de Gruyer, Berlin, 1979, p. 473.
24. Lynch, D. V. and Thompson, G. A., *Plant Physiology*, 1982, **69,** 1369.
25. Harwood, J. L. and Jones, A. L., in *Advances in Botanical Research. Vol.* 16, ed. J. A. Callow. Academic Press, London, 1989, p. 1.
26. Nishida, I. and Murata, N., *Annual Review of Plant Physiology and Plant Molecular Biology*, 1996, **47,** 541.
27. Thompson, P. A., Guo, M., Harrison, P. J. and Whyte, J. N. C., *Journal of Phycology*, 1992, **28,** 488.
28. Davies, D. L. and Finkner, V. C., *Plant Physiology*, 1972, **52,** 324.
29. Wilmott, C., *Phytochemistry*, 1980, **19,** 1071.
30. Jusaitis, M., Paleg, L. G. and Aspinall, D., *Phytochemistry*, 1981, **20,** 417.
31. Yeagle, P. L., *Biochimica Biophysica Acta*., 1985, **322,** 267.
32. Nabil, S. and Cosson, J., *Hydrobiologia*, 1996, **326/327,** 511.
33. Jones, A. L. and Harwood, J. L., *Journal of Experimental Botany*, 1993, **44,** 1203.
34. Smith, K. L., Bryan, G. W. and Harwood, J. L., *Journal of Experimental Botany*, 1985, **36,** 663.
35. Jones, G. J., Nichols, P. D., Johns, R. B. and Smith, J. D., *Phytochemistry*, 1987, **26,** 1343.
36. Nealon, D. G., Sorensen, E. M. B. and Acosta, D., *Journal Tissue Culture Methods*, 1984, **9,** 11.
37. Ahluvalia, A. S. and Kaur, M., *Microbios*, 1988, **53,** 37.
38. Patterson, J. W. and Minear, R. A., in *Heavy Metals in the Aquatic Environment*, ed. P. A. Krenkel. Pergamon Press, Oxford, 1975, p. 262.
39. Dojlido, J. and Best, G. A., *Chemistry of Water and Water Pollution*. Ellis Horwood Ltd., Chichester, 1993.
40. Kent, R. A. and Caux, P. Y., *Canadian Journal of Botany*, 1995, **73,** 45.
41. Tompkins, J., De Ville, M. M., Day, J. G. and Turner, M. F., *Culture Collection of Algae and Protozoa Catalogue of Strains*. NERC Ambleside, U.K., 1995.
42. Rolph, C. E., Moreton, R. S. and Harwood, J. L., *Lipids*, 1989, **24,** 715.
43. Parkin, E., Goad, J. L. and Rolph, C. E., *Physiologia Plantarum*, 1995, **94,** 643.