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Lead and copper effects on lipid metabolism in cultured lichen photobionts with different phosphorus status

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Dedicated to Professor Rodney Croteau on the occasion of his 60th birthday.

Abstract

We evaluated the ability of heavy metals (copper, lead) to alter lipid metabolism in four algal lichen photobionts following short term exposure. Metal concentrations (10 μM) were equivalent to environmentally relevant levels that have been reported to have effects on intact algae. The algae were grown under normal or deficient phosphate conditions to assess any interactions with the heavy metal stress. Given the frequent sensitivity of lichens to copper and lead, there were surprisingly small changes on lipid metabolism, as assessed by radiolabelling from [1-^{14}C]acetate. The main effects, which were seen in a number of cases, were an overall inhibition of total lipid labelling and a relative increase in the labelling of triacylglycerols in the non-polar fraction. Both of these changes can be viewed as reflecting general toxicity of heavy metals. The *Coccomyxa* photobiont species were more sensitive than *Trebouxia* species, which fits with the general distribution of the latter in lichens inhabiting harsh environments.

Keywords: Lichen photobionts; *Coccomyxa mucigena*; *Coccomyxa peltigera variolosae*; *Trebouxia erici*; *Trebouxia aggregata*; Lipid metabolism

1. Introduction

Lichens are symbiotic associations between heterotrophic fungi (mycobiont) and photosynthetic prokaryotic (cyanobacterium) or eukaryotic (alga) organisms referred to as a photobiont. Lichens are important components of the vegetation of many ecosystems in the world from the tropics to the polar regions (approximately 8% of terrestrial ecosystems are lichen-dominated). They include about 17,000 species and are often especially important in extreme environments (Nash, 1996a; Hale, 1983). Around 85% of lichen-forming fungi are symbiotic with green algae, approximately 10% with cyanobacteria (blue-green algae) and 3–4% are cephalodiate species which associate simultaneously with cyanobacteria and green algae (Honegger, 1996).

Lichens are useful as biomonitors of environmental pollution, including raised heavy metal levels for several reasons. First, they have a wide geographic distribution and as perennial, slow-growing and long-lived organisms, maintain a fairly uniform morphology over a long period of time (Ahmadjian, 1993). Second, lichens can accumulate many compounds in high concentrations due to the absence of a wax cuticle and stomata on the surface of lichen thalli that allows diffusion of contaminants into the tissue. Third, because lichens lose water as a result of evaporation during dry periods this may lead to pollutant concentration and thus increase lichen sensitivity. Fourth, photosynthesis (which is often a primary target of pollution) can occur at low temperatures in lichens which are, thus, sensitive throughout the year (Nash, 1996b; Gries, 1996). Finally, the sensitivity of lichens to pollution may also be related to their symbiotic nature that requires that

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a metabolic balance between symbionts is maintained and a disturbance may readily lead to a breakdown of the whole association (Gries, 1996).

Several reviews (e.g., Tyler, 1989; Puckett and Burton, 1981; Garty, 1993) have dealt with aspects of accumulation, tolerance, and toxicity of heavy metals in lichens. Generally, reductions in photosynthetic and respiration rates are found, often with increased membrane permeability and degradation. With some lichens additional, more specific, metabolic responses have been noted (Puckett, 1976; Brown and Beckett, 1983; Brown, 1995; Branquinho et al., 1997b). The photobiont and fungal components of the lichens may respond differently (Tarhanen, 1998). However, the photobionts are clearly key components because of their role in photosynthesis (Ahmadjian, 1993) and, also, because molecular mechanisms causing sensitivity are often confined to the photobiont (e.g., Pawlik-Skowrońska et al., 2002).

The specific effects of heavy metals on photosynthesis has been studied in algae and cyanobacteria where Cu, Ni, Pb are all compounds which can cause inhibition at environmentally-relevant concentrations (Nalewajko and Olaveson, 1995; Rai et al., 1995, 1996; Rai and Rai, 1997; Lu and Zhang, 1999). Other membrane-located activities which are also adversely affected by heavy metal exposure are cation leakage (Lage et al., 1996), ATP production (Rai and Rai, 1997) and ion uptake (Nalewajko and Olaveson, 1995).

Lipids are key components of membranes and, therefore, are vital for their function (Murata and Siegenthaler, 1998). Furthermore, lipid metabolism is known to be affected by environmental stress and, in resistant organisms, to be able to adjust appropriately (see Thompson, 1996; Harwood, 1998; Rama Deli and Prasad, 1999). Following heavy metal stress, several aspects of lipid biochemistry have often been noted to change. These include qualitative and quantitative alterations in lipids, inhibition of biosynthetic pathways and a reduction in unsaturated fatty acids due to metal-enhanced peroxidation (Harwood, 1994; Rama Deli and Prasad, 1999; Dietz et al., 1999).

Although abundant information on lipids of metal-stressed higher plants is available (for review see Rama Deli and Prasad, 1999), much less is known of the effects of heavy metals on algal or cyanobacterial lipids. Matson et al. (1972) showed the drastic reduction in galactolipid biosynthesis, via an inhibition of galactosyl-transferase activity, in two green algae (*Monoraphidium braunii, Euglena gracilis*) treated with mercury. Effects of cadmium on *Euglena gracilis* membrane lipids resulted in a lowering of sterol content but an increase in phosphatidylglycerol and cardiolipin in light-exposed cells (Einicker-Lamas et al., 1996). Similarly, cells of the marine diatom *Asterionella glacialis* treated with mercury and cadmium also had decreased sterols and their total polyunsaturated fatty acid content was reduced (Jones et al., 1987). Exposure to heavy metals (Cu, Zn and Cd) also changed fatty acid patterns in *Selenastrum capricornutum* (McLarnon-Riches et al., 1998) and fatty acid synthesis, as estimated by labelling from [^{14}C]acetate, in the marine brown algae, *Fucus serratus* and *F. vesiculosus* (Harwood and Jones, 1989).

It should be noted, however, that eukaryotic algae use phytochelatins to complex heavy metals whereas cyanobacteria may utilize metallothioneins. So the exact molecular mechanisms to ensure some resistance to heavy metals exposure may be somewhat different. Nevertheless, the response of algae, cyanobacteria and lichens to heavy metals, such as copper, is often very similar (Bačkor et al., 2004).

Some complex interactions between other nutrients and heavy metal toxicity have been well documented, including phosphorus status. Thus, the efficiency of phosphate utilization or the ability to tolerate phosphate deficiency often influences metal sensitivity in many plants and algae (Foy et al., 1978; Rai et al., 1981). Cd has been reported to inhibit phosphate uptake in *Anacystis nidulans* (Singh and Yadav, 1984), while Al affected only its mobilization in *Anabaena cylindrical* (Pettersson et al., 1988). Conversely, the important role of phosphate in regulating cellular uptake of copper has been established using the nutritionally starved cyanobacterium *Nostoc calcicola* (Verma et al., 1991) and for Zn and Cd in *Chlorella autotrophica* (Wang and Dei, 2001). In a following study, Verma et al. (1993), presented evidence that Cu toxicity in cyanobacteria was due to the Cu-induced phosphate starvation and that exogenous addition of phosphate could antagonize the Cu-effect in *N. calcicola* (Verma et al., 1993). Phosphorus metabolism was also shown to influence cadmium toxicity to *N. linckia* (Husaini and Rai, 1991) and a significant amelioration of Cr and Pb toxicity has been observed in *Nostoc muscorum* at high concentrations of phosphate (Singh et al., 1993). Some of these interactions may include involvement of polyphosphate granules or bodies (PPB) in intracellular metal accumulation. These granules may maintain low cytoplasmic levels of metals, thus reducing their toxicity, as has been demonstrated in the diatoms *Amphora* and *Navicula* exposed to copper (Daniel and Chamberlain, 1981). In the cyanobacterium *Nostoc muscorum*, PPB have been suggested to be the main sink for nickel (Singh et al., 1992). Moreover, a copper-resistant *Anabaena variabilis* strain contained more PPB and also had higher internal phosphorus levels than the sensitive strain (Hashemi et al., 1994) while Torres et al. (1998) showed Zn, Pb, Mg and Al sequestering to be important in the PPB of *Plectonema boryanum.*

Unfortunately, there have been few studies of the effect of metals on lichen photobionts. Backor et al. (1998) studied the effect of metals (Cd, Cu, Hg) on the lichen photobiont, *Trebouxia irregularis* while copper tolerance was evaluated in *Trebouxia erici* (Bačkor and Váczi, 2002). The same group also studied the effect of the above metals on chlorophyll levels in *Coccomyxa* sp. and *Myrmecia biatorellae* (Bačkor and Dzubaj, 2004).

At the same time, heavy metals, like Pb and Cu, are important industrial pollutants and their environmental effects may be increasing (Branquinho et al., 1997b; Maksymiec, 1997). Because of the importance of lichens in the environment and their reported sensitivity to heavy metals, we wished to know more about the biochemistry of Cu and Pb on lipid metabolism. In order to provide a simpler system for the experiments we used cultured algal photobionts and examined the action of Cu and Pb in organisms of different phosphorus status. The heavy metal levels used were chosen because they were reported to be toxic to lichens but, surprisingly, they caused relatively little immediate effect on the photobionts alone.

2. Results

2.1. Algal photobionts show variable sensitivity to heavy metal exposure

We exposed the isolated photobionts to levels of Cu and Pb which have been reported to be toxic to lichens in general (Branquinho et al., 1997a,b; Cabral, 2003) and including those containing *Coccomyxa* and *Trebouxia* spp. (Branquinho et al., 1997a,b; Pawlik-Skowrońska et al., 2002). They were also similar to these used for experiments with isolated *Coccomyxa* and *Trebouxia* photosymbionts (Bačkor and Dzubaj, 2004; Bačkor et al., 2004). Exposure was for 24 h in order to follow acute effects of heavy metal exposure. This time has been shown to be sufficient for effects on lichens containing *Trebouxia* (Pawlik-Skowrońska et al., 2002) and for isolated *Trebouxia* spp. (Bačkor and Váczi, 2002; Bačkor et al., 2004) to be seen.

We used [1-^{14}C]acetate for labelling of lipids. This has been shown to be a highly sufficient and suitable precursor for such purposes and also has an advantage that radioactivity is almost entirely confined to the acyl chains (see Roughan and Slack, 1982). As noted before (Guschina et al., 2003), all four algal photobionts were well-labelled in normal phosphate medium but radiolabelling was markedly decreased in the low phosphate medium (Table 1).

The algae showed different sensitivities of their lipid labelling to the heavy metals but seemed to be more sensitive to Pb than Cu. *C. mucigena* showed no effect when grown in the normal media but lipid labelling was reduced by Pb exposure in the low phosphate medium. In contrast, *C. peltigera variolosae* was affected by both Cu and Pb in the normal medium and by Pb in the low phosphate medium. *Trebouxia erici* labelling was affected by Pb in normal phosphate medium only. In contrast, lipid labelling in *Trebouxia aggregata* was not reduced by any of the heavy metal treatments. Polar lipids were predominantly labelled and accounted for 67–85% of the total lipid radioactivity depending on the algal species and conditions. The relative labelling of polar versus non-polar lipids was not altered noticeable by heavy metal exposure (data not shown).

Table 1
Effect of heavy metals on the incorporation of radioactivity from [1-^{14}C]acetate into the lipids of cultured lichen photobionts of different phosphorus status

	1.7 mM P_i	0.017 mM P_i
Coccomyxa mucigena		
Control	2873 ± 134	341 ± 25
+Lead	2726 ± 26	$264 \pm 36^{*}$
Control	1502 ± 143	388 ± 71
+Copper	1750 ± 82	359 ± 84
Coccomyxa peltigera variolosae		
Control	3859 ± 209	804 ± 63
+Lead	$2378 \pm 126^{**}$	$663 \pm 34^{*}$
Control	4228 ± 224	1111 ± 152
+Copper	$3659 \pm 231^{*}$	966 ± 157
Trebouxia erici		
Control	1305 ± 179	308 ± 85
+Lead	$924 \pm 83^{*}$	295 ± 88
Control	1308 ± 85	465 ± 27
+Copper	$1086 \pm 84^{*}$	$181 \pm 43^{**}$
Trebouxia aggregata		
Control	794 ± 79	344 ± 54
+Lead	765 ± 83	501 ± 50
Control	1434 ± 162	424 ± 22
+Copper	1433 ± 143	496 ± 97

Data as d.p.m. × 10^{-4}/g fresh wt and show means ± s.d., where $n = 3$ for independent samples. Cells were in the log phase of growth (see Section 4). $^{**}P < 0.005$, $^{*}P < 0.05$ on statistical analysis by one-way ANOVA using a Tukey test for comparison of controls with metal-treated samples.

2.2. Heavy metal exposure does not alter fatty acid labelling patterns

The main fatty acids present in the lipids of the four algal photobionts have been previously reported (Guschina et al., 2003). As with that study, (following a 2 h incubation with [1-^{14}C]acetate) palmitic, palmitoleic, stearic, oleic and linoleic acids were the main radiolabelled products. Although, as expected, there were some differences in the pattern of labelled fatty acids produced in the four algae (see Guschina et al., 2003), exposure to heavy metals had almost no effect on the relative distributions of radioactivity. Data for the two *Coccomyxa* spp. are shown in Table 2. The control patterns, including those for *Trebouxia* spp., were similar to those reported before (Guschina et al., 2003).

Although there have been, to our knowledge, no other studies on radiolabelling of fatty acids, it is pertinent to add that the endogenous fatty acid patterns were similar to those reported for other photobionts (e.g. Torres et al., 2003) as discussed before (Guschina et al., 2003).

2.3. Alterations in non-polar lipid labelling were seen for algal photobionts under certain conditions

Although neither Cu nor Pb changed the ratio of polar to non-polar lipid labelling even when total lipid synthesis was impaired (data not shown), the pattern of non-polar labelling was altered for all photobionts except *T.*

Table 2
Heavy metals do not change fatty acid labelling patterns in cultured lichen photobionts

	1.7 mM P_i				0.017 mM P_i			
	Control	+Lead	Control	+Copper	Control	+Lead	Control	+Copper
C. mucigena								
C16:0	30 ± 2	28 ± 2	36 ± 3	33 ± 1	32 ± 2	29 ± 2	30 ± 1	28 ± 3
C16:1	9 ± 2	8 ± 2	7 ± 2	9 ± 1	7 ± 2	7 ± 1	7 ± 1	5 ± 1
C18:0	5 ± 1	3 ± 1	5 ± 1	6 ± 1	5 ± 1	5 ± 1	5 ± 1	6 ± 1
C18:1	46 ± 3	50 ± 4	44 ± 3	41 ± 2	43 ± 4	46 ± 3	42 ± 2	43 ± 2
C18:2	10 ± 3	11 ± 2	10 ± 2	11 ± 1	13 ± 1	13 ± 3	16 ± 2	18 ± 1
C. peltigera variolosae								
C16:0	40 ± 4	36 ± 3	37 ± 2	36 ± 3	37 ± 1	38 ± 1	42 ± 1	39 ± 4
C16:1	6 ± 1	7 ± 2	6 ± 1	5 ± 2	3 ± 1	3 ± 1	5 ± 2	5 ± 1
C18:0	2 ± 1	1 ± 0	6 ± 1	7 ± 1	4 ± 1	3 ± 1	4 ± 1	5 ± 1
C18:1	39 ± 2	40 ± 3	40 ± 2	40 ± 2	48 ± 1	48 ± 3	45 ± 4	46 ± 1
C18:2	13 ± 2	16 ± 2	12 ± 1	13 ± 1	8 ± 1	9 ± 2	5 ± 1	6 ± 1

Data as % total fatty acid labelling and show means ± s.d., where $n = 3$ for independent samples.

aggregata. In fact, as noted above, the latter alga was insensitive to heavy metal exposure at least in terms of its lipid metabolism (see Table 1).

The alteration in non-polar lipid labelling which was seen repeatedly was a reduction in the relative labelling of diacylglycerol and an increased proportion in triacylglycerol. Situations where these changes were statistically significant are shown in Fig. 1. Alterations in the labelling patterns tended to be found in conditions where the metal exposure also inhibited total lipid labelling but there were exceptions. For example, *C. mucigena* grown in normal phosphate media had its total lipid labelling unaffected by Cu but its non-polar pattern was still altered. In the TLC system we used the 1,3 and 1,2-isomers of DAG are clearly separated so we were able to confirm that the 1,2-isomer was radiolabelled . This isomer is an intermediate of the Kennedy pathway for TAG synthesis whereas the 1,3-isomer is produced by catabolism.

2.4. Polar lipid labelling patterns are generally unaltered by heavy metal exposure

Most of the common polar lipids in both *Coccomyxa* and *Trebouxia* spp. were well-labelled from [1-^{14}C]acetate. Phosphatidylglycerol (PG) was the best labelled phosphoglyceride and phosphatidylcholine (PC) the next (Figs. 2 and 3). Monogalactosyldiacylglycerol (MGDG) was the major glycosylglyceride labelled, although this only represented about 9% of the total labelling in *T. erici* (Fig. 3). Diacylglycerol trimethylhomoserine (ether) lipid (DGTS) was a significant component in three species and contained 13% of the total radioactivity in *C. mucigena* while both *Coccomyxa* species contained a rhamnose-glycerolipid whose complete structure was not fully defined.

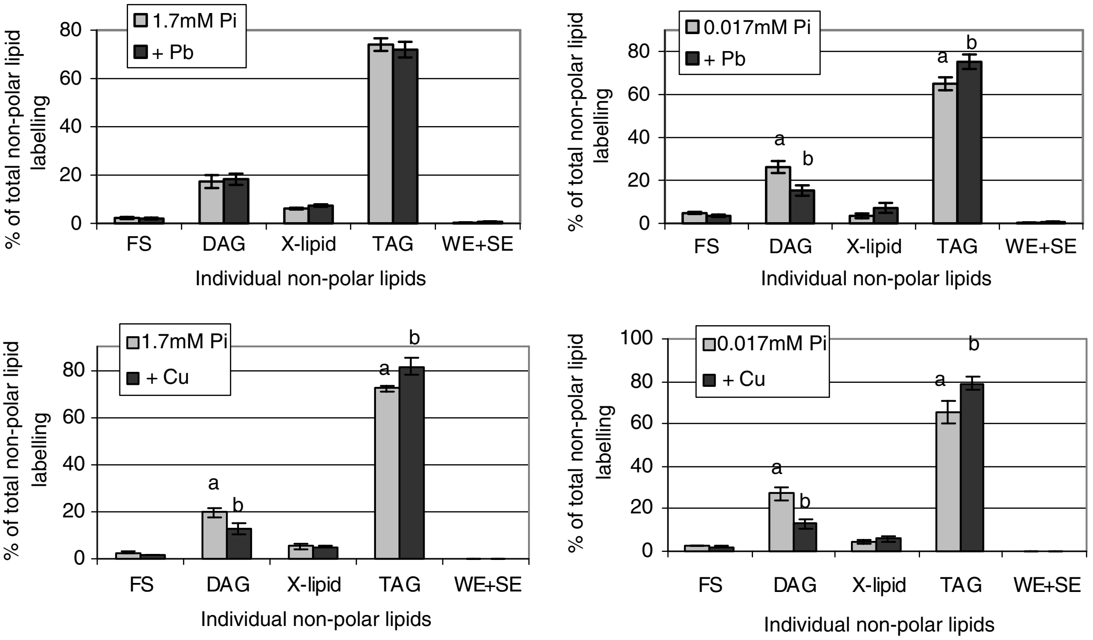
There were hardly any alterations in polar lipid labelling patterns under either normal or reduced phosphate growth that were produced by heavy metal exposure (Figs. 2 and 3). This was despite the fact that 75–90% of the total label went into polar lipids and that incorporation could be reduced up to 80% (Table 1) following exposure to heavy metals. The lack of effect of heavy metals on the relative distribution of radioactivity between the polar lipid classes was in contrast to the effect on non-polar lipids (Fig. 1).

3. Discussion

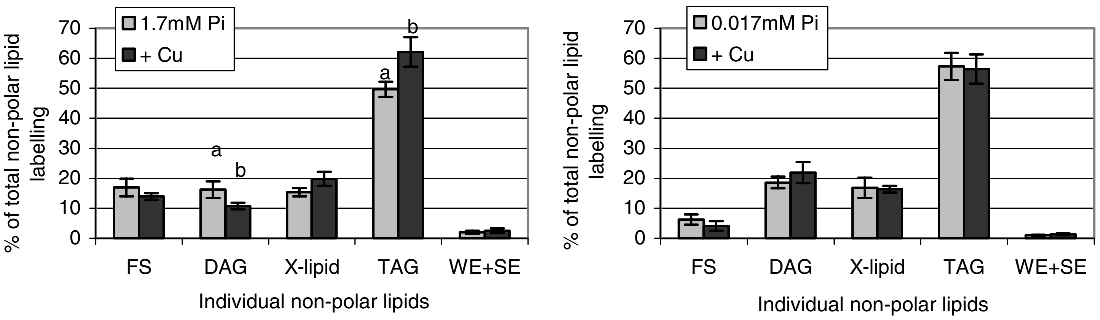
In the present study the role of lipid metabolism in the response of taxonomically close lichen photobionts to heavy metal stress has been evaluated. The species investigated were chosen by the different ability of lichens containing them to tolerate harsh environments. The *Trebouxia* spp. represent an example of photobionts which usually associated with the ecologically most successful lichen species of extreme environments (arctic-alpine, antarctic, and desert ecosystems) (Honegger, 1991). On the other hand, green algal photobionts of *Coccomyxa* are often symbiotic partners in *Peltigera* lichens which are relatively sensitive to environment and grow exclusively in mild moist habitats. Although *Trebouxia* and *Coccomyxa* species have close taxonomical relationship, they form the lichens with different structural and functional mycobiont–photobiont interfaces. The physiological integration between symbionts is closer in lichens with *Trebouxia* photobionts compared to lichens with *Coccomyxa*. This partly may be explained by fact, that *Trebouxia* is rarely found free-living in natural habitats, whereas *Coccomyxa* also comprises non-symbiotic species (Honegger, 1991).

Our results demonstrate some notable differences in lipid metabolism of the studied species. A domination of phospholipid metabolism was revealed in *Trebouxia* spp. where the ^{14}C-incorporation into the main phospholipids, PG and PC, reached up to about 30–35% and 20–25% of total polar lipid labelling, respectively. In *Coccomyxa* species, the common chloroplast glycosylglycerides, MGDG, DGDG and SQDG, were well labelled, while the betaine lipid, DGTS, was significant, especially in *C. mucigena.*

Coccomyxa mucigena



Coccomyxa peltigera variolosae



Trebouxia erici

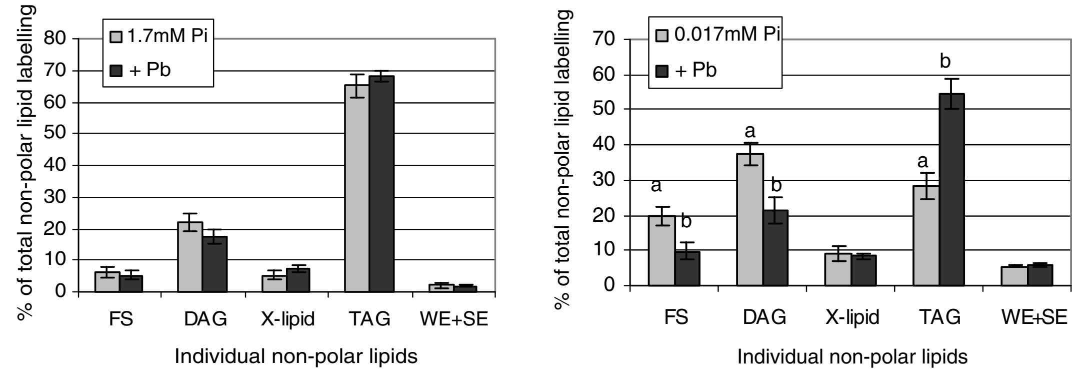


Fig. 1. Heavy metals can produce a change in the pattern of non-polar lipid labelling from [1-^{14}C]acetate in cultured lichen photobionts. Abbreviations: FS, free sterols; DAG, diacylglycerols; X-lipid, unidentified lipid; TAG, triacylglycerols; WE + SE, wax esters + steryl esters. Incubations were ±Cu or Pb at 10 μM for 24 h. Bars with different letters are statistically different at $P < 0.05$ on statistical analysis by one-way ANOVA using a Tukey test in comparison of control and metal-treated samples.

The two *Coccomyxa* species could also synthesize two unusual glycoglycerolipids containing rhamnose and an unidentified sugar. The presence of rhamnose (and mannose), which might partly replace the galactose of MGDG and DGDG, was found previously in glycolipids of the red algae, *Chondrus crispus* and *Polysiphonia lanosa* (Pettitt and Harwood, 1986; Harwood and Jones, 1989). However, in spite of their good labelling and the effect of heavy metals on this (Table 1), the pattern of polar lipid labelling and, also, that of fatty acids (Table 2) was not significantly

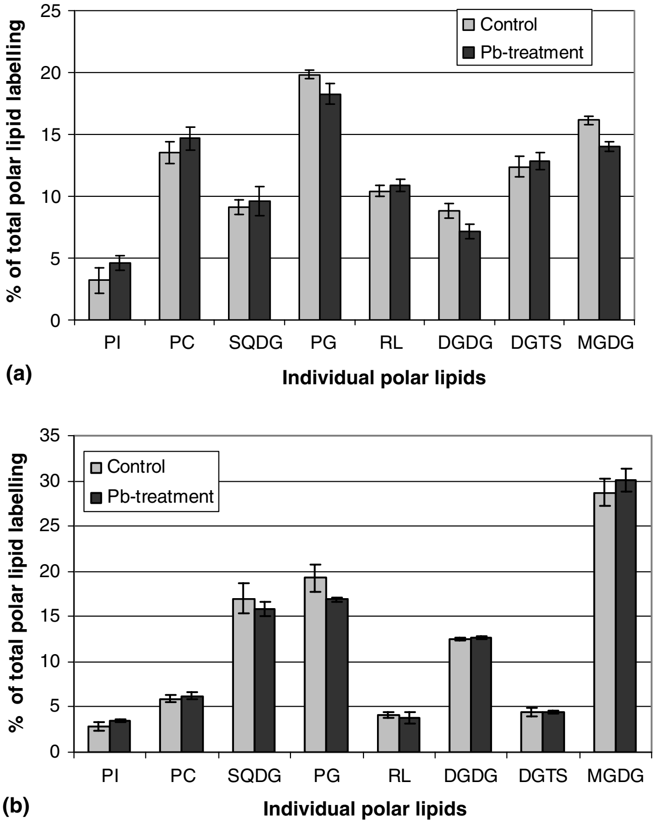


Fig. 2. Effect of lead treatment (24 h, 10 μM) on the relative labelling of individual polar lipids of the green algae *Coccomyxa mucigena* (a) and *Coccomyxa peltigera variolosae* (b) in phosphorus-sufficient medium. RL, rhamnose-containing lipid.

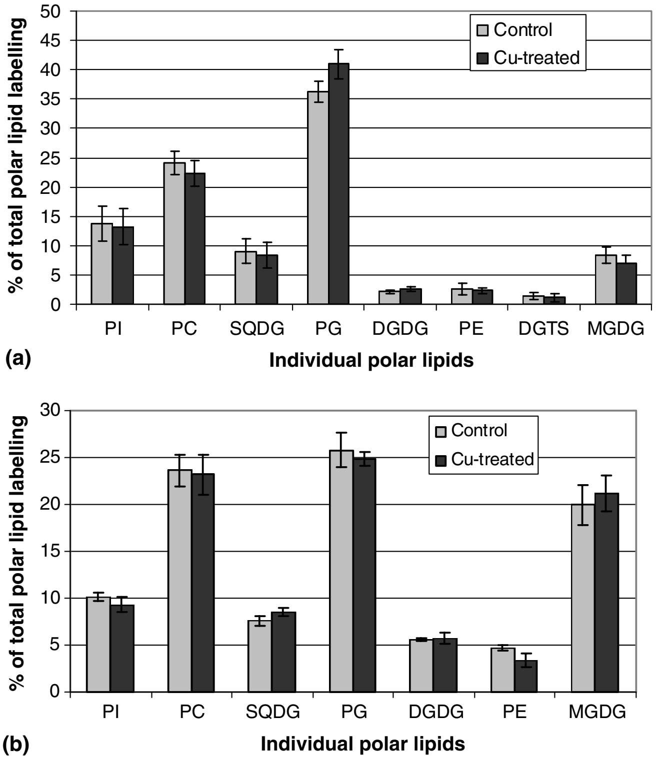


Fig. 3. Effect of copper treatment (24 h, 10 μM) on the relative labelling of individual polar lipids of the green algae *Trebouxia erici* (a) and *Trebouxia aggregata* (b) in phosphorus-sufficient medium.

altered by exposure of the photobionts to Cu or Pb (Figs. 2 and 3).

In contrast, a general increase in the relative labelling of triacylglycerols at the expense of its immediate precursor in the Kennedy pathway, diacylglycerol (Gurr et al., 2002) was found (Fig. 1). The context for this percentage increase should be remembered when trying to interpret the changes. That is, for *Coccomyxa* spp. significant alterations in non-polar lipid patterns are caused by heavy metals when the latter also inhibit total labelling. In short, they are commensurate with a measure of acute toxicity. Accumulation of triacylglycerol under such circumstances has been recorded for a wide variety of species (Gurr et al., 2002) including photosynthetic organisms (Harwood, 1994). It may reflect a preferential use of diacylglycerol for triacylglycerol formation rather than membrane lipid (phosphatidylcholine, glycosylglyceride) production.

Perhaps the most surprising aspect of our results is the robustness of isolated photobionts towards acute heavy metal exposure. When in a symbiotic relationship, the species studied are rather sensitive to Cu and Pb. Moreover, *Trebouxia*-containing lichens react to micromolar concentrations of heavy metals within 24 h (Pawlik-Skowrońska et al., 2002). Of course, given the unusual habitat of lichens, any pollutant can be easily concentrated under dehydrating conditions. Also, in the lichen, the delicate symbiotic relationship may be critical and may be especially sensitive to pollution (Gries, 1996). It is possible that some of the heavy metals were unavailable due to the formation of insoluble complexes in the Bold's medium. However, we did not see any evidence for this by microscopy and atomic absorption analysis of *Coccomyxa* species confirmed uptake of heavy metals.

Given the known effects of Cu and Pb on photosynthesis (Puckett, 1976; Nalewajko and Olaveson, 1995; Rai et al., 1995, 1996) then it is not surprising that the main consequence of heavy metals in our experiments is on total lipid labelling. This means that the supply of carbon for fatty acid synthesis and lipid assembly is altered, rather than the particular enzymes within the lipid biosynthetic pathways. The relative tolerance of lichens containing *Trebouxia* spp. to environmental stress (Pawlik-Skowrońska et al., 2002; Bačkor and Váczi, 2002) is also reflected in the data we obtained for isolated photobionts.

4. Experimental

4.1. Experimental material

Authentic strains of the unicellular green algae *Coccomyxa mucigena* Jaag (215-4), *C. peltigera variolosae* Jaag (216-6), *Trebouxia aggregata* (Archibald) Gärtner (219-1d) and *T. erici* Ahmadjian (32.85), originating from the SAG-

Sammlung von Algenkulturen at the University of Göttingen were cultured in Bold's medium (Ahmadjian, 1967) photoorganotrophically with 1% w/v glucose at 24 °C under a 14 h photoperiod and 100 μE m^{-2} s^{-1} illumination. Growth of the cultures was monitored by measuring the optical density of the cell suspension spectrophotometrically at 560 nm.

4.2. Chemicals

Fatty acid standards were from Nu-Chek Prep. Inc. (P.O. Box 172, Elysian, MN 56028, USA) and silica gel G plates from Merck. Complex lipid standards were from Sigma (Poole, Dorset, UK) and were checked for purity before use. [1-^{14}C]Acetate, Na salt (sp. act. 1.85–2.29 GBq/mmol) was from Amersham Life Sciences Ltd. (Bucks HP7 9NA, UK). Other reagents were of the best available grades and were from Sigma (Poole, Dorset, UK) or from BDH (Poole, Dorset, UK).

4.3. Incubations

To produce a biomass of algal cells at a similar exponential growth phase, 3 ml aliquots of algae cells from stock cultures were transferred to 100 ml flasks containing 50 ml fresh Bold's medium with normal phosphate (1.7 mM) or low phosphate concentrations (0.017 mM) and then incubated for 14 days under conditions as in Section 4.1. At the end of 14 days, the cultures were subjected to heavy metal treatments by adding either $Cu(NO_3)_2$ or $Pb(NO_3)_2$ at a final concentration of 10 μM for 24 h with gently shaking. The algae were then separated by centrifugation (600 r.p.m.), supernatants were decanted and 20 ml fresh glucose-free medium (with normal or limited phosphate supply) were added, following by gently stirring, to each flask.

These cultures were used for lipid labelling by adding 5 μCi [^{14}C]acetate with further incubation for 2 h at 24 °C with 200 μE m^{-2} s^{-1} continuous illumination. All experiments were performed in triplicate. At the end of the incubation period, cell pellets (about 300 mg of fresh weight) were harvested by centrifugation, washed twice with 5 ml of distilled H_2O to remove excess radiolabel and metabolism was terminated by the addition of hot *iso*-propanol and heating at 70 °C for 30 min. This method ensures that lipid catabolic enzymes are inactivated. Lipids were extracted twice more with hot *iso*-propanol (three extractions) and finally with a mixture of *iso*-propanol:chloroform (1:1, v/v) (Kates, 1986). Garbus solution (2 M KCl in 0.5 M potassium phosphate buffer, pH 7.4) was used to wash the extraction mixture (Garbus et al., 1963). Fatty acid methyl esters were prepared by transmethylation with 2.5% H_2SO_4 in dry methanol.

4.4. Lipid analysis

Non-polar lipids were separated by 1-D TLC on 10 × 10 cm silica gel G plates with double development, first with toluene–hexane–formic acid (140:60:1, by vol.) for the whole plate height followed by hexane–diethyl ether–formic acid (60:40:1, by vol.) to half height (Hansen and Rossi, 1990).

Polar lipids were separated by 2-D TLC on 10 × 10 cm silica gel G plates using chloroform–methanol–water (65:25:4, by vol.) in the first dimension and chloroform–acetone–methanol–acetic acid–water (50:20:10:10:5, by vol.) in the second direction (Benning et al., 1995).

Plates were sprayed with 0.05% 8-analino-4-naphthosulphonic acid in methanol and viewed under UV light to reveal lipids or visualized under iodine vapour. Identification was made by reference to authentic standards and confirmed using specific colour reagents such as Dittmer for phospholipids, Dragendorff for quaternary ammonium compounds, ninhydrin for amino-lipids etc. (Kates, 1986).

The moiety of a rhamnose-containing glycerolipid (with a positive α-naphthol reaction) was identified after hydrolysis and TLC of the carbohydrate portion as described by Roy and Harwood (1999). The glycerol backbone of this lipid was also confirmed by monitoring incorporation from [^{14}C]glycerol and subsequent hydrolytic analysis.

Fatty acid methyl esters (FAMEs) were prepared by acid-catalyzed methanolysis (2.5% H_2SO_4 in dry methanol) (Christie, 2003) and analyzed by radio-GLC using a Unicam GCD gas chromatograph connected via an effluent splitter to a LabLogic RAGA (LabLogic, Sheffield, UK) gas flow proportional counter. Glass columns (1.5 m × 4 mm int. diam.) were packed with 10% SP-2330 on 100/120 Supelcoport (Supelco, Bellefonte, PA, USA). The SP-2330 column was run isothermally at 180 °C. Routine identification was by reference to standards and quantification (Rachel Software, LabLogic) was made using an internal standard of heptadecanoate. Other details of fatty acid analysis are given in Guschina et al. (2003).

Radioactive counting was made using Opti-Fluor (Packard Bioscience B.V., Groningen, The Netherlands) scintillant and a Beckman 1209 Rackbeta liquid scintillation counter. Quench correction was by the external standard channels ratio method.

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