

Comparative study of metal induced phospholipid modifications in the heavy metal tolerant filamentous fungus *Paecilomyces marquandii* and implications for the fungal membrane integrity*

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In this work we compared the effect of five heavy metals: Zn, Pb, Cd, Ni and Cu on phospholipid composition of the ubiquitous soil fungus *Paecilomyces marquandii*, originating from a strongly metal polluted area and characterized by high tolerance to these elements. Cd, Ni and Cu caused an increase in phosphatidylcholine (PC). Only Pb decreased PC content, which was accompanied by a significant rise in the phosphatidic acids (PA) level, probably due to activation of phospholipase D which hydrolyzes PC to PA. This could result in membrane fluidity disturbance, and thus affect its integrity. The assessment of propidium iodide influx showed strong disturbance of membrane integrity for Cu and Pb stressed mycelia, whereas mycelia treated with Ni were impermeable to this dye. The results obtained revealed a strong Cu and Pb toxicity involving disruption of membrane integrity. Pb action was reflected by lipid composition, whereas changes in Cu treated mycelia did not completely elucidate its harmful effect on the membrane, which was most probably caused by Cu induced lipid peroxidation. Zn did not induce quantitative changes in PC and phosphatidylethanolamine (PE) but caused changes in phospholipid lipid saturation, which appears to be important for fungus adaptation to the presence of metals. The enhanced PC content balanced by higher PC saturation can help in the maintenance of proper membrane fluidity and result in alleviating the Cd and Ni induced stress. These results will allow to clarify the mechanism of Pb toxicity and help to elucidate the cellular basis of fungal membrane adaptation to heavy metals.

Key words: heavy metals, membrane integrity, phospholipids, *Paecilomyces marquandii*

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INTRODUCTION

Phospholipids (PLs), constituting about 70% of all membrane lipids, are the major structural lipids in eukaryotic membranes and play a pivotal role in the membrane fluidity and permeability regulation (de Kroon *et al.*, 2013). Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the most abundant PLs in the eukaryotic cells including yeast and fungi and perform crucial functions in the maintenance of membrane integrity. Although fungal PLs are synthesized *via* pathways common to higher eukaryotic organisms, differences in their composition and functions have been found. For example, in contrast to plants, phosphatidylglycerol in fungi

is produced in negligible amounts, mainly as a metabolic precursor of cardiolipine, a lipid important for cell growth, anaerobic metabolism and mitochondrial function (Šimočková *et al.*, 2008). Phosphatidylserine (PS) is crucial in biology of higher eukaryotes contributing to PE and PC synthesis, whereas PS does not play such an essential role in yeast (Vance & Streenbergen, 2005; de Kroon *et al.*, 2013). Phosphatidic acid (PA), a lipid signal, is synthesized in a minor amount in normal growth conditions, but its pool can significantly rise under stress conditions (Quartacci *et al.*, 2001). It was documented that PA plays a central role in the transcriptional regulation of phospholipid synthesis in *Saccharomyces cerevisiae* (Carman & Henry, 2007). Phosphatidylinositol (PI) is a minor membrane component, but it serves as a precursor of sphingolipids, ceramides and glycosylphosphatidylinositol anchors of proteins which are essential in eukaryotic microorganisms (Orlean & Menon, 2007; Reynolds, 2009).

PLs are considered to be the most sensitive to alterations under environmental stress exposure (Dercová *et al.*, 2004; de Kroon *et al.*, 2013).

Heavy metal toxicity to living cells can be manifested by an increase in membrane permeability leading to disruption of the membrane function. However, the mechanism involved in heavy metal induced toxicity has not been fully understood. Azavedo *et al.* (2007) reported that Cu induced oxidative damage to the fungal cell membrane. Lipid peroxidation was also involved in the toxic action of Cu and Cd against the yeast membrane (Howlett & Avery, 1997; Chmielowska-Bąk *et al.*, 2013). Cd, Cu and Hg promoted enhanced membrane permeability and generated reactive oxygen species in mitochondria (Belyaeva *et al.*, 2004).

Additionally, heavy metals can interrupt synthesis of lipids and fatty acids in microscopic fungi (Čertík *et al.*, 2005; Paraszkiwicz *et al.*, 2009; Słaba *et al.*, 2013). An increase in the *trans/cis* ratio of unsaturated fatty acids in the presence of metals was observed. *Trans* isomers are more stable than *cis* and can counteract the membrane disturbance (Kaur *et al.*, 2005). A reduction in polyunsaturated fatty acid production caused by some heavy metals was noted (Garcia *et al.*, 2005; Paraszkiwicz *et al.*, 2010). On the other hand, microorganisms, espe-

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Abbreviations: DBI, double-bond index; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PLs, phospholipids; PS, phosphatidylserine

cially fungi inhabiting polluted environments, can evolve many adaptive mechanisms to limit heavy metal toxicity and simultaneously contribute to their removal (e.g. extracellular polymer production, cell wall and membrane modification or metal sequestration) (Paraszkiewicz *et al.*, 2007; Gadd, 2010; Ślaba & Długoński, 2011). Therefore, a better understanding of the mechanisms allowing fungi to survive at high metal concentrations has a great environmental and biotechnological importance.

In our earlier report, details of Zn and Pb toxicity to germinating spores of *Paecilomyces marquandii* were described (Ślaba *et al.*, 2005). It was also found that this filamentous fungus can effectively bind Zn and Pb (Ślaba & Długoński, 2011).

In this work we studied comparatively the effect of zinc, lead, cadmium, nickel and copper ions on phospholipids of *P. marquandii* strain and the metal treatment implications for fungal membrane integrity.

MATERIALS AND METHODS

Chemicals. Metal salts (acetates) were purchased from Sigma–Aldrich Chemical Co. (Germany). Propidium iodide used to demonstrate the condition of the membrane was purchased from Molecular Probes, Eugene (Oregon, USA). Five analytical standards of a given class of phospholipids (>99% purity): 1,2-dimyristoyl-sn-glycero-3-phospho-rac-(1-glycerol) (sodium salt) (14:0/14:0 PG); 1,2-dilauroyl-sn-glycero-3-phosphoethanolamine (12:0/12:0 PE), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (14:0/14:0 PC); L-R-phosphatidylinositol (PI) sodium salt from bovine liver; 1,2-dimyristoyl-sn-glycero-3-phospho-L-serine sodium salt (14:0/14:0 PS), and 1,2-dimyristoyl-sn-glycero-3-phosphate (sodium salt) (14:0/14:0 PA) were obtained from Avanti Polar Lipids, Inc. (Alabaster, USA). Other chemicals used in this study were from J.T. Baker, Sigma and POCH.

Microorganism cultures. A filamentous fungus *Paecilomyces marquandii* isolated from post-flotation tailings of Non-Ferrous Metal Works ‘Szopienice’ (Katowice, Poland), strongly polluted with heavy metals, was the subject of this work. This strain was stored at ZT slants (composition in g l⁻¹: glucose, 4; Difco yeast extract, 4; agar, 25; malt extract 6–11°Blg (1° Balling = 1 g of soluble substances extracted from the grain per 100 ml malt extract); pH 7.0) in the collection of the Department of Industrial Microbiology and Biotechnology, University of Lodz, Poland (IM 6003). 7-day spores were introduced into 20 ml Sabouraud medium (composition described by Lisowska *et al.*, 2006) in 100 ml Erlenmeyer flasks. Preculture was prepared as presented previously (Ślaba *et al.*, 2013). After 24 h, 15% of inoculum was added to Sabouraud medium, supplemented with the following concentrations of Zn, Cu, Pb, Ni and Cd, inhibiting fungal growth by 50%: 12, 4.5, 3.7, 3.0, 2.5 mM, respectively. Control samples without the metal supplementation were prepared simultaneously. The cultures were grown for 5 days on a rotary shaker (160 r.p.m.) at 28°C. Next, mycelia samples were washed and separated for further analyses. To check the permeability of the fungal membrane, the samples (1 ml) were collected after 24 h incubation, washed twice with deionized water and phosphate buffer and stained with propidium iodide.

Phospholipid extraction and sample preparation. Samples were prepared according to the method described by Folch *et al.* (1957) with modifications. Fresh wet mycelia (1.0 g) were homogenized in a mortar with 10 ml of a mixture containing chloroform : methanol (2

: 1, v/v). After 18 h incubation at room temperature the samples were vortexed for 1 min. Next, 5 ml of a fresh extraction mixture was added and vials were vortexed again for 1 min. After filtration through a paper filter, 0.02 ml of lipid standards (one standard from each class: PA, PE, PC, PS, PI, PG at the concentration of 0.1 mg ml⁻¹) and 3.0 ml of 0.8% NaCl were introduced into the supernatants and the samples were again vortexed for 1 min. The organic phases were collected, dried up with anhydrous sodium sulfate and evaporated under reduced pressure at 40°C. Evaporated residues were dissolved in 2 ml of methanol and 0.2 ml was transferred onto chromatography plates for analysis.

HPLC-MS/MS analysis. The LC-MS analysis of phospholipids was carried out on an Agilent 1200 HPLC system (Santa Clara CA, USA) coupled to a 3200 Q-Trap mass spectrometer (Ab Sciex, Framingham, MA, USA). The total phospholipid sample was separated on an Allure® PFP Propyl column (50 mm × 2.1 mm i.d., 5 µm, Restek, Bellafonte, PA, USA) with a column temperature of 40°C. Mobile phase A consisted of water/5 mM ammonium formate, and mobile phase B consisted of methanol/5 mM ammonium formate. A linear gradient program was set as follows: 0–2 min, 80–100% B; 2–4 min, 100% B; 4–6 min, 100–80% B. Injection volume was 5 µl, and the flow rate was 0.8 ml min⁻¹.

The nitrogen drying gas and turbo gas were at 50 and 60 psi, respectively. The curtain gas that prevents contamination of the ion was set at 25 psi. Different phospholipid classes were identified using a Precursor ion scan (m/z 153) as a survey scan (range m/z 650–950, scan speed 1000 Da s⁻¹, collision energy 40 eV); an enhanced resolution (ER) scan was used to confirm phospholipid mass assignments. An EPI scan was used as a dependent scan (range m/z 100–950, scan speed 1000 Da s⁻¹, collision energy 40 eV). Based on the product ion and precursor ion analysis of the obtained phospholipid species, a list of multiple reaction monitoring (MRM) transitions was constructed. The MRM mode was used for relative quantitation of individual phospholipids.

Membrane integrity assay. For the membrane integrity assay, hyphae suspended in phosphate buffered saline (0.1 M, pH = 7.4) were stained with propidium iodide at the final concentration of 1 mg ml⁻¹, for 5 min, at room temperature in the dark. The residual dye was removed by centrifugation (15000 r.p.m.) and samples were washed three times in the same buffer as described above. The images of stained hyphae were captured using a Confocal Laser Scanning Microscope (Pascal 2, Zeiss) combined with an Axiovert 200M (Zeiss, Germany) inverted microscope equipped with a Plan-Apochromat objective (63×/1.25 Oil). All settings were held constant during the course of all experiments. The PI fluorescence was detected with a He-Ne laser (543 nm) and an LP filter set (560–615 nm) and images of the hyphae were collected at Nomarski DIC at the excitation of 488 nm of an Ar laser.

The percentages of membrane damaged hyphae were calculated as follows: (total area of PI stained hyphae/total area of hyphae) × 100. The values were calculated from at least 10 microphotographs from 3 independent experiments.

Statistical analysis. All experiments were done in triplicate. One-way analysis of variance (ANOVA) was performed to determine the significance of differences between the samples using Excel 2007 (Microsoft Corporation, USA).

Table 1. Relative content (mol%) of major phospholipid classes extracted from heavy metal stressed *P. marquandii* mycelia.

Class of phospholipids	control	Zn	Pb	Cd	Ni	Cu
PA	3.83±0.36	5.60±1.87	16.12**±5.28	4.25±2.36	3.71±1.53	2.59±1.21
PE	47.49±2.57	54.80**±2.69	48.57±3.10	33.18**±3.59	43.64±6.00	38.58**±3.32
PS	10.16±1.08	2.23**±0.59	10.18±6.03	2.18**±1.30	1.36**±0.65	1.13**±0.34
PC	34.31±3.55	36.76±2.79	22.62**±6.64	59.40**±4.19	50.42**±5.06	56.75**±3.49
PI	4.21±2.11	0.71**±0.05	2.51±0.97	1.00*±0.58	0.85**±0.56	0.95**±0.25
PC/PE ratio	0.73±0.11	0.70±0.08	0.48**±0.16	1.82**±0.32	1.18**±0.27	1.46**±0.16

PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine. Data are mean values ± S.D. * **Indicate values that differ significantly from the control at $P < 0.05$ and < 0.01 respectively. Metal concentrations (mM) inhibiting fungal growth by ca. 50% were used as follows: Cd 2.5, Ni 3.0, Pb 3.7, Cu 4.5, Zn 12.

A double-bond index (DBI), which indicates the saturation level of lipids, was calculated according to the equation described by Su *et al.* (2009).

RESULTS

Phospholipids composition in *P. marquandii* cultures supplied with heavy metals

Lipidomic analysis of organic extracts of *P. marquandii* cultures not supplemented with heavy metals revealed the presence of 30 phospholipids from five classes (Table 1, Fig. 1). Phosphatidylcholines were the most numerous (9) represented: 16:0/18:2, 16:0/18:1, 16:0/18:0, 18:2/18:2, 18:1/18:2, 18:0/18:2, 18:1/18:1, 18:0/18:1, 18:0/18:1, followed by phosphatidylethanolamines (6): 16:0/18:2, 16:0/18:1, 18:1/18:0, 18:2/18:2, 18:1/18:2, 18:1/18:1, phosphatidic acids (6): 16:0/18:2, 16:0/18:1, 18:2/18:2, 18:2/18:1, 18:1/18:1, 18:1/18:0, phosphatidylserines (6): 16:0/18:2, 16:0/18:1, 18:1/18:2, 18:1/18:1, 18:1/18:1, 18:0/18:0 and (not shown in Fig. 1) phosphatidylinositols (3): 16:0/18:2, 16:0/18:1, 18:0/18:1. Fungal phospholipids contained 4 fatty acids (16:0, 18:0, 18:1, 18:2). The same fatty acids dominated in total fatty acids isolated from cultures of the *P. marquandii* treated with heavy metals (Słaba *et al.*, 2013).

The percentage composition of the main class of PLs is shown in Table 1. PE and PC were the most abundant phospholipids in *P. marquandii* and constituted around 55 and 32% of total phospholipids present in the control samples. 5-day heavy metal exposure at the concentrations which inhibited growth by about 50% induced different changes in the phospholipid composition.

Only Zn did not affect the PC and PE content. An elevated PC level was observed after Cd, Ni and Cu treatment. In the case of Cd and Cu a rise in PC was correlated with a significant drop in PE. In contrast,

only Pb induced a decrease in the PC level and the PC/PE ratio. All the metals caused a decrease in the PI level and almost all (with the exception of Pb) reduced the PS abundance. Additionally, an increase in PA was noticed for Pb supplemented cultures (from 3.83 to 16.12%). The above mentioned PL alteration and its implications will be discussed in the Discussion Section.

Changes in phospholipid fatty acids and saturation

Quantitative differences among major fatty acids, which are part of phospholipids isolated after metal exposure are illustrated in Fig. 1. Although no qualitative changes were observed, significant quantitative variations were related to the fatty acid abundance in PC and PE and caused an increase or decrease in the DBI value, depending on a particular metal (Table 2).

Zinc induced a statistically significant rise in fatty acid unsaturation in PE, PC and PI. DBI variations in zinc supplemented cultures were mainly caused by an increased content of PE 16:0/18:2, PE 18:2/18:2 and PC 18:2/18:2 (Fig. 1). The amounts of more unsaturated species significantly decreased in the fungal cultures cultivated in the presence of Pb, Cd and Ni, which resulted in a DBI decline in the PC class (also in PE and PS in Cd treated mycelia). Interestingly, the marked changes in the composition of particular PL classes did not correlate with changes in their saturation in Cu stressed samples (Table 1 and Table 2).

Membrane integrity of metal stressed mycelia

The condition of a fungal membrane exposed to heavy metals was estimated by the measurement of propidium iodide intracellular accumulation (Table 3). This dye is impermeable to intact cells, whereas its influx into hyphae demonstrates disruption of membrane integrity. Cu and Pb caused a strong disruption of *P. marquandii* membrane integrity. Propidium iodide was present in

Table 2. A double-bond index of phospholipids isolated from *P. marquandii* cultures exposed to heavy metal ions at the concentration inhibiting growth by about 50%.

Class of phospholipids	control	Zn	Pb	Cd	Ni	Cu
PA	2.24 ± 0.14	2.32 ± 0.09	2.22 ± 0.15	2.20 ± 0.14	2.12 ± 0.06	3.05 ± 1.65
PE	2.31 ± 0.04	2.50** ± 0.02	2.39 ± 0.17	2.14** ± 0.07	2.27 ± 0.08	2.32 ± 0.04
PS	1.81 ± 0.43	1.67 ± 0.05	1.76 ± 0.10	1.42 ± 0.09	1.47 ± 0.16	1.59 ± 0.09
PC	2.97 ± 0.03	3.11** ± 0.05	2.80** ± 0.07	2.57** ± 0.03	2.88 ** ± 0.04	3.00 ± 0.12
PI	1.55 ± 0.06	1.71** ± 0.02	1.31 ± 0.20	1.57 ± 0.13	1.58 ± 0.05	1.76* ± 0.13

PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine. Data are mean values ± S.D. * **Indicate values that differ significantly from the control at $P < 0.05$ and < 0.01 respectively.

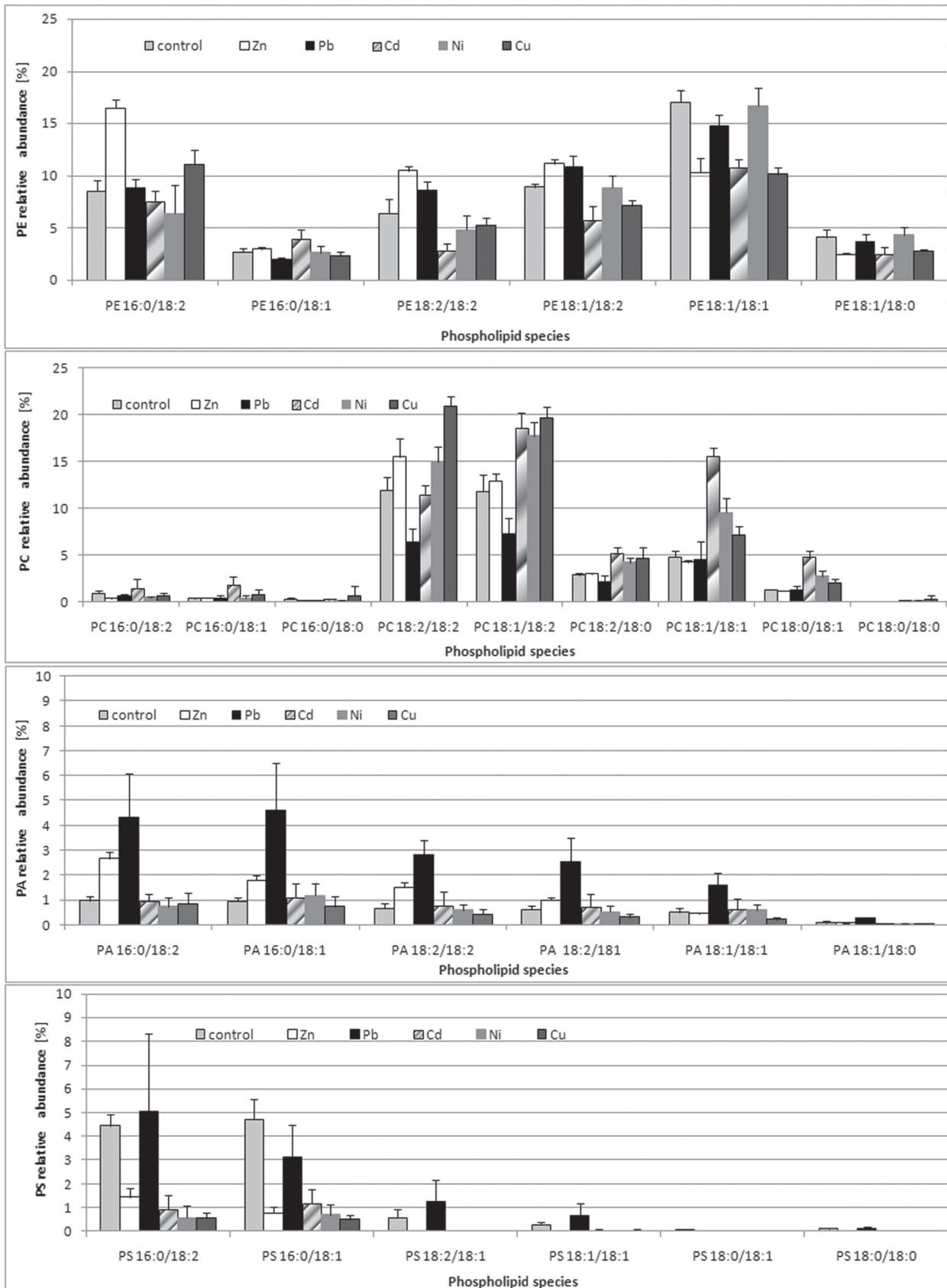


Figure 1. Fatty acid composition in a particular class of phospholipids (PE, PC, PA and PS) isolated from *P. marquandii* cultured in the presence of Zn, Pb, Cd, Ni and Cu ions.

Table 3. *P. marquandii* hyphae permeability determined with propidium iodide after heavy metal treatment.

Samples	Permeability to propidium iodide (%)
Control	1.10±0.85
Zn	3.01±1.22
Pb	31.69±4.75
Cd	3.47±0.34
Ni	0.00±0.00
Cu	41.62±6.78

Data are mean values ±S.D. All samples differ significantly from the control at $P \leq 0.02$

41.6 and 31.7% of hyphae surface for Cu and Pb treated samples, in comparison to 1.10% in the case of control hyphae. Cd and Zn treated mycelia maintained an intact membrane (permeability reached 3.47 and 3.01% for this dye, respectively). Interestingly, after Ni exposure the mycelia were totally impermeable to propidium iodide (0.0%).

DISCUSSION

The phospholipid composition, lipid acyl chain length and saturation determine the physical properties of the membrane (de Kroon, 2007; de Kroon *et al.*, 2013). Because PC and PE play a crucial role in the maintenance of proper fluidity and permeability of the membrane, their variability seems to be particularly important (Quartacci *et al.*, 2001; Bou Khalil *et al.*, 2010). Niebergal & Vance (2012) found that the total cellular amount of PC and PE influences growth and membrane integrity of animal cells. It was documented that PC tends to form a bilayer, whereas PE has a strong propensity to build a nonbilayer hexagonal phase (Quartacci *et al.*, 2001; Bou Khalil *et al.*, 2010). Welti *et al.* (2002) explained that the transformation of a membrane lipid bilayer into a nonbilayer structure deteriorates membrane integrity and cell functions. The results obtained here, revealed a reduced level of PC and an increased level of PA in Pb supplemented mycelia, combined with enhanced propidium iodide influx, indicating a strong membrane permeabilisation. In our earlier work we documented peroxidation of *P. marquandii* lipids as a result of the toxic action of Pb, Ni and Cu. Although all tested metals induced a statistically important increase in the electrolyte leakage, the efflux level was two-fold higher in Pb treated mycelia than in mycelia treated with other metals (Slaba *et al.*, 2013). This work extends the knowledge of Pb toxicity towards membrane lipids of *P. marquandii*. We conclude that a drop in PC and a rise in PA destabilises the balance between the bilayer and nonbilayer structure and promotes nonbilayer phase formation, leading to decreased viability through membrane integrity perturbation. A similar mechanism for a freezing stress was documented in *Arabidopsis* plants (Welti *et al.*, 2002). The authors had shown that changes in the PC and PA levels were caused by phospholipase D, which hydrolysed PC to PA. Additionally, our study concerning Pb toxicity to phospholipids is in line with the supposition of Li *et al.* (2006) suggesting that a PC/PE ratio can regulate membrane integrity and a drastic drop in PC/PE may lead to cell death.

An elevated level of PC enhancing membrane fluidity (Quaracci *et al.*, 2001; Bernat *et al.*, in press) was observed in mycelia exposed to Cd, Ni and Cu, but the effect of these metals on membrane integrity varied. Cd and Ni most strongly inhibited growth of the tested fun-

gus (Slaba *et al.*, 2013), but a measurement of propidium iodide influx, impermeable to intact cells, showed that membrane integrity was not disturbed. As observed for Cd and especially for Ni, enhanced PC content, together with its higher saturation than in the control samples, can counteract PC fluidizing effect and allow to maintain proper fluidity of the membrane. In contrast, Cu treatment, apart from causing a rise in PC level and PC/PE ratio, simultaneously led to greater damage to the *P. marquandii* membrane. Similar results were obtained by Azevedo *et al.* (2007), who found that Cu induced a strong disruption of membrane integrity in aquatic hyphomycetes fungi. The mechanism of Cu toxicity to microorganisms by inducing membrane perturbation is well documented (Stojs & Bagchi, 1995; Azevedo *et al.*, 2007). It depends on the fatty acid membrane composition (Avery *et al.*, 1996). On the other hand, Cu, as a transition metal, contributes to oxidative membrane damage (Azevedo *et al.*, 2007). These observations are in accord with our earlier results showing enhanced lipid peroxidation in mycelia under Cu exposure (Slaba *et al.*, 2013).

In the case of Zn, changes in the lipid fatty acid saturation appear to be important for metal adaptation. A significant rise in phospholipid unsaturation was detected for PE, PC and PI mainly due to linoleic acid enrichment. Probably, enhanced production of unsaturated fatty acids balanced the elevated level of PE, which was more saturated than PC in all fungal samples. A high level of PE enhanced membrane rigidity (Quaracci *et al.*, 2001; Bernat *et al.*, in press). A rigidification of membrane lipids in the rat liver was involved in the membrane damage by cisplatin (Martins *et al.*, 2008).

There is a discrepancy in the attitude towards the role of linoleic acid in microorganism adaptation. Howlett & Avery (1997) documented that *Saccharomyces cerevisiae* sensitivity to cadmium ions increased with the higher degree of fatty acids unsaturation and was supported by metal induced membrane permeability, leading to the reduction in yeast viability. In contrast, Masia *et al.* (1998) reported that linoleic acid supplementation partially neutralizes the toxicity of tributyltin to *S. cerevisiae*. Alteration in the cellular level of a single polyunsaturated fatty acid, especially in the linoleic acid content, may also lead to changes in metal sensitivity (Avery *et al.*, 1996).

In summary, we suggest that linoleic acid abundance diminishes the membrane perturbation. Cu and Pb strongly affect the membrane integrity. The toxicity of Cu against the membrane occurs probably as a consequence of lipid peroxidation, whereas Pb toxicity is caused by both oxidative membrane damage and deterioration of the membrane integrity due to a drop in PC level.

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