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Effects of lead on growth, photosynthetic characteristics and production of reactive oxygen species of two freshwater green algae

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HIGHLIGHTS

- *Chlorella* was more tolerant to free Pb^{2+} than *Scenedesmus*.
- Long-term Pb treatments resulted in decreased chlorophyll.
- Pb stimulated ROS production though this depended on exposure time and Pb concentration.
- Photosynthetic impairment by Pb was likely responsible for the growth decrease in *Chlorella*.
- The causes of growth inhibition in *Scenedesmus* were related to lead-induced ROS.

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ABSTRACT

In the natural environment, heavy metal contamination can occur as long-term pollution of sites or as pulses of pollutants from wastewater disposal. In this study two freshwater green algae, *Chlorella* sp. FleB1 and *Scenedesmus* YaA6, were isolated from lead-polluted water samples and the effects of 24 h vs 4 and 8 d exposure of cultures to lead on growth, photosynthetic physiology and production of reactive oxygen species (ROS) of these algae were investigated. In *Chlorella* sp. FleB1, there was agreement between lead impacts on chlorophyll content, photosynthesis and growth in most case. However, in *Scenedesmus acutus* YaA6 growth was inhibited at lower lead concentrations ($0.03–0.87 \times 10^{-9}$ M), under which ROS, measured by 2′,7′ dichlorodihydrofluorescein diacetate fluorescence, were 4.5 fold higher than in controls but photosynthesis was not affected, implying that ROS had played a role in the growth inhibition that did not involve direct effects on photosynthesis. Effects of short-term (5 h, 24 h) vs long-term (4 d and 8 d) exposure to lead were also compared between the two algae. The results contribute to our understanding of the mechanisms of lead toxicity to algae.

1. Introduction

Lead has been recognized as potentially causing severe effects on human health and ecosystem function and this has led to the almost global phasing out of leaded gasoline and the recycling of lead acid batteries (Vest, 2002) in attempts to decrease the amount of the metal released into the environment. However, the demand for lead used in human activities has still been increasing steadily, from ca. 5 million metric tons in 1990 to more than 11 million metric tons in 2014 (Rich, 1994; ILZSG, 2015). Therefore, lead pollution arising from mining or from its consumption by other industries is still an issue of concern. Polluted waters may still be found around the world with concentrations of lead that are much higher than wastewater discharge limits for this metal, which range from 0.01 mg/L–1 mg/L depending on country (Helmer et al., 1997; Deng et al., 2007). For instance, soluble lead was found to be in the range of 5 mg/L–15 mg/L in storage battery industry wastewater in Italy (Macchi et al., 1993) or at 551 mg/L (Namasivayam and Yamuna, 1995) to 709 mg/L (Kadirvelu et al., 2001) in wastewater from radiator manufacturing industries in India.

There is a large body of research investigating the potential of algae for lead bioremediation, though most of the studies have used dead biomass (Dönmez et al., 1999; Jalali, 2002; Deng et al., 2007; Gupta and Rastogi, 2008; Kumar et al., 2008; Andrade et al., 2010). Living cells however are able to produce new biomass

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through growth, thus increasing the metal absorption capacity. They also have a greater capacity for heavy metal uptake by involving both active and passive uptake mechanisms rather than only the passive sorption found in dead material. However, to use live algae for the purpose of bioremediation, factors affecting their growth and physiology should first be investigated. Among these, it is critical that the inhibitory effects of the metal need to be assessed. Heavy metals can affect algae in various ways by inhibiting different physiological processes. Since algae are photoautotrophic, effects on photosynthesis are of primary concern as this process is often the most sensitive to environmental stress. There have been some studies on lead toxicity to algal photosynthesis but the results were based on nominal lead concentration (Overnell, 1975; Woolery and Lewin, 1976; Rashid et al., 1994; Belatik et al., 2013) not on the concentration of free ionic lead, which is the form actually taken up and affects aquatic organisms (Campbell, 1995).

Reactive oxygen species (ROS), including superoxide radicals ($^{\cdot}O_2$), hydroxyl radicals ($^{\cdot}OH$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), are normal by-products of oxidative metabolism in aerobic organisms but are frequently stimulated by stress, including metal toxicity. The unpaired electron in ROS species, especially $^{\cdot}OH$, makes them highly reactive and they can aggressively attack other molecules including DNA, proteins and fatty acids. Under non-stressful conditions, cells maintain a balance in ROS levels by protective antioxidant mechanisms. When exposed to stresses caused by various factors, which include heavy metals, temperature or high salt levels, the balance is altered, leading to a series of changes in metabolic processes related to stress acclimation, development, or programmed cell death (Gechev et al., 2006). However, work on the direct measurement of ROS in algae under lead exposure is very scarce in the literature. To our knowledge, there has been only one report about lead-induced ROS production (for *Chlamydomonas reinhardtii*; Szivák et al., 2009). Instead, ROS have been indirectly indicated by changes in ROS scavenging compounds, and these responses have been investigated mostly in lead-treated vascular plants (Malecka et al., 2001; Reddy et al., 2005), with only very few studies on algae (Okamoto et al., 2001).

Lead reacts with S and N in cells and is also capable of displacing other metal ions (Nieboer and Richardson, 1980). Therefore, lead may affect photosynthesis by replacing metal ions such as Mg^{2+}, Ca^{2+} and Fe^{2+} in the photosynthetic apparatus or by interacting with SH groups and/or metal ions in enzymes involved in this process. Consequently, ROS production could be affected by lead via impairment of photosynthesis involving oxygen-consuming processes such as the Mehler reaction, photorespiration and chlororespiration (Beardall et al., 2003; Pospíšil, 2009). However, ROS are also produced in mitochondria and peroxisomes (Apel and Hirt, 2004), which could thus also be impacted by lead. In this study, we aimed to assess the impacts of lead on growth, photosynthetic physiology and production of ROS of two newly isolated microalgae. The responses were investigated in long-term and short-term treatments due to the fact that in the natural environment, algae may live in long-term heavy metal polluted sites or experience a sudden increase of heavy metal concentration from pulses of toxic waste discharge.

2. Materials and methods

2.1. Isolation of lead tolerant algae

Algae were isolated from creeks known, from data provided by Melbourne Water (MW, 2012), to be contaminated by lead. The highest lead concentration at Stony Creek, Yarraville (37°49′36.4″S 144°53′39.8″E) was 1.30×10^{-7} M and at Moonee Ponds Creek, Racecourse Road, Flemington (37°47′14.2″S 144°56′22.6″E) was 1.45×10^{-6} M for measurements conducted in 2010. Water samples were spread on agar plates containing Bold's Basal medium (BB) (Nichols and Bold, 1965; Barsanti and Gualtieri, 2006) and different concentrations of $Pb(NO_3)_2$ ($3–240 \times 10^{-6}$ M). Unialgal colonies were isolated from colonies appearing on these plates by checking and transferring everyday when they appeared, using a combination of spray plating and/or streak plating methods. Selected strains were purified to obtain axenic cultures by antibiotic treatment (a mixture of penicillin, streptomycin and chloramphenicol) and/or repeated streak plating (Hoshaw et al., 1973). Bacteria-free cultures were tested by plating them on BB medium supplemented with 0.05% yeast extract and 0.05% glucose for 2 weeks in the dark, axenic cultures were confirmed if no bacteria/fungi grew (Hoshaw et al., 1973).

Two green algal strains that grew on agar plates containing 240×10^{-6} M $Pb(NO_3)_2$ were selected and purified to axenicity as described above. The algae were identified by the online algal key ALGKEY (Yee and Entwisle, 2013) used to classify Australian freshwater algae based on morphological features. Official identification was also done by Microalgae Services (http://microalgal.com.au/). These isolates have been designated *Chlorella* sp. FleB1 and *Scenedesmus acutus* YaA6 and are referred to hereafter as *Chlorella* and *Scenedesmus*.

2.2. Culture conditions, experimental setup and assay of toxicity to algal growth

The algae were maintained in liquid culture in BB medium, pH = 6.5, at 18 °C, at ambient CO_2 levels under a continuous irradiance of 60 μmol photons.m^{-2}.s^{-1} (LED panel LP41, ENTTEC, Australia). Under these light conditions, growth was close to light saturated. Cultures were agitated by magnetic stirring at 200 rpm.

Long-term and short-term treatments were set up as in Fig. 1. For long-term treatments, BB medium supplemented with different $Pb(NO_3)_2$ concentrations (6.04×10^{-5} M– 5.74×10^{-4} M) were prepared in advance, then inoculated with algae. Samples for measurements of chlorophyll content, ROS production and photosynthesis were taken aseptically using disposable sterile pipettes in the exponential phase of growth i.e. after 4 d (though sampling was more frequent and extended to stationary phase i.e. 7 d or 8 d for some ROS measurements). Short-term toxicity was investigated by growing algae in lead-free BB medium until the middle of the exponential phase, then lead was added to the cultures at one concentration lower than, and one concentration higher than, IC_{50} and the measurements were conducted after 24 h of exposure. In some cases, additional sampling was carried out at 5 h to monitor early responses to lead.

In all experiments, the initial inoculum was taken from log-phase stock cultures prepared by growing cells in BB medium for 4 d. Initial cell density for all cultures was 10^4 cells/mL. The control was BB media without added lead. Levels of free Pb^{2+} were calculated using MINEQL + version 4.6 (Schecher and McAvoy, 2007) from the added amounts of lead pH and the other constituents of the medium. For ease of presentation, the free ionic lead concentrations in nM were transformed, and are presented in all figures as $\log_{10}$ (free $[Pb^{2+}] + 1$), referred to as LgPb.

For studies on the lead toxicity effect on growth, cell numbers were measured daily using an Improved Neubauer Haemocytometer. Maximum growth rate K_e (d^{-1}) was calculated based on equation 1

$$Ke = \frac{lnN2 - lnN1}{t2 - t1} \quad (1)$$

where N2 and N1 are cell concentrations at time t2 and t1