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Contrasting ecotoxicity effects of zinc on growth and photosynthesis in a neutrophilic alga (*Chlamydomonas reinhardtii*) and an extremophilic alga (*Cyanidium caldarium*)

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HIGHLIGHTS

- Growth in *C. reinhardtii* was inhibited at much lower concentrations than *C. caldarium*.
- Zn treatments affected pigment production in both species.
- Zn toxicity does not greatly impair PSII activity in *C. reinhardtii*.
- Zn affected PSII centers of *C. caldarium*, the more Zn tolerant species.
- PSII in acclimated *C. reinhardtii* was more Zn-resistant than in *C. caldarium*.

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ABSTRACT

This study aimed to determine the contrasting ecotoxicity effects of zinc on growth and photosynthesis in a neutrophilic (*Chlamydomonas reinhardtii*) and an extremophilic (*Cyanidium caldarium*) alga. Experiments were carried out to see if cells acclimated to zinc would respond differently to cells that were unexposed to zinc. The study also aimed to see if extremophiles displayed different acclimation properties to neutrophiles. Results showed that the neutrophilic alga *C. reinhardtii*, was more susceptible to free zinc and had a lower IC_{50} value than the extremophile, however its stress response protected the photosynthetic apparatus. Upon acclimation, the photosynthetic abilities of *C. reinhardtii* were not significantly compromised when exposed to toxic levels of free zinc. On the other hand, *C. caldarium* had a stress response which allowed it to tolerate significantly higher amounts of free zinc in its environment compared to *C. reinhardtii*, however the stress response did not protect the photosynthetic apparatus, and upon acclimation *C. caldarium* was no better equipped to protect its photosynthetic integrity than unexposed cells.

1. Introduction

Low pH environments occur naturally and were probably crucial for emergence of life on Earth. Anthropogenically created low pH sites, such as those subjected to acid mine drainage, also have the added complication of spoil and metals (Novis and Harding, 2007). Algae possess a number of physiological responses to stressful environments, including induction of protective mechanisms and enhanced repair capabilities (Pinto et al., 2003).

Environments with pH values below 2.5 foster the establishment of unique ecosystems (Gross, 2000; Aguilera et al., 2012). In such habitats, the evolution of novel metabolic pathways enables resident flora to survive the extreme conditions (Gimmler, 2001). Such metabolic pathways are often catalysed by enzymes with higher thermo-tolerance and an ability to perform at extreme pH values, and extremophiles often produce macromolecules with functions that guard the cell from the impacts of environmental extremes (Morozkina et al., 2010).

Low pH sites often contain high levels of metals, as metal ions readily dissolve in acid. Such metal-enriched sites are often rich in extremophiles, and some species of acidophilic algae are

Abbreviations: PSII, photosystem II; PAM, pulse-amplitude-modulated; $rETR_{max}$, maximum relative electron transport rate; F_v/F_m, maximum quantum yield of photosynthetic energy conversion in PSII; α, light harvesting efficiency; NPQ, non-photochemical quenching; F_0, minimum fluorescence yield; F_m, maximum fluorescence; F_m', maximum fluorescence yield in the light; Chl *a*, chlorophyll *a*; Chl *b*, chlorophyll *b*; PC, phycocyanin; AP, allophycocyanin; ROS, reactive oxygen species; Hsps, heat shock proteins.

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extremely tolerant of high metal levels (Whitton, 1970). Biofilms of metal tolerant algae may have a potential role in bioengineering solutions for water remediation of metal-polluted sites.

Physiological acclimation can increase metal tolerance (Klerks and Weis, 1987; Muyssen and Janssen, 2001; Bossuyt and Janssen, 2004), specifically or generally. Thus pre-exposure of *Chlamydomonas reinhardtii* to cadmium stimulated production of phytochelatins, which resulted in a higher tolerance of the alga to arsenic (Kobayashi et al., 2006). Biological remediation of metal polluted sites may benefit from insights into the metabolic mechanisms by which extremophilic organisms acclimate to metal-enriched environments, in comparison to their neutrophilic counterparts.

The key cellular response to metal stress in algae is inhibition of photosynthesis; thus metal stress is considered to be the main factor limiting primary productivity at low pH (Rai et al., 1981; Lu et al., 2000; Hanikenne, 2003; Spijkerman et al., 2007). There have been reports of metal ecotoxicity to photosynthetic organisms increasing with decreasing pH (Monahan, 1976; Hart and Scaife, 1977; Muller and Payer, 1979), although the evidence is equivocal (Gutknecht, 1961; Kamp-Nielsen, 1971; Parry and Hayward, 1973; Hargreaves and Whitton, 1976; Peterson et al., 1984). Low pH itself is not considered to be a limiting factor to photosynthetic activity (Guyre et al., 1987). Adaptations by microalgae to avoid metal build-up at important cellular locations include exclusion mechanisms, efflux mechanisms, and the ability to carry out biotransformations or sequestration and detoxification of toxins within the cell (Gimmler, 2001; Hall, 2002; Bertrand and Poirier, 2005).

Despite being an essential metal and important cofactor for many biochemical processes, at high levels of exposure, zinc damages photosystem II (PSII) (Van Assche and Clijsters, 1986; Gimmler, 2001; Spijkerman et al., 2007) and decreases efficiency of the Calvin cycle (Bertrand and Poirier, 2005). By interacting with thiol (–SH) protein groups, metals interfere with many proteins, including those involved in PSII (Clijsters and Van Assche, 1985; Gimmler, 2001). Excess zinc also results in molecular mimicry; thus Zn competes with other metals at different sites in the cell, altering protein function (Singh and Singh, 1987; De Filippis and Ziegler, 1993). In photosynthesis, Zn has been found to displace Mg in the active ternary Rubisco–CO_2 metal complex, and will substitute for Mn in the water splitting complex of PSII (Clijsters and Van Assche, 1985; Van Assche and Clijsters, 1986).

Understanding how metals affect photosynthesis in acidophiles compared to neutrophiles may shed some light on the ecological importance of the primary production of these extreme and unique ecosystems. Some algae will be more susceptible to metal damage than others, and will also be more susceptible to certain metals and less so to others. Understanding the ecosystem structure and function in these environments is vital for the development of effective remediation strategies.

The extremophile *Cyanidium caldarium* is abundant in some of the harshest known environments (Allen, 1959). It can tolerate high metal concentrations, extraordinarily low pH, and high temperatures. Contrastingly, *C. reinhardtii* is found in neutral environments and grows best at moderate temperatures. We investigated the differential Zn ecotoxicity to photosynthesis in *C. caldarium* and the less tolerant neutrophile *C. reinhardtii* by quantifying their growth and measuring photosynthetic parameters at different zinc concentrations, and measuring oxygen evolution rates and pigments under different zinc treatments. Our aims in this regard were:

(1) To compare the effects of Zn on growth and photosynthesis of a neutrophilic and an acidophilic microalga.
(2) To determine if pre-exposure to Zn altered the responses of cells to Zn additions i.e. do the microalgae studied exhibit acclimation to elevated zinc?

2. Materials and methods

2.1. Cultures and maintenance

C. caldarium (Tilden) Geitler was obtained from the NIES culture collection in Tsukuba, Japan (strain number NIES-2137). *C. reinhardtii* (Dangeard) was obtained from the Australian National Algae Culture Collection, Hobart, Australia (strain number CS-51). *C. caldarium* was maintained in M-Allen's medium, pH 2.5 at 37 °C. *C. reinhardtii* was maintained in MLA medium, pH 7.5 at 18 °C. Illumination (Enttec 66.27 mW cool white light LED lamps, Melbourne, Victoria, Australia) was continuous at 100 μmol photons m^{-2} s^{-1} and 80 μmol photons m^{-2} s^{-1} for *C. caldarium* and *C. reinhardtii*, respectively. Cultures were magnetically stirred at 250 rpm. Cell growth was monitored by cell counts using a Neubauer haemocytometer.

The study species originate in very different environments. Optimal growth conditions replicating these environments were provided. Trace metals in M-Allen's are reduced to the same order found in MLA.

Zn concentrations, expressed as free ionic zinc Zn^{2+}, have been calculated using MINEQL+ version 4.6 from the added amounts of Zn to media.

2.2. Ecotoxicity assays – growth and pulse-amplitude-modulation (PAM) measurements

C. caldarium cells were grown with 0.002–13.0 mg L^{-1} free ionic Zn in M-Allen's media, supplied as $ZnCl_2$ and $ZnSO_4{\cdot}7H_2O$ (Merck). *C. reinhardtii* was grown with 2.3×10^{-6}–0.2 mg L^{-1} free ionic Zn in MLA medium, supplied as $ZnSO_4{\cdot}7H_2O$ (Merck).

Inoculum was prepared for ecotoxicity assays from exponentially growing cells in control medium. Inoculum was then transferred to a Nalgene flask containing medium. Zn was added to the cultures at the above-mentioned concentrations. The starting cell concentration in ecotoxicity assays was 10^4 cells mL^{-1}.

Growth at different zinc concentrations was monitored daily until stationary phase. Culture samples were fixed with Lugol's iodine for cell counts. The specific growth rate (μ) was calculated from changes in cell concentration during the exponential phase according to Eq. (1) where μ is expressed as [h^{-1}], N_2 and N_1 are the cell concentrations [cell mL^{-1}] at times t_2 and t_1 ([h]), respectively, between which an exponential rise in cell number is observed. Growth expressed as a percentage of the observed maximum growth rate p [%] (Eq. (2)), caused by Zn concentration, C_{Zn2+} [mg L^{-1}], was modelled according to the four-parameter logistic fit described by Eq. (3), from which IC_{50} was calculated.

$$\mu\ [h^{-1}] = \frac{\ln N_2 - \ln N_1}{t_2 - t_1} \quad (1)$$

$$p_\mu\ [\%] = \frac{\mu_{Zn\text{-}treatment}}{\mu_{control}} \times 100 \quad (2)$$

$$p_\mu\ [\%] = p_{\mu_{min}} + \frac{p_{\mu_{max}} - p_{\mu_{min}}}{1 + \left[\frac{C_{Zn^{2+}}}{IC_{50}}\right]^h} \quad (3)$$

Pulse-amplitude-modulated (PAM) fluorescence measurements of photosynthetic parameters were made using a PhytoPAM Analyser (Heinz Walz). Rapid light curves were measured during exponential phase for all Zn concentrations. Non-linear regression was used to fit models to describe the relationship between p_μ [%] (Eq. (2)) and the value of photosynthetic parameters as a percentage of the observed maximum value. The photosynthetic parameters observed include maximum quantum yield of photosynthetic energy conversion in PSII (R_{F_v/F_m} [%], Eq. (4)), maximum relative