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A rapid screening method to assess soil algal toxicity: Non-destructive sampling of algal cells using culture medium extraction

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ABSTRACT

Populations of soil-living algae serve as important proxies for the level of metal toxicity in contaminated soils, therefore, there is a need for efficient bioassays to effectively monitor these populations. This study evaluated two approaches for the extraction of algal cells from soil for further analysis. One method employed direct solvent extraction of chlorophyll from the algae. A second method utilized indirect isolation of algal cells from culture media incubated for 24 h. The utility of both methods was compared by estimating the relative viability of *Chlorococcum infusionum* and *Chlamydomonas reinhardtii* grown in the presence of varying concentrations of Ni. The biomass of *C. infusionum* and *C. reinhardtii* decreased as a result of both extraction approaches. The direct extraction of algal chlorophyll with ethanol proved to be the simpler and more direct procedure; however, the process destroyed the cells and, therefore, only allowed for the measurement of a single endpoint. The indirect culture medium extraction was at least as sensitive as the direct extraction method in the measurement of chlorophyll, but allowed for the analysis of multiple endpoints. The culture medium method was used to characterize the biomass, photosynthetic activity, cell size, cell granularity, cell membrane permeability, and esterase activity of *C. infusionum* and *C. reinhardtii* to assess cell viability of cultures grown in increasing concentrations of Ni. Factors were measured by flow cytometry. Photosynthetic activity was inhibited, and cell size, membrane permeability, and esterase activity of *C. reinhardtii* were all affected when exposed to Ni-treated soil. This study demonstrated that the culture medium extraction method is preferable; allows for the analysis of multiple endpoints, such as photosynthetic and flow cytometric analyses of soil algae; has a shorter extraction period (24 h); and can, therefore, be used as an efficient and effectively screening method for soil toxicity.

1. Introduction

Algae are distributed on and beneath the soil surface, and are considered a vital source of food for soil microfauna and mesofauna (Metting, 1981). Despite their ubiquity in the soil, little attention has been paid to algae as bioindicators for soil pollutants that are potentially toxic to soil organisms such as earthworms, nematodes, springtails, and plants. Classical methods used to monitor the growth of soil algae, i.e., to detect toxicity of metals in terrestrial ecosystems, are based on the most-probable-number (MPN) method as is common practice in cell culture (Megharaj et al., 1986a,b, 1989, 1999a,b; Muralikrishna and Venkateswarlu, 1984), and the measurement of algal chlorophyll that is extracted with organic solvents (Hammel et al., 1998; Nam and An, 2015a). However, these methods are time consuming and have several disadvantages, such as the requirement for long-term incubation, the limited utility of singular endpoint analysis, or cellular disruption caused by extraction with organic solvents (Hosikian et al., 2010). Other methods, such as those outlined by the Organization for Economic Co-operation and Development (OECD, 2011), European Community (EC) (1992), American Society for Testing and Materials (ASTM, 2012), and the International Organization for Standardization (ISO, 2012), have been used to analyze algal growth inhibition, and are based on cell counts, cell volume, fluorescence, optical density, and dry weight. These methods indirectly determine the toxicity of contaminated soil extracts or suspensions by relying on algal cell counts (Maisto et al., 2011; Marques et al., 2011; Robidoux et al., 2004; Thomas et al., 1990), chlorophyll fluorescence (Aruoja et al., 2004; Baun et al., 2002; Nam and An, 2015b), and absorbance (Antunes et al., 2010). Although these methods are based on organisms that are affected by pollutants contained in the water within soil pores (Loureiro et al., 2005), they do not consider the ecological relationships within the soil matrix as a whole. Therefore, a culture media extraction method that is based on flow cytometry may present a more promising solution to evaluate algal photosynthesis and bioassays. Moreover, this method could easily be applied to measure other relevant parameters of soil algae to create other bioassays to characterize the effects of soil

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toxins on algal growth and physiology.

The aim of this study was to validate the use of non-destructive sampling of algal cells to assess soil algal toxicity. The green algae, *Chlorococcum infusionum* and *Chlamydomonas reinhardtii* were chosen as the model species because they are commonly found in freshwater and in soils (Graham and Wilcox, 2000). Flow cytometry was used to measure cell size, granularity, membrane permeability, and enzyme activity. Indirect culture media extraction allowed for the analysis of other endpoints, including chlorophyll fluorescence and photosynthetic activity. We selected Ni as the representative heavy metal found in soil. To the best of our knowledge, this is the first study to use axenic soil algae in combination with an efficient screening method to directly evaluate the heavy-metal toxicity of soil.

2. Materials and methods

2.1. Test species and pre-culture

The model algal species, *C. infusionum* and *C. reinhardtii*, were purchased from the culture collection of algae from the University of Göttingen in Germany, and the UTEX (University of Texas, Austin) in USA, respectively. Both species were sub-cultured in 250 mL borosilicate glass flasks with air-permeable stoppers, in Bold's Basal Medium (BBM) and Tris-Acetate-Phosphate (TAP) mediums, at 24 ± 2 °C, shaken at 100 rpm, and under a photoperiod regime of 16:8 h (light:dark). Illumination was provided by cool-white fluorescent lamps (approximately 4000 Lux).

2.2. Test soil and chemicals

LUFA 2.2 (LUFA-Speyer, Sp 2121, Germany) soil was used for all experiments. The physicochemical properties of LUFA 2.2 soil are presented in Table 1. Ni was purchased from Sigma-Aldrich (USA) as $NiCl_2$ (purity 98%) and used without further purification. Serial dilutions of the Ni^{2+} (5000 mg L^{-1}) stock solution were prepared in deionized water.

2.3. Experimental design

2.3.1. Experimental arrangement and treatments

The detailed experimental protocol used to assess the effect of Ni toxicity on the soil algae, *C. infusionum* and *C. reinhardtii*, is shown in Fig. 1. Autoclaved dry soil (0.5 g) was placed in the wells (each well diameter: 22 mm, height: 18 mm, volume: 6.0 mL) of flat-bottomed, 12-well microplates. Serial dilutions of Ni (0–160 mg kg^{-1} of dry soil weight for *C. infusionum,* and 0–220 mg kg^{-1} of dry soil weight for *C. reinhardtii*) were prepared with deionized water, and 0.27 mL of each dilution was added to the microplate wells, in triplicate. After the soil in each well was saturated with Ni, the soil surface was inoculated with 0.13 mL of *C. infusionum* or *C. reinhardtii* cultures grown to the log phase (initial density of 1×10^5 cells g^{-1} for *C. infusionum* and 2×10^5 cells g^{-1} for *C. reinhardtii*). Overall, 0.4 mL of solution was added to each well, which approximated 80% of the dry weight of the soil. Deionized water was added in place of the algal cultures as a negative control to correct for background fluorescence or light scattering from the soil. Microplates were incubated, without shaking, for 6 days under the same culture conditions used for sub-culturing.

Table 1
Physicochemical properties of test soil.

Physicochemical properties		LUFA 2.2 soil
Texture		Loamy sand
pH		5.6
WHC (mL g^{-1})		0.44
Available phosphate (mg kg^{-1})		24.00
Total nitrogen (mg kg^{-1})		375.00
Organic matter (%)		3.39
Exchangeable cations (Cmol$^+$ kg^{-1})	Ca	9.43
	K	0.18
	Mg	0.40
Total metals (mg kg^{-1})	As	11.24
	Cd	ND*
	Cu	2.41
	Ni	2.61
	Pb	19.17
	Zn	16.36

ND*: means not detected.

2.3.2. Biomass analysis

2.3.2.1. Biomass analysis by direct ethanol extraction. To monitor algal biomass, chlorophyll was extracted from *C. infusionum* and *C. reinhardtii* cultures grown on each test soil. Ethanol was added (2.5 mL per well) to each plate, which were then kept at 24 °C in the dark, and shaken for 3 h at 100 rpm. After a 5-min precipitation period, the algal chlorophyll a fluorescence of the supernatant was measured at an excitation wavelength of 420 nm and emission wavelength of 671 nm (Baun et al., 2002) with a fluorescence microplate reader (Gemini Molecular Devices, USA).

2.3.2.2. Biomass analysis by indirect culture medium extraction. Algal cells were also extracted indirectly by using the culture medium, BBM, which was added (2.5 mL) to each well that contained *C. infusionum* or *C. reinhardtii* growing in each test soil. The plates was shaken for 24 h under the standard algal growth conditions. After a 5-min precipitation period, 0.05 mL of supernatant was mixed with 0.2 mL of ethanol, kept at 24 °C in the dark, and shaken for 3 h at 100 rpm. Algal chlorophyll a fluorescence was measured as per the materials and methods described in Section 2.3.2.1.

2.3.3. Photosynthetic activity analysis

To extract *C. infusionum* and *C. reinhardtii* grown on each test soil, we performed an indirect extraction of algal cells as described in Section 2.3.2.2. After adaptation in the dark for 15 min, the photosynthetic activities of *C. infusionum* and *C. reinhardtii* were measured with a Handy Plant Efficiency Analyzer (PEA, Hansatech Instruments Ltd., UK). Photosystem II parameters were analyzed with regard to the total complementary area (Area), maximum quantum yield of primary photochemistry (at t = 0) (Fv/Fm), reaction center per absorption flux (RC/ABS), reaction center per trapped energy flux (at t = 0) (RC/TRo), electron transport flux per reaction center (at t = 0) (ETo/RC), and reaction center per dissipated energy flux (at t = 0) (RC/Dio) (Nam and An, 2015b; Strasser et al., 2000).

2.3.4. Analysis by flow cytometry

To extract *C. infusionum* and *C. reinhardtii* cells that were grown on each test soil, BBM (2.5 mL) was added to each well, shaken for 24 h under standard growth conditions, and algal cells were collected. Cell size, cell granularity, cell membrane permeability, and esterase activity of *C. infusionum* and *C. reinhardtii* were measured by flow cytometry using a FACSCalibur flow cytometer (Becton-Dickinson, USA) equipped with an ion argon laser (excitation wavelength: 488 nm). Immediately after collection, the cell size and cell granularity of the algae were measured by forward scatter and side scatter. To measure cell membrane permeability and esterase activity, cells were stained for 20 min with fluorescein diacetate (FDA) and for 30 min with calcein acetoxymethyl ester (calcein-AM), respectively. The fluorescent stains were prepared according to Michels et al. (2010) and Brussaard et al. (2001). Stock solutions of FDA (11 mM) and calcein-AM (0.5 mM) were prepared in acetone and dimethyl sulfoxide, respectively. They were stored in a dark freezer at −50 °C until use. Algal aliquots were added to obtain a final concentration of 0.11 mM for FDA and 0.01 mM for calcein-AM. FDA was cleaved by intracellular hydrolysis into non-