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# Assessing applicability of the paper-disc method used in combination with flow cytometry to evaluate algal toxicity☆

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ABSTRACT

Soil algal bioassays have been limited by their inability to evaluate several toxic endpoints because it is difficult to collect pure soil algae growing on and beneath the soil surface. This study describes the extension of a previously developed paper-disc method for analyzing soil toxicity to algae. The method can be used in conjunction with flow cytometric analysis and facilitates the assessment of previously proposed toxicity endpoints, such as the growth zone, biomass, and photosynthetic activity. We assessed the applicability of this paper-disc soil method using the green algae *Chlamydomonas reinhardtii* and *Pseudokirchneriella subcapitata* exposed to nickel-contaminated soil; examined cell sizes, cell granularity, enzyme activity, and oxidative stress as new toxicity endpoints using flow cytometry; and identified morphological changes in green algae assayed. The results showed that, used in conjunction with flow cytometry, the extended paper-disc soil method is sufficiently sensitive to detect decreases in cell granularity in *C. reinhardtii* and esterase activity in *P. subcapitata*. The method also revealed decreases in growth zone, biomass, and electron transfer from the reaction center to the quinone pool. Collectively, the results of this study indicate that soil algal bioassays using nonspecific algae can be used to assess soil quality, to derive several toxicity endpoints for individual cells, and to evaluate previously established flow cytometric toxicity endpoints.



## 1. Introduction

In a previous study (Nam and An, 2016), we investigated the growth zone, chlorophyll fluorescence, and photosynthetic activity of the soil alga *Chlorococcum infusionum* after exposure to copper- and nickel-treated soils using a paper-disc method. The results of that study indicated that the paper-disc method is an efficient, user-friendly assay for evaluating metal toxicity to soil algae. In this study, we extended our evaluation of the paper-disc method by assessing its validity when applied to nonspecific test species.

In soil ecosystems, soil algae are primary producers that are extensively distributed on and beneath the soil surface (Metting, 1981). They constitute an important food source for nematodes, earthworms, and springtails, among others, and should be considered in soil-toxicity assessments. Given the paucity of soil-toxicity data (due to the limited number of soil test species), available test species should be evaluated to provide a better determination of soil quality. However, despite the ecological relevance of these species, there is limited available information on soil algae. Therefore, there is a need to standardize a soil test using soil algae, based on high testability with test species.

Previously, non-identified soil algal populations were used to assay agricultural chemicals (Muralikrishna and Venkateswarlu, 1984; Megharaj et al., 1986a, 1986b, 1989, 1999a, 1999b). The algal toxicity of antimony-, copper-, and nickel-contaminated soils have been assessed using the green alga *C. infusionum* (Hammel et al., 1998; Nam and An, 2015a, 2016). However, these studies provided few soil algal-toxicity values. Although a few studies have reported soil algal-toxicity values for *C. infusionum* exposed to heavy metal-contaminated soils, as well as chlorophyll analysis after organic solvent extraction (Hammel et al., 1998; Nam and An, 2015a), limited information is available on toxicity values for various endpoints, which are conventionally provided in freshwater or marine water algal bioassays (e.g., cell size, cell granularity, photosynthetic activity, enzyme activity, membrane integrity) (Perron and Juneau, 2011; Nestler et al., 2012; Hyka et al., 2013).

The aims of the present study were to (1) assess the validity of

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the paper-disc method using the green algae *Chlamydomonas reinhardtii* and *Pseudokirchneriella subcapitata*; (2) examine the cell size, cell granularity, enzyme activity, and oxidative stress using flow cytometry as new toxicity endpoints in the soil algal bioassay based on the paper-disc soil method; and (3) identify morphological changes in green algae based on the paper-disc soil method. *C. reinhardtii* is widely distributed in freshwater and soil, and is routinely used as a test species. *P. subcapitata* is also a typical test species, is recommended in international guidelines for testing chemical toxicity using algal bioassays (OECD, 2011; ASTM, 2012; ISO, 2012), and is used to assess algal toxicity in contaminated soil extracts (Thomas et al., 1990; Baun et al., 2002; Aruoja et al., 2004; Robidoux et al., 2004; Antunes et al., 2010; Maisto et al., 2011; Marques et al., 2011). To our knowledge, this is the first study to perform flow cytometric analysis of the green algae *C. reinhardtii* and *P. subcapitata* in combination with an efficient paper-disc method for evaluating heavy metal toxicity in soils.

## 2. Materials and methods

### 2.1. Test species and pre-culturing

*C. reinhardtii* and *P. subcapitata* were purchased from the University of Texas (Austin, TX, USA) and sub-cultured in Tris-acetate-phosphate and OECD media. Cultures were incubated in 250-mL borosilicate glass flasks fitted with air-permeable stoppers at 24 ± 2 °C and shaken at 100 rpm, under 16: 8 h (light: dark) illumination cycles provided by cool-white fluorescent lamps (approximately 4000 lux). Aliquots of the algal samples were taken during the exponential growth phase and used as described below.

### 2.2. Test soil and heavy metals

A natural loamy sand soil, LUFA 2.2 (LUFA-Speyer, Sp 2121, Germany), composed of 93% sand, 6.8% silt, and 0.2% clay, was autoclaved and used as the test soil. The physicochemical properties of the test soil are presented in Table SD 1 (Supplementary Data). Soil pH was measured using a pH probe in soil samples diluted 1: 2 (w/v) in water. The gravimetric water-holding capacity was analyzed after 2 h of saturation and free drainage. Available phosphate ion ($PO_4^{3-}$) and exchangeable cations ($Ca^{2+}$, $K^+$, and $Mg^{2+}$) were analyzed using the Lancaster method (NAAS, 2010). Total nitrogen (N) and organic matter were evaluated using the Kjeldahl and loss-on-ignition methods (Harding and Ross, 1964). Total metals, including arsenic (As), cadmium (Cd), copper (Cu), nickel (Ni), lead (Pb), and zinc (Zn), were analyzed in an inductively coupled plasma-atomic emission spectrophotometer following acid digestion. Ni was purchased as nickel chloride ($NiCl_2$; purity 98%) from Sigma–Aldrich (USA) and used without further purification. Stock solutions of $Ni^{2+}$ (5000 mg/L) were prepared using deionized water, and exposure solutions were prepared by serial dilution in deionized water.

### 2.3. Experimental method for evaluating Ni toxicity to C. reinhardtii and P. subcapitata

#### 2.3.1. Experimental design and treatments using the paper-disc soil method

The paper-disc soil method used here was previously described by Nam and An (2016). In this study, we used different test species and an expanded range of toxicity endpoints (cell morphology, esterase activity, and oxidative stress). The detailed protocol used for evaluating Ni toxicity to *C. reinhardtii* and *P. subcapitata* is shown in Fig. SD 1 (Supplementary Data). Autoclaved soil (1 g) was placed in each well of a 24-well flat-bottom microplate (height 15.6 mm, volume 3.4 mL for each well). $Ni^{2+}$-exposed soil media (0, 40, 50, 60, 70, 80, 90, and 100 mg/kg of dry soil weight) were prepared using deionized water. Aliquots (0.325 mL) of the different exposure solutions were dispensed into each well of three microplates in nine replicates. The first microplate was used for analyzing the growth zone, biomass, photosynthetic activity, and cell morphology, whereas the second and third microplates were used for measuring esterase activity and oxidative stress, respectively. After 1 h, we transferred one paper disc (diameter 10 mm, thickness 1.1 mm; Advantech, USA) to each well and pressed it with a glass rod so that it adhered to the soil surface. Then, 0.025 mL of *C. reinhardtii* and *P. subcapitata* in exponential growth phase (with an initial density of $3 \times 10^4$ cells/g of dry soil weight for *C. reinhardtii* and $5 \times 10^4$ cells/g of dry soil weight for *P. subcapitata* in the soil media) was inoculated onto each paper disc. The microplate was incubated for 6 days under the same conditions used to culture the algae, but without shaking.

#### 2.3.2. Growth-zone analysis

*C. reinhardtii* and *P. subcapitata* growth zones were photographed using a camera (Canon, Japan) and analyzed using Motic Images Advanced software, version 3.2 (Motic, USA).

#### 2.3.3. Biomass analysis using chlorophyll a fluorescence

To extract the *C. reinhardtii* and *P. subcapitata* grown in each well, we brushed the soil particles adhered to paper discs and incubated the paper discs in 1 mL Bold's basal medium (BBM) for 1 day, in a 24-well microplate, under the conditions described for algal culture. The algal suspension was extracted from each well. Ethanol was added to the *C. reinhardtii* and *P. subcapitata* biomass (algal suspension: ethanol = 1: 4) and incubated in a heated shaker under the algal culture conditions described above, but without light. Algal chlorophyll *a* fluorescence was measured with a fluorescence microplate reader (Gemini Molecular Devices, USA), using 420 nm as the excitation wavelength and 671 nm as the emission wavelength (Baun et al., 2002).

#### 2.3.4. Photosynthetic activity analysis

The *C. reinhardtii* and *P. subcapitata* grown in each well were extracted as described in section 2.3.3. After a 15-min dark adaptation, their photosynthetic activities were evaluated using a Handy Plant Efficiency Analyzer (PEA; Hansatech Instruments Ltd., UK) with a liquid-phase chlorophyll-fluorescence adapter (Hansatech Instruments Ltd., UK). The following fluorescence parameters related to photosystem II were measured: total complementary area; maximum quantum yield; and specific energy fluxes per quinone A-reducing photosystem II reaction center (RC). The latter parameter included the RC per absorption flux (RC/ABS), RC per trapped energy flux (at t = 0; RC/TRo), electron transport flux per RC (at t = 0; ETo/RC), and RC per dissipated energy flux (at t = 0; RC/Dio) (Strasser et al., 2000; Nam and An, 2015b, 2016).

### 2.4. Flow cytometric analysis

#### 2.4.1. Cell size and granularity

*C. reinhardtii* and *P. subcapitata* grown in each well were extracted as described in section 2.3.3. To assess morphological changes in algae induced by Ni exposure, cell size and granularity were measured via flow cytometric analysis. Forward-scattered light (FSC) and side-scattered light (SSC) parameters indicated cell size and cell granularity, respectively. The gated region in dot plots of FSC *versus* SSC was set to separate algal populations from background signals, and data were then acquired from 10,000 events in the gated population (Fig. SD 2, Supplementary Data). Because algal cell numbers at the higher exposure concentrations