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Toxicity of fuel-contaminated soil to antarctic moss and terrestrial algae

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Abstract

Fuel pollution is a significant problem in Antarctica, especially in areas where human activities occur, such as at scientific research stations. Despite this, there is little information on the effects of petroleum hydrocarbons on Antarctic terrestrial biota. The authors demonstrate that the Antarctic mosses Bryum pseudotriquetrum, Schistidium antarctici, and Ceratodon purpureus, and the Antarctic terrestrial alga Prasiola crispa are relatively tolerant to Special Antarctic Blend (SAB) fuel-contaminated soil (measured as total petroleum hydrocarbons). Freshly spiked soils were more toxic to all species than were aged soils containing degraded fuel, as measured by photosynthetic efficiency (variable fluorescence/maximum fluorescence [Fv/Fm]), pigment content, and visual observations. Concentrations that caused 20% inhibition ranged from 16 600 mg/kg to 53 200 mg/kg for freshly spiked soils and from 30 100 mg/kg to 56 200 mg/kg for aged soils. The photosynthetic efficiency of C. purpureus and S. antarctici was significantly inhibited by exposure to freshly spiked soils with lowest-observed-effect concentrations of 27 900 mg/kg and 40 400 mg/kg, respectively. Prasiola crispa was the most sensitive species to freshly spiked soils (Fv/Fm lowest-observed-effect concentration 6700 mg/kg), whereas the Fv/Fm of B. pseudotriquetrum was unaffected by exposure to SAB fuel even at the highest concentration tested (62 900 mg/kg). Standard toxicity test methods developed for nonvascular plants can be used in future risk assessments, and sensitivity data will contribute to the development of remediation targets for petroleum hydrocarbons to guide remediation activities in Antarctica

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Toxicity of fuel contaminated soil to Antarctic moss and terrestrial algae

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ABSTRACT

Fuel pollution is a significant problem in Antarctica, especially in areas where human activities occur, such as at scientific research stations. Despite this, there is little information on the effects of petroleum hydrocarbons on Antarctic terrestrial biota. This paper demonstrates that the Antarctic mosses *Bryum pseudotriquetrum*, *Schistidium antarctici, Ceratodon purpureus* and the Antarctic terrestrial alga *Prasiola crispa* are relatively tolerant to Special Antarctic Blend (SAB) fuel contaminated soil (measured as total petroleum hydrocarbons, TPH). Freshly-spiked soils were more toxic to all species than were aged soils containing degraded fuel, as measured by photosynthetic efficiency (Fv/Fm), pigment content and visual observations. Inhibitory concentration (IC20) values ranged from 16,600 to 53,200 mg/kg for freshly-spiked soils and from 30,100 to 56,200 mg/kg for aged soils. Photosynthetic efficiency of *C. purpureus* and *S. antarctici* was significantly inhibited by exposure to freshly-spiked soils with lowest observable effective concentrations (LOECs) of 27,900 and 40,400 mg/kg, respectively. *Prasiola crispa* was the most sensitive species to freshly-spiked soils (Fv/Fm LOEC of 6,700 mg/kg), whereas Fv/Fm of *B. pseudotriquetrum* was unaffected by exposure to SAB even at the highest concentration tested (62,900 mg/kg). Standard toxicity test methods developed here for non-vascular plants can be used in future risk assessments and sensitivity data will contribute to the development of remediation targets for petroleum hydrocarbons to guide remediation activities in Antarctica.

Key words

Chlorophyll fluorescence, Ecological risk assessment, Petroleum hydrocarbon, Soil contamination, Toxic effects

INTRODUCTION

Most contaminated sites in Antarctica occur around research stations located in ice-free coastal areas [1]. These ice-free areas comprise < 0.3% of the Antarctic land mass yet support the majority of the terrestrial ecosystem biota [2]. Despite this, there is little information on the sensitivity of local species and the toxic effects of contaminants including petroleum hydrocarbons on Antarctic terrestrial biota, with the exception of soil microbial communities [1, 3]. In addition, no soil quality guidelines for Antarctica currently exist.

Data from toxicity tests are crucial in the development of site-specific environmental quality guidelines, including remediation targets for contaminated sites. Establishing relevant remediation targets for petroleum hydrocarbons in Antarctica is necessary to inform remediation activities, facilitate soil re-use, and enable a site to be recognised as no longer posing significant environmental risk. In temperate regions, seed germination as well as shoot and root growth in vascular plants have commonly been used as endpoints in toxicity tests using plants to identify their tolerance thresholds to contaminated soils [Macoustra et al. unpublished results; 4, 5]. However, these toxicity tests and endpoints cannot be applied to Antarctic regions, where plant life is largely limited to cryptogams, such as moss and algae, which do not have roots nor produce seeds [6]. These cryptogams are specially adapted to survive in extreme environments, with an ability to withstand low temperatures and periodic desiccation [e.g. 7, 8]. Mosses are the main components of the Antarctic flora, and grow on ice-free refuges often in combination with terrestrial algae [6]. Moss and terrestrial algae therefore represent ideal test species for toxicity bioassays evaluating contaminated Antarctic soils.

Mosses are known to accumulate elements, as well as some inorganic and organic compounds in their tissue [e.g. 9, 10, 11, 12]. While some studies have investigated the effects of metals [e.g. 11, 12], there are no published investigations into the effect of petroleum hydrocarbons on moss. Furthermore, there is only one published study on the effect of petroleum hydrocarbons on terrestrial algae, namely soil microalgae [13].

Petroleum hydrocarbons are considered persistent hazardous pollutants that can have both direct and indirect toxic effects on soil ecosystems [2, 3, 14]. Hydrocarbons may accumulate in the membrane lipid bilayer of cells, affecting their structural and functional properties, including membrane fluidity and function, which may lead to leakage of cell contents and cell death [3, 15].

The toxicity of petroleum hydrocarbons in soil may decrease with time as a result of weathering and aging processes [16]. Aging changes the composition and concentration of petroleum hydrocarbons through biological and physico-chemical processes, such as volatilisation, sorption, and microbial degradation [17]. In Antarctic soils, these processes are significantly slower than in temperate and tropical regions and consequently, natural attenuation is extremely slow [2, 18]. As the toxicity of petroleum hydrocarbons in soils change through time, testing the toxicity of both aged and fresh fuel contamination provides a better understanding of toxicity to terrestrial biota [14].

The most commonly used fuel at Australian Antarctic Research Stations for power generation and station equipment is Special Antarctic Blend (SAB) diesel. Special Antarctic Blend fuel is primarily composed of aliphatic alkanes in the range n-C9 to n-C14 (80-90%) with trace amounts of n-C15 to n-C23 and aromatics, such as polycyclic aromatic hydrocarbons (PAHs, 10-20%) [19, 20].

Chlorophyll fluorescence has been used in previous toxicity studies to determine the impacts of petroleum hydrocarbons on vascular plants [21], the impact of PAHs on aquatic plants [22], and the toxicty of metals to moss [12]. The variable to maximum chlorophyll fluorescence ratio (Fv/Fm) is indicative of the photosynthetic efficiency of a plant and a decrease in Fv/Fm implies a decrease in the potential efficiency of photosystem II (PSII) photochemistry. Damage to PSII is often the first sign of stress in a plant, thus Fv/Fm provides a good measurement of plant health [23]. Under optimal conditions, Fv/Fm is around 0.8 for most species. Chlorophyll fluorescence measurements are non-destructive and can be used repeatedly to rapidly assess contaminant effects throughout the exposure duration [22].

Chlorophyll content can also provide valuable information regarding the physiological status of photosynthetic organisms. Pigment content and composition is closely associated with environmental conditions, and is affected by stressors such as metal contamination [24] and ultraviolet light (UV) [25].

The aim of this research was to determine the toxicity of fuel contaminated soil to Antarctic moss and terrestrial algae using photosynthetic efficiency, pigment content and visual health as endpoints. As the composition and concentration of fuel in the environment changes through time, the toxicity of both aged and freshly-spiked soils was investigated. This research provides ecologically relevant toxicity data that can be used in the development of site-specific environmental quality guidelines and to establish remediation targets for contaminated soils for use throughout Antarctica.

MATERIALS AND METHODS

Field site and test species

Field collections were conducted in the Windmill Islands region of East Antarctica, in the vicinity of Australia’s Casey Station (66°17'S, 110°32'E). Ice-free areas in this region support vegetation that is exceptionally well developed and diverse [26]. There are a number of petroleum hydrocarbon contaminated sites close to Casey Station [19, 27] and *in situ* remediation of a previous fuel spill is currently underway.

Four cryptogamic species commonly found in ice-free refuges near Casey Station were selected for the present study. These include three mosses: *Bryum pseudotriquetrum* (Hedw.) P. Gaertn., B. May. & Scherb, *Schistidium antarctici* (Cardot) L. I. Savicz & Smirnova, and *Ceratodon purpureus* (Hedw.) Brid, and one terrestrial green alga, *Prasiola crispa* (Lightfoot) Kützing. *Schistidium antarctici* is endemic to Antarctica, *C. purpureus* has a cosmopolitan distribution, while *B. pseudotriquetrum* occurs throughout polar regions [28]. These are the only moss species known to occur in the Windmill Islands and availability of free water is believed to be the primary driver of their distributions. *Schistidium antarctici* is restricted to relatively wet habitats, *C. purpureus* is more abundant in drier sites and *B. pseudotriquetrum* has a wide distribution, co-occurring with the other two species across these two extremes [8, 26, 29]. *Prasiola crispa* is a cosmopolitan thalloid terrestrial green alga. It is abundant on the upper shorelines on the coast of Antarctica, often around penguin colonies [30]. These four test species, together with a range of lichens, comprise the majority of the macroflora of this region.

Bryum pseudotriquetrum, *S. antarctici* and *C. purpureus* were collected from moss turfs near Casey Station, and *P. crispa* was collected from Clark Peninsula, 2.5 km north of Casey Station, in January 2013. Live samples were desiccated, and stored at -18ºC during transport to Australia.

General procedures

All glass and plasticware for chemical analyses were cleaned by soaking in 10% (v/v) nitric acid (BDH, Analytical Reagent grade) for a minimum of 24 h followed by thorough rinsing with Milli-Q deionised water (18 MΩ/cm; Merck Millipore). All glassware used for analyses of hydrocarbons and extraction techniques were cleaned by rinsing with acetone and dichloromethane (Suprasolv, Merck).

Test soils

A laboratory-formulated substrate was used in toxicity tests, providing a standardised soil matrix for spiking with contaminants [5] and preventing unnecessary disturbance and removal of Antarctic soil. The matrix was modified from standard methods [5] in order to closely represent field soil properties, and was comprised of 20% (w/w) kaolin clay (particles <40 µm) and 80% (w/w) propagating sand (sieved to 1 mm). Soils were characterised for physico-chemical parameters as reported in a companion paper (Macoustra et al., unpublished results). Two spiked stock soils were prepared to nominal concentrations of 30,000 and 60,000 mg TPH/kg (soil dry mass) using SAB fuel obtained from Casey Station. Spiked soils were homogenised overnight in a mechanical sample rotator (Environmental Express, 12 places LE rotator).

Toxicity of fresh fuel in soils

A concentration series of seven soils was prepared by mixing the freshly-spiked soil stock with uncontaminated control soil to produce nominal treatment concentrations of 0, 10,000, 20,000, 30,000, 40,000, 50,000 and 60,000 mg TPH/kg. The highest test concentration was based on the maximum concentrations of petroleum hydrocarbons reported from contaminated sites at Casey Station [18, 27]. Aged soils were prepared by storing spiked stock soils in 2.5 L amber schott bottles at 15 ± 1°C for two weeks before being diluted with controls. Whilst two weeks of aging may not accurately represent an older fuel spill, it is sufficient time for most of the volatile hydrocarbons to have evaporated [17]. Concentrations with a geometric or logarithmic dilution series are generally used for toxicity testing. However, due to the current lack of information available on the toxicity of petroleum hydrocarbons to moss and algae, and the high tolerance all species demonstrated to SAB fuel in pilot studies, a greater number of higher SAB fuel concentrations were tested in the present study.

There were six replicates per treatment, each with approximately 50 mL of soil spread evenly within a glass petri dish (90 x 15 mm) containing 10 mm stainless steel washers that encased approximately 0.5 cm^2 of each plant species placed in an indentation in the soil (Figures 1 and 2). Plant material was desiccated prior to testing, to mimic field conditions (periodic lack of free water, causing desiccation of moss and terrestrial algae), and to facilitate uptake of contaminants upon rehydration. Petri dishes were placed within sealable transparent plastic containers (©Sistema, 5 L, 21.0 x 24.2 x 10.5 cm). Two water level treatments were investigated. These consisted of 17% moisture content, hereafter referred to as ‘low water’; and fully saturated soils, with MilliQ water almost up to the tip of the moss shoots, hereafter referred to as ‘high water’. The moisture content in low water treatments was based on standard protocols, calculated on a dry-weight basis, by dividing the mass of water by the mass of dry soil and expressed as the percentage of water retained in a saturated subsample of soil [5]. High water levels were used to simulate natural field conditions in Antarctica during a melt event.

Bioassay treatments were incubated for 28 days in a temperature controlled cabinet (Thermoline Scientific) at 15 ± 1°C on a day/night photoperiod of 16/8 hours. Maximum light intensity inside the plastic containers was 55 $\mu mol/m^2/s$. The temperature and photoperiod were chosen to mimic conditions in moss turf during summer months in Antarctica, but also to stimulate photosynthesis and growth. Antarctic mosses only grow during the summer, when moss beds and surface soils are commonly >20°C above ambient air temperatures [31]. Furthermore, 15°C has been shown to be the optimal temperature for photosynthesis in both *B. pseudotriquetrum* and *C. purpureus* [32]. Since Antarctic mosses grow exceptionally slowly under natural conditions [0.1 to 4.6 mm.yr-1 for these species; 33], optimised culture conditions were used to enable responses to be observed within a reasonable test period.

Toxicity of aged fuel in soils

Aged fuel bioassays were set up per the fresh fuel bioassays, with the following four modifications: (i) soils were aged for two weeks, (ii) exclusion of the 10,000 mg TPH/kg treatment, (iii) use of high water level treatment only; and (iv) a 21-day exposure period.

Toxicity test end points

Visual health and photosynthetic efficiency

Any colour change or other obvious visual change, such as growth of moss shoots, was noted throughout the exposure period. Photosynthetic efficiency measurements (Fv/Fm) were made using a pulse-amplitude modulated fluorometer (MINI PAM, Heinz Walz GmbH, Effeltrich, Germany), following 20 minutes of dark-adaptation. Fv/Fm measurements were taken daily during the first week, then subsequently every third day.

Photosynthetic pigment content

Total chlorophyll and carotenoid pigments extracted from moss leaf tip and algal material were determined spectrophotometrically in 80% acetone using the methodology of Lichtenthaler & Buschmann [34].

Total petroleum hydrocarbon (TPH) analysis

The concentration of SAB in spiked soils was measured as TPH by Gas Chromatography with Flame Ionisation Detector (GC-FID) [as per 14]. Samples were extracted in hexane, mixed with internal standard (containing 50 µg/mL 1,4-dichlorobenzene, 50 µg/mL p-terphenyl, 250 µg/mL cyclooctane, 50 µg/mL $C_{24}D_{50}$ and 250 µg/mL bromoeicosane) and MilliQ water. Samples were tumbled (17 h), centrifuged (1,000 rpm, 5 min) and 3 µL aliquots were analysed (GC oven temperature 50°C for 3 min, then increased to 320°C at 18°C/min, detector temperature was 330°C). Reported concentrations of TPH were for the SAB fuel hydrocarbon range of n-C9 to n-C18, calculated on a soil dry weight basis (mg TPH/kg).

Statistical analysis

Dose-response analysis was performed on photosynthetic efficiency and pigment data using the software ToxCalc for Microsoft Excel (Version 5.0.23, TidePool Scientific Software, California, 1994), with significance set at an alpha level of 0.05. Data were tested for normality using the Shapiro-Wilk W test, and for homogeneity of variance using Bartlett's test. Data were power^2 or square root transformed where necessary. Point estimates including inhibitory concentrations (IC10 and IC20) were calculated using linear interpolation and one tailed distribution. Where IC10 and IC20 estimates were extrapolated outside the range of concentrations tested, results are not reported. No observed effective concentration (NOEC) and lowest observable effective concentration (LOEC) values were determined using Steel's Many-One Rank test, with significance set at $P < 0 .05$.

RESULTS

Total petroleum hydrocarbons in test soils

Measured TPH concentrations in freshly-spiked soils were within 1 to 33 % of target nominal concentrations (Table 1). Following two weeks of aging, measured TPH concentrations deviated between 2 and 15% from target nominal concentrations. In both fresh and aged soils, deviation between the measured and nominal concentrations generally decreased with increasing concentration (Table 1).

Table 1 Nominal and measured total petroleum hydrocarbon (TPH) concentrations for freshly-spiked and aged (two weeks) soils. Soils were spiked with Special Antarctic Blend (SAB) fuel and measured on a dry weight basis. Measured concentrations were determined for soils at the commencement of tests.

Test soil type	Nominal TPH (mg/kg soil)	Measured TPH (mg/kg soil), day 0.	Deviation from nominal (%)
Freshly-spiked	10,000	6,700	33
	20,000	16,300	18
	30,000	27,900	7
	40,000	40,400	1
	50,000	51,900	3
	60,000	61,800	3
Aged	20,000	17,200	14
	30,000	25,500	15
	40,000	35,800	10
	50,000	48,800	2
	60,000	62,900	4

Visual health

Physiological impacts were observed in many samples of moss and algae following 28-day exposures to freshly-spiked soils up to 61,800 mg TPH/kg (Figure 1). *Ceratodon purpureus* demonstrated considerable chlorophyll bleaching (chlorosis) as indicated by yellowing of tissue, following exposure to concentrations ≥ 16,300 mg TPH/kg. Chlorosis was less severe in *B. pseudotriquetrum* and *S. antarctici* which instead turned dark brown in concentrations of ≥ 16,300 mg TPH/kg. Growth was apparent in all control treatments for *B. pseudotriquetrum* with new shoots growing up to 6 mm over 28 days, compared to less than 2 mm in fuel treatments (Figure 2). *Prasiola crispa* showed no visual response to freshly-spiked soils. Exposure to aged SAB fuel showed no clear visual impact on any of the species tested.

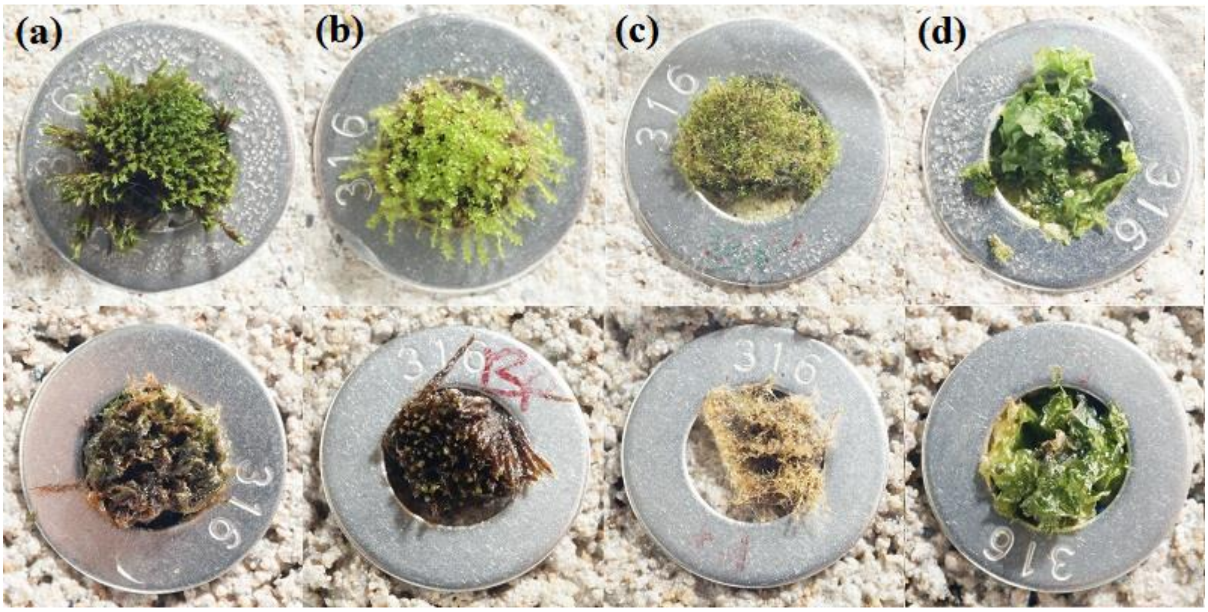

Figure 1 Representative samples of the Antarctic mosses (a) *Schistidium antarctici*, (b) *Bryum pseudotriquetrum,* (c) *Ceratodon purpureus,* and the (d) Antarctic terrestrial alga *Prasiola crispa* from low water treatments following 28-day exposures to control soils (0 mg TPH/kg) (top row) and to soils freshly spiked with Special Antarctic Blend (SAB) fuel at 61,800 mg TPH/kg soil (bottom row). Similar responses were observed in the high water treatments.

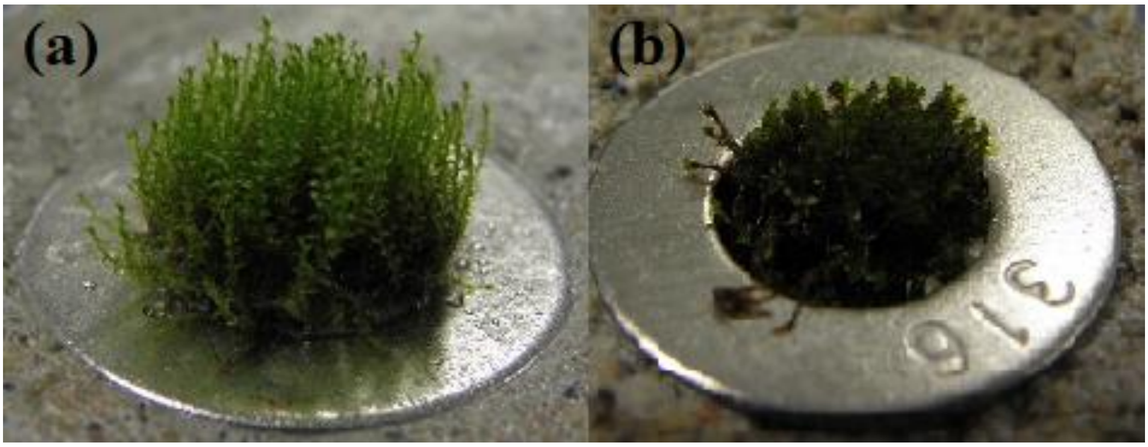

Figure 2 Representative samples of the Antarctic moss *Bryum pseudotriquetrum* from low water treatments following 28-day exposures to (a) control soils (0 mg TPH/kg) and (b) soils freshly spiked with Special Antarctic Blend (SAB) fuel at 61,800 mg TPH/kg soil.

Toxicity of fresh fuel in soils

Exposure to freshly-spiked soils up to 61,800 mg TPH/kg had no impact on Fv/Fm of *B. pseudotriquetrum* in either low or high water treatments following 28-day exposures (Table 2, Figure 3). Similarly, there was no impact on Fv/Fm of *S. antarctic*i in high water treatments, however in low water treatments a 20% decrease was observed with an IC20 estimated at 33,700 mg TPH/kg. The IC20 for *C. purpureus* exposed to freshly-spiked soils was 33,500 mg TPH/kg in low water treatments, whereas the IC20 in high water treatments could not be calculated as the inhibitory effect of freshly-spiked soils on Fv/Fm was less than 20% at the highest concentration tested. Freshly-spiked soils had a greater impact on Fv/Fm of *P. crispa* in low water than in high water treatments, with IC20 values of 21,300 and 53,200 mg TPH/kg, respectively.

Total chlorophyll and carotenoid content of *B. pseudotriquetrum* was significantly inhibited by exposure to freshly-spiked soils at 6,700 mg TPH/kg in both high and low water treatments (LOEC values, Table 2). The average decrease in chlorophyll content was 72 and 73% (relative to the control) in high and low water treatments, respectively (Figure 3). In high water treatments, freshly-spiked soils up to 61,800 mg TPH/kg had no impact on *S. antarctici*, whereas low water treatments have an estimated 20% decrease in total chlorophyll and carotenoid content at 20,400 and 20,600 mg TPH/kg, respectively. Freshly-spiked soils significantly inhibited total chlorophyll content of *C. purpureus*, with a LOEC of 27,900 mg TPH/kg in both high and low water treatments (Table 2). In high water, freshly-spiked soils up to 61,800 mg TPH/kg had no impact on *P. crispa*, whereas low water treatments have an estimated LOEC of 16,300 mg TPH/kg (Table 2). Total chlorophyll of *P. crispa* in high water treatments was nearly three times higher in 61,800 mg TPH/kg than in 27,900 mg TPH/kg (Figure 3).

Toxicity of aged fuel in soils

Exposure to aged soils over 21 days significantly inhibited photosynthetic efficiency (Fv/Fm) of *C. purpureus* and *P. crispa*, with LOEC values of 25,500 and 48,800 mg TPH/kg, respectively (Table 2). A 20% decrease in Fv/Fm is predicted to occur at 39,000 mg TPH/kg for *P. crispa*, however, there was no change in Fv/Fm response for *B. pseudotriquetrum* or *S. antarctici* for concentrations up to 62,900 mg TPH/kg (Table 2, Figure 4).

Table 2 Toxicity estimates for soils spiked with Special Antarctic Blend (SAB) fuel in tests with the Antarctic mosses *Bryum pseudotriquetrum*, *Schistidium antarctici*, *Ceratodon purpureus*, and the Antarctic terrestrial alga *Prasiola crispa*. Exposure response was measured as change in photosynthetic efficiency (Fv/Fm) and total chlorophyll and carotenoid contents relative to the control following 28-day exposures to freshly-spiked soils (Fresh) at six concentrations from 6,700 to 61,800 mg TPH/kg, and 21-day exposures to spiked soils aged for 2 weeks (Aged) at five concentrations from 17,200 to 62,900 mg TPH/kg, under different waters levels (High and Low). Point estimates were calculated from Day 0 measured total petroleum hydrocarbon (TPH) concentrations (range n-C9 to n-C18).

Species	**Fv/Fm**				**Total chlorophyll**				**Total carotenoid**			
SAB/Water level	**NOEC**	**LOEC**	**IC10**	**IC20**	**NOEC**	**LOEC**	**IC10**	**IC20**	**NOEC**	**LOEC**	**IC10**	**IC20**
B. pseudotriquetrum												
Fresh/High	61,800	N/A	N/A	N/A	<6,700	6,700	N/D[a]	N/D[a]	<6,700	6,700	N/D[a]	N/D[a]
Fresh/Low	61,800	N/A	N/A	N/A	<6,700	6,700	N/D[a]	N/D[a]	<6,700	6,700	N/D[a]	N/D[a]
Aged/High	62.900	N/A	N/A	N/A	<17,200	17,200	N/D[a]	56,200	<17,200	17,200	N/D[a]	58,200
S. antarctici												
Fresh/High	61,800	N/A	N/A	N/A	61,800	N/A	N/D[a]	N/D[a]	61,800	N/A	N/D[a]	54,800
Fresh/Low	27,900	40,400	29,800	33,700	16,300	27,900	16,600	20,400	16,300	27,900	18,100	20,600
Aged/High	62,900	N/A	N/A	N/A	62,900	N/A	30,800	N/A	25,500	35,800	N/A	N/A
C. purpureus												
Fresh/High	27,900	40,400	56,000	N/A	16,300	27,900	N/D[a]	N/D[a]	61,800	N/A	N/A	N/A
Fresh/Low	16,300	27,900	28,500	33,500	16,300	27,900	N/D[a]	N/D[a]	16,300	27,900	N/D[a]	N/D[a]
Aged/High	17,200	25,500	N/A	N/A	62,900	N/A	N/D[a]	30,100	62,900	N/A	N/D[a]	N/D[a]
P. crispa												
Fresh/High	<6,700	6,700	N/D[a]	53,200	61,800	N/A	N/A	N/A	61,800	N/A	N/A	N/A
Fresh/Low	<6,700	6,700	N/D[a]	21,300	6,700	16,300	N/D[a]	N/D[a]	6,700	16,300	N/D[a]	N/D[a]
Aged/High	35,800	48,800	N/D[a]	39,000	62,900	N/A	N/A	N/A	62,900	N/A	N/A	N/A

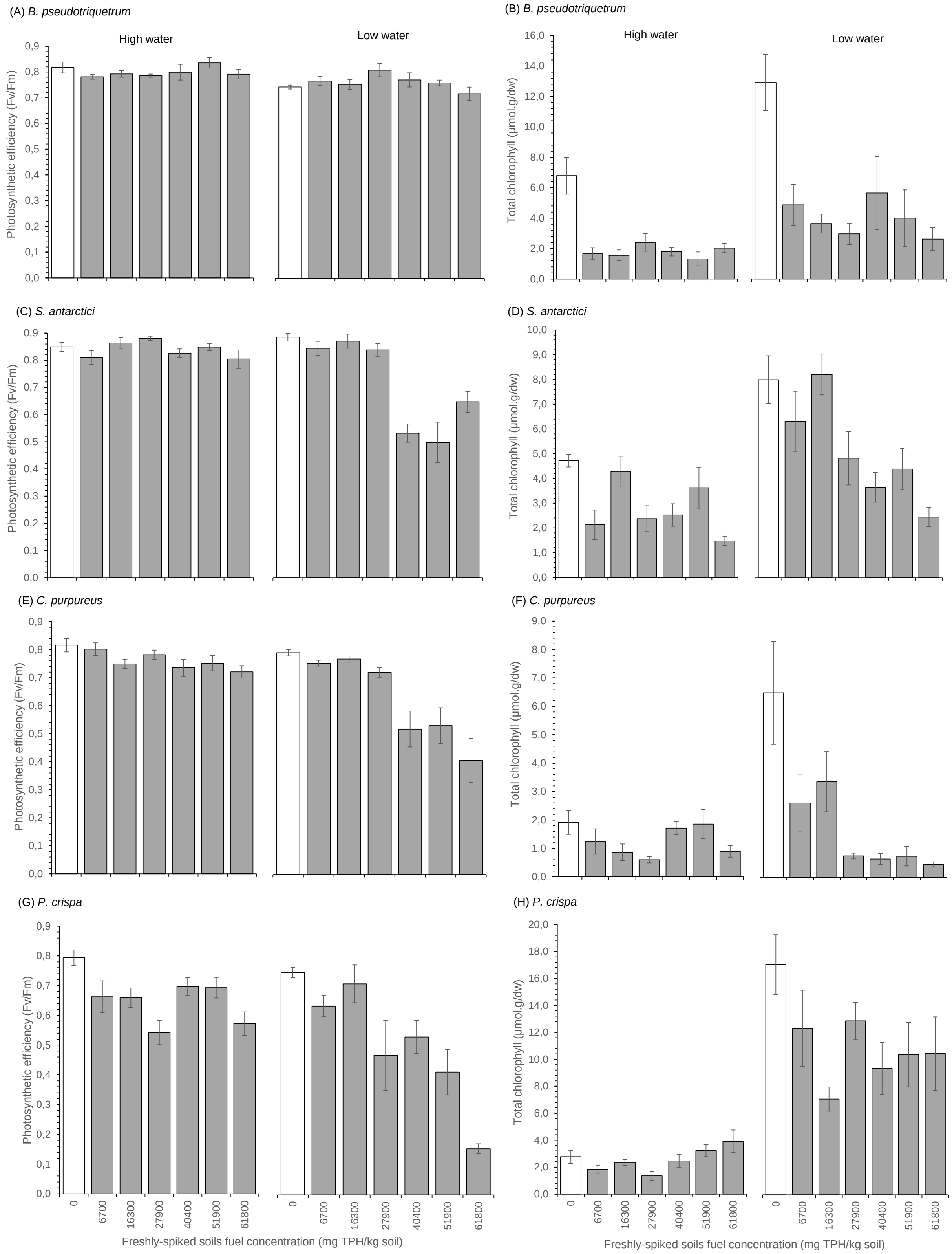

Figure 3 Photosynthetic efficiency (left column) and total chlorophyll content (right column) of the Antarctic mosses (a,b) *Bryum pseudotriquetrum*, (c,d) *Schistidium antarctici*, (e,f) *Ceratodon purpureus* and the (g,h) Antarctic terrestrial alga *Prasiola crispa* following 28-day exposures to soils freshly-spiked with Special Antarctic Blend (SAB) fuel, under different water levels (high and low). White bars represent control (0 mg TPH/kg soil) and grey bars represent fuel treatments. Values are mean (±SE, *n*=6). Response in total carotenoids was similar to total chlorophylls for all species tested, therefore, only results from total chlorophylls are presented.

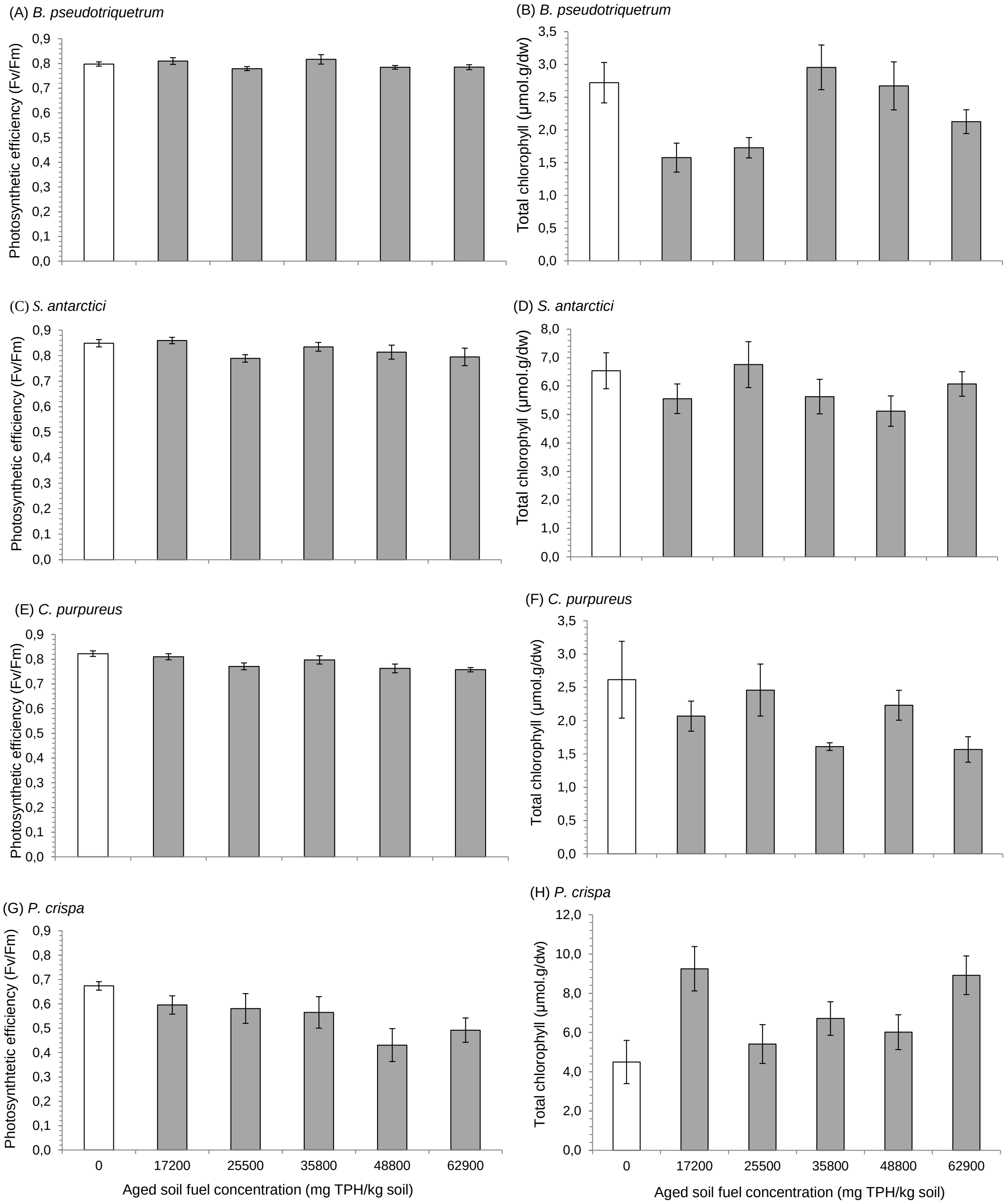

Figure 4 Photosynthetic efficiency (left column) and total chlorophyll content (right column) of the Antarctic mosses (a,b) *Bryum pseudotriquetrum,* (c,d) *Schistidium antarctici*, (e,f) *Ceratodon purpureus* and the (g,h) Antarctic terrestrial alga *Prasiola crispa* following 21-day exposures to aged soils spiked with Special Antarctic Blend (SAB) fuel, under high water levels. White bars represent control (0 mg TPH/kg soil) and grey bars represent fuel treatments. Values are mean (±SE, *n*=6). Response in total carotenoids was similar to total chlorophylls for all species tested, therefore, only results from total chlorophylls are presented.

The aged soils inhibited total chlorophyll and carotenoid content in *B. pseudotriquetrum* following exposure to ≥ 17,200 mg TPH/kg (LOEC (Table 2). At approximately 30,000 mg TPH/kg, a 20% and 10% decrease in chlorophyll content was predicted for *C. purpureus* and *S. antarctici*, respectively (Table 2). Chlorophyll and carotenoid content of *P. crispa* was unaffected by exposure to aged soils up to 62,900 mg TPH/kg (Figure 4).

DISCUSSION

Soils containing fresh SAB fuel were generally more toxic than aged SAB fuel to the test species, as observed in a number of endpoints including photosynthetic efficiency, pigment content and visual health. The low response observed following exposure to aged SAB fuel may be explained by the change in petroleum hydrocarbon composition resulting from two weeks of aging. It has been demonstrated that at an average temperature of 15°C, 95% of volatile aromatic hydrocarbons evaporate in just two weeks following a fuel spill [20]. Therefore it is feasible that the majority of the more toxic and volatile aromatic hydrocarbons had evaporated during the two week aging period in the present study leaving the less toxic (and/or less labile) residual compounds.

The high tolerance demonstrated by all species exposed to aged SAB fuel may depend on the ability of the moss and algae to uptake hydrocarbons from the soil. Mosses and algae lack the developed root and vascular systems found in higher plants, and this is thought to limit their access to soil nutrients [9]. It has consequently been argued that the uptake of nutrients and pollutants by mosses occurs exclusively via atmospheric deposition [35]. However, the moss *C. purpureus* has been found to accumulate metals including copper, lead and zinc from contaminated soil (T. Gibson, 2000, Honour’s thesis, University of Wollongong, Wollongong, Australia). In addition, stable isotopic techniques (^{15}N) have been used to establish that the two moss species *Polytrichum alpinum* and *Racomitrium lanuginosum* are able to derive nitrogen from the soil [10]. While it has been shown that mosses have the ability to derive nutrients and metals from soil, it is unknown whether mosses have the ability to similarly acquire organic compounds.

The higher toxicity of fresh SAB fuel to moss and algae exposed to the low water treatment, compared to high water treatment, also suggests that the more volatile hydrocarbons may have caused the greatest impact. In low water treatments, moss and algae did not have access to free water, and therefore had to obtain moisture either from the soil or the atmosphere. The majority of mosses are ectohydric, mostly absorbing water from precipitation or from flowing water. Only a few species are endohydric, transporting water up from the underlying substrate by means of water-conducting hydroids [36]. Unlike the other two moss species in the present study, *C. purpureus* has been shown to possess such hydroids, and may consequently be capable of hydrocarbon uptake from the soil [37]. This may explain why *C. purpureus* was the only moss species impacted by exposure to aged SAB fuel. Furthermore, terrestrial algae do not have water-conducting hydroids, suggesting that they mostly obtain water from the atmosphere, from precipitation or flowing water [38]. Since moss and algae take up gases for photosynthesis from the atmosphere, it is likely that they also take up some volatile hydrocarbons, particularly as both taxa lack cuticles such that cells are in close contact with the atmosphere. However, further studies quantifying and analysing the uptake, and possible bioaccumulation, of petroleum hydrocarbons into the moss and algal tissue are required to determine their mechanism of tolerance, whether these species can detoxify accumulated hydrocarbons, or exclude hydrocarbons from their cells.

Although all species, except *B. pseudotriquetrum*, showed reduced photosynthetic efficiency in response to fresh SAB fuel exposure, all species were quite tolerant to fuel contaminated soil, with IC20 values (when these could be calculated) ranging from 21,300 to

61,500 mg TPH/kg. The high tolerance of moss and algae to fuel observed in the present study contrasts strongly with the high sensitivities reported for Antarctic and subantarctic microbial communities and invertebrates [1, 14]. For example, Schafer et al. [22] found that the Antarctic microbial community was sensitive to SAB fuel contaminated soil, with an EC25 (effective concentration causing a 25% effect) for community composition and for microbial biomass of 800 and 2,400 mg TPH/kg, respectively. Furthermore, following toxicity tests with native earthworms, concentrations of 50 to 200 mg TPH/kg were suggested as appropriate protective remediation targets for SAB fuel contamination at subantarctic Macquarie Island [14]. In comparison with these previous studies on Antarctic and subantarctic biota, Antarctic moss and terrestrial algae appear exceptionally tolerant to petroleum hydrocarbons.

Antarctic mosses are known to be extremely tolerant to a range of environmental stressors, being able to withstand extreme weather conditions with sub-zero temperatures, lack of free water and nutrients, diurnal freeze/thaw cycles, high levels of UV radiation and periodical desiccation [31]. *Prasiola crispa* is also exceptionally tolerant to many environmental stressors, such as hypersaline conditions and desiccation, and is capable of photosynthesis at sub-zero temperatures [30]. Antarctic moss and terrestrial algae must be able to survive the transition from a desiccated or frozen state to a thawed and hydrated state without loss of cellular integrity and viability. During the Antarctic summer, these transitions often occur repeatedly over short time spans [7]. It has been shown that terrestrial algae possess a number of genes that code for specific substances that promote cellular integrity, structure, and viability through these extreme transitions [38]. It is possible that Antarctic mosses have similar genes protecting their cells from damage due to environmental stress and also making them highly tolerant to SAB fuel.

The observed declines in photosynthetic efficiency of moss and algae in response to contaminant exposure are in agreement with previous toxicity studies [12, 22]. For example, photosynthetic efficiency of Australian *C. purpureus* was found to decrease following exposure to copper, lead and zinc contaminated water as well as when exposed to zinc contaminated soil (T. Gibson, 2000, Honour's thesis, University of Wollongong, Wollongong, Australia). Furthermore, photosynthetic efficiency of the algal species *Lemna gibba* and the aquatic plant *Myriophyllum spicatum* decreased following exposure to PAHs [22].

Decreasing photosynthetic efficiency in response to contaminant exposure is a strong indicator of plant stress and could be a consequence of the breakdown of photosynthetic pigments or of damage to PSII [39]. Since hydrocarbons are lipophilic, it is reasonable to hypothesise that cellular membranes are the site of cellular disruption [40]. Hydrocarbons may dissolve in the plasma membrane and make it more permeable by displacing membrane-bound lipids, leading to leakage of cell contents, and enabling hydrocarbons to enter the cells and affect intracellular organelles [40]. Therefore, the decrease in photosynthetic efficiency observed in the present study could be due to disruption of chloroplast and/or thylakoid membranes. The inhibitory effect of fuel contamination on photosynthesis may also be due to increased membrane permeability as this would impact on proton gradients within the cell [15]. A change in the proton gradient would compromise the electrochemical gradient across the thylakoid membrane and, in turn, decrease the photosynthetic yield of PSII [39].

For all moss species in the present study there was generally a significant breakdown or inhibition of biosynthesis of both chlorophylls and carotenoids in response to increasing concentrations of freshly-spiked soils when water levels were low. However, when water levels were high, this response was observed in *B. pseudotriquetrum* only. A decrease in pigment content in moss and algae when exposed to increasing concentrations of contaminants has been observed in previous toxicity studies [22, 24, 39]. For example, chlorophyll content in the alga *L. gibba* decreased in a concentration-dependent manner in response to PAH exposure [22], and chromium exposure caused extensive chlorophyll degradation in the alga *Scenedesmus obliquus* [39]. Similarly, chlorophyll content in the mosses *Thuidium delicatulum* and *T. sparsifolium* was reduced following exposure to copper [24]. Although, the mechanisms of hydrocarbon and copper toxicity may differ, it has been argued that both contaminants alter membrane structure and function [40]. Thus, the decrease in pigment content observed in the present study may be due to damage to chloroplast membranes, or to damage of cell membranes resulting in leakage of cell content.

Interestingly, *B. pseudotriquetrum* was the only species showing a response in pigment content to soils freshly-spiked with SAB fuel at the lowest concentration of 6,700 mg TPH/kg, while there was no impact on photosynthetic efficiency in concentrations up to 61,800 mg TPH/kg. This breakdown of pigments, without any impact on photosynthetic efficiency, suggests that the available chlorophyll was not affected by the hydrocarbons, and there was no damage to PSII. A likely explanation is that the total chlorophyll levels of *B. pseudotriquetrum* under control conditions actually increased during the experiment, thus even though there appears to be an impact on chlorophyll content in response to exposure to SAB fuel, the pigment levels could potentially have remained relatively consistent throughout the experiment. *Bryum pseudotriquetrum* is the fastest growing of the three moss species tested [33] and was the only species that demonstrated new growth during the course of the experiments and mainly in the control treatment. Therefore, the higher total chlorophyll content observed in *B. pseudotriquetrum* on uncontaminated soil is likely due to this new growth containing much higher levels of pigment than the older shoots.

When there was free water available on the surface of the soil, photosynthetic efficiency of all mosses was unaffected by exposure to high concentrations of fresh SAB fuel.

This suggests that when melt-water runs through contaminated sites in Antarctica during the summer, and petroleum hydrocarbons and other contaminants become mobilised, the impact of exposure on these mosses may be reduced. The high tolerance of the test species to fresh SAB fuel in high water treatments may be explained by the moss and algae quickly becoming fully hydrated with no need for further water uptake, and limited diffusion of volatile hydrocarbons from the surrounding water. Furthermore, the hydrated tissue may repel the petroleum hydrocarbons due to their hydrophobicity. Thus the rapid hydration of moss and algae that occur when free water is available may provide protection from fuel contaminants within melt-water. However, Antarctica is the driest continent in the world, and it is believed the East Antarctic climate is becoming drier as a result of climate change [33]. This is of concern given that in the present study, the greatest response to SAB fuel demonstrated by both moss and algae occurred when free water was limited (in low water treatments). Consequently, the impact of fuel contamination on Antarctic terrestrial flora may become more severe in the future as the climate changes and Antarctic terrestrial environments become drier.

In conclusion, all species tested appeared to experience little physiological damage as a result of exposure to SAB fuel in highly contaminated soils under the controlled test conditions used in the present study. Therefore, Antarctic moss and algal communities may be resilient to the range of concentrations of hydrocarbons currently reported at many contaminated sites in Antarctica. However, results suggest that interactions with environmental parameters associated with climate change are potentially important, and that fuel contamination may impact on Antarctic terrestrial flora in the future, particularly if the Antarctic climate continues to get drier. The toxicity test procedures presented in the present study can readily be used on site in Antarctica to assess the risk of contaminants, including petroleum hydrocarbons and metals in soils to Antarctic terrestrial flora. Toxicity estimates reported here will be used along with toxicity data from other species across multiple taxonomic groups, to establish site specific protective concentrations and remediation targets for petroleum hydrocarbons in Antarctica. These guidelines will inform and guide remediation activities at contaminated sites in Antarctica, and enable sites to be signed off as no longer posing significant environmental risk.

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REFERENCES

[1] Schafer AN, Snape I, Siciliano SD. 2009. Influence of liquid water and soil temperature on petroleum hydrocarbon toxicity in Antarctic soil. *Environmental Toxicology and Chemistry* 28:1409-1415.

[2] Aislabie JM, Balks MR, Foght JM, Waterhouse EJ. 2004. Hydrocarbon spills on Antarctic soils: effects and management. *Environmental Science and Technology* 38:1265-1274.

[3] Schafer AN, Snape I, Siciliano SD. 2007. Soil biogeochemical toxicity end points for sub-Antarctic islands contaminated with petroleum hydrocarbons. *Environmental Toxicology and Chemistry* 26:890-897.

[4] Banks MK, Schultz KE. 2005. Comparison of plants for germination toxicity tests in petroleum- contaminated soils. *Water, Air, and Soil Pollution* 167:211-219.

[5] EC. 2005. Biological test method: test for measuring emergence and growth of terrestrial plants exposed to contaminants in soil. Vol Method Development and Applications section, Environmental Science and Technology centre. Environment Canada, Ontario, Canada, p. EPS 1/RM/45.

[6] Lewis Smith RI. 1984. *Terrestrial plant biology of the sub-Antarctic and Antarctic*. Academic press, London.

[7] Lovelock CE, Osmond CB, Seppelt RD. 1995. Photoinhibition in the Antarctic moss *Grimmia antarctici* Card. when exposed to cycles of freezing and thawing. *Plant, Cell and Environment* 18:1395-1402.

[8] Robinson SA, Wasley J, Popp M, Lovelock CE. 2000. Desiccation tolerance of three moss species from continental Antarctica. *Australian Journal of Plant Physiology* 27:379-388.

[9] Zechmeister HG, Grodzinska K, Szarek-Lukaszewska G. 2003. Bryophytes. In Markert BA, Breure AM, Zechmeister HG, eds, *Bioindicators and biomonitors: principles, concepts and applications*. Vol 6. Elsevier Science Ltd, Kidington, Oxford, pp 329-376.

[10] Ayres E, Van Der Wal R, Sommerkorn M, Bardgett RD. 2006. Direct uptake of soil nitrogen by mosses. *Biology Letters* 2:286-288.

[11] Basile A, Sorbo S, Pisani T, Paoli L, Munzi S, Loppi S. 2012. Bioaccumulation and ultrastructural effects of Cd, Cu, Pb and Zn in the moss *Scorpiurum circinatum* (Brid.) Fleisch. & Loeske. *Environmental Pollution* 166:208-211.

[12] Rau S, Miersch J, Neumann D, Weber E, Krauss GJ. 2007. Biochemical responses of the aquatic moss Fontinalis antipyretica to Cd, Cu, Pb and Zn determined by chlorophyll fluorescence and protein levels. *Environmental and Experimental Botany* 59:299-309.

[13] Megharaj M, Singleton I, McClure NC, Naidu R. 2000. Influence of petroleum hydrocarbon contamination on microalgae and microbial activities in a long-term contaminated soil. *Archives of Environmental Contamination and Toxicology* 38:439-445.

[14] Mooney TJ, King CK, Wasley J, Andrew NR. 2013. Toxicity of diesel contaminated soils to the subantarctic earthworm *Microscolex macquariensis*. *Environmental Toxicology and Chemistry* 32:370-377.

[15] Sikkema J, De Bont JAM, Poolman B. 1995. Mechanisms of membrane toxicity of hydrocarbons. *Microbiological Reviews* 59:201-222.

[16] Fernández MD, Cagigal E, Vega MM, Urzelai A, Babín M, Pro J, Tarazona JV. 2005. Ecological risk assessment of contaminated soils through direct toxicity assessment. *Ecotoxicology and Environmental Safety* 62:174-184.

[17] Tang J, Lu X, Sun Q, Zhu W. 2012. Aging effect of petroleum hydrocarbons in soil under different attenuation conditions. *Agriculture, Ecosystems and Environment* 149:109-117.

[18] Snape I, Ferguson SH, Harvey PM, Riddle MJ. 2006. Investigation of evaporation and biodegradation of fuel spills in Antarctica: II-extent of natural attenuation at Casey Station. *Chemosphere* 63:89-98.

[19] Snape I, Harvey PM, Ferguson SH, Rayner JL, Revill AT. 2005. Investigation of evaporation and biodegradation of fuel spills in Antarctica: I-a chemical approach using GC-FID. *Chemosphere* 61:1485-1494.

[20] Serrano A, Tejada M, Gallego M, Gonzalez JL. 2009. Evaluation of soil biological activity after a diesel fuel spill. *Science of the Total Environment* 407:4056-4061.

[21] Bramley-Alves J, Wasley J, King CK, Powell S, Robinson SA. 2014a. Phytoremediation of hydrocarbon contaminants in subantarctic soils. *Journal of Environmental Management* 142:60-69.

[22] Marwood CA, Solomon KR, Greenberg BM. 2001. Chlorophyll fluorescence as a bioindicator of effects on growth in aquatic macrophytes from mixtures of polycyclic aromatic hydrocarbons. *Environmental Toxicology and Chemistry* 20:890-898.

[23] Björkman O, Demmig B. 1987. Photon yield of O_2 evolution and chlorophyll fluorescence characteristics at 77 K among vascular plants of diverse origins. *Planta* 170:489-504.

[24] Shakya K, Chettri MK, Sawidis T. 2008. Impact of heavy metals (copper, zinc, and lead) on the chlorophyll content of some mosses. *Archives of Environmental Contamination and Toxicology* 54:412-421.

[25] Robinson SA, Turnbull JD, Lovelock CE. 2005. Impact of changes in natural ultraviolet radiation on pigment composition, physiological and morphological characteristics of the Antarctic moss, *Grimmia antarctici*. *Global Change Biology* 11:476-489.

[26] Lewis Smith RI. 1986. Plant ecological studies in the fellfield ecosystem near Casey Station, Australian Antarctic Territory, 1985-86. *British Antarctic Survey Bulletin* 72:81-91.

[27] Ochyra R, Lewis Smith RI, Bednarek-Ochyra H. 2008. *The illustrated moss flora of Antarctica*. Cambridge University Press, Cambridge.

[28] Deprez PP, Arens M, Locher H. 1999. Identification and assessment of contaminated sites at Casey Station, Wilkes Land, Antarctica. *Polar Record* 35:299-316.

[29] Wasley J, Robinson SA, Turnbull JD, King DH, Wanek W, Popp M. 2012. Bryophyte species composition over moisture gradients in the Windmill Islands, East Antarctica: Development of a baseline for monitoring climate change impacts. *Biodiversity* 13:257-264.

[30] Becker EW. 1982. Physiological studies on Antarctic *Prasiola crispa* and *Nostoc commune* at low temperatures. *Polar Biology* 1:99-104.

[31] Bramley-Alves JE, King DK, Miller RE, Robinson SA. 2014b. Dominating the Antarctic environment: bryophytes in a time of change. In Hanson DT, Rice SK, eds, *Photosynthesis of bryophytes and seedless vascular plants*. Vol X-Advances in photosynthesis and respiration. In series: Advances in photosynthesis and respiration, Volume X.

[32] Lewis Smith RI. 1999. Biological and environmental characteristics of three cosmopolitan mosses dominant in continental Antarctica. *Journal of Vegetation Science* 10:231-242.

[33] Clarke LJ, Robinson SA, Hua Q, Ayre DJ, Fink D. 2012. Radiocarbon bomb spike reveals biological effects of Antarctic climate change. *Global Change Biology* 18:301-310.

[34] Lichtenthaler HK, Buschmann C. 2001. Chlorophylls and carotenoids: measurement and charcterization by UV-vis spectroscopy. *Current Protocols in Food Analytical Chemistry*:F4.3.1-F4.3.8.

[35] Holoubek I, Kořínek P, Šeda Z, Schneiderová E, Holoubková I, Pacl A, Tříska J, Cudlín P, Čáslavský J. 2000. The use of mosses and pine needles to detect persistent organic pollutants at local and regional scales. *Environmental Pollution* 109:283-292.

[36] Buck WR, Goffinet B. 2000. Morphology and classification of mosses. In Shaw AJ, Goffinet B, eds, *Bryophyte biology*. Cambridge University Press, Cambridge, UK, pp 77-123.

[37] Stanton DE, Merlin M, Bryant G, Ball MC. 2014. Water redistribution determines photosynthetic responses to warming and drying in two polar mosses. *Functional Plant Biology* 41:178-186.

[38] Graham LE, Graham JM, Wilcox L. 2009. *Algae*. Pearson Education Inc., University of Wisconsin, Madison.

[39] Khalida Z, Youcef A, Zitouni B, Radovvan P. 2012. Use of chlorophyll fluorescence to evaluate the effect of chromium on activity photosystem II at the alga *Scenedesmus obliquus*. *International Journal of Research and Reviews in Applied Sciences* 12:304-314.

[40] Hutchinson TC, Hellebust JA, Mackay D, Turn D, Kauss P. 2005. Relationship of hydrocarbon solubility to toxicity in algae and cellular membrane effects. *International Oil Spill Conference Proceedings* 1:541-547.