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Effects of heavy metals on bioremediation of diesel by Antarctic microalga *Tritostichococcus* sp.

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Abstract: Hydrocarbon spillage has long been a concern in Antarctica as it can result in detrimental effects on Antarctic biota and ecosystems. Bioremediation, using microorganisms such as microalgae, represents one of the most effective and least damaging methods developed to remove pollutants from the environment. However, the effectiveness of bioremediation in eliminating diesel can be influenced by co-contamination of the spill area by heavy metal ions, as is often the case. This study assessed the effects of zinc (Zn), lead (Pb), copper (Cu) and cadmium (Cd) on the bioremediation of diesel by a freshwater Antarctic microalga isolated from soil, *Tritostichococcus* sp. WCY_AQ5_1 (GenBank accession number: OQ225631), under laboratory conditions. Toxicity testing of heavy

metals (1 to 16 ppm) on *Tritostichococcus* sp. showed that microalgal specific growth rates and pigment ratios remained constant up till 8 ppm for all four heavy metals, an implication of toxicity at 16 ppm. In subsequent experiments, where diesel was introduced, sub-lethal Zn and Cd ion concentrations (2 to 10 ppm) did not significantly affect the biodegradation ability of *Tritostichococcus* sp. In contrast, sub-lethal Pb and Cu levels led to reduced diesel biodegradation at higher concentrations (8 to 10 ppm) by approximately 33% and 55%, respectively. Intriguingly, patterns of microalgal growth were not correlated with those of biodegradation efficiency as a prominent increase in growth of Zn-exposed cultures was observed at 8 and 10 ppm, and growth of Cu-exposed cultures peaked at 6 ppm. On the other hand, microalgal growth in Pb and Cd-exposed cultures (2 to 10 ppm) generally remained the same as control (0 ppm).

Keywords: Antarctic, Greenwich Island, polar algae, remediation, hydrocarbon pollution.

Introduction

Since the first human contact with Antarctica over two centuries ago, the region has been a magnet for marine exploitation industries, explorers, researchers and, most recently, tourists (Tin *et al*. 2014). Today, the fishing industry, shipping related with the tourism industry and the support and logistics of national research station operation and marine research in Antarctica rely almost entirely on the use of diesel hydrocarbons (Aislabie *et al*. 2004; Wong *et al*. 2021). This creates risks of accidental spillage during transfer and storage of fuel ashore, aircraft and vehicle refuelling and shipping accidents (Kerry 1993; Tin *et al*. 2010; Raymond *et al*. 2017; Wong *et al*. 2021). It has been recorded that from 1980 to 1989 alone, fuel spillages at Williams Field, an airfield in Antarctica which provides logistical support to McMurdo Station, amounted to approximately 380,000 litres (Tumeo and Larson 1994). Notably, the size of the accumulated spillage even surpassed the *Nella Dan* vessel incident which occurred in 1987 and had a spill size of 270,000 litres (Pople *et al*. 1990). Fuel leaks can also occur due to weathering and damage to containment facilities and pipelines as a result of freezing temperatures, corrosion, lack of maintenance of infrastructure at abandoned or mothballed facilities and historical waste disposal sites (Hughes and Stallwood 2005; Tin *et al*. 2010; Raymond *et al*. 2017).

Diesel is a complex mixture of approximately 10 groups of hydrocarbons (Sazhin *et al*. 2014; Wong *et al*. 2021). A large proportion typically comprises aromatic hydrocarbons, which are refractile and resistant to biodegradation due to the presence of C=C bonds (Roslee *et al*. 2021). In Antarctica, diesel persistence is further enhanced by the chronically low temperatures, which result in increased viscosity, decreased volatilisation and low water solubility (Aislabie *et al*. 2006; Roslee *et al*. 2021). Inaccessible emulsions may be formed in Antarctic marine

oil spill events due to strong sea surface turbulence (McGenity *et al*. 2012). The number of publications related to diesel pollution in Antarctica has been increasing with an annual growth rate of 5.84% in the past four decades, highlighting the expanding recognition of the importance of this issue (Lim *et al*. 2021).

The *Bahia Paraiso* oil spill incident in 1989, still one of the largest single oil spills to have occurred in Antarctica, led to several hundred deaths of affected penguins and shags over the subsequent three-week period primarily due to feather fouling (Kennicutt 1990; Matcott *et al*. 2019). In penguins for example, oiled feathers diminish their thermoregulatory ability and increase body drag during swimming, ultimately accelerating energy consumption in penguins while greatly limiting forage time due to increased risk of hypothermia in freezing Antarctic waters (Culik *et al*. 1991; Goldsworthy *et al*. 2000). Importantly, the refractile nature of diesel pollution as discussed earlier and its toxic properties also pose chronic threats to Antarctic biota. In particular, high and persistent aromatic content in the toxicity-determining water-accommodated fractions (WAFs) of several commonly used Antarctic fuels under polar conditions as compared with temperate conditions has been reported (Brown *et al*. 2016; Puasa *et al*. 2021). Furthermore, the high levels of polycyclic aromatic hydrocarbons (PAHs) of up to 1588 ng g^{-1} lipid noted in fat tissues of deceased penguins is an indication of their incorporation in the Antarctic food chain (Taniguchi *et al*. 2009). While there is limited information about the association of tissue PAH concentrations with biochemical responses and genetic damage in seabirds (Albers 2006), it is well-documented that rehabilitated oiled seabirds have significantly lower post-release survival rates than their non-oiled counterparts, likely due to PAH toxicity from ingested fuel affecting their overall health (Goldsworthy *et al*. 2000). PAH toxicity have caused endocrine disruption, impaired immunity, and reproductive failure in seabirds (Franci *et al*. 2014), posing a significant threat to the restoration and maintenance of population sizes, especially among Antarctic-exclusive species.

The long-term adverse impacts of persisting residual hydrocarbon pollution highlight the necessity for remediation, which can take the form of physical, chemical and biological methods. The use of bioremediation, which eliminates environmental pollutants by utilising living organisms including microbes and plants, has gained increasing momentum in recent years as it is more efficient, cost-effective and ecologically friendly compared to other methods (Banerjee *et al*. 2016; Sharma 2020; Zakaria *et al*. 2021). Briefly, bioremediation at hydrocarbon polluted sites can take place via biosorption which involves the removal of hydrocarbons that are passively bound to the biosorbent, or more commonly, biodegradation whereby hydrocarbons are internalised and undergo a transformation process to become harmless products (Pathak *et al*. 2018). At present, the most well-studied candidate organisms in hydrocarbon degradation are bacteria (Zakaria *et al*. 2021). However, the use of microalgae may provide

several advantages including significantly reduced production of secondary waste (sludge) (Touliabah *et al*. 2022) and the potential for use in biofuel production due to the high lipid and carbohydrate content of microalgal biomass (Vo *et al*. 2018). Merit should also be given for their high carbon fixation capacity which assists in reduction of global warming (Sydney *et al*. 2014). In addition, although most microalgae cannot directly fix atmospheric nitrogen (N) to be used biologically (Coale *et al*. 2024), they are highly efficient in uptaking and assimilating inorganic N forms compared to bacteria because their phototropic nature permits them to generate energy and use water as the ultimate reductant to convert up-taken nitrates/nitrites into ammonium, the fundamental prerequisite for many biologically important molecules, in the presence of sunlight (Kumar and Bera 2020). Examples of hydrocarbon-degrading microalgae include *Chlorella vulgaris*, which is capable of removing 82–100% of oil at concentrations of 10 to 20 g/L within two weeks (Kalhor *et al*. 2017). *Nannochloropsis oculata* also showed ability to remove 66.5% of oil in oilfield produced water-containing medium within three weeks (Ammar *et al*. 2018).

Oil pollution often does not occur in isolation, and co-contamination with heavy metals is another common feature of pollution events in Antarctica (Chu *et al*. 2019). Heavy metal pollution in Antarctica is often associated with anthropogenic activities such as fuel combustion, handling and spills, and waste disposal and incineration (Goldsworthy *et al*. 2003; Chu *et al*. 2019). Natural sources of heavy metals can also contribute, from sources such as geological and glaciological weathering, wind-blown crustal dust, sea-spray, volcanism, and marine vertebrate excreta and carcasses (Chu *et al*. 2019). For instance, high levels of anthropogenically-sourced heavy metal contamination have been reported in the McMurdo Sound region of Victoria Land in Antarctica, including zinc (Zn), lead (Pb), copper (Cu) and cadmium (Cd) (Claridge *et al*. 1995) and similar findings have been reported more widely around human facilities and centres of operation (Zakaria *et al*. 2021).

Locally, Zn can be found at high levels at rubbish dump sites because of its presence in compounds commonly employed as protective coatings for fuel drums as well as in man-made accessories such as batteries, wires and nails (Claridge *et al*. 1995; Gasparon and Burgess 2000). Inappropriate disposal of Pb-containing batteries can lead to elevated concentrations of Pb in surrounding soils as their metallic layer and electrolyte solution contain PbO and Pb^{2+}, respectively, while other sources include paints and cans (Claridge *et al*. 1995). High levels of both metals have also been reported in sewage outfalls (Kennicutt *et al*. 1995). Soils near buried Cu wires and corroding Cu pipes can also contain high Cu concentrations (Claridge *et al*. 1995). Finally, transportation of Cd to Antarctica from other regions can take place through the global distribution of volatile methylated Cd generated by marine prokaryotes (Emnet 2009). Recently, analyses of Zn, Pb, Cu, and Cd in snow samples collected along transverse route from coastal research station to Antarctic ice sheet summit have

shown decreasing annual deposition flux towards inland regions, suggesting that anthropogenic activities at ice-free coastal areas are a significant source of these elements (Jiang *et al*. 2018; Herath *et al*. 2024). On the other hand, elements in inland regions of Antarctica are primarily contributed by long-distance transport of anthropogenic emissions from South American continent in addition to local research stations (Herath *et al*. 2024). Importantly, higher concentrations of Pb and Cu have been observed in sea-surface microlayer than underlying water which confirm maritime Pb and Cu input from the atmosphere (Frache *et al*. 2001). It has also been suggested that maritime Zn and Cd input can occur from glacial meltwaters previously trapped in ice via atmospheric deposition (Sieber *et al*. 2019, 2020).

Although some heavy metals (*e.g.*, Zn and Cu) are essential to microalgae, exposure to heavy metals at concentrations above their maximum tolerance level will incur negative impacts (Monteiro *et al*. 2012). For instance, Zn concentrations below 15 ppm improved growth of *Dunaliella tertiolecta* while at 25 ppm the microalga greatly suffered from toxic effects (El-Agawany and Kaamoush 2023). Generally, excessive Zn can induce toxicity in microalgae by disrupting membrane integrity, altering intracellular sodium and potassium concentrations, and inhibiting photosynthesis (Wong and Chau 1990). Pb toxicity is often associated with diminished photosynthetic ability, elevated oxidative stress and defects in cell division in microalgae (Küpper 2017). Microalgae can also experience reduced ability to photosynthesise and respire, at the same time develop organelles of abnormal size and morphology, when exposed to high Cu concentrations, thus reducing growth rates (Rocha *et al*. 2021). Exposure to elevated Cd concentration can lead to dilation of membrane-containing organelles, granule accumulation and the substitution of essential metal components in enzymes in microalgae (Vymazal 1987).

Heavy metal exposure can also alter the way that cells and hydrocarbon fractions interact, as shown by PAH biodegradation rates best fitting first-order kinetics when heavy metals are present in a study performed with soil microcosms (Baltrons *et al*. 2018). Previously, it was also reported that biodegradation of low molecular weight PAHs by green microalga, *Selenastrum capricornutum*, were significantly enhanced under exposure to a mixture of heavy metals (Ke *et al*. 2010). It is therefore crucial to understand the role that heavy metals play during hydrocarbon biodegradation (Lim *et al*. 2021). As *Tritostichococcus* sp. WCY_AQ5_1 is the first Antarctic green microalga confirmed to possess diesel degradation ability (Lim *et al*. 2023), the primary aim of the current study was to assess the impact of the heavy metal ions, Zn, Pb, Cu and Cd, on this Antarctic microalga and how these co-contaminants affect its ability to bioremediate diesel, based on changes in microalgal growth, pigment content, oxidative stress responses and diesel biodegradation efficiency.

Material and methods

Study alga and culture conditions. — The freshwater green unicellular microalgal strain, *Tritostichococcus* sp. WCY_AQ5_1 (GenBank accession number: OQ225631) used in this study was originally isolated from soil samples obtained from Greenwich Island (South Shetland Islands; 62.4692°S, 59.7963°W) (Lim *et al*. 2023). The alga was grown in optimised Bold's Basal Medium (BBM) and maintained in an incubator at 10°C (Lim *et al*. 2023) under illumination with cool white fluorescent lamps (Philips, TLD 18W/54-765 providing 42 μmol m^{-2} s^{-2} PAR) on a 12h:12h light:dark cycle.

Heavy metals and diesel source. — Atomic absorption spectroscopy grade heavy metal stock solutions containing 1000 ppm of one of the following heavy metals: zinc (Zn), lead (Pb), copper (Cu), or cadmium (Cd), were obtained from Sigma-Aldrich Chemicals, USA. Dilution was used to obtain the required heavy metal test concentrations using optimised BBM. Petronas diesel (Diesel Euro B10) was purchased from a local fuel station in Kuala Lumpur. The diesel was filter-sterilised using an 0.22 μm nylon membrane filter and stored in an amber Scott bottle at room temperature.

Toxicity testing of heavy metals. — In initial trials, the microalga was exposed to a broad range of heavy metal concentrations to establish a suitable test concentration range. An exponential phase inoculum of *Tritostichococcus* sp. was standardised at OD_{620} (absorbance value) to 0.5. Fifty millilitre cultures were grown in 100 mL flasks consisting of 20% microalgae and one of each of the heavy metal standard solutions (Zn, Pb, Cu, Cd), with concentrations varying across the range 0, 1, 2, 4, 8, 16 ppm. Incubation took place under standard controlled conditions as described for algal culture maintenance for 7 days. During this period, microalgal growth was assessed on days 0, 1, 3, 5 and 7 through determination of chlorophyll *a* (chl-*a*) content.

Determination of photosynthetic pigment contents. — As a measure of algal growth, the concentrations of chl-*a*, chlorophyll *b* (chl-*b*) and carotenoids (car) in the microalgal cultures on day 7 were determined by spectrophotometry after methanol extraction. Five mL of resuspended culture from each flask were harvested and centrifuged at 4,000 rpm at 4°C for 20 min. The cell pellet was resuspended in 2 mL analytical grade 100% methanol (Sigma-Aldrich, USA) and sonicated in iced water for 1 h. The resulting samples were stored in the dark for 24 h at 4°C before being centrifuged at 4,000 rpm and 4°C for 25 min followed by OD measurement of the supernatants at 665.2, 652.4 and 470 nm. Concentrations of chl-*a*, chl-*b*, and car were calculated using the following equations (Lichtenthaler and Buschmann 2001):

$$\mathrm{Chl-a\ (mg/L)} = 16.72\mathrm{A}_{665.2} - 9.16A_{652.4}$$

$$\mathrm{Chl-b\ (mg/L)} = 34.09\mathrm{A}_{652.4} - 15.28\mathrm{A}_{665.2}$$

$$\text{Car (mg/L)} = \frac{1000\text{A}_{470} - 1.63 \times (\text{Chl} - \text{a}) - 104.96 \times (\text{Chl} - \text{b})}{221}$$

Determination of specific growth rate. — Using the values of chl-*a* obtained, the microalgal specific growth rates (μ) expressed in unit reciprocal days (d^{-1}) under each heavy metal treatment concentration were determined using the following formula (Krzemińska *et al*. 2014):

$$\mu\ (d^{-1}) = \frac{lnN_t - lnN_0}{t - t_0}$$

where N_t and N_0 represent chl-*a* concentrations at time t and t_0 respectively.

Assessment of the influence of heavy metals on diesel hydrocarbon bioremediation. — Fifty millilitres of microalgal culture were grown in 100 mL flasks containing 20% microalgal inoculum (OD_{620} = 0.5), 1% v/v diesel, and a single heavy metal standard solution (Zn, Pb, Cu, Cd) at concentrations 0, 2, 4, 6, 8 or 10 ppm. An abiotic control test was run using optimised BBM and 1% v/v diesel. Aeration with syringe-filtered ambient air was implemented for all cultures and incubation took place under controlled conditions as described in Section 2.1 for 7 d. Biodegradation efficiency was measured at the end of the experiment, and a sub-sample of culture was harvested for the determination of growth by assessment of photosynthetic pigment content (method described previously), and measurement of oxidative stress responses.

Assessment of microalgal diesel biodegradation efficiency. — To measure biodegradation efficiency, the experimental microalgal cultures were subjected to *n*-hexane extraction (1:1 medium to solvent ratio). The mixtures were thoroughly swirled and allowed to settle for 10 min until two separated layers were formed (culture layer and *n*-hexane layer). The *n*-hexane layer containing residual diesel was recovered onto a pre-weighed Petri dish followed by 24 h drying under a fume hood at room temperature to ensure complete vaporisation of the extraction solvent. The residual diesel mass was obtained via subtracting the original mass of the Petri dish from its final mass and the % biodegradation efficiency (BE) of the microalga was calculated as follows (Chen *et al*. 2013; McFarlin *et al*. 2018):

$$BE\% = \frac{(Residual\ diesel\ in\ abiotic\ control\ (g) - Residual\ diesel\ in\ experimental\ cultures(g))}{Diesel\ introduced\ (g)}\ x\ 100$$

Determination of reactive oxygen species levels. — Reactive oxygen species (ROS) analysis was carried out using the cell permeable indicator 2, 7-dichlorodihydrofluorescein diacetate (H2DCFDA) (Invitrogen™, Thermo Fisher Scientific, USA). Two hundred microlitres of experimental culture were added to 4 μL of 20 mM H2DCFDA in a 96-well Nucleon black plate (ThermoFisher Scientific, USA) in triplicates and resuspended thoroughly. The plate was left in the dark for 15 min at room temperature. Then, fluorescence was

measured at 495 nm excitation and 530 nm emission wavelengths using a microplate reader (Tecan Infinite F200).

Determination of lipid peroxidation. — Lipid peroxidation (LP) analysis used the cell permeable indicator C11-BODIPY$^{581/591}$ (Life Technologies®, USA). Two hundred microlitres of experimental culture were added to 5 µL of 5 mM C11-BODIPY$^{581/591}$ in a 96-well Nucleon black plate in triplicates and resuspended thoroughly. The plate was left in the dark for 30 min at room temperature. Fluorescence was then measured at 485 nm excitation and 530 nm emission wavelengths using a microplate reader.

Statistical analyses. — All statistical analyses were performed using GraphPad Prism 9. Means and standard deviations were calculated and compared using one-way analysis of variance (ANOVA) and, where significant, pair-wise Tukey *post-hoc* tests were then carried out. Differences were considered significantly different at $p < 0.05$. Where non-parametric tests were necessary, Kruskal-Wallis test was applied.

Results

Toxicity testing of heavy metals on *Tritostichococcus* sp. WCY_AQ_1

Growth response. — As shown in Fig. 1, results suggest that exposure of microalgal cultures to heavy metal concentrations from 1 to 8 ppm, regardless of the type of heavy metal, does not negatively impact cell growth because their growth trends largely overlapped with the trend exhibited by their respective control groups. In line with this, although one-way ANOVA revealed significant differences in the µ over 7 d among cultures subjected to different concentrations of Zn ($F = 22.94$, $p < 0.0001$), Pb ($F = 6.017$, $p = 0.0052$), or Cu ($F = 222.5$, $p < 0.0001$), subsequent Tukey's *post-hoc* tests revealed that the microalgal cultures only showed a significant drop in their µ when exposed to these heavy metals at 16 ppm (Table 1), relative to µ obtained at other test concentrations, hence inferring toxicity at 16 ppm. In contrast, comparatively good tolerance of exposure to Cd was apparent up to 16 ppm, with µ values not differing significantly across the concentrations tested.

Photosynthetic pigment content. — No significant changes in chl-*a*, chl-*b* or car contents were observed under exposure to 0–8 ppm concentrations all four heavy metals (Fig. 2). The pigment ratio, chl-*b*/chl-*a*, remained relatively constant across all concentrations tested, suggesting that any changes in chl-*b* and chl-*a* content were of similar magnitude. However, one-way ANOVA identified significant differences in the car/chl-*a* ration of microalgal cells when subjected to different heavy metal test concentrations (Zn: $F = 20.23$, $p < 0.0001$; Pb: $F = 17.49$, $p < 0.0001$; Cu: $F = 46.38$, $p < 0.0001$; Cd: $F = 22.61$, $p < 0.0001$). Subsequent Tukey's *post-hoc* tests confirmed that only at 16 ppm were the car/

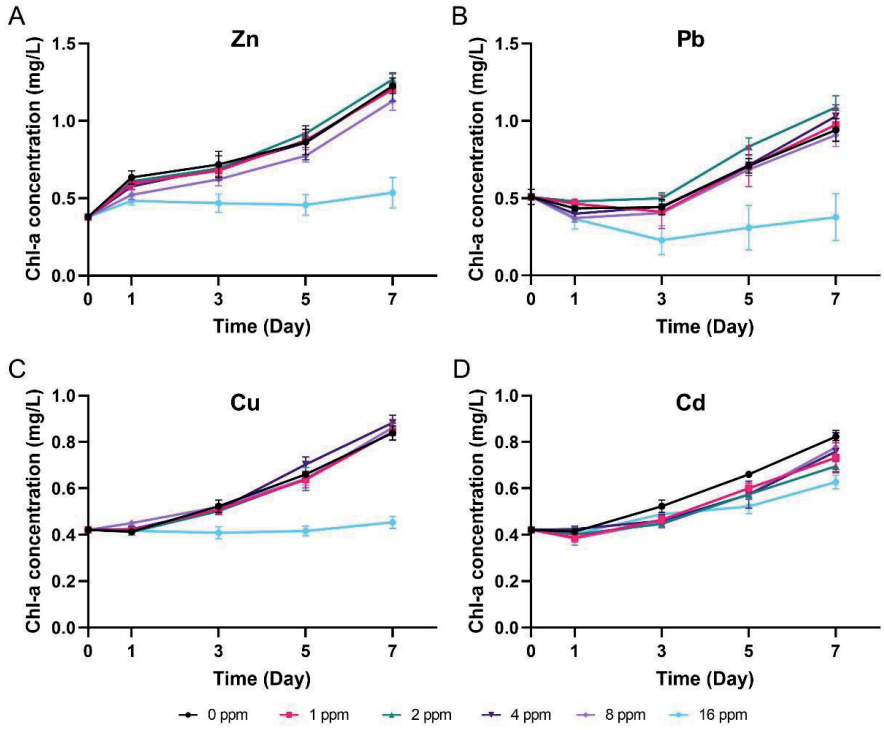

Fig. 1. Growth of *Tritostichococcus* sp. WCY_AQ5_1 over 7 d exposure to (**A**) Zn, (**B**) Pb, (**C**) Cu, and (**D**) Cd at various concentrations. Data shown are mean values of three replicates with error bars indicating standard deviation.

Table 1.

Specific growth rate (μ) of *Tritostichococcus* sp. WCY_AQ5_1 under exposure to different concentrations of one of four heavy metals over a 7 d culture period.

Treatment (ppm)	Specific growth rate (μ), d^{-1}			
	Zn	Pb	Cu	Cd
0	0.135 ± 0.022	0.189 ± 0.013	0.115 ± 0.003	0.115 ± 0.003
1	0.144 ± 0.017	0.221 ± 0.044	0.116 ± 0.006	0.106 ± 0.009
2	0.151 ± 0.011	0.194 ± 0.003	0.116 ± 0.007	0.092 ± 0.011
4	0.141 ± 0.010	0.209 ± 0.012	0.121 ± 0.006	0.107 ± 0.011
8	0.149 ± 0.003	0.202 ± 0.025	0.108 ± 0.001	0.117 ± 0.021
16	0.033 ± 0.026*	0.128 ± 0.018*	0.018 ± 0.01*	0.073 ± 0.011

Data shown are mean values ± standard deviation of three replicates. Asterisks (*) indicate significant differences at $p < 0.05$.

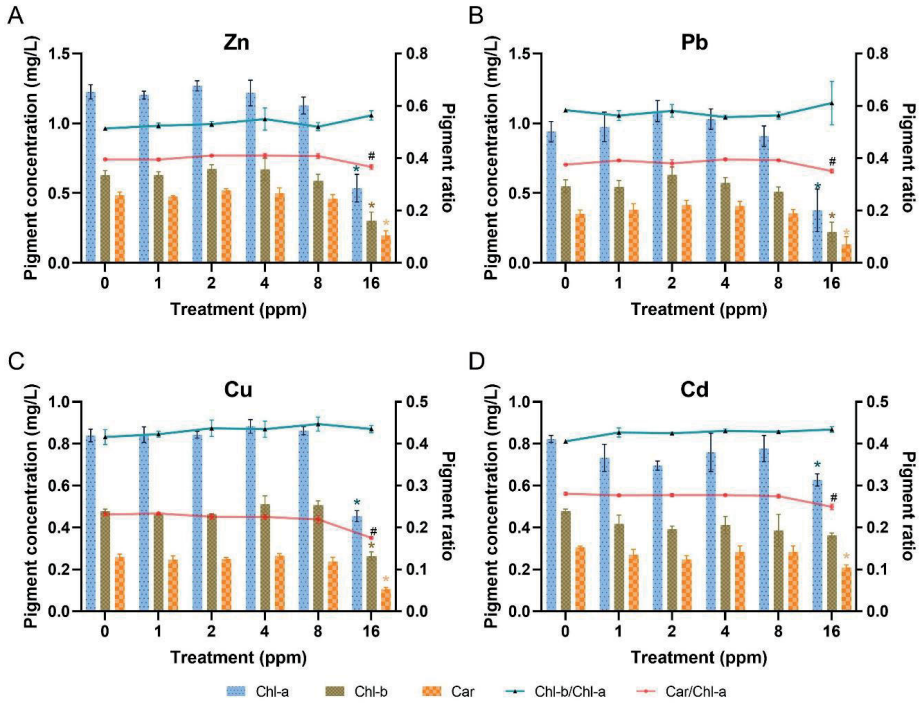

Fig. 2. Photosynthetic pigment content of *Tritostichococcus* sp. WCY_AQ5_1 on day 7 of exposure to different concentrations of (**A**) Zn, (**B**) Pb, (**C**) Cu and (**D**) Cd. Data shown are mean values of three replicates with error bars indicating standard deviation. Asterisks in blue (*), brown (*), orange (*) and hashtag (#) denote significant differences at $p < 0.05$ for chl-*a*, chl-*b*, car and car/chl-*a* ratio, respectively.

chl-*a* ration of microalgal cells significantly lower than cultures tested with lower heavy metal concentrations (0–8 ppm) and this observation was consistent for all four heavy metals.

Effect of heavy metals on diesel bioremediation by *Tritostichococcus* sp. WCY_AQ5_1

Growth response and biodegradation efficiency. — In the presence of diesel, the microalgal cultures exposed to Zn, Pb and Cd showed overall lower chl-*a* concentration ranges on day 7 (Zn: 0.398–0.879 mg/L, Pb: 0.364–0.519 mg/L, Cd: 0.169–0.221 mg/L) as compared to those of cultures exposed to heavy metals alone (Zn: 1.128–1.268 mg/L, Pb: 0.908–1.087 mg/L, Cd: 0.696–0.777 mg/L) (Fig. 3). A similar pattern was seen in the combined diesel and Cu treatment, except for cultures exposed to Cu at 6 ppm where a peak in growth (Fig. 4C) was seen which surpassed those achieved with Cu treatment alone (Fig. 2C).

One-way ANOVA comparing the effects of different heavy metal concentrations on microalgal growth revealed significant differences in growth response

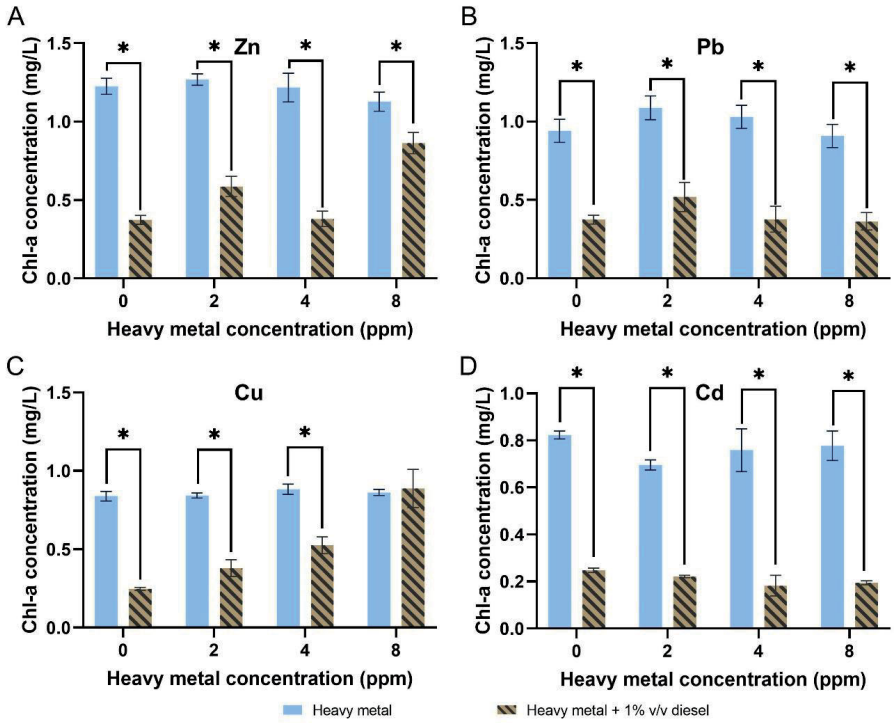

Fig. 3. Growth of *Tritostichococcus* sp. WCY_AQ5_1 on day 7 under (**A**) Zn, (**B**) Pb, (**C**) Cu, and (**D**) Cd treatment at various concentrations with and without diesel co-treatment. Data shown are mean values of three replicates with error bars indicating standard deviation. Asterisks denote significant differences at $p < 0.05$.

for diesel-exposed cultures under Zn ($F = 49.53$, $p < 0.0001$) and Cu ($F = 39.71$, $p < 0.0001$) treatment. Tukey's *post-hoc* test results further revealed that Zn significantly enhanced microalgal growth at 8 and 10 ppm compared to other test concentrations (Fig. 4A). In cultures exposed to Cu, microalgal growth was significantly greater at 6 ppm than at the other concentrations tested (Fig. 4C). Cu concentrations above 6 ppm resulted in reduced growth. Pb and Cd concentration did not have a significant influence on microalgal growth across all concentrations tested. Nevertheless, as shown in Fig. 4D, increasing Cd concentration was associated with reduced microalgal growth.

One-way ANOVA also identified significant effects of different heavy metal concentrations on biodegradation efficiency (BE, %) (Pb: $F = 17.91$, $p = 0.0004$, Cu: $F = 16.87$, $p = 0.0005$). *Post-hoc* pairwise tests confirmed significantly lower BE in cultures exposed to 8 and 10 ppm Pb (Fig. 4B) and Cu (Fig. 4C) as compared to groups exposed to lower concentrations. However, BE of cultures exposed to different concentrations of Zn and Cd were not significantly different although, again, a slight reduction in BE with increasing Cd concentration can be observed in Fig. 4D.

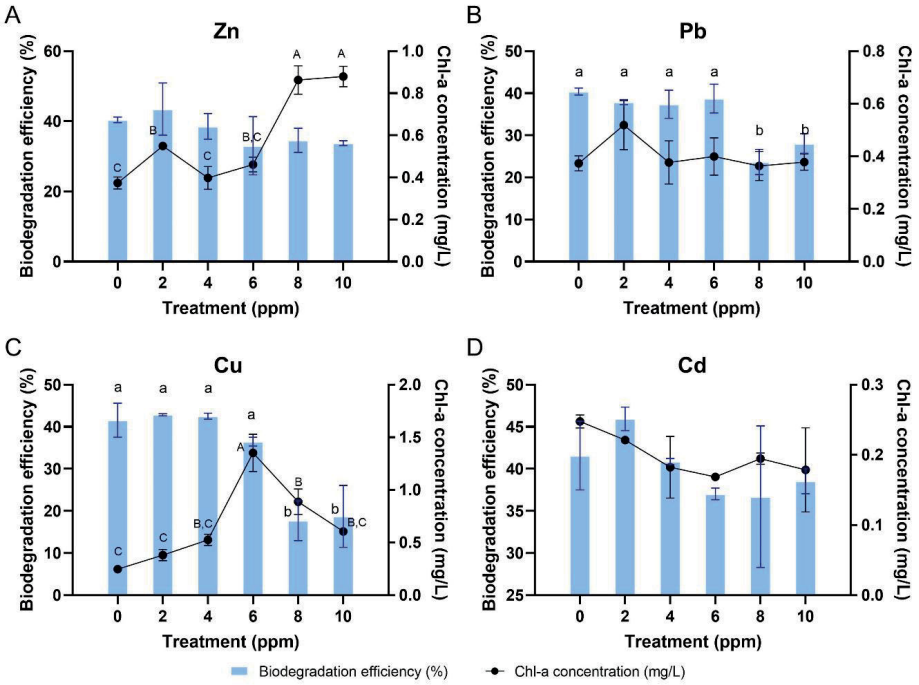

Fig. 4. Biodegradation efficiency of diesel and growth of *Tritostichococcus* sp. WCY_AQ_1 in BBM that contained diesel and (**A**) Zn, (**B**) Pb, (**C**) Cu, or (**D**) Cd, measured on day 7. Data shown are mean values of three replicates with error bars indicating standard deviation. Uppercase letters (A, B, C) and lowercase letters (a, b, c) denote significant differences at $p < 0.05$ for chl-*a* and BE, respectively, between treatment groups for each heavy metal.

Photosynthetic pigment content. — One-way ANOVA confirmed significant differences in all three pigment contents in diesel-exposed microalgal cells when subjected to different concentrations of Zn (Fig. 5A) (chl-*a*: $F = 49.53$, $p < 0.0001$; chl-*b*: $F = 18.88$, $p < 0.0001$; car: $F = 9.308$, $p = 0.0011$) and Cu (Fig. 5C) (chl-*a*: $F = 39.71$, $p < 0.0001$; chl-*b*: $F = 26.52$, $p < 0.0001$; car: $F = 78.51$, $p < 0.0001$), but no significant differences for Pb (Fig. 5B) and Cd-treated (Fig. 5D) cultures. The changes in individual photosynthetic pigment content (Fig. 5) were consistent with the measure of cell growth (Fig. 4). In addition, one-way ANOVA of pigment ratios showed significant changes in the chl-*b*/chl-*a* ($F = 10.65$, $p = 0.0009$) ratio of Zn-treated cultures, and in both chl-*b*/chl-*a* ($F = 5.179$, $p = 0.0163$) and car/chl-*a* ($F = 29.77$, $p < 0.0001$) ratios for Cu-treated cultures in the presence of diesel, with a peak at 2 ppm (Fig. 5A) and 0 ppm (Fig. 5C), respectively; minimal changes in chl-*b*/chl-*a* and car/chl-*a* ratios were observed when treatment concentrations of Zn and Cu were further increased.

Oxidative stress response. — Figure 6 illustrates the ROS and LP levels in microalgal cells after exposure to diesel in combination with various

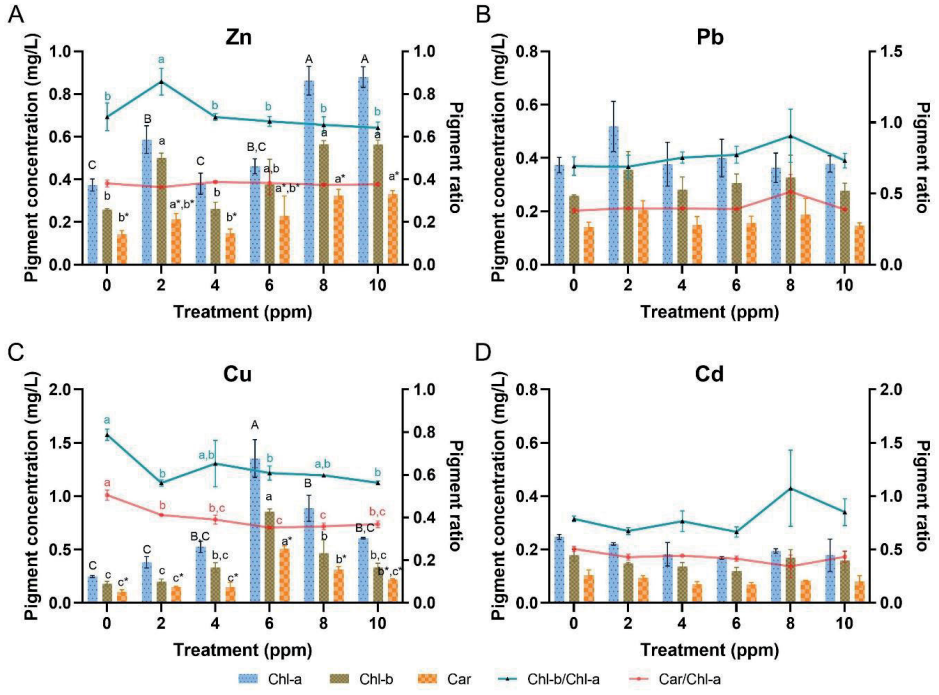

Fig. 5. Photosynthetic pigment content of *Tritostichococcus* sp. WCY_AQ5_1 on day 7 in BBM that contained 1% v/v diesel and different concentrations of (**A**) Zn, (**B**) Pb, (**C**) Cu, and (**D**) Cd. Data shown are mean values of three replicates with error bars indicating standard deviation. Uppercase letters (A, B, C), lowercase letters (a, b, c), lowercase letters with asterisk (a*, b*, c*), lowercase letters in green (a, b, c) and pink (a, b, c) denote significant differences at $p < 0.05$ in chl-*a*, chl-*b* and car contents, and chl-*b*/chl-*a* and car/chl-*a* ratios, respectively, between treatment groups for each heavy metal.

concentrations of different heavy metals. One-way ANOVA revealed significant differences in ROS for diesel-exposed cultures subjected to different concentrations of Zn (Fig. 6A) ($F = 23.48$, $p < 0.0001$), Cu (Fig. 6C) ($F = 204.8$, $p < 0.0001$) and Cd (Fig. 6D) ($F = 68.22$, $p < 0.0001$), while significant differences in LP were only observed for those exposed to Zn (Fig. 6E) ($F = 78.65$, $p < 0.0001$) and Cu (Fig. 6G) ($F = 695.6$, $p < 0.0001$). In Cu-treated cultures, ROS and LP levels decreased as Cu concentration increased (Figs. 6C, G). In contrast, increasing ROS levels (Fig. 6A) were found in combination with decreasing LP levels (Fig. 6E) in Zn-exposed samples. Cells subjected to Cd treatment showed increased ROS levels as the concentration of Cd increased (Fig. 6D) but with no significant changes in LP levels (Fig. 6H). Pb treatment resulted in ROS and LP levels that did not differ significantly from those observed in the control (0 ppm) (Figs. 6B, F).

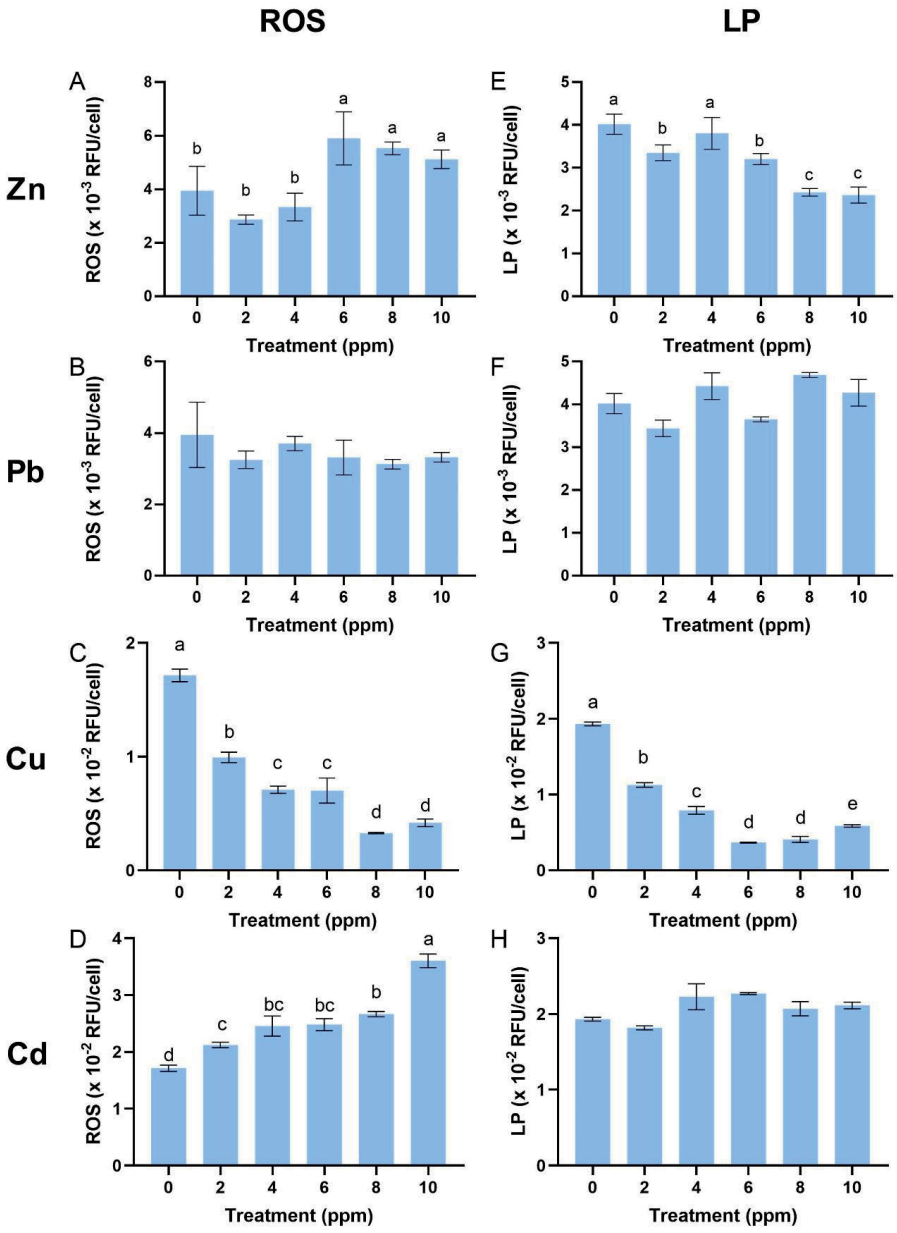

Fig. 6. Reactive oxygen species (ROS) levels (**A**–**D**) and lipid peroxidation (LP) levels (**E**–**H**) in *Tritostichococcus* sp. WCY_AQ5_1 on day 7 in culture in BBM containing 1% v/v initial diesel concentration in combination with different concentrations of Zn, Pb, Cu, or Cd. Data shown are mean values of three replicates with error bars indicating standard deviation. Lowercase letters (a, b, c, d, e) denote significant difference at $p < 0.05$ in ROS and LP between treatment groups for each heavy metal.

Discussion

Zn and Cu are essential micronutrients for algal growth as they are components of a diverse group of enzymes required for algal metabolism and photosynthesis-associated electron transport reactions (Coleman 1998; Raven *et al*. 1999). Conversely, Pb and Cd are non-essential metals and are considered as toxic. Notably, pigment concentrations and ratios of *Tritostichococcus* sp. subjected to 1–8 ppm of Zn, Pb, Cu or Cd showed no significant differences from the control group and no metal-specific effects (Fig. 2). Nonetheless, co-treatment of microalgal cultures with sublethal heavy metal concentrations (1–10 ppm) and diesel generally resulted in lower growth response as compared to that under heavy metal treatment alone (Fig. 3) as well as noticeable changes in microalgal growth patterns (Fig. 4).

The drastically suppressed microalgal growth (Fig. 3) could indicate diesel toxicity, as a negative correlation between diesel exposure and chl-*a* content has previously been reported in *Pseudokirchneriella subcapitata* and *Chlorella* sp. MM3 (Ramadass *et al*. 2017). The study also noted greater toxicity induced by the diesel WAF, *i.e.*, the water-soluble proportion of diesel that were made bioavailable to organisms, as compared to diesel itself. Other mechanisms involved could include reduced light penetration as a result of slick formation, impaired photosynthetic ability due to membrane distortion, and extracellular leaching of chlorophyll (O'Brien and Dixon 1976; González *et al*. 2009).

The presence of some heavy metals may alleviate diesel-induced toxicity experienced by microalgal cells. Our data suggest Zn may play such a role, as significant growth of microalgal cells was observed when exposed to 8 or 10 ppm of Zn in combination with diesel (Fig. 4A). Consistent with this, a marked decrease in LP was observed in microalgal cells at higher Zn concentrations (Fig. 6E) despite the concomitant increment in ROS levels (Fig. 6A). Many polar algae, such as *Ulothrix zonata* and Antarctic strains of *Stichococcus* sp., have been reported to possess high proportions of unsaturated fatty acids in their membranes as compared to temperate algae, highlighting their importance in membrane fluidity maintenance at low temperatures (Osipova *et al*. 2009; Chen *et al*. 2012). Given that unsaturated fatty acid chains are the primary targets of ROS attack (Rezayian *et al*. 2019), the antioxidative defence system of polar microalgae may be robust, as low temperatures have been observed to induce expression of glutathione *S*-transferase, an efficient antioxidative enzyme, in *Chlamydomonas* sp. and other Antarctic microbes (Kan *et al*. 2006; Hou *et al*. 2019). Zn treatment has also been observed to increase synthesis of superoxide dismutase (Coleman 1998) and upregulate ROS scavenging enzyme activities of peroxidases in *Chlorella sorokiniana* and *Scenedesmus acuminatus* (Hamed *et al*. 2017). Notably, it has been reported that, unlike temperate microalgae and non-photosynthetic polar microorganisms, polar microalgae have a higher demand for Zn as they hold greater diversity of Zn-binding proteins involved in regulating

photosynthesis-related processes and other primary metabolism (Ye *et al*. 2022). In our study, it is possible that much of the Zn supplied was utilised in processes that combat ROS instead of diesel catabolism, thereby causing the lack of change in BE despite increased microalgal growth at 8 and 10 ppm Zn (Fig. 4A).

An increasing ability of microalgal cells to overcome diesel toxicity in the presence of heavy metals could also be observed in Cu-treated cultures but at lower concentrations (0–4 ppm) (Fig. 4C). Combined diesel and Cu treatment at a higher concentration of 6 ppm resulted in higher chl-*a* concentration (1.353 mg/L) in microalgal cells than those subjected to Cu only (0.843–0.883 mg/L). This phenomenon could be due to the synergistic effects of diesel and Cu in a co-contaminated environment which can enhance the secretion of extracellular polymeric substances (EPS) (Ali *et al*. 2022). EPS functions as a protective barrier that prevents direct exposure of the microalga to diesel by neutralising cell surface charges, thereby enhancing the hydrophobicity of microalgal cells towards diesel whilst evading its toxic effects (Wang *et al*. 2008; Ali *et al*. 2022). However, the interactive chemistry between microalgae and co-contaminants has received little study. Further increase in Cu concentrations up to 8 ppm and 10 ppm in diesel-containing culture resulted in decreased microalgal growth (Fig. 4C). The toxic effects of Cu may arise at high concentrations through oxidative stress, as oxidation of Cu produces ROS which can in turn lead to degradation of photosynthetic pigments, oxidation of proteins and membrane lipids and DNA damage (Sujetovienė 2014; Kaamoush *et al*. 2022). However, such a mechanism is unlikely in the current study given the reduced ROS and LP levels observed at higher Cu concentrations (Figs. 6C, G). Instead, high Cu levels may have resulted in reduced permeability of the microalgal cell membrane and impeded the binding of other essential microelements such as manganese that also serve as cofactors in photosynthesis (Sunda and Huntsman 1983).

In contrast with Zn and Cu, addition of the non-essential heavy metals, Pb and Cd, to diesel-containing culture resulted in insignificant impact on microalgal growth at the concentrations tested (Fig. 3). Oxidative stress response tests were consistent with this observation, as ROS and LP levels were not significantly different to those observed in the control cultures (Figs. 6B, F), implying absence of impact of Pb treatment up to 10 ppm. It is notable, however, that BE dropped significantly under exposure to Pb at concentrations of 8 and 10 ppm (Fig. 4B). This could be a result of the powerful enzyme-binding ability of Pb causing enzyme activity inhibition (Souza *et al*. 2012). Supporting this suggestion, dehydrogenase activity in soil has been shown to be inhibited in areas with high Pb contamination (Łukowski and Dec 2021). Dehydrogenase is one of the main classes of microbial enzyme involved in oxidative activities, including the catabolism of diesel hydrocarbons into organic carbon-containing metabolites (Errington *et al*. 2018; Łukowski and Dec 2021). Other enzymes which may be affected include dioxygenases, an important enzyme group that plays a role in bio-transforming benzo[a]pyrene hydrocarbon and has been recently reported to

be present in the hydrocarbon-degrading alga, *Selenastrum capricornutum* (García de Llasera *et al*. 2022). Other than the protein level, Pb could also interfere with the transcription of hydrocarbon-degrading enzymes. In bacteria, for example, cytochrome P108J1 has previously been reported to play an important role in the initial breakdown of PAHs (Luo *et al*. 2016). Since Pb and some other heavy metals have also been shown to alter the expression of cytochrome P450 genes, and an increasing library of cytochrome p450 genes have been identified in microalgae, Pb may play a role in downregulating the expression of these genes resulting in degradation of hydrocarbons becoming less effective (Korashy and El-Kadi 2005; Zheng *et al*. 2022). However, functional studies on these microalgal enzymes remain limited (Zheng *et al*. 2022).

In comparison with the other heavy metals tested, the impact of Cd addition to diesel-containing cultures were minimal in terms of both microalgal growth and BE (Fig. 4D). Cd is generally regarded as toxic towards microalgae as it leads to mitochondrial damage, disrupts cell motility, affects cellular components involved in photosynthesis, and inhibits protein synthesis and growth of microalgae (Trevors *et al*. 1986). Nevertheless, the current study demonstrated relatively strong tolerance of *Tritostichococcus* sp. towards Cd, with LP levels that differed minimally from the control treatment across all tested concentrations in cultures containing diesel (Fig. 6H) and insignificant impact on μ resulting from Cd addition up to 16 ppm (Fig. 1D). As Cd has been reported to be one of the most effective inducers of phytochelatins in microalgae (Ahner and Morel 1995), increased production of phytochelatins may be a possible mechanism that contributes to such strong tolerance as it enhances ROS scavenging, prevents heavy metal build-up in the cytoplasm and the non-specific binding of heavy metals to important biomolecules by sequestering the peptide-bound heavy metals into the microalgal vacuole (Scarano and Morelli 2002; Tsuji *et al*. 2002). In addition, good metal-phytochelatin binding properties of Cd (>80%) in *Phaeodactylum tricornutum* compared to other non-essential heavy metals such as Pb (40%) have previously been reported (Scarano and Morelli 2002). Nonetheless, there was a noticeable but non-significant gradual decrease in both chl-*a* and BE with increasing Cd concentration (Fig. 4D). Similar findings have been reported in *Rhodococcus* sp., whereby inhibitory effects of Cd were reflected in the growth of bacteria and their oil-degrading ability (Ibrahim *et al*. 2020). This phenomenon could be due to the slight downregulation of antioxidative enzyme cofactors such as glutathione, which have a role in phytochelatin formation (Tsuji *et al*. 2003; Zhao *et al*. 2019).

In comparison to other studies which assessed the impact of various heavy metals on hydrocarbon degradation ability of Antarctic microbes, it was previously reported that 1 ppm of Zn and Cd were sufficient to reduce the degradation of waste canola oil by a bacterial community isolated near an Antarctic research station by approximately 40% and 25%, respectively (Zahri *et al*. 2020). In another similar study which used the same bacterial community, 1 ppm of Cu also slightly

hindered degradation of waste canola oil compared to control whereas the inhibitory effects of Pb were observed at 10 ppm (Zahri *et al*. 2021). *Rhodococcus* sp. (AQ5-07) which waste canola oil degradation ability was unaffected by 1 ppm of Zn, Pb, and Cu, was significantly influenced at 0.4 ppm of Cd concentration with an approximate 20% drop in oil removed (Ibrahim *et al*. 2020). For a fair comparison considering the type of hydrocarbon being degraded, diesel degradation ability of an undefined Antarctic bacterium strain AQ5-AO1 was significantly impaired with exposure to Zn and Cd at 1 ppm (Zakaria *et al*. 2020). Degradation of phenanthrene, a PAH, was almost completely inhibited at 5 ppm of Cd in another Antarctic bacterium, *Sphingobium xenophagum* (Gran-Scheuch *et al*. 2017). In this respect, *Tritostichococcus* sp. has outperformed many reported Antarctic bacteria in maintaining control (0 ppm)-comparable BE up to 10 ppm for Zn and Cd-exposed cultures, and 6 ppm for Cu-exposed cultures whereas tolerance to Pb was similar for the bacterial community reported by Zahri *et al*. (2021). The same studies also observed that bacterial growth was generally inhibited at around 1–5 ppm of Zn, Pb, Cu, and Cd (Gran-Scheuch *et al*. 2017; Ibrahim *et al*. 2020; Kai *et al*. 2020; Zahri *et al*. 2020; Zakaria *et al*. 2020). This suggests that hydrocarbon-degrading Antarctic bacteria are more sensitive to heavy metal interference than *Tritostichococcus* sp., where growth inhibition only became evident above 6 ppm of Cu (Fig. 4C) whereas impacts of other heavy metals were either minimal (Figs. 4B, D) or even positive on growth (Fig. 4A).

Content of heavy metals in soils of Antarctic anthropogenically-affected sites can differ drastically with range 11.3–116.2 ppm for Zn, 0.05–68.7 ppm for Pb, 2.56–145.2 ppm for Cu, and 0.005–0.308 ppm for Cd reported in Larsemann Hills and Mirny station (Alekseev and Abakumov 2021). In another study, samples from topsoil of Mirror Peninsula that is exposed to a wide range of weathering processes, anthropogenic impacts, and biological activity ranged 1.05–15.3 ppm for Zn, 6.68–98.9 ppm for Pb, and 0.071–24.8 ppm for Cu (Xu *et al*. 2020). Sediments in vicinity of a research station from Hangar Cove, South Cove, and Lagoon Island found range 29.6–54.3 ppm for Zn, 4.8–5 ppm for Pb, 19.6–44.3 ppm for Cu, and 0.2–0.5 ppm for Cd (Webb *et al*. 2020). Meanwhile, positive correlation was found between organic matter and levels of Zn and Cd in soil samples collected from Admiralty Bay, indicating that selective enrichment of these heavy metals is also influenced by the presence of marine vertebrates and their feces (Souza-Kasprzyk *et al*. 2022). Given the substantial difference in heavy metal concentrations within each site and from site-to-site, the higher the microbial tolerance towards heavy metals, the higher the chances for the microbe to thrive and subsequently carry out bioremediation activities in various environmental conditions. This preliminary study has shown outstanding ability of *Tritostichococcus* sp. to withstand Zn, Pb, Cu, and Cd at concentrations that are several folds of other reported Antarctic hydrocarbon-degrading microbes to remove diesel, making them promising bioremediation candidates for the co-contaminated Antarctic land.

Conclusions

The current study confirmed that different heavy metals have different impacts on *Tritostichococcus* sp. WCY_AQ5_1, reflected in changes in microalgal growth and diesel degradation ability. Cultures supplemented with the essential heavy metals, Zn and Cu, generally demonstrated improved microalgal growth in diesel-containing medium and these metals likely played a role in limiting diesel toxicity. Conversely, the influence of Pb on microalgal growth was insignificant although BE was reduced at higher concentrations. The microalga showed strong tolerance of exposure to Cd even at the highest concentrations tested here compared to the other tested heavy metals, which caused only a slight decline in microalgal growth and BE. Overall, the microalga demonstrated relatively robust survival resilience and retention of diesel biodegradation ability compared to other existing Antarctic hydrocarbon-degrading microbes in an environment where considerable amount of heavy metals have been added. Moreover, these promising results were obtained under controlled laboratory conditions with temperatures slightly higher than those typically found *in situ*. This suggests that *Tritostichococcus* sp. might show optimal bioremediation activity during the Antarctic summer or potentially for extended periods annually due to increasing global warming.

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