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Are root elongation assays suitable for establishing metallic anion ecotoxicity thresholds?

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ABSTRACT

Metallic anions including antimonate (Sb^{V}), arsenate (As^{V}), chromate (Cr^{VI}), molybdate (Mo^{VI}), selenate (Se^{VI}), tungstate (W^{V}) and vanadate (V^{V}) are important pollutants in the terrestrial environment due to their impacts on human and ecological health. It is essential that appropriate assays are used for derivation of toxicity models and guidance values, and to assess potential impacts on a site-specific basis. Root elongation is a simple and quick method for assessment of metal toxicity, yet there has been little to no validation. This study outlines results demonstrating low sensitivity of metallic anions in the often used 4-d root elongation test relative to 28-day nutrient culture assays. Therefore, root elongation assays may not be suitable for As^{V}, Cr^{VI}, Mo^{VI}, Sb^{V}, Se^{VI}, and W^{V} toxicity studies based on estimated toxicity parameters given the sensitivity of longer test assays. Only vanadate showed equivalent toxicity in the 4 d and 28 d assays. These results have significant implications for development of toxicity models and derivation of safe soil guidance values involving metallic anions.

Introduction

Metallic anions (arsenate (As^{V}), chromate (Cr^{VI}), molybdate (Mo^{VI}), selenate (Se^{VI}), tungstate (W^{V}) and vanadate (V^{V})) are important stressors in the terrestrial environment (Smedley and Kinniburgh, 2002, 2017; Winkel et al., 2015). They tend to exist predominantly as oxyanions at most environmental pH values, but may form polynuclear species at high concentrations or neutrally charged species at lower oxidation states (Winkel et al., 2015; Feng et al., 2013; Lamb et al., 2016; McGrath et al., 2010). The charge characteristics of a metallic ion determines the nature of interactions with root cell walls and plasma membranes. Toxicity of cationic stressors such as aluminium (Al^{3+}) and copper (Cu^{2+}) appear to exert rapid toxicity via sorption to cell walls and/or plasma membranes, thus modifying plant root function (Kopittke et al., 2016). Metallic anions are unlikely to exert their toxic action from direct surface impacts to cells due to their anionic characteristics and the negative charge of roots. However, knowledge of ecotoxicity of metallic anions tends to lag behind important cationic contaminants, including the development of appropriate testing protocols (Lamb et al., 2016; Ji et al., 2020; Di Toro et al., 2001; Balistrieri and Mebane, 2014; Mebane et al., 2020).

In the field of terrestrial ecotoxicity it is common to use rapid root elongation assays for metals (Kopittke et al., 2011), and more recently metallic anions, for parameterising toxicity models (Wang et al., 2011, 2001; Council, 1999; NEPC, N. E. P. C., 2013). Yet the appropriateness of this particular assay for metallic anions has not been validated in the literature. Root elongation studies of metal contaminants have been widely adopted since the Wong and Bradshaw Wong and Bradshaw (1982) study. However, there has been a very disproportionate focus on cationic toxicants such as Cu, Ni, Pb, Zn (Balistrieri and Mebane, 2014; Mebane et al., 2020; Thakali et al., 2006; Farley et al., 2015). The root elongation assays for cationic metals have also been developed without comparison with toxicological data from longer term assays Wong and Bradshaw (1982) and thus the validity of root elongation assays for use as a model of chronic responses is unknown. In some cases, studies indicated data had been validated but not the accuracy of the derived values (Wang et al., 2001; Wong and Bradshaw, 1982).

Root elongation assays have principally been applied to rhizotoxic metals (e.g. Al, Cu, Pb) which hinder root cell proliferation and cell elongation in the root elongation zone (Kopittke et al., 2016, 2009; Bojórquez-Quintal et al., 2017). However, it remains unknown whether short term studies are suitable for contaminants which do not strongly

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disturb cell development and elongation in plant roots. Many root elongation assays are <100 h in exposure (Ji et al., 2020; Song and Ma, 2017; Guzman-Rangel et al., 2018), and their appropriateness for deriving chronic toxicity values may differ across contaminants depending on the mechanism of toxic action. To the best of our knowledge, the application of short-term root elongation assays to study metallic anions toxicity has not yet been demonstrated in the literature.

The aim of this study was to assess the appropriateness of root elongation assays for the derivation of phytotoxicity thresholds. We present results from 4 d root elongation with *Cucumis sativus* L. conducted in water and nutrient solution compared against 28-day nutrient culture assays for arsenate (As^{V}), chromate (Cr^{VI}), molybdate (Mo^{VI}), antimonate (Sb^{V}), selenate (Se^{VI}), tungstate (W^{V}) and vanadate (V^{V}) species.

Materials and methods

Toxicity bioassays

Sodium or potassium salts of representative metals were used for experiments. The three tests included root elongation at (i) 4 d in deionised water (18.2 MΩ.cm) (ii) 4 d in nutrient culture (abbreviated as 4 d NS), and (iii) 28 d exposure in full nutrient culture. Cucumber (*Cucumis sativus* L.) was selected a model plant for this investigation as it is a recommended species for toxicity assays (OECD, 2006; USEPA, 2012). Initially seeds of cucumber were sterilised with 1 % NaOCl solution for 10 min and rinsed with ultrapure water thoroughly before germination. Seeds were germinated in petri dishes lined with Whatman filter #1 filter papers with ultrapure water (Lamb et al., 2010a). For the root elongation experiments, 2 days after initial addition of seeds to petri-dishes lined with Whatman filter # 1 papers, root lengths were carefully measured and 5 seedlings were transferred into pots (polypropylene) containing 130 mL of solutions (n = 3). A small piece of Sellotape on the seed was used to keep in the seedling in place. Exposure concentrations varied between toxicants due to vastly different sensitivity. Exposure concentrations were (μM): As^{V}, Cr^{VI}, W^{V} 0–250, Sb^{V} 0–800, Se^{VI} 300, Mo^{VI} 0–1600 and V^{V} 0–400. After 4 d exposure, roots were separated and lengths were measured. The toxic response was assessed by final-initial root length during exposure.

The nutrient solution used in the 4 d root elongation study was identical to the 28 day study (Lamb et al., 2012). The composition of the nutrient solution was (μM) nitrate (NO_3^-) 750, sulfur (S) 100, chlorine (Cl), phosphorus (P) 10, boron 3, Mo 0.02, potassium (K) 250, calcium (Ca) 250, magnesium (Mg) 100, ammonium (NH_4) 100, manganese (Mn) 1, zinc (Zn) 0.5, cobalt (Co) 0.04, iron (as Fe-EDTA) 2 and Cu 0.1. For the nutrient culture study, the same procedure was followed for germination, except seedlings were transferred to complete nutrient culture (Asher and Loneragan, 1967; Reichman et al., 2001). Washed samples were dried at 65 °C for 72 h and dry weights were measured.

The response-data from all assays were fitted using logistic regression, as has been previously described (Kader et al., 2015, 2016a):

$$y = \frac{100}{1 + e^{k(x-c)}} \quad (1)$$

where y is the tested parameter (% RG, relative growth), k is slope factor, x is the logarithm of the concentration (μM) and c is $\log_{10}EC_{50}$ value, respectively. The EC_x refers to effective concentration causing x% reduction of the measured parameter (where x = 10 or 50 %). The estimated values of log EC_{50} and k were used to estimate the EC_{10}. Eq. (1) was fitted in SAS (version 9.4; 100 SAS Campus Road, Cary, North Carolina, USA) using non-linear regression.

Results and discussion

Root elongation (4 d) was inhibited with increasing concentrations of Cr^{VI}, Mo^{VI} and Se^{VI}; however, even at 200 μM there was limited impact on

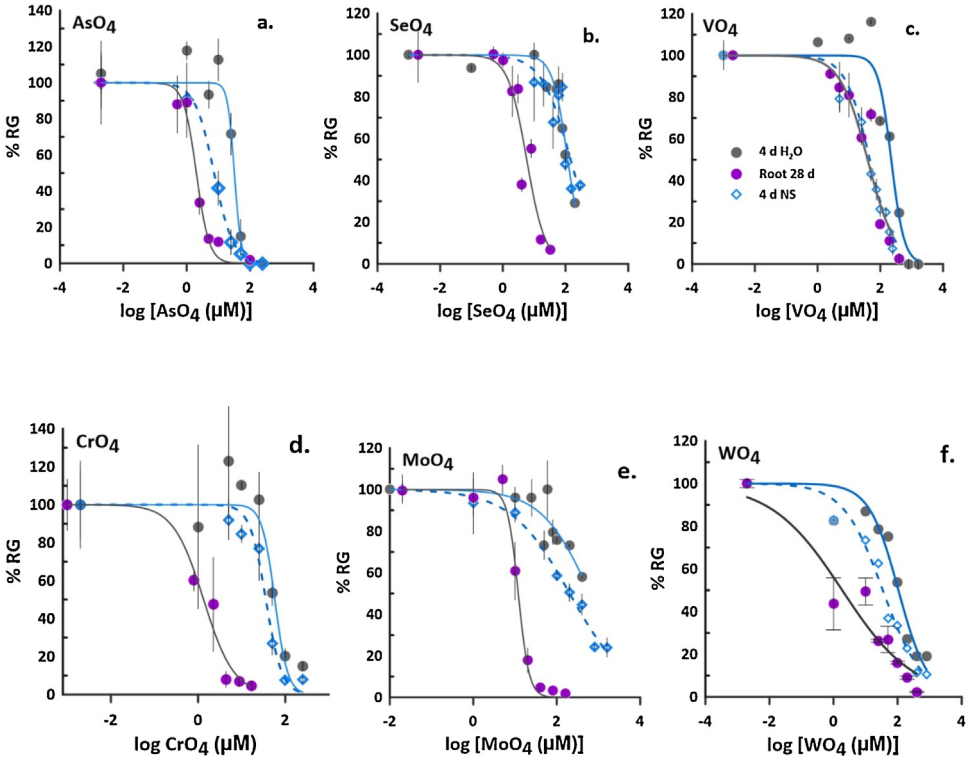

Fig. 1. Comparison of metallic anion dose-response data for 4 d root elongation in water (H_2O 4 d) and nutrient solution (NS 4 d) with the 28-d growth study in nutrient solution (root 28 d) for *Cucumis sativus*. Depicted is the dose response for arsenate (a), selenate (b), vanadate (c), chromate (d), molybdate (e) and tungstate (f). %RG indicates relative growth to control (%) and vertical bars indicated standard errors.

Table 1
Estimated Effect Concentrations (EC_{50}, EC_{20} and EC_{10}) for root elongation in 4 d water, and nutrient solution root elongation tests and full 28-d growth studies for arsenate, chromate, molybdate, antimonate, selenate, vanadate and tungstate. Values are estimate parameters with 95 % confidence intervals in parenthesis. RMSE – root mean square error.

		As^V	Cr^{VI}	Mo^V	Sb^V	Se^{VI}	V^V	W^V
4 d w	EC_{10} (μM)	18.6 (1.36–30.6)	27.4 (0.015–60.06)	33.0 (0.003–388)	36.6 (0.05–456)	34.8 (14.3–58.4)	78.9 (5.74–173)	10.6 (1.50–30.7)
	EC_{20} (μM)	22.7 (3.96–34.2)	36.0 (0.27–68.4)	98.5 (0.189–730)	85.9 (0.8–661)	54.5 (28.9–81.2)	116 (19.4–218)	24.7 (6.20–56.4)
	EC_{50} (μM)	31.9 (24.6–41.3)	57.42 (38.54–85.6)	637 (189-2,150)	370 (2.04–1250)	118 (96.9–143)	223 (155–321)	106 (70.3–160)
	RMSE (%)	**9.76**	**13.8**	**8.37**	**13.8**	**5.88**	**11.0**	**8.06**
	R^2adj	**0.96**	**0.89**	**0.660**	**0.711**	**0.94**	**0.945**	**0.934**
4 d NS	EC_{10} (μM)	1.59 (0.73–2.74)	12.0 (3.21–25.8)	5.77 (1.48–15.7)	257 (10.7–519)	15.0 (0.06–66.3)	4.25 (1.76–8.04)	1.52 (0.3–4.31)
	EC_{20} (μM)	2.79 (1.57–4.26)	14.2 (7.11–32.0)	21.5 (8.20–45.6)	355 (42.6–624)	34.6 (0.895–109)	9.66 (5.10–15.7)	4.82 (1.58–10.6)
	EC_{50} (μM)	7.29 (5.83–9.10)	35.8 (27.6–46.5)	204 (153–284)	619 (448–855)	143 (81.4–252)	39.3 (31.5–49.1)	35.5 (24.0–49.6)
	RMSE (%)	**2.80**	**7.50**	**3.70**	**16.7**	**11.7**	**8.56**	**5.40**
	R^2adj	**0.999**	**0.97**	**0.985**	**0.612**	**0.791**	**0.923**	**0.973**
28 d roots	EC_{10} (μM)	0.72 (0.15–1.42)	0.231 (0.002-0.85)	6.23 (3.41–8.68)	>250	1.28 (0.06–3.64)	6.45 (0.35–21.9)	0.0007 (<0.1-0.29)
	EC_{20} (μM)	1.06 (0.35–1.81)	0.44 (0.0172–1.25)	7.97 (5.15–10.3)	>250	2.22 (0.27–5.11)	13.4 (1.76–35.1)	0.05 (0.0001–1.06)
	EC_{50} (μM)	2.02 (1.48–2.75)	1.34 (0.747–2.40)	12.0 (10.4–13.8)	>250	5.74 (3.61–9.13)	46.3 (7.37–78.3)	1.86 (0.4–9.83)
	RMSE (%)	**7.28**	**8.82**	**5.12**	**n.a.**	**12.7**	**11.2**	**9.64**
	R^2adj	**0.97**	**0.948**	**0.988**	**n.a.**	**0.885**	**0.91**	**0.904**

root elongation (Fig. 1, Table 1, Supporting Information). In contrast, in the 28-day assays, close to 100 % growth inhibition occurred between 5 and 40 μM for Cr^{VI}, Mo^{VI} and Se^{VI}.

The root elongation data in water produced the highest estimated EC_x parameters for all species investigated. In water, the EC_{50} values for As^V, Cr^{VI}, Mo^{VI}, Sb^V, Se^{VI}, V^V and W^V were 32, 57 640, 370, 118, 220 and 106 μM, respectively (Table 1). Root elongation toxicity parameters were substantially lower in nutrient solution. The EC_{50} values after 4 d in nutrient solution for As^V, Cr^{VI}, Mo^{VI}, Sb^V, Se^{VI}, V^V and W^V were 7, 36, 204, 619, 143, 39 and 36 μM, respectively (Table 1).

Differences observed between water and nutrient solution are to be expected, since, in addition to the presence of essential nutrients, Ca and B are well established to be required for healthy cell division and cell elongation in roots (Hawkesford et al., 2012; Broadley et al., 2012). The rate of observed root growth in control groups were also notably greater in nutrient solutions compared to water alone (Fig. 2; Table 1). Aside from greater cell division, the presence of nutrients avoids negative osmotic impacts on root cells and promotes greater growth, thus producing greater discrimination of toxic impacts.

The sensitivity of the root elongation assay in water (based on the ratio between 4 d: 28 day) was between 25 and 92 times less sensitive than the 28-d assay (Table 1 and Fig. 2). The trend for water paralleled the 4 d nutrient solution data compared to the 28 d growth data, but the magnitude of difference was reduced (Table 1; Fig. 2). However, it is the comparison between the 4 d nutrient solution and the 28 d growth data that is most relevant given the requirement for Ca and B.

Fig. 2 compares the EC_{50} values as a ratio of 4 d NS:28 d results. In general, the greatest differences between the 4 d NS and 28 d was observed in the most toxic metallic anions (As^V, Cr^{VI}, W^V)(based on 28 d results). The 28 d assay for As^V, Cr^{VI}, Mo^{VI}, Se^{VI} and W^V was 10, 29, 17, 20 and 19 times more sensitive than the 4 d NS root elongation assay. As these are nutrient solution toxicity parameters, the differences could potentially be more disparate when expressed as total concentrations in soil. Previous reports indicate significant, though less severe, variations based on total soil concentrations (Kader et al., 2016b). The only report we are aware for this group of elements was reported for As^V in cucumber. Based on soil total concentrations (expressed as mg/kg As), As phytotoxicity was only 2–3 fold different across different soils (Kader et al., 2016b).

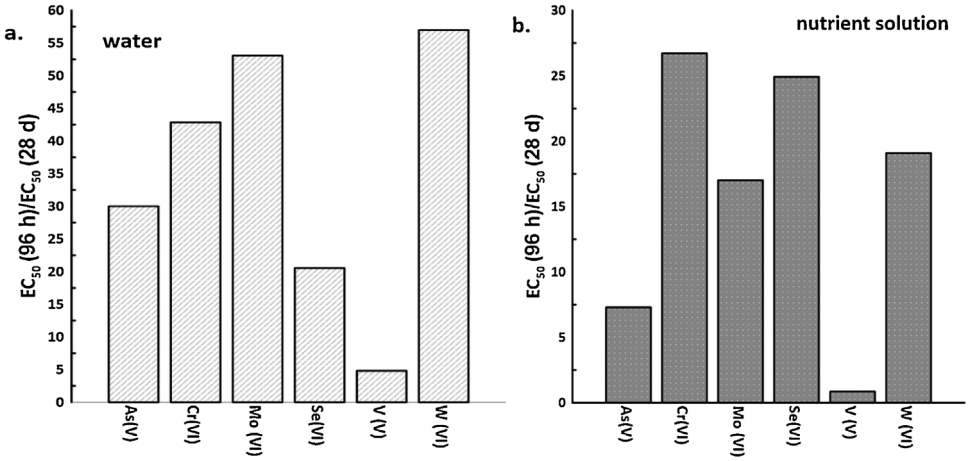

Fig. 2. Ratio of root elongation data (4 d exposure) in water (a) and nutrient solution (b) to the 28 d growth study (EC_{50}) for *Cucumis sativus*.

There were small differences between 4 d root elongation and the 28 d assay nutrient solution assay for Sb^{V} and V^{V}. In the case of Sb^{V}, the lack of variation corresponded to a lack of toxicity observed for Sb^{V} in both root elongation and 28 d assays. In addition, it is rare to find Sb^{V} levels higher than 250 μM in soil solution (Okkenhaug et al., 2012, 2013). The limited toxicity observed for Sb^{V} was consistent with other plant species investigated (Lin et al., 2020). This agrees with recent literature that shows that Sb^{V}, as opposed Sb^{III}, tends to be of relatively low toxicity in plants (e.g. Lin et al., 2020).

The similarity of EC_{50} values of V^{V} among different assays contrasts with the other metallic anions studied. Indeed, the V^{V} root elongation assay in the 4 d nutrient solution was slightly lower than that observed in the 28 d assay. Even in water the difference between EC_{50} values was minor in comparison to other metallic anions. Roots exposed to V^{V} showed obvious blackening of the root tips and stunted lateral root growth more typically associated with cationic contaminants (Lamb et al., 2010b; Kopittke and Menzies, 2006). The distinctive response of V^{V} to cucumber underlines a potentially unique toxicity mechanism within plant roots that was not observed for other metallic anions.

Vanadate is used as a phosphate-blocking agent at high concentrations in phosphorylation processes and uptake processes to membranes (Asard and Bérczi, 1998; Ullrich-Eberius et al., 1989). However, the V^{V} is also known to cause cell death through stimulation of reactive oxidative species (Imtiaz et al., 2018, 2015; Capella et al., 2007). The latter agrees with the noticeable blackening of root tips observed under vanadate exposure. Enhanced lipid peroxidation may also be caused by As^{V}, Cr^{VI}, W^{V} and Sb^{V}. However, typically lipid peroxidation occurs at levels well above those of direct ecological relevance and may not be an important toxicity mechanism. In addition, Se^{VI}-induced production of H_2O_2 in roots during exposure may be associated with reduced lipid peroxidation and oxidative stress (Silva et al., 2020).

Toxicity of aluminium is known to begin in very short time frames (<10 min) and appears to be partially linked to sorption of Al to the cell wall, primarily as Al^{3+}[7]. In addition, polymeric species of Al are also highly rhizotoxic within short time frames. The rapid vanadate toxicity could be associated with dimeric or polymeric V species interacting with the root cell wall, although formation of polymeric V species may not be significant at <50 μM V (Huang et al., 2015). A more likely explanation for the rapid V toxicity is sorption to root cell walls. The vanadium III, IV and V oxidation states are known to bind with fulvic and humic acids, and may, therefore, also bind with the cell walls of plant roots (Huang et al., 2015; Lu et al., 1998). The ability of V to bind with cell walls may explain the comparative toxicity between the 4 d and 28-d exposure periods, a process not likely to be significant for the other metallic anions studied. Further investigation is needed to ascertain the kinetically constrained toxicity of metallic anions observed in this study.

These results indicate that, unlike cationic metals (e.g. Cu^{II}), root elongation assays applied to metallic anions may not be suitable for deriving chronic toxicity guidelines for metallic anions. In deriving toxicity values for derivation of soil quality guidelines or parametrisation of toxicity models (Ji et al., 2020), it is essential that suitable indices and endpoints are used. Development of toxicity models using assays of low sensitivity may result in poor decision-making regarding risk and potential remediation efforts. However, the basis for the use of root elongation assays as representative of more chronic responses has not demonstrated in the literature.

One of the first reports in the use of root elongation as an appropriate test was reported by Wong and Bradshaw Wong and Bradshaw (1982). In this study, root elongation as a toxicity indicator was investigated using 10 mM calcium nitrate for cationic metals and chromate. However, this study did not validate the use of root elongation against longer term assays that provide chronic data needed for guideline setting. Therefore, although root elongation assays may be appropriate for relative comparisons under controlled conditions, they may not be appropriate for deriving toxicity models or guidelines without prior validation. In this study, of the 7 metallic anions investigated, only vanadate exhibited comparable toxicity parameters between root elongation and long-term assays. Prior to application of toxicity assays, the assay should be determined to be of sufficient sensitivity to reflect environmental exposure of the contaminant of concern. Indeed, despite the widespread use of root elongation assays, regulatory agencies including the USEPA and OECD (Organization for Economic Co-operation and Development) tend not to recommend root elongation assays, but rather a minimum 14 d exposure period (OECD, 2006; USEPA, 2012). We have also investigated a 14 d exposure period for As^{V} and Se^{VI} and found comparable data to the 28 d exposure period (14 d EC_{50} As^{V} = 1.70 μM; Se^{VI} was 6.92). Unfortunately, methodologies from guidelines such as the USEPA (or OECD) are often cited but not adhered to with respect to the exposure time (Wang et al., 2001). This further complicates the applicability of root elongation methodologies.

In conclusion, root elongation assays (4 d exposure) in this study were between 2 and 92 times less sensitive than 28 d exposure periods. Based on the data presented, short term root elongation assays may not suitable for As^{V}, Cr^{VI}, Mo^{VI}, Sb^{V}, Se^{VI} and W^{V} toxicity studies. These findings have significant implications for application to metal(loid) mixture toxicity studies and derivation of safe soil guidance values involving metallic anions. If short term assays are to be used to represent chronic responses, it is necessary that future studies demonstrate the sensitivity of the assays to metallic anions, and, indeed, other contaminants, prior to implementation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.hazl.2021.100024.

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