Integument colour change : Tracking delayed growth of Oppia nitens as a sub-lethal indicator of soil toxicity

Environmental Pollution

Jegede, Olukayode O.; Fajana, Hamzat O.; Adedokun, Adedamola; Najafian, Keyhan; Lingling, Jin et al

https://doi.org/10.1016/j.envpol.2023.122772

Environmental Pollution 339 (2023) 122772

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Integument colour change: Tracking delayed growth of *Oppia nitens* as a sub-lethal indicator of soil toxicity☆

Olukayode O. Jegede [a,b,*], Hamzat O. Fajana [c], Adedamola Adedokun [c], Keyhan Najafian [d], Jin Lingling [d], Ian Stavness [d], Steven D. Siciliano [a,c]

[a] Department of Soil Science, University of Saskatchewan, Saskatoon, SK, S7N 5A8, Canada
[b] Soil Physics and Land Management Group, Wageningen University & Research, Droevendaalsesteeg 4, 6708, PB Wageningen, the Netherlands
[c] Toxicology Centre, University of Saskatchewan, Saskatoon, SK, S7N 5B3, Canada
[d] Department of Computer Science, University of Saskatchewan, Saskatoon, SK, S7N 5C9, Canada

ARTICLE INFO

Keywords:
Oppia nitens
Growth
Toxicity endpoint
Colour

ABSTRACT

Growth is an important toxicity end-point in ecotoxicology but is rarely used in soil ecotoxicological studies. Here, we assessed the growth change of *Oppia nitens* when exposed to reference and heavy metal toxicants. To assess mite growth, we developed an image analysis methodology to measure colour spectrum changes of the mite integument at the final developmental stage, as a proxy for growth change. We linked the values of red, green, blue, key-black, and light colour of mites to different growth stages. Based on this concept, we assessed the growth change of mites exposed to cadmium, copper, zinc, lead, boric acid, or phenanthrene at sublethal concentrations in LUFA 2.2 soil for 14 days. Sublethal effects were detected after 7 days of exposure. The growth of *O. nitens* was more sensitive than survival and reproduction when exposed to copper (EC_{50}growth = 1360 mg/kg compared to EC_{50}reproduction = 2896 mg/kg). Mite growth sensitivity was within the same order of magnitude to mite reproduction when exposed to zinc (EC_{50}growth = 1785; EC_{50}reproduction = 1562 mg/kg). At least 25% of sublethal effects of boric acid and phenanthrene were detected in the mites but growth was not impacted when *O. nitens* were exposed to lead. Consistent with previous studies, cadmium was the most toxic metal to *O. nitens*. The mite growth pattern was comparable to mite survival and reproduction from previous studies. Mite growth is a sensitive toxicity endpoint, ecologically relevant, fast, easy to detect, and can be assessed in a non-invasive fashion, thereby complimenting existing *O. nitens* testing protocols.

1. Introduction

Growth is an important toxicity endpoints that are often suggested as an additional endpoint in toxicity testing because they are fundamental/primary life-history parameter (Van Gestel et al., 1992; Banszegi et al., 2014; Guimarães et al., 2023). When growth is adversely affected, it signifies that an individual's fitness is adversely affected (Fountain and Hopkin, 2001). Although reproduction is often a more sensitive endpoint than survival in most ecotoxicological studies, growth could be equally as sensitive, less sensitive or in some cases, more sensitive than reproduction (Van Straalen et al., 1989). Like reproduction, growth or maturation is a relevant endpoint that translates to population-level effects (Van Gestel, 2012; Alves and Cardoso, 2016). For example, Moe et al. (2001) reported that delayed growth in blowfly exposed to cadmium, resulted in impaired adult fitness (in terms of reproduction) and significantly reduced population growth rate. Growth inhibition in soil invertebrates has been assessed in some ecotoxicology studies. For example, the growth of the earthworm, *Eisenia fetida*, was inhibited by 47% when exposed to 800 mg/kg of perfluorooctanoic acid (PFOA) (Zheng et al., 2016). Cadmium, copper, zinc and lead significantly inhibited the growth of the collembolan, *Folsomia candida* after 28 days of exposure with EC_{50} ranging from 2290 to 27500 mg/kg (Fountain and Hopkin, 2001). It is noteworthy that there has been no study on the growth inhibition of oribatid mites when exposed to contaminants. The lack of this study is probably due to the difficulty in measuring growth changes in mites by size or weight.

The growth rate/change assessment method in soil invertebrates exposed to contaminants has always been by measuring their body size

☆ This paper has been recommended for acceptance by Charles Wong.

* Corresponding author. Department of Soil Science, University of Saskatchewan, Saskatoon, SK, S7N 5A8, Canada.
E-mail address: kayode.jegede@usask.ca (O.O. Jegede).

https://doi.org/10.1016/j.envpol.2023.122772
Received 23 May 2023; Received in revised form 21 September 2023; Accepted 16 October 2023
Available online 17 October 2023

or weight changes. For example, earthworm growth is often assessed by measuring body weight or size changes which is easy because of their relatively large size. The collembolan, *F. candida*'s length have also been used as a metric for growth in ecotoxicological studies. Early ecological and ecotoxicological studies used magnified ocular micrometers to view and measure the length of collembolans manually (Bengtsson et al., 1983). Subsequent studies used cameras mounted on stereo microscopes and viewed the pictures on a computer monitor. They manually measured the length or width of the animal on the monitor. In recent times, image analytic software is used for manual or automatic measuring of the length of the organisms on the picture transmitted to a screen (Banszegi et al., 2014).

Oppia nitens is an oribatid mite that feeds on fungi, decomposes organic matter and is involved in nutrient cycling in soil (Jegede et al., 2019a). They are one of the most abundant microarthropods in boreal forests (Princz et al., 2010). Although a standardized species, growth inhibition as a toxicity endpoint has never been assessed for *O. nitens* likely because juveniles are morphologically different from adults (i.e. heteromorphism) and the pre-ecdysial stages that occur between mite growth (Fajana et al., 2019; Environment and Climate Change Canada, 2020). This life-history pattern makes using length or width to measure growth cumbersome. However, at the adult (post-eclosion) stage, where shape and size are somewhat similar, there is a difference in coloration of newly emerged adults and fully matured adults. *Oppia nitens* like most oribatid mites are typically medium to dark brown. As the oribatid mites grow and mature, they go through five stages of development after hatching from eggs (Behan-Pelletier, 1999; Environment and Climate Change Canada, 2020). At the different growth stages, the mites are morphologically different but maintain a semi-translucent white coloration except at adulthood (Fajana et al., 2019). Common to arthropods, the mite sheds their exoskeleton as they grow from stage to stage (Environment and Climate Change Canada, 2020). At and after the tritonymph stage, there is a period of prolonged immobility where the mites rest and develop a sclerotized and hardened exoskeleton to become a newly emerged adult mite. The newly emerged adult mites are usually ambered coloured, and through melanization, the colour changes to medium brown and finally to dark brown as the mites grow to full maturity (Fajana et al., 2019; Environment Canada, 2020). The difference in coloration is a spectrum from semi-translucent golden--brown or light amber coloration (newly emerged adult) to rich chestnut or dark brown colour (matured adult) (Fajana et al., 2019; Environment and Climate Change Canada, 2020). The adult's darkened brown colour is due to melanization and sclerotization, which is common to many arthropods. The dark brown mites are the mites that have attained the age to start laying eggs.

Image analysis is the extraction and analysis process of meaningful features from digital images using image processing techniques. These types of analysis include a wide variety of tasks such as object detection, image segmentation, texture and color investigation. Considering the improvement of technological devices such as high-resolution digital cameras, fast and computationally powerful computers, image analysis techniques are more efficient. Image analysis for data collection is considerably fast, cost-effective, and reliable in estimating important biological parameters such as abundance, morphology, and behavior (Pennekamp and Schtickzelle, 2013).

A standard test species that can also benefit from image analysis to generate observational data is *Oppia nitens* because of its distinct colour change at some points in its lifecycle. For this study, we explored the colour spectrum, and with computer-aided image analysis, we assessed the colour change as a proxy for growth. We hereby defined the maturity of the mites to dark brown adults as the full growth of the mite species. We then used this method to assess the growth rate/change or growth inhibition of *O. nitens* when exposed to contaminants. We used Cd, Cu, Zn, Pb, phenanthrene and boric acid, all known to be toxic to *Oppia nitens* (Princz, 2014; Owojori and Siciliano, 2012; Fajana et al., 2019; Jegede et al., 2020). We hypothesized that the darkening and sclerotization of the cuticle, hence growing would be slowed down by toxicants.

2. Materials and methods

2.1. Test species

The specimens of *O. nitens* used for his study were taken from the already established cultures in the Soil Toxicology Laboratory at the Department of Soil Science, University of Saskatchewan, Canada. The mites were cultured as described in Jegede et al. (2019a).

2.2. Determination of growth of mites through integument colours

From the laboratory cultures, adult mites at three different growth stages were selected. Adult mites were selected because they have the same shape unlike the juveniles. Having the same shape makes it easy to measure any growth differences at this stage. The photos of the mites were captured at magnification of 3 (mag 3x), using a Amscope camera attached to a stereo microscope (Fig. S1). The captured image was then viewed in the computer. The image was repeatedly taken to ensure a clear and sharp image of the mites was captured. The image files were saved in portable network graphics (PNG) format. The image was then uploaded on https://color.adobe.com/create/image where the colour of the mites were extracted and matched with a corresponding hexadecimal (Hex) colour code (Fig. S2). The Hex colour code was then interpreted at https://color-hex.org/. The colours represented were red, green, blue (RGB) cyan, magenta, yellow, key-black (CMYK), hue, saturation, value, light (HSVL).

2.3. Validation of the growth of mites using colour instead of body length

Using a different microscope and different lightning set-up, we observed a new set of mites (15 mites, divided into 3 replicates of 5). The mite growth due to possible body length changes and integument colour changes were observed for 30 days. The mites were fed with baker's yeast and watered as when needed. To view the images, the numerical aperture of the microscope objective was set at a constant (1) throughout the test. The microscope lighting was not used, instead we used two Monoprice light devices (placed within 30 cm range) to provide lighting for clear images. The light device has 5 degrees of brightness, so for the test the degree of brightness used was 3. The mite photographs were taken daily but in some cases at intervals of 3 days (considering that the mites were not checked on weekends). The body sizes of the mites were measured from the captured images, using the ImageJ program to measure length of the images on screen. The measured length of the mites were based on the idiosoma. And the colour changes were measured as described in the previous section.

2.4. Advanced image processing pipeline

To validate our experiment computationally, we devised a semi-automatic methodology to analyze the captured images from different growth stages. The proposed methodology included data preprocessing, image segmentation, object extraction, and information extraction. The pipeline was implemented in Python version 3.9 employing OpenCV (Villán, 2019), Scikit-image (Van der Walt et al., 2014), and NumPy (Supplemental Data, Section S1).

2.5. Test soil

The soil used was the natural standard soil, LUFA 2.2 (Lufa Speyer, Germany). Soils were air-dried prior to use. LUFA 2.2 is a sandy loam soil and other physicochemical properties are summarized as follows: pH = 4.97, water holding capacity (WHC) = 47.5%.

2.6. Test chemicals

Six chemicals were used for this test. The chemicals are cadmium as $CdSO_4$ (Sigma Aldrich), copper as $CuCl_2$ (Sigma Aldrich, 97%), zinc as $Zn(NO_3)_2$ (Sigma Aldrich, 98% reagent grade), lead as $Pb(NO_3)_2$ (Sigma Aldrich, ACS reagent ≥99%), boric acid (Merck KGaA, Analar 99.8%) were weighed into water to make stock solutions for each of the concentrations tested. Phenanthrene (Alfa Aesar, 98%) was dissolved at tested concentrations, in acetone and then added to the soil. The acetone-phenanthrene-soil mixture was then placed in the fume-hood to allow the soil to be dried and free of acetone.

2.7. Toxicity tests

LUFA 2.2 soil was dosed at three concentrations excluding control at 60% WHC (water holding capacity) of the soil. The highest concentrations were equivalent to literature EC_{50} reproduction values for each of the chemicals used (Table 1). The concentrations we used in this study are nominal concentrations. The cadmium concentrations used were 50, 150 and 350 mg/kg; copper concentrations used were 750, 1500, 3000 mg/kg; zinc concentrations used were 450, 900, 1800 mg/kg; lead concentrations used were 500, 1000 and 2000 mg/kg; boric acid concentrations used were 45, 90, 180 mg/kg and phenanthrene concentrations used were 35, 70 and 140 mg/kg. For the control soils, no chemical was applied but the soil was moistened with water to 60% WHC. The pH of the dosed soils were measured to ensure that the pH does not affect the result (Table S4). Three grains of yeast were added to each soil. Ten newly emerged adults were put in 3 g of dosed or control soils and three replicates were made. Before the ten newly emerged adult mites were put in each soil, their photos were taken and stored in the computer to determine the colour value at day zero (0-day). The test was kept under constant conditions for the duration of the test; 21 °C, 50–60 % humidity, >800 lx, day to light; 16: 8 h regime (Princz et al., 2010; Jegede et al., 2020). Between the 0 and 14 days of test, the mites were extracted from the soil using a paint brush and the mite photos were captured and stored on the computer. This was done two times (6-day and 9-day) before day fourteen (14-day). On the final day of test (14-day), the mite photos were also captured and stored on the computer. The images were then analyzed and the colour values they represented were computed to determine colour change (see Table 2).

2.8. Procedure for growth change and growth change per day derivation from mite colour change

For each of the colours, colour change was derived by subtracting the value of the colour at day-14 from the value of the colour at day-0. This was done for red, green, blue and light (RGBL) colours because colour change at day-14 was less than day-0 colour value. However, for the key-black, the value of colour from day-0 was subtracted from day-14 colour value because day-14 colour value was more than the day-0 colour value. To derive the growth change, after the colour change has been determined, each of the colour change was normalized by dividing by the highest colour change (often the control) and multiplying by 100%. See below example of how the colour change was determined for the exposed mites:

2.8.1. Step by step determination of growth change from red the changes in the red colour

At the control (0 mg/kg) on day zero (Day 0), the red value = 150, after 14 days (Day 14), the red value = 90, therefore the colour change = 150–90 = 60.

At 50 mg/kg, on day 0, the red value = 150 and on day 14 = 110, therefore colour change = 150–110 = 40.

At 150 mg/kg, on day 0, the red value = 150 and on day 14 = 120, therefore colour change = 150–120 = 30.

At 350 mg/kg, on day 0, the red value = 150 and on day 14 = 135, therefore colour change = 150–135 = 15.

Using the control value as the base line;

Growth change at 0 mg/kg = (60 ÷ 60) x 100 = 100

Growth change at 50 mg/kg = (40 ÷ 60) x 100 = 67

Growth change at 150 mg/kg = (30 ÷ 60) x 100 = 50

Growth change at 350 mg/kg = (15 ÷ 60) x 100 = 25

This procedure is followed for the other four colours. The values for each colour were pooled together and averaged as five estimates of colour for each concentration.

For the growth change per day derivation, see example below in the table:

To derive the growth change per day at 0 mg/kg (Control), the means of the colour values (in bold letters) were regressed with the days. The slope of the regression line (after transformation) was the growth change per day.

The steps (i) and (ii) were repeated for the other concentrations. After the growth change per day have been derived for 0 mg/kg to the highest concentration. A dose response was modelled using the growth change per day as response on y axis and concentrations on x axis.

Table 1
The highest concentrations of the toxicants (in mg/kg) representing sublethal concentrations on growth of *Oppia nitens*, based on the sublethal concentrations (EC_{50}) on reproduction of *O. nitens*.

Toxicant	Highest concentration used for mite growth test (in mg/kg)	EC_{50} reproduction (in mg/kg)	Reference
Cadmium	350	345	Keshavarz Jamshidian et al. (2017)
Copper	3000	2896 (2375–2938)	Owojori and Siciliano (2012)
Zinc	1800	1562 (1363–1952)	Owojori and Siciliano (2012)
Lead	2000	1678 (1066–2290)	Owojori and Siciliano (2012)
Boric acid	180	118 (91-138)	Princz et al. (2010)
Phenanthrene	140	95 (26–345)	Owojori et al. (2011)

2.9. Compared with other studies

The toxicity of the toxicants on growth of *O. nitens* were compared with results from earlier studies of the toxicants on reproduction and survival of *O. nitens* (Owojori et al., 2011; Owojori and Siciliano, 2012; Keshavarz-Jamshidian et al., 2017; Fajana et al., 2020; Jegede et al., 2020). The pattern of *O. nitens'* response to the toxicants as it affects growth was compared with the pattern of responses of *O. nitens* as it affects its reproduction and survival from earlier studies.

2.10. Statistics

The mite growth change (Growth-14D) was based on subtracting the colour at day 14 from the colour at day 0. Data for the mite growth change in the graphs are presented as mean ± standard error. Normality was checked for the data and to check the differences between the mite colour changes per days, one-way analysis of variance (ANOVA) was used. To determine the concentration causing 50% effect (EC_{50}), 25% effect (EC_{25}) or 20% effect (EC_{20}), three or four parameter non-linear log-logistics was used with the drm package in R (Ritz, 2016). The slopes of the dose response curves were determined for each toxicant to assess if there are differences in how the mite growth was influenced on

Table 2
The steps in determination of growth change per day using the red, green, blue, key-black and light colour mode values.

Step (i)							
	Concentration	Red	Green	Blue	Key-black	Light	
Day 0	0 mg/kg	171.50	158.25	63.75	32.50	46.50	
Day 6	0 mg/kg	133.75	110.50	31.25	47.50	32.50	
Day 9	0 mg/kg	121.33	94.42	31.25	52.50	29.92	
Day 14	0 mg/kg	107.73	85.82	29.73	55.00	26.82	
Then the colour values were normalized to 100% as seen in step (ii) below							
Step (ii)							**Mean**
Day 0	Control	100.00	100.00	100.00	67.50	100.00	**93.50**
Day 6	Control	77.99	69.83	49.02	52.50	69.89	**63.85**
Day 9	Control	70.75	59.66	49.02	47.50	64.34	**58.25**
Day 14	Control	62.81	54.23	46.63	45.00	57.67	**53.27**

exposure to the toxicants. The differences in slope value were determined using a one-tailed *t*-test. The mite growth change per day (Growth PD) was based on incorporating the colour changes between intervals of 0, 6, 9 and 14 days, instead of just day 14 and day 0 only (as was the case with mite growth change). The growth at (0, 6, 9, 14 days) followed a non-linear pattern for all chemicals tested. The growth was then square-root transformed for regression analyses. The slope of the regression line was used to determine the growth change per day for each chemical at each concentration tested to detect how the chemicals influenced the pattern of mite growth for the total duration of 14 days. Variance ratio tests were performed to determine similarities or differences of lethal and sublethal responses from previous mite studies with the sublethal responses in the present study.

3. Results

3.1. Determination of maturity of non-exposed mites

The red, green, blue, and light colour values followed a pattern such that the values are lower as the mite matures (Table 3, Fig. 1). However, the key black followed an opposite pattern showing that the values are higher as the mite matures (Table 3, Fig. 1). The cyan, magenta, yellow, hue and saturation were not consistent with the maturity of the mites (Table 3).

3.2. Validation of O. nitens growth using body length and colour changes

For the 30-day duration, mite length did not change (Fig. 2a), however the mite colours changed as they age from 100% at day zero to about 20% at the end of 30 days. The mite colour change showed about three different curves within the first 21 days. The first 7 days (0 - 7 d) and the last 7 days (14 - 21 d) showed steep curves but the 7 days in-between (7–14 d) showed a less steep slope. From 21 days to 30 days, there was no change in colour of the mites as the curve flattened (Fig. 2b). This implies that the mites grew to full maturity 21 days after eclosion.

3.3. Growth changes in O. nitens over 30 days observation

The colours changed based on the days of maturity from day 0 to day 4, there were significant ($P < 0.05$) changes in maturity (Table 4). Although day 1 was not different from day 4, but it was different from day 7. From day 17, the mites attained full growth (maturity) that was not different as the days increased, where day 17 and day 30, was not significantly different in the maturity in terms of colour change (Table 4). All the days of growth were compared to each other (Table S1).

3.4. Advanced image analysis

The red, green, and blue (RGB) colours of the mites were highest at day zero and reduced as the age of the mites increased (Fig. 3). At day zero, the red colour had the highest value and the blue colour had the lowest. However, at about 14 days post-eclosion of the mites, the blue colour increased and was at the same value with the red colour after 30 days post-eclosion of the mites. The green colour value was the lowest of the RGB at the end of the 30 days post-eclosion of the mites. The RGB curves flattened from about 18 days post eclosion (Fig. 3).

3.5. Toxicity test results with Cd, Cu, Zn, Pb, boric acid and phenanthrene

After 14 days exposure, mite growth was impaired after exposure to the chemicals except lead where there was no effect on mite growth even at the highest concentration (Fig. 4). Within the range of concentrations used for each chemical, cadmium, copper, and zinc impaired the growth of the mites by about 50% (Fig. 4). Hormetic response was observed when the mites were exposed to copper and zinc where the lowest concentrations after the control were significantly ($p < 0.05$) higher than control and other concentrations. Boric acid and phenanthrene impaired the growth by at least 25% (Fig. 4). Based on 50% effect level

Table 3
Colour changes according to growth or maturity stage of *Oppia nitens*. CMYKSV are normally expressed as percentages (%), and H expressed as degree (°).

Mite stage	**RGB colour mode**			**CMYK colour mode**				**HSV colour mode**			**HSL colour mode**		
	R	G	B	C (%)	M (%)	Y (%)	K (%)	H (°)	S (%)	V (%)	H (°)	S (%)	L (%)
Adult 1A	64	58	31	0	9	52	75	49	52	25	49	35	19
Adult 1B	64	59	33	0	8	48	75	50	48	25	50	32	19
Adult 1C	64	58	32	0	9	50	75	49	50	25	49	33	19
Adult 2A	140	125	50	0	11	64	45	50	64	55	50	47	37
Adult 2B	140	125	48	0	11	66	45	50	66	55	50	49	37
Adult 2C	140	116	43	0	17	69	45	45	69	55	45	53	36
Adult 3A	188	191	94	2	0	51	25	62	51	75	62	43	56
Adult 3B	187	191	69	2	0	64	25	62	64	75	62	49	51
Adult 3C	188	191	96	2	0	50	25	62	50	75	62	43	56

RGB = Red Green Blue, CMYK = Cyan Magenta Yellow Key/Black, HSV = Hue Saturation Value, HSL = Hue Saturation Lightness.

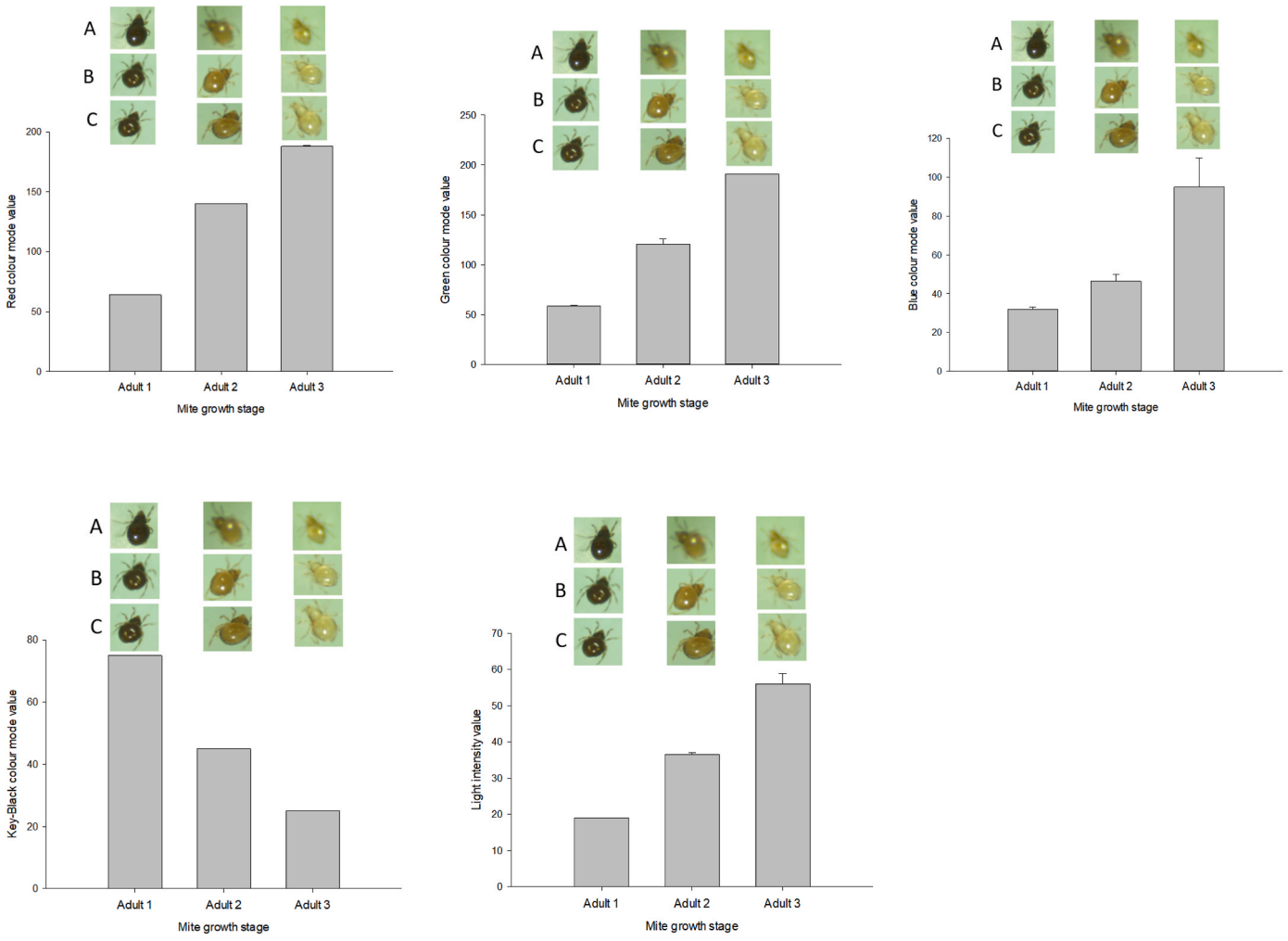

Fig. 1. The plots of the red, green, blue, key-black and lightness colour mode values ± standard deviation, based on three *Oppia nitens'* growth stages (adult 1 = oldest, adult 2 = older and adult 3 = youngest) in three replicates per growth stage. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

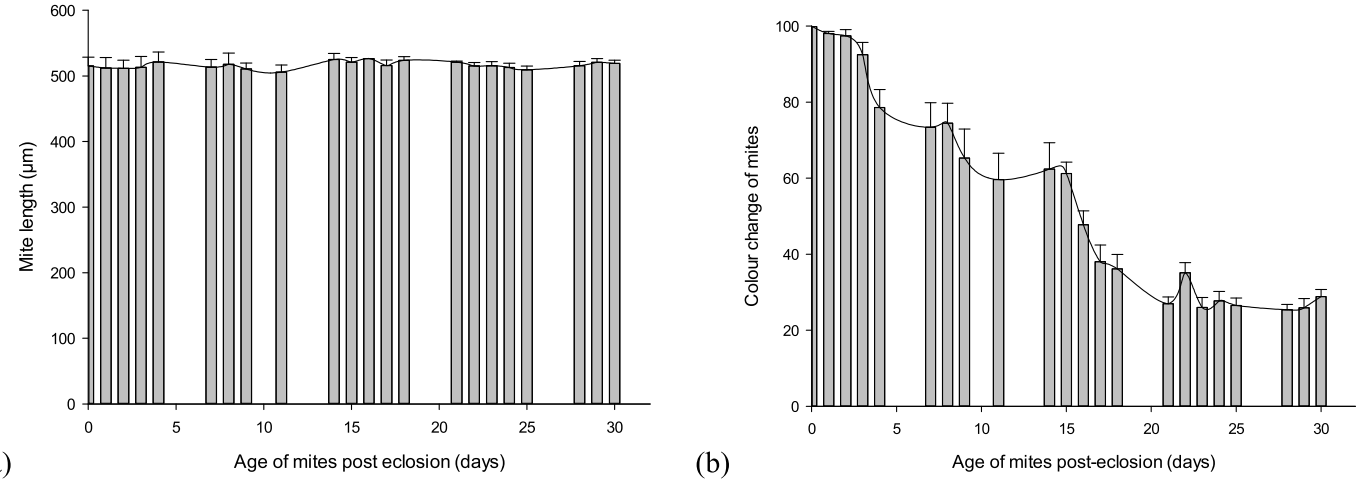

Fig. 2. Post-eclosion of fifteen (n = 15) *Oppia nitens* growth ± SE monitored for 30 days measuring the (a) body length in µm (b) colour changes from light brown to dark brown expressed in percentages relative to day zero.

on growth, cadmium was the most toxic of the toxicants (EC_{50} = 262 ± 39 mg/kg) whereas boric acid was the most toxic based on 25% effect level (EC_{25} = 111 ± 32 mg/kg) on growth. The growth change per day (growthPD), which was modelled by accounting for growth changes at 0-, 6-, 9- and 14-days intervals was also used to derive EC_{50} values for the toxicants indicative of higher sensitivity (Fig. S3-9).

Table 4
The Analysis of variance (ANOVA) pairwise multiple comparison of observed days of growth to maturity of *Oppia nitens* measured through the integument colour change of the mites. A Tukey post hoc test was used to detect significant differences in the observed days.

Days comparison	Diff of Means	n	q	P
Day 0 vs. Day 4	21.403	22	5.343	**0.041**
Day 0 vs. Day 3	7.525	22	1.879	0.999
Day 1 vs. Day 7	24.675	22	6.16	**0.006**
Day 1 vs. Day 4	19.479	22	4.863	0.105
Day 2 vs. Day 7	24.049	22	6.004	**0.009**
Day 2 vs. Day 4	18.853	22	4.707	0.138
Day 3 vs. Day 9	27.141	22	6.776	**0.001**
Day 3 vs. Day 8	18.015	22	4.498	0.196
Day 4 vs. Day 16	30.832	22	7.698	**<0.001**
Day 4 vs. Day 15	17.344	22	4.33	0.253
Day 7 vs. Day 16	25.636	22	6.4	**0.004**
Day 7 vs. Day 15	12.148	22	3.033	0.858
Day 9 vs. Day 17	27.323	22	6.822	**0.001**
Day 9 vs. Day 16	17.569	22	4.386	0.233
Day 14 vs. Day 17	24.366	22	6.083	**0.008**
Day 14 vs. Day 16	14.612	22	3.648	0.574
Day 16 vs. Day 25	21.256	22	5.307	**0.044**
Day 16 vs. Day 24	20.065	22	5.009	0.08
Day 17 vs. Day 30	9.191	22	2.295	0.99

The **bolded numbers** show significance ($p < 0.05$).

3.6. Slope of the dose response curves of growth change and growth change per day, differed for metals

For the growth change, dose response curve (DRC) slopes around the effective concentrations for phenanthrene and boric acid were similar ($p > 0.01$) (Table 5). However, all the DRC slopes for the metals were significantly different ($p < 0.01$) when compared to phenanthrene and boric acid. Of the metals, zinc's slope (2.68 ± 0.75) was significantly ($p < 0.01$) higher than slopes of cadmium and copper (Table 5).

3.7. Patterns of response of mites to toxicity of Cd, Cu, Zn, boric acid and phenanthrene on growth, reproduction, and survival

The patterns of toxic response between growth change (growth-14D) and growth change per day (growth PD) with survival and reproduction of the mites showed a clear difference between the toxic effect by essential metals (copper and zinc) and other toxicants (Fig. 5). On exposure of the mites to copper and zinc, growth-14D, growth PD, survival and reproduction almost perfectly followed the same pattern. Interestingly, cadmium and phenanthrene elicited similar responses in terms of the overall shapes of their curves. For boric acid, growth PD and reproduction followed similar pattern (Fig. 5). On the exposure of the mites to lead, growth-14D, growth PD, survival and reproduction all had different patterns. To assess if response patterns were similar or different, variance ratio tests (F-tests) were determined (Table S2).

4. Discussion

Protection of population level effects is a key goal of soil risk assessment. Reproduction is one method to assess population level effects (Van Gestel, 2012; Alves and Cardoso, 2016), but reproduction tests in site soils can confound habitat suitability with toxicity. Mites, are selective about soil conditions in which to lay eggs (Princz et al., 2010; Jegede et al., 2019b). Thus, investigators are often faced with a dilemma in which mites will not reproduce in a soil of interest because it lacks enough fibrous organic matter (Princz et al., 2010). Van Gestel et al. (1992) reported a trade-off between reproduction and growth, thereby suggesting that growth change could be used as a response to toxicants.

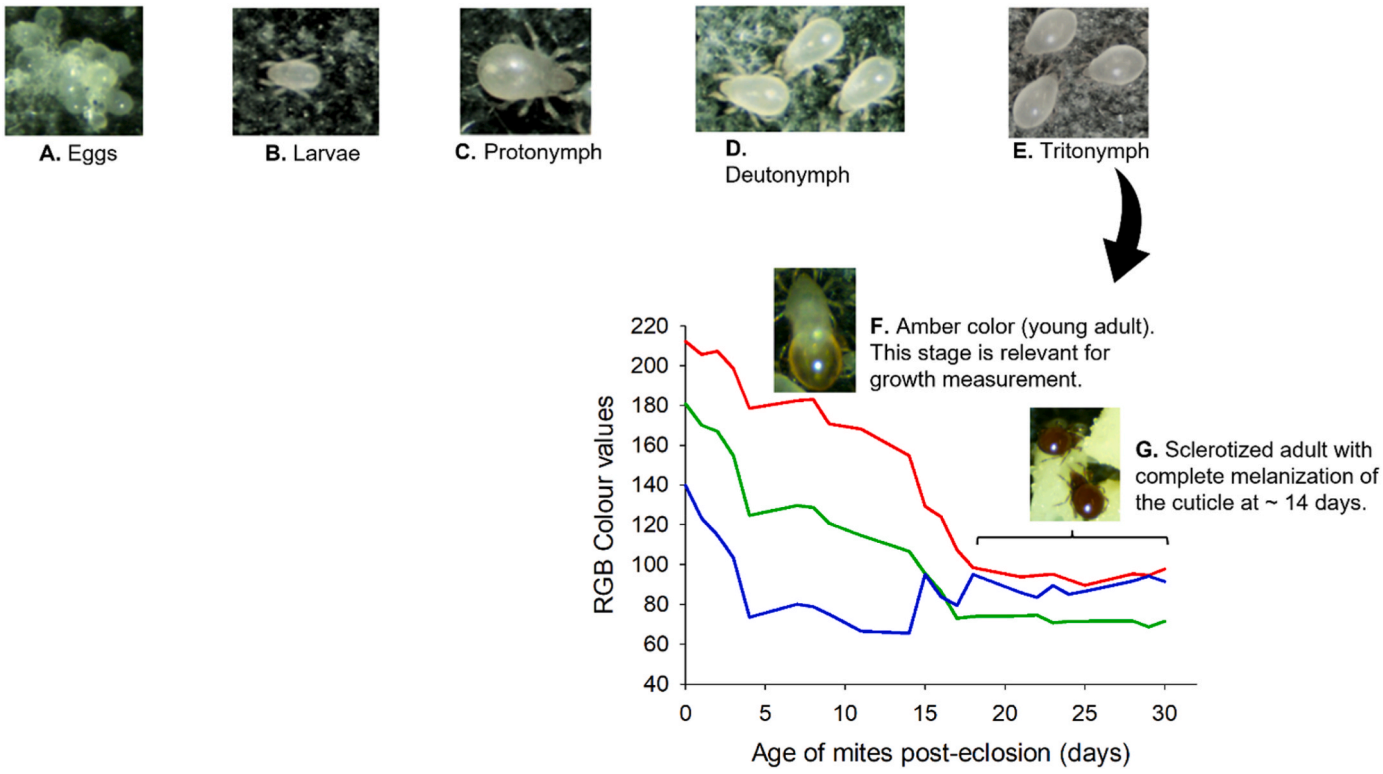

Fig. 3. Developmental stages of *Oppia nitens* from the eggs to tritonymph before eclosion to the ambered colour stage (young adult) that is relevant for growth measurement (Adapted from Fajana et al., 2019). Upon post-eclosion, *Oppia nitens* monitored for 30 days showing red, green and blue (RGB) colour change from day 0 to day 30. The RGB colours were derived from the mite photos after the images have been segmented from the background, and erosion method was thereafter applied to remove the overlap between the segmented mite image and background. This was implemented in Python 3.9. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

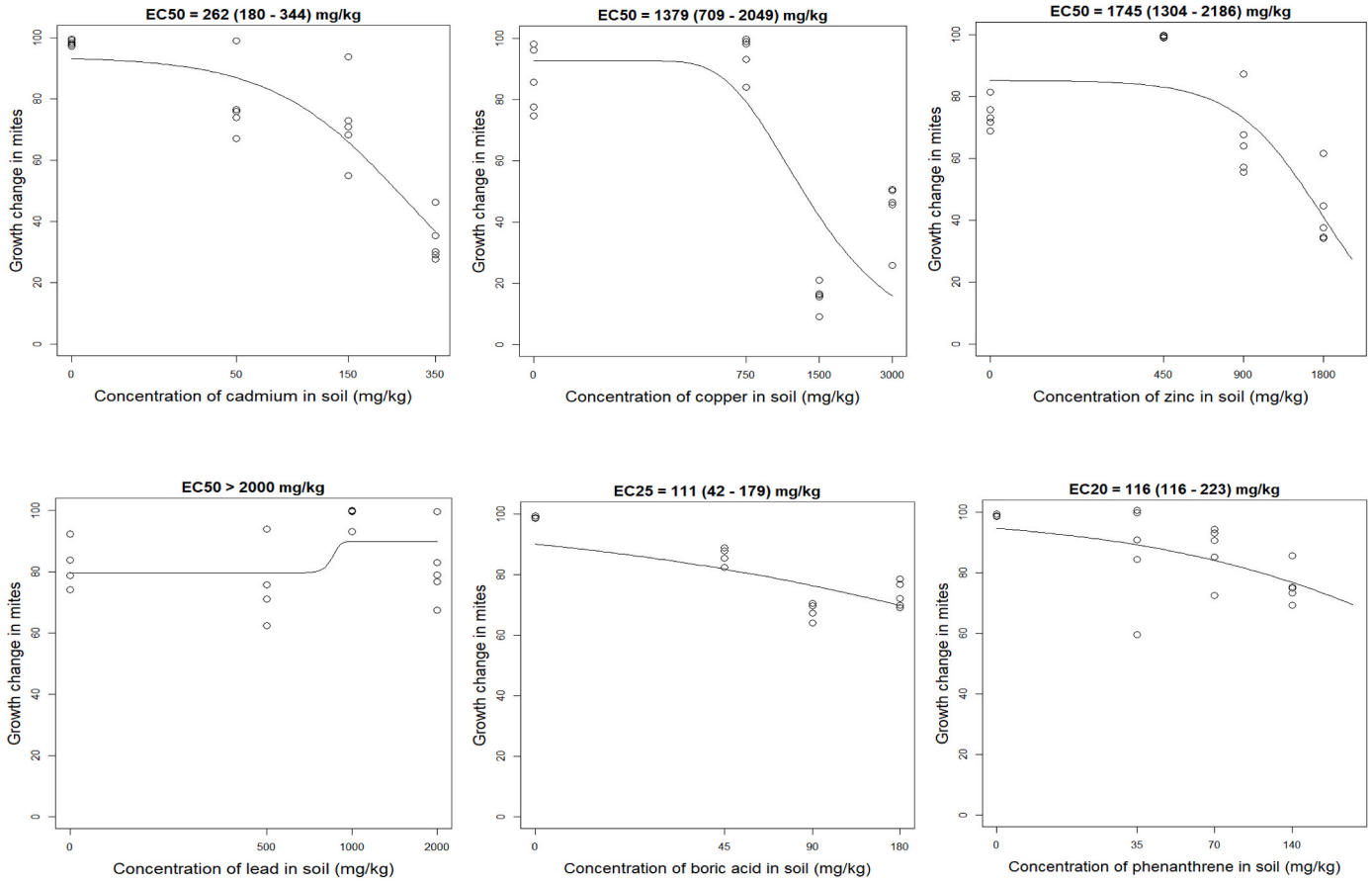

Fig. 4. Dose response of mite to cadmium, copper, zinc, lead, boric acid and phenanthrene after 14 days exposure. The effective concentration inhibiting mite growth at 50% effect (EC_{50}) were determined for cadmium, copper, and zinc. EC_{25} were determined for boric acid and EC_{20} for phenanthrene. EC_{50}, EC_{25} nor EC_{20} could be determined for lead. All concentrations are nominal.

Table 5
The comparison of slopes around the EC_{50} ± standard error (SE) for growth change of the mites after exposure to phenanthrene, boric acid, cadmium, copper and zinc. Growth change = growth change in mites after 14 days exposure. *Significant differences ($p < 0.01$).

Contaminant	Slope ±SE	Phenanthrene	Boric acid	Cadmium	Copper	Zinc
		0.72 ± 0.46	0.49 ± 0.16	1.53 ± 0.65	1.68 ± 0.89	2.68 ± 0.75
Phenanthrene	0.72 ± 0.46		0.885	0.002**	0.001**	0.001**
Boric acid	0.49 ± 0.16	0.885		0.001**	0.001**	0.001**
Cadmium	1.53 ± 0.65	0.002**	0.001**		0.913	0.001**
Copper	1.68 ± 0.89	0.001**	0.001**	0.913		0.001**
Zinc	2.68 ± 0.75	0.001**	0.001**	0.001**	0.001**	

**$p < 0.001$.

Moreover, like reproduction, growth is better for assessing population-level effects (Alves and Cardoso, 2016). Our method of colour change detected sublethal effects within seven (7) days of exposure at levels close to those that inhibit reproduction. A key consideration is that this method can be readily added to other sublethal toxicity tests, providing investigators with additional information on the mechanisms by which chemicals alter population survival of one of the world's most abundant soil invertebrate groups, the mites.

Metals and other xenobiotics disrupt moult hormone signaling, thereby eliciting endocrine-disrupting effects (Zou, 2005; Luo et al., 2015; Chen et al., 2019) either by direct inhibition of key energy enzymes like chitin synthetase or tyrosinase production of melanin. Growth is likely inhibited by the reduction in cuticle thickening and melanization that is linked to moulting and is the characteristic of many invertebrates, including arthropods. For arthropods like *Daphnia,* decreased chitobiose inhibits moulting and alters growth, reproduction, and survival in *D. pulex* and *D. Magna* (Duchet et al., 2011; Chen et al., 2019).). The inhibition of the chitin synthetase (enzyme responsible for chitin synthesis from glucose) in collembolans, prevents moulting and promotes collembola mortality (Campiche et al., 2006). Copper is a co-factor for the tyrosinase enzyme responsible for pigmentation in insects (Zhou et al., 2003; Wang et al., 2018; Theotoki et al., 2019). The *Drosophila melanogaster*'s copper content correlated positively with cuticle darkening due to increased melanin production from the upregulation of tyrosinase (Vásquez-Procopio et al., 2020). In our study, the toxicants interfered with the melanization of *O. nitens* thereby inhibiting growth and consequently will lead to delayed reproduction.

Based on the dose-response curve slope around the effective concentrations, zinc had a significantly higher slope than the other metals and contaminants, indicative of more potency (Jegede et al., 2019b). Although zinc was more potent, it was not the most toxic metal. As expected, the most toxic metal is cadmium. Cadmium was more toxic

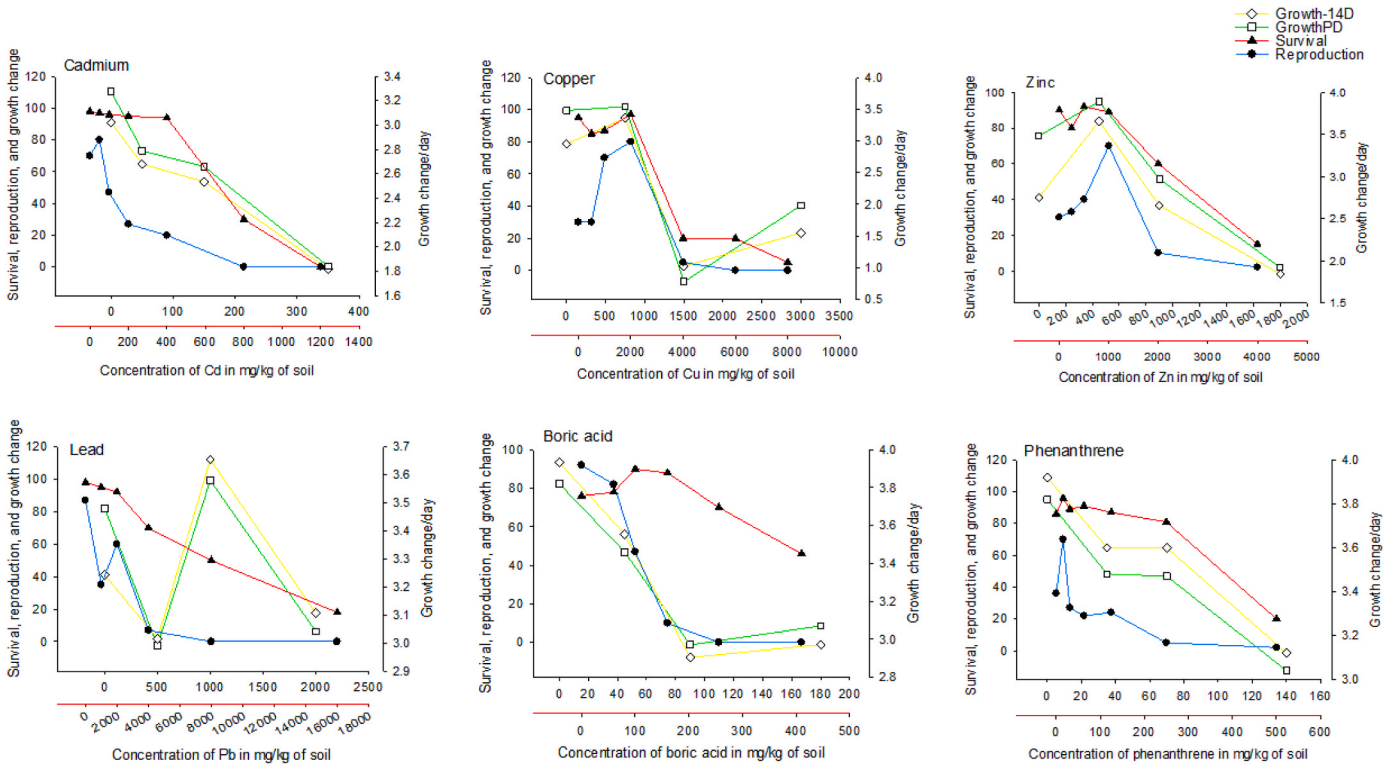

Fig. 5. The toxic effect pattern of cadmium, copper, zinc, lead, boric acid, and phenanthrene on survival, reproduction, growth change (Growth-14D) and growth change per day (Growth PD) of *Oppia nitens*. The survival and reproduction data was adapted from Owojori and Siciliano (2012). The growth change and growth change per day data was based on present study. The chemical concentration axes (in red) below each graph, represent the concentrations for the adapted study of Owojori and Siciliano (2012). All concentrations are nominal. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

than zinc, lead, copper on reproduction of *Oppia nitens* (Owojori and Siciliano, 2012). Fountain and Hopkin (2001) reported that cadmium was the most toxic of metals among copper, zinc, and lead to the growth of *F. candida*. In our study, lead was not toxic to the mites at the concentrations that caused toxic effects on mite reproduction. For example, lead EC_{50}growth based on one-day exposure modelling was calculated as 2727 mg/kg against 1678 mg/kg reported by Owojori and Siciliano (2012). However, Owojori et al. (2011) also demonstrated that *O. nitens* did not avoid 2000 mg/kg lead. Therefore, it could be that the mites were not avoiding lead at that concentration because it was not having any deleterious effect on the mite growth. Based on EC_{50}growth, the toxicity of the metals on mite was cadmium > copper > zinc > lead, following the same pattern for *F. candida* as reported by Fountain and Hopkin (2001). Although we could not determine EC_{50} of phenanthrene and boric acid, we could detect 25% growth inhibition. Phenanthrene and boric acid effect on the mite growth were not as sensitive as their effect on mite reproduction implying that the mode of toxic action differs for these contaminants.

When we factored time into modelling the toxic effects of the toxicants to derive the growth change per day (growthPD), we were able to account for the EC_{50} of phenanthrene and boric acid. With time factored, it appeared that the EC_{50} value of cadmium reduced, indicating higher sensitivity. This observation with cadmium demonstrates that cadmium sublethal toxic effect is delayed at least within 14 days probably due to toxicokinetics. This contrasts with Keshavarz Jamshidian et al. (2017) who reported increased cadmium lethal toxicity over time in *O. nitens*. The difference could be that sublethal and lethal toxicity of cadmium to *O. nitens* follow differing processes. The EC_{50} values of copper and zinc derived by factoring time (growthPD), remained similar if time was not considered (growth-14D). Also, the pattern of the mite growth response (growth-14D or growthPD) was similar to the pattern of mite reproduction and survival. Owojori and Siciliano (2012) suggested that copper and zinc have the same mode of action on *O. nitens*. Interestingly, lower concentrations of copper and zinc elicited a hormetic response of the mite growth, just as observed in mite reproduction by Owojori and Siciliano (2012). These observations with copper and zinc could be related to their essentiality and efficient regulation of these metals by soil organisms (e.g., earthworms, springtails, isopods, ants) (Heikens et al., 2001; Ardestani et al., 2014). For example, Ardestani and Van Gestel (2013) reported that *F. candida* regulated copper when exposed to copper spiked LUFA soil for 14 days. Zinc was reported to attain a steady state in earthworms, *Eisenia fetida* and *Eisenia andrei* (Peijnenburg et al., 1999; Spurgeon and Hopkin, 1999; Smith et al., 2010). Therefore, growthPD could be a surrogate for growth-14D in determining growth change in mites in some instances. However, it is important to note that growthPD is better suited for experiments when toxicokinetics of toxicants are considered.

When compared to three other studies, only Owojori and Siciliano (2012) reported higher *O. nitens* sensitivity to cadmium (137 mg/kg) as against the present study (262 mg/kg) (Table 6). The other two studies (Keshavarz-Jamshidian et al., 2017; Jegede et al., 2020) reported lesser sensitivity. Based on the present study, mite growth was a more sensitive endpoint (EC_{50} = 1360 mg/kg) than mite reproduction (≥2896 mg/kg) from Owojori and Siciliano (2012), when the mites were exposed to copper. For zinc, growth rate of the mites was more sensitive than reproduction when compared to Jegede et al. (2020), however was slightly less sensitive than reproduction as reported by Owojori and Siciliano (2012). No lead toxicity to *O. nitens* was observed at the concentrations tested, implying that the EC_{50} mite growth is above 2000 mg/kg (Table 6). Therefore, growth was a less sensitive endpoint than reproduction when exposed to lead. For mites exposed to boric acid, growth was a slightly less sensitive endpoint when compared to reproduction at 25% effect level. When growth is expressed as growth change per day, it was more sensitive at EC_{50} of 47 mg/kg. Although, toxicity

Table 6
Comparison of *O. nitens'* growth change (Growth-14D) and growth change per day (Growth PD), as a sublethal toxicity endpoints at 50%, 25%, 20% and 10% effect concentrations (EC_{50} and EC_{25}, EC_{20}, EC_{10} in mg/kg) of cadmium, copper, zinc, lead, boric acid and phenanthrene in this study to reproduction EC_{50} of *O. nitens* in literature.

Cadmium				
Endpoint	**Substrate**	**Exposure duration (days)**	**EC_{50} (mg/kg)**	**Reference**
Growth-14D	Natural soil[a]	14	270	Present study
Growth PD	Natural soil[a]	14	144	Present study
Reproduction	Natural soil[a]	42	301	Keshavarz-Jamshidian et al., 2017
Reproduction	Artificial soil	35	137	Owojori and Siciliano (2012)
Reproduction	Artificial soil	28	392	Fajana et al. (2020)
Copper				
Growth-14D	Natural soil[a]	14	1360	Present study
Growth PD	Natural soil[a]	14	1461	Present study
Reproduction	Artificial soil	35	2896	Owojori and Siciliano (2012)
Reproduction	Natural soil[b]	28	3466	Jegede et al. (2020)
Zinc				
Growth-14D	Natural soil[a]	14	1803	Present study
Growth PD	Natural soil[a]	14	1608	Present study
Reproduction	Artificial soil	35	1562	Owojori and Siciliano (2012)
Reproduction	Natural soil[b]	14	8121	Jegede et al. (2020)
Lead				
Growth-14D	Natural soil[a]	14	>2000	Present study
Growth PD	Natural soil[a]	14	>2000	Present study
Reproduction	Artificial soil	35	1678	Owojori and Siciliano (2012)
Reproduction	Natural soil[b]	28	1404	Jegede et al. (2020)
Boric acid				
Growth-14D	Natural soil[a]	14	111*	Present study
Growth PD	Natural soil[a]	14	47	Present study
Reproduction	Artificial	35	314	Owojori et al. (2011)
Phenanthrene				
Growth-14D	Natural soil[a]	14	116[c]	Present study
Growth PD	Natural soil[a]	14	60[d]	Present study
Reproduction	Artificial soil	35	95	Owojori et al. (2011)

[a] Standard natural soil (LUFA 2.2).
[b] Non-standard natural soil (Loamy sand), *EC_{25}.
[c] EC_{20}.
[d] EC_{10}.

was observed at 25% effect levels when mites were exposed to phenanthrene (173 mg/kg), growth was a less sensitive endpoint than reproduction at 50% effect level (95 mg/kg) (Table 6).

The biological process level characteristics of oribatid mites need to be explored more for soil quality assessment (Lebrun and Van Straalen, 1995; Behan-Pelletier, 1999). Assessing mite growth changes with image analytic methods is a step in the right direction in exploring additional toxicity endpoints. Moreover, this test procedure is non-invasive because it avoids using heat for mite extractions. Heat extractions may be a source of additional heat stress on mites and could interfere with measuring mite biochemical changes. Therefore, the non-invasiveness of the test makes it well suited to be combined with emerging test methods such as genomic and omic technologies in toxicological assessment of chemicals on *Oppia nitens*. Doing more toxicity tests with new test methods and developing more toxicity endpoints that are ecologically relevant should be prioritized in soil ecotoxicology (Van Gestel, 2012). Mite growth change is a sensitive toxicity endpoint, ecologically relevant yet fast and straightforward, as demonstrated in our study.

5. Conclusions

Assessing mite growth changes with image analytic methods is a step in the right direction in exploring additional toxicity endpoints. Our method of mite colour change detected sublethal effects within seven days of exposure at levels close to those that inhibit reproduction. At sublethal concentrations, cadmium, copper, zinc, phenanthrene and boric acid delayed the growth of *Oppia nitens*. As expected, cadmium was the most toxic metal on the growth of the mites. The influence of copper and zinc on growth changes followed the same pattern as their influence on survival and reproduction. This influence of copper and zinc was peculiar to these two metals and alludes to their essentiality. The method used in this study is non-invasive, efficient, yet fast. The method can be readily added to other sublethal toxicity tests, providing investigators with additional information on the mechanisms by which chemicals alter the fitness of one of the most abundant soil invertebrate groups, the mites.

CRediT authorship contribution statement

Olukayode O. Jegede: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Hamzat O. Fajana:** Investigation, Methodology, Formal analysis, Visualization, Writing – review & editing. **Adedamola Adedokun:** Investigation, Methodology, Writing – review & editing. **Keyhan Najafian:** Investigation, Methodology, Writing – review & editing. **Jin Lingling:** Investigation, Methodology, Writing – review & editing. **Ian Stavness:** Investigation, Methodology, Writing – review & editing. **Steven D. Siciliano:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2023.122772.

References

Alves, P.R.L., Cardoso, E.J.B.N., 2016. Overview of the standard methods for soil ecotoxicology testing. Invertebrates–Experimental Models in Toxicity Screening 35–56.
Ardestani, M.M., van Gestel, C.A., 2013. Dynamic bioavailability of copper in soil estimated by uptake and elimination kinetics in the springtail *Folsomia candida*. Ecotoxicology 22 (2), 308–318.

Ardestani, M.M., van Straalen, N.M., van Gestel, C.A., 2014. Uptake and elimination kinetics of metals in soil invertebrates: a review. Environ. Pollut. 193, 277–295.
Banszegi, O., Kosztolányi, A., Bakonyi, G., Szabó, B., Dombos, M., 2014. New method for automatic body length measurement of the collembolan, *Folsomia candida* Willem 1902 (insecta: collembola). PLoS One 9 (6), e98230.
Behan-Pelletier, V.M., 1999. Oribatid mite biodiversity in agroecosystems: role for bioindication. Agric. Ecosyst. Environ. 74, 411–423.
Bengtsson, G., Gunnarsson, T., Rundgren, S., 1983. Growth changes caused by metal uptake in a population of *Onychiurus armatus* (Collembola) feeding on metal polluted fungi. Oikos 40, 216–225.
Campiche, S., Becker-van Slooten, K., Ridreau, C., Tarradellas, J., 2006. Effects of insect growth regulators on the nontarget soil arthropod *Folsomia candida* (Collembola). Ecotoxicol. Environ. Saf. 63, 216–225.
Chen, H., Gu, X., Zeng, Q., Mao, Z., 2019. Acute and chronic toxicity of carbamazepine on the release of chitobiase, molting, and reproduction in *Daphnia similis*. IJERPH 16, 209.
Duchet, C., Inafuku, M.M., Caquet, T., Larroque, M., Franquet, E., Lagneau, C., Lagadic, L., 2011. Chitobiase activity as an indicator of altered survival, growth and reproduction in *Daphnia pulex* and *Daphnia magna* (Crustacea: Cladocera) exposed to spinosad and diflubenzuron. Ecotoxicol. Environ. Saf. 74, 800–810.
ECCC (Environment and Climate Change Canada), 2020. Biological Test Method: Test for measuring reproduction of oribatid mites exposed to contaminants in soil STB 1/RM/61. https://publications.gc.ca/collections/collection_2020/eccc/En83-10-1-61-2020-eng.pdf.
Fajana, H.O., Gainer, A., Jegede, O.O., Awuah, K.F., Princz, J.I., Owojori, O.J., Siciliano, S.D., 2019. *Oppia nitens* CL Koch, 1836 (Acari: Oribatida): current status of its bionomics and relevance as a model invertebrate in soil ecotoxicology. Environ. Toxicol. Chem. 38, 2593–2613.
Fajana, H.O., Jegede, O.O., James, K., Hogan, N.S., Siciliano, S.D., 2020. Uptake, toxicity, and maternal transfer of cadmium in the oribatid soil mite, *Oppia nitens*: implication in the risk assessment of cadmium to soil invertebrates. Environ. Pollut. 259, 113912.
Fountain, M.T., Hopkin, S.P., 2001. Continuous monitoring of *Folsomia candida* (Insecta: collembola) in a metal exposure test. Ecotoxicol. Environ. Saf. 48, 275–286.
Guimarães, B., Römbke, J., Amorim, M.J.B., 2023. On the importance of longer-term exposure to stressors-A critical review and proposal for multigenerational testing in standard soil invertebrates. Sci. Total Environ. 854, 158680.
Heikens, A., Peijnenburg, W.J.G.M., Hendriks, A.J., 2001. Bioaccumulation of heavy metals in terrestrial invertebrates. Environ. Pollut. 113, 385–393.
Jegede, O.O., Awuah, K.F., Renaud, M.J., Cousins, M., Hale, B.A., Siciliano, S.D., 2020. Single metal and metal mixture toxicity of five metals to *Oppia nitens* in five different Canadian soils. J. Hazard Mater. 392, 122341.
Jegede, O.O., Hale, B.A., Siciliano, S.D., 2019a. Multigenerational exposure of populations of *Oppia nitens* to zinc under pulse and continuous exposure scenarios. Environ. Toxicol. Chem. 38, 896–904.
Jegede, O.O., Awuah, K.F., Fajana, H.O., Owojori, O.J., Hale, B.A., Siciliano, S.D., 2019b. The forgotten role of toxicodynamics: how habitat quality alters the mite, *Oppia nitens*, susceptibility to zinc, independent of toxicokinetics. Chemosphere 227, 444–454.
Keshavarz Jamshidian, M., Verweij, R.A., Van Gestel, Van Straalen, 2017. Toxicokinetics and time-variable toxicity of cadmium in *Oppia nitens* Koch (Acari: Oribatida). Environ. Toxicol. Chem. 36 (2), 408–413.
Lebrun, P., van Straalen, N.M., 1995. Oribatid mites: prospects for their use in ecotoxicology. Exp. Appl. Acarol. 19, 361–379.
Luo, J., Pei, S., Jing, W., Zou, E., Wang, L., 2015. Cadmium inhibits molting of the freshwater crab *Sinopotamon henanense* by reducing the hemolymph ecdysteroid content and the activities of chitinase and N-acetyl-β-glucosaminidase in the epidermis. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 169, 1–6.
Moe, S.J., Stenseth, N.C., Smith, R.H., 2001. Effects of a toxicant on population growth rates: sublethal and delayed responses in blowfly populations. Funct. Ecol. 712–721.
Owojori, O.J., Healey, J., Princz, J., Siciliano, S.D., 2011. Can avoidance behavior of the mite *Oppia nitens* be used as a rapid toxicity test for soils contaminated with metals or organic chemicals? Environ. Toxicol. Chem. 30, 2594–2601.
Owojori, O.J., Siciliano, S.D., 2012. Accumulation and toxicity of metals (copper, zinc, cadmium, and lead) and organic compounds (geraniol and benzo [a] pyrene) in the oribatid mite *Oppia nitens*. Environ. Toxicol. Chem. 31, 1639–1648.
Peijnenburg, W.J., Baerselman, R., De Groot, A.C., Jager, T., Posthuma, L., van Veen, R. P., 1999. Relating environmental availability to bioavailability: soil-type-dependent metal accumulation in the Oligochaete *Eisenia andrei*. Ecotoxicol. Environ. Saf. 44, 294–310.
Pennekamp, F., Schtickzelle, N., 2013. Implementing image analysis in laboratory-based experimental systems for ecology and evolution: a hands-on guide. Methods Ecol. Evol. 4, 483–492.
Princz, J.I., Behan-Pelletier, V.M., Scroggins, R.P., Siciliano, S.D., 2010. Oribatid mites in soil toxicity testing—the use of *Oppia nitens* (CL Koch) as a new test species. Environ. Toxicol. Chem. 29, 971–979.
Princz, J., 2014. Development of a New Test Suite of Ecologically-Relevant Species for the Assessment of Contaminants in Boreal Soils-Special Emphasis on Oribatid Mites. Doctoral dissertation, University of Saskatchewan.
Ritz, C., 2016. Analysis of Dose-Response Curves. CRAN. 2016-08-30. http://www.r-project.org.Version.3.0-1.
Smith, B.A., Egeler, P., Gilberg, D., Hendershot, W., Stephenson, G.L., 2010. Uptake and elimination of cadmium and zinc by *Eisenia andrei* during exposure to low concentrations in artificial soil. Arch. Environ. Contam. Toxicol. 59, 264–273.
Spurgeon, D.J., Hopkin, S.P., 1999. Comparisons of metal accumulation and excretion kinetics in earthworms (*Eisenia fetida*) exposed to contaminated field and laboratory soils. Appl. Soil Ecol. 11, 227–243.
Theotoki, E.I., Velentzas, A.D., Katarachia, S.A., Papandreou, N.C., Kalavros, N.I., Pasadaki, S.N., Giannopoulou, A.F., Giannios, P., Iconomidou, V.A., Konstantakou, E.G., Anastasiadou, E., Papassideri, I.S., Stravopodis, D.J., 2019. Targeting of copper-trafficking chaperones causes gene-specific systemic pathology in *Drosophila melanogaster*: prospective expansion of mutational landscapes that regulate tumor resistance to cisplatin. Biol. Open 8 bio046961.
Van der Walt, S., Schönberger, J.L., Nunez-Iglesias, J., Boulogne, F., Warner, J.D., Yager, N., Gouillart, E., Yu, T., 2014. scikit-image: image processing in Python. PeerJ 2, e453.
Van Gestel, C.A.M., Dirven-Van Breemen, E.M., Baerselman, R., 1992. Influence of environmental conditions on the growth and reproduction of the earthworm *Eisenia andrei* in an artificial soil substrate. Pedobiologia 36109–36120.
van Gestel, C.A., 2012. Soil ecotoxicology: state of the art and future directions. ZooKeys (176), 275.
van Straalen, N.M., Schobben, J.H., de Goede, R.G., 1989. Population consequences of cadmium toxicity in soil microarthropods. Ecotoxicol. Environ. Saf. 17, 190–204.
Vásquez-Procopio, J., Rajpurohit, S., Missirlis, F., 2020. Cuticle darkening correlates with increased body copper content in *Drosophila melanogaster*. Biometals 33 (6), 293–303.
Villán, A.F., 2019. Mastering OpenCV 4 with Python: a Practical Guide Covering Topics from Image Processing, Augmented Reality to Deep Learning with OpenCV 4 and Python 3.7. Packt Publishing Ltd.
Wang, X., Yin, S., Yang, Z., Zhou, B., 2018. *Drosophila* multicopper oxidase 3 is a potential ferroxidase involved in iron homeostasis. Biochim. Biophys. Acta 1862 (8), 1826–1834.
Zheng, X.Q., Shi, Y.J., Lu, Y.L., Xu, X.B., 2016. Growth inhibition and DNA damage in the earthworm (*Eisenia fetida*) exposed to perfluorooctane sulphonate and perfluorooctanoic acid. Chem. Ecol. 32, 103–116.
Zhou, H., Cadigan, K.M., Thiele, D.J., 2003. A copper-regulated transporter required for copper acquisition, pigmentation, and specific stages of development in *Drosophila melanogaster*J. Biol. Chem. 278, 48210–48218.
Zou, E., 2005. Impacts of xenobiotics on crustacean molting: the invisible endocrine disruption. Integr. Comp. Biol. 45, 33–38.