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A pilot *in vivo* evaluation of Sb(III) and Sb(V) genotoxicity using comet assay and micronucleus test on the freshwater fish, silver perch Bidyanus bidyanus (Mitchell, 1838)

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ABSTRACT

Antimony (Sb) is a priority water pollutant known to be toxic to aquatic organisms at high concentrations. Environmental exposure, however, occurs most often at sub-lethal concentrations but very limited information is available on effects of sub-lethal, chronic exposure to Sb, which hinders reliable risk assessment and the setting of protective guidelines. In this pilot study, *in vivo* screening for Sb genotoxicity in the erythrocytes of the freshwater fish, silver perch (Bidyanus bidyanus) was conducted where fish were exposed to environmentally relevant and sub-lethal Sb concentrations of 0.4, 0.9 and 1.8 mg L^{-1} Sb(III), and 0.9, 2 and 5 mg L^{-1} Sb(V), for 14 d. Genotoxicity was assessed by both a single cell gel electrophoresis (SCGE) assay and a micronucleus (MN) test. The SCGE assay showed that all Sb(III) exposure concentrations induced a statistically significant non-dose-related increase in DNA damage after 2 d of exposure after which there was no further increase in DNA damage evident in relation to the control. Mortality of fish was 100 % in all Sb(III) exposures by 14 d. Clastogenic and/or aneugenic effects were not observed. The 1.8 mg L^{-1} Sb(III) exposure was the only Sb concentration at which a significant increase in the cytotoxicity index as measured by the ratio of polychromatic erythrocytes (PCEs) to normochromatic erythrocytes (NCE) was induced at 2 d exposure. For Sb(V) exposures, no significant genotoxic effects were observed using either assay, nor was the PCE/NCE altered. This pilot investigation has indicated that sub-lethal waterborne Sb(III) exposure manifests in genotoxic effects in freshwater fish species, which may have consequences for resilience and survival. Further study is needed for deeper insight into the relationship between Sb(III) and genotoxicity and the multiple biomarker responses that need assessment to evidence effects.

1. Introduction

Global use of antimony (Sb) is increasing (Obiakor et al., 2017a; USGS, 2020) with the consequence of increased contamination of aquatic environments (Telford et al., 2009; Zhou et al., 2015; Dovick et al., 2016; Fu et al., 2016; He et al., 2019; USGS, 2020). Typical background concentrations of Sb are less than 1 μg L^{-1} in unpolluted stream water, and 0.2 μg L^{-1} in ocean water (Filella et al., 2002). Antimony is prioritised as a water pollutant in many jurisdictions (USEPA, 1988; CEC, 1998; ANZECC/ARMCANZ, 2000; CCME, 2014) and both acute and chronic exposure to Sb is known to result in fish mortality (Nam et al., 2009; Obiakor et al., 2017a, 2017b; Obiakor, 2017c). Nevertheless, the limited exposure-dose response studies for Sb in aquatic systems mostly focus on acute effects (EURAR, 2008; Obiakor et al., 2017a, 2017b; Obiakor, 2017c) with effects from exposure to sub-lethal concentrations little explored. There is some evidence, however, that cumulative exposure to low Sb concentrations can manifest in sub-lethal responses. For example, Chen and Yang (2007) reported that the common carp fish, Cyprinus carpio, exposed to sub-lethal concentrations of Sb(III) ($SbCl_3$, 1.0 – 8.0 mg L^{-1}) for 28 days showed significant decreases in oxygen consumption at 4.0 and 8.0 mg L^{-1}. Similarly, Yang et al. (2010) found that 0.8 and 1.2 mg L^{-1} of Sb(III) significantly

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reduced oxygen consumption and induced histopathological alterations of the hepatopancreas in freshwater swamp shrimp (Macrobrachium nipponense) over a 7 day exposure. Sub-lethal genotoxic effects also include DNA damage, chromosome breakage, and also cellular changes (Kumar et al., 2010; Kumar et al., 2014; Obiakor et al., 2014) and although genotoxic effects of Sb have been reported in mammalian cells *in vitro* (Kuroda et al., 1991; Gebel et al., 1997; Elliott et al., 1998; Gebel et al., 1998; Huang et al., 1998; Schaumloffel and Gebel, 1998; Migliore et al., 1999) and *in vivo* (Gurnani et al., 1992a, 1992b; Hantson et al., 1996; Cavallo et al., 2002), little or no information is available for aquatic organisms, despite the growing knowledge on adverse genetic effects from metalloids such as arsenic (As) (Ramírez and García, 2005; Ahmed et al., 2011b; Selvaraj et al., 2013; Kumar et al., 2014; Kumari et al., 2017; Obiakor et al., 2019). The consequences of sub-lethal responses may include increased environmental stress, lower resilience, developmental and reproductive changes that could impact the ecosystem biodiversity and sustainability (Nam et al., 2009; Obiakor et al., 2017a, 2017b). The dearth in data on Sb in freshwater systems limits comprehensive assessment and the establishment of reliable protective guidelines (Chen and Yang, 2007; Nam et al., 2009; Obiakor et al., 2017b).

Antimony occurs with four oxidation states (-3, 0, +3, and +5), with the +3 (antimonite, denoted as Sb(III)) and +5 (antimonate, denoted as Sb(V)) states of known environmental and biological importance (Filella et al., 2002; Multani et al., 2016; He et al., 2019). These two oxidation states can exist in environmental systems in organic, inorganic and methylated forms, but the inorganic forms are usually dominant (EURAR, 2008; Wilson et al., 2010; Multani et al., 2016). The toxic effects of Sb depend strongly on the chemical form and oxidation state and can be highly variable, with the underlying mechanisms of toxicity not clear (De Wolff, 1995; Filella et al., 2007; Sun, 2010; Sundar and Chakravarty, 2010; Obiakor et al., 2017a). For example, bacteria exposed to Sb(III) and Sb(V) produced genotoxic (recombination) effects in the recombination assay, but showed no mutagenic effects in the Ames, Chromotest, and Umu tests (Kuroda et al., 1991; De Boeck et al., 2003), leading to the assumption that Sb may exert genotoxicity indirectly rather than through direct DNA alteration (Gebel et al., 1997; Schaumloffel and Gebel, 1998; Beyersmann and Hartwig, 2008; NTP, 2010). Thus, improved knowledge of a range of cumulative sub-lethal effects for the different Sb forms to which organisms are exposed would advance our understanding of the biological consequences of Sb exposure.

Detection of genotoxicity is gaining importance as an early measure of effects in biological systems (Bücker et al., 2012; Obiakor et al., 2014). These effects are used as specific biomarkers in evaluating the ecosystem health, organism exposure, and adverse outcome (Nwani et al., 2011; Obiakor et al., 2014; Kumari et al., 2017). A range of different assays have been applied for genotoxicity assessment in fish. Two common assays used are the single cell gel electrophoresis (SCGE) and the micronucleus (MN) assay. The SCGE measures small DNA alterations induced by genotoxic chemicals that can arise from DNA-single and double strand breaks, DNA-DNA, and DNA-protein crosslinks, as indicated by a DNA migration (tail) on photomicrographs (Tice et al., 2000; De Lapuente et al., 2015; Gajski et al., 2019). One of the strengths of the SCGE assay is its capacity to detect in target tissue acute DNA damage in the absence of any clinical signs of stress or toxicity. This acute DNA damage can be repaired or induce apoptosis, thereby decreasing the amount of damage measurable by the SCGE assay at later exposure times when clastogenic and/or aneugenic effects are most probably detected (Recio et al., 2010; Vasquez, 2010; Gajski et al., 2019). The MN assay measures clastogenicity (chromosome breakage events and rearrangements) and aneugenicity (whole chromosome loss or lagging) due to dysfunctions in mitotic spindle apparatus, as indicated by the frequency of detected MN in cell cytoplasm (Fenech, 2007; Saleh and Sarhan, 2007; Corvi et al., 2008; Zelazna et al., 2011). The ratio of the polychromatic erythrocytes (PCE) to the normochromatic erythrocytes (NCE) is a complementary metric, to indicate cell cytotoxicity produced by suppression of erythropoiesis, for example, by exposure to contaminants (Suzuki et al., 1989).

Silver perch, Bidyanus bidyanus (Mitchell, 1838), is a subtropical freshwater fish indigenous to Australia and its distribution spans much of the Murray-Darling Drainage of Queensland, New South Wales, Victoria and South Australia (NSW-DPI, 2006; Trueman, 2007; Wells et al., 2007). Silver perch generally has a longer life span, slower growth, and slower maturation than many other freshwater fish in Australia (NSW-DPI, 2006) and thus, maybe a good subject for assessment of cellular damage resulting from chronic exposure to moderate-to-low levels of aquatic freshwater contaminants. In this work, we evaluated the effects of Sb(III) and Sb(V) on silver perch DNA integrity in circulatory erythrocytes. The aims of this study were to combine SCGE and MN techniques in a small pilot trial examination to understand DNA damage, clastogenic and/or aneugenic effects, and cytotoxicity evident with Sb(V) and Sb(III) exposure in silver perch, to provide information on biological effects and for ecological risk.

2. Materials and methods

2.1. Chemicals

All chemicals were analytical grade. Antimony potassium tartrate (CAS no. 28300-74-5, purity $\geq$ 98.5 %, Ajax Chemicals, Australia) and potassium hexahydroxyantimonate (CAS no. 12208-13-8, purity 99.99 %, Sigma-Aldrich Inc. USA) were used as test chemicals to supply Sb(III) and Sb(V), respectively for the *in vivo* SCGE and MN tests. Other chemicals were purchased from the suppliers as follows: Comet Low Melting Point (LMP) agarose (Catalog #4250-050-02), 200 mM ethylenediaminetetraacetic acid (EDTA) pH 10 (Catalog #4250-050-04) and lysis solution (Catalog #4250-050-01) from Trevigen, Gaithersburg, Maryland, USA; sodium hydrogen phosphate (CAS no. 7558-79-4, purity 99-102 %) and sodium dihydrogen phosphate (CAS no. 7558-80-7, purity 98–100.5 %) for preparation of 0.1 M phosphate buffer saline (PBS) 7.2 from Ajax Chemicals Australia; hydrochloric acid (HCl) 32 % (CAS no. 7647-01-0) from RCI Labscan, Australia; sodium hydroxide (NaOH) (CAS no. 1310-73-2, purity > 98 %), 0.4 % Trypan blue solution (CAS no. 72-57-1), Giemsa stain modified G5500, and May-Grünwald solution 63590 from Sigma-Aldrich Inc. USA; and dimethyl sulfoxide (DMSO) (CAS No. 67-68-5, purity $\geq$ 99.9 %) from Research Organics, Inc. USA. Chemical preparations were carried out using ultrapure water (TKA Micropure, Germany, electrical conductivity: 0.05 μS cm^{-1}).

2.2. Fish husbandry and management

Juvenile silver perch aged 8–10 weeks old, weight 0.8–1.1 g, with standard length range (distance between snout and caudal peduncle depth) and total length (distance between snout and tail fin) of 1.8–2.9 cm and 3.5–5.6 cm, respectively were procured from Aquablue Seafoods, New South Wales, Australia, and transported to the laboratory holding facility for acclimatisation. The fish were maintained in 2 × 500 L holding tanks filled with dechlorinated water for 14 days under controlled photoperiod (12/12 light/dark). Temperature was measured using a submersible aquarium thermometer (JW Pet Fusion Smart Temp Non-Standing Magnet) and pH by a TPS plastic body pH sensor and digital meter (WP-81s V6.0 W1543, TPS, Australia). Dissolved oxygen (DO) was measured at a depth of 15 cm using a Vernier Labquest® 2 meter optical DO sensor probe (Beaverton, USA). Hardness and ammonia were measured using API® hardness and ammonia test kits, respectively (Mars Fishcare North America, USA). Water temperature was maintained at 24.6–26.2 °C. Fish were fed ad libitum daily with commercial (Aquablue Seafoods) floating feed pellets (made up of > 35 % crude protein, > 5 % fat, 10 % moisture, minerals, and vitamins) as per breeder's fish handling instructions due to sensitivity of silver perch under controlled conditions. Accumulated materials in the tanks were

siphoned off and 70–95 % of water was renewed daily. Mortality of fish was less than 3 % in the four days before the exposure experiment commenced. Fish were transferred and retained in experimental exposure tanks for 5 days prior to Sb dosing. Fish exposure occurred in three replicates for the control and also for exposure doses of Sb(III) and Sb (V). The experimental protocol and animal handling procedures were approved by the Animal Ethics Committee of the University of New England in accordance with guidelines of the Australian Code for the Care and Use of Animals for Scientific Purposes (Authority No. AEC14-113).

2.3 Genotoxic assay design and rationale for exposure concentrations

Hierarchical experimental design was used for the fish exposures and SCGE and MN tests. Briefly, for each Sb dose and control treatment, two fish were collected at each sampling time (2, 6, and 14 days) from each treatments, and each fish was sampled to produce two SCGE gel slides and two MN slides per fish sample. In this pilot study we sampled two fish in three replicates for each exposure duration to maximise exposure times and concentrations tested with fish numbers available at the time, but scale-up would require additional investigation with multi-genotoxic methodologies. The sampling times were selected to examine genotoxic effects of Sb across a standard chronic exposure period with increasing intervals from first exposure (Kumar et al., 2010; GHS, 2011; Nwani et al., 2011; Bhatnagar et al., 2016). Our protocol integrated both SCGE and MN assays to provide concurrent data for both endpoints in blood sampled from the same fish in each treatment group (Recio et al., 2010; Vasquez, 2010; OECD, 2014; Hansen, 2018).

The Sb exposure concentrations encompassed sub-lethal but environmentally relevant concentrations (Telford et al., 2009; Obiakor et al., 2017b; Obiakor, 2017c). For Sb(III), the three exposure concentrations were based on fractions of the 96 h LC50 (18 mg L^{-1}) estimated for silver perch exposed to antimony potassium tartrate in previous studies (Obiakor, 2017c), and included 0.4 mg L^{-1} (1/50 of 96 h LC50), 0.9 mg L^{-1} (1/20 of 96 h LC50) and 1.8 mg L^{-1} (1/10 of 96 h LC50). Antimony (III) is less detected in the environment since Sb(V) appears to occur in more concentration than Sb(III) in predominantly oxic environmental systems (Wilson et al., 2010; Obiakor, 2017c). The concentrations of 0.9, 2 and 5 mg L^{-1} were used for Sb(V). The 0.9 mg L^{-1} Sb(V) was reflective of the maximum Sb concentration detected in an Sb contaminated freshwater ecosystem in New South Wales Australia (Telford et al., 2009), while 2 mg L^{-1} and 5 mg L^{-1} Sb(V) were sub-lethal concentrations at 1/125 and 1/50, respectively of the approximate Sb(V) 96 h LC50 value (~ 259 mg L^{-1}) previously determined for silver perch (Obiakor, 2017c). Antimony concentrations and speciation in water were confirmed by instrumental analysis with a PSA 10.055 Millennium Excalibur coupled to a PSA 10.820 Speciation Modular Interface for automation (P S Analytical). Confirmation of water concentrations showed within 83 % (82.2–84.1 %) Sb(III) and 89 % (87.9–90.4 %) Sb (V) of the nominal concentrations.

2.4. Test fish, dosing, and sampling

Exposures of fish to the three selected exposure concentrations of Sb (III) and Sb(V) and one untreated control without Sb were performed in a semi-static system under a controlled environment with a 12 h light-dark cycle. Each 20 L tank (35 × 22 × 25 cm) in three replicates had 15 L of test chemical. The fish were randomly distributed to tanks containing the Sb solutions, or the untreated control without Sb. Each tank received 12 fish. Tanks were fitted with an aquarium aeration pump. Renewal of Sb solutions was undertaken two times daily to reduce ammonia and waste build up in exposure media, and fish were fed once a day for the 14 day period. The water quality parameters (pH, dissolved oxygen, temperature, and hardness) were checked prior to and after introduction of fish. Fish were monitored at least ten times a day for behavioural anomalies that make them distinguishable from the control group (e.g. different swimming behaviour, changes in appearance, reduction or cessation of feed intake) and mortality, and where mortality occurred, dead fish were removed and recorded. For each sampling interval (2nd, 6th and 14th day after Sb exposure) 2 fish were randomly collected from each treatment group for the SCGE and MN assays. Peripheral blood was collected (~ 50 μL) with a small heparin-wetted syringe, through a cardiac puncture, and diluted in 80 μL of PBS in 2 mL Eppendorf Tubes® (Australia). Blood samples were suspended in PBS as it has been reported that cells suspended in PBS show fewer nonviable cells compared to other solutions (e.g. EDTA) (Ramsdorf et al., 2009; Strober, 2015).

2.5. Cell viability

Cell viability (indicated by intact cell membranes) was undertaken by the Trypan blue exclusion method before undertaking the genotoxic assays. A 0.4 % solution of Trypan blue in PBS at pH 7.2 was prepared and 15 μL added to 15 μL of the sampled blood cells suspended in the PBS, followed by gentle homogenisation. With the coverslip situated evenly, 10 μL aliquots of the homogenised mixture was applied to each chamber of a haemocytometer (Neubauer® chamber) which was immediately placed in the microscope (Olympus BX41TF, Japan). Using 100 × magnification, viable (clear) and nonviable/dead (blue) cells were counted within the grids on the haemocytometer. Percentage (%) cell viability was calculated (total viable cells/total cell count × 100). Multiple samples were prepared and only samples with cell viability ≥ 90 % were used for SCGE and MN assays.

2.6. In vivo alkaline (pH > 13) SCGE

The alkaline SCGE was performed according to the consistent methods described by Olive and Banath (2006), OECD (2014), Møller et al. (2020), and the manufacturer of the CometAssay® kit (Trevigen, Gaithersburg, Maryland, USA) with additional modifications based on our preliminary studies on silver perch. Briefly, a 50 μL aliquot of the blood-PBS diluted mixture was mixed with 500 μL of molten Low Melting Point (LMP) agarose at 37 °C (1:10 v/v), and 50 μL pipetted onto a two-well Trevigen CometSlide™ (Catalog #4250-050-03, Trevigen, Gaithersburg, Maryland, USA) per fish. Slides were placed at 4 °C in a refrigerator in the dark and allowed to gel for 30 min. Slides were gently removed and immersed in pre-chilled CometAssay® lysis solution supplemented with 10 % v/v DMSO to induce heme lysis. The slides were left on ice and protected from light and allowed to stand overnight (~ 16 h) for optimal cell lysis. All slides were carefully positioned horizontally at the anode end of the cold electrophoresis system (Trevigen® CometAssay® ES II, Catalog #4250-050-ES) while submerged in a freshly prepared pre-chilled alkaline electrophoresis buffer, pH > 13 (200 mM NaOH, 1 mM EDTA, prepared from a stock solution of 5 M NaOH and 200 mM EDTA, pH 10). Slides were then covered with a slide tray overlay and left in the solution for 1 h at room temperature (14–16 °C) for DNA unwinding and conversion of alkali-labile sites to single strand breaks. The electrophoresis run was conducted using the same electrophoresis buffer for 40 min at 21 V (1 V per cm) and 500 mA. The slides were drained of excess electrophoresis solution, immersed twice in distilled water for 5 min, and the samples were fixed in 70 % ethanol for 5 min before drying at 37 °C for 20 min.

For the staining procedure, each circle of dried agarose on slides was stained with 100 μL of SYBR Green I and placed in a 4 °C refrigerator for 10 min. Excess stain solution was drained off by tilting the slides, and then allowed to dry completely at room temperature in the dark. Immediate evaluation of the slides was at 200 × and 400 × magnification on an epifluorescence microscope (Nikon Eclipse 90i, Japan) with a 450–490 nM excitation filter, a 520 nM barrier filter, and a 505 dichromatic mirror. The distribution of DNA damage shown by the SCGE assay was assessed by scoring 50 randomly selected cell images for each gel slide, with a total of 100 images per fish sample. Only individual

nucleoids and non-overlapping cells were scored for each fish and images were photographed (camera Digital Sight DS-Ri1, Japan). Images were evaluated in a blind analysis with no identifiable labelling during analysis using image analysis software, OpenComet (Gyori et al., 2014). The percent tail DNA (% Tail DNA = 100–Head DNA) as determined by the software was used for quantification of % DNA damage.

2.7. Micronucleus test

A thin, uniform smear of the PBS diluted blood, obtained from the same fish of each treatment used in the SCGE assay, was placed on clean microscope slides and left to air-dry at room temperature overnight in a dust-free and moisture-free environment. Two primary slides were prepared for each fish. The slides were then fixed by submerging in absolute methanol for 20 min and left to air-dry at room temperature for 2 h.

For the staining process, the slides were stained in May-Grünwald's solution for 5 min, placed in working phosphate buffer (pH 7.2) for 1.5 min, and counter stained with Giemsa stain in distilled water (1:20 v/v) for 20 min to quantify and discriminate between PCE and NCE. All slides were rinsed in deionised water for 4 min and air-dried overnight. Cells were blindly scored using a light transmission microscope (Olympus BX41TF, camera DP71 U-TVO-63XC, Japan) at 100 × magnification. A minimum of 2000 erythrocytes per fish (1000 cells for each of the duplicate slides) were examined for the incidence of MN. Only cells with an intact cellular and nuclear membrane were scored. For the scoring of MN, the criteria were adopted from Fenech (2007). From the same microscope fields for screening MN, at least 1500 cells were scored to determine the PCE to NCE ratio as an index of cytotoxicity (Krishna and Hayashi, 2000).

Immature erythroblasts in the sampled blood on the third slide prepared were counted to determine frequency as a measure of their release into the circulatory blood stream following fish exposure to Sb (Abdel-Aziz et al., 2010).

$$\%\ \mathrm{MN} = \frac{\text{Number of micronucleated erythrocytes}}{\text{Total number of erythrocytes examined}} \times 100$$

$$\text{Cytotoxicity index} = \frac{\text{Number of polychromatic erythrocytes(PCE)}}{\text{Number of normochromatic erythrocytes (NCE)}}$$

2.8. Statistics

Statistical analysis on DNA migration as determined by the % tail DNA (in SCGE), incidence of MN (%) and PCE/NCE ratio was conducted with an IBM SPSS Statistics computer software program (version 22, SPSS Inc., Chicago, IL, USA) and Microsoft Excel (version 2016). The summary statistics including mean, median (50th), 75th, 85th, and 95th percentile values were calculated from the % tail DNA values of 50 cells scored for each SCGE gel to characterise the data distribution and minimise data loss since genotoxic stress (such as DNA migration) is frequently indicated by an increase in both median values and data dispersion (Duez et al., 2003). Arithmetic means were estimated for MN frequency and PCE/NCE ratios. These summary statistics were obtained first by averaging the SCGE gel and MN data for each SCGE and MN slide, with two slides prepared per fish, and from these values the cumulative means and standard deviations were calculated for each exposure concentration.

Data normality was first checked using normal probability plots and the Shapiro-Wilk test (Shapiro and Wilk, 1965), while Levene's test was used to analyse homogeneity of variance. Parametric tests were conducted at $P < 0.05$, and nonparametric tests analysed at $P < 0.01$ because nonparametric methods are often sensitive to high within-sample heterogeneity for SCGE data distribution (Duez et al., 2003; Lovell and Omori, 2008; Vasquez, 2010; Møller and Loft, 2014; Verd and Rottmann, 2015; Braafladt et al., 2016). For normally distributed data, a parametric independent Student's t-test was used to compare Sb exposure concentration group means, medians, 75th, 85th and 95th percentile values to that of the concurrent untreated control. A one-way analysis of variance (ANOVA) and a Tukey HSD's post hoc test was used to determine the presence of a dose response.

For data that could not be normalised, a nonparametric Mann-Whitney test (Mann and Whitney, 1947) was used to compare the summary statistics of Sb exposure concentration groups (i.e., means, medians, 75th, 85th or 95th percentile values) to the untreated control, while the presence of a dose response was determined by Kruskal-Wallis test, a nonparametric ANOVA based on ranks transformation (Conover, 1999) with Dunn's post hoc pairwise comparison test using a Bonferroni correction. The Kruskal-Wallis tests used Monte Carlo approximation (1, 000,000 random observations) to estimate a *P* value. Mann-Whitney test was also performed to determine significant difference in immature erythroblast counts between Sb treated fish and those of the untreated control.

Either a parametric ANOVA and Tukey HSD's post hoc or Kruskal-Wallis test with Dunn's post hoc pairwise comparison test and Bonferroni correction was used to compare dose groups and untreated controls for the MN frequency and PCE/NCE ratios depending on the distributional assumptions. Association between % MN and DNA damage (% tail DNA) was determined using Pearson or Spearman's correlation coefficients depending on data distribution. A linear regression between MN incidence and corresponding PCE/NCE ratios was used to determine the association between the two endpoints.

3. Results

3.1. Cumulative mortality in exposed fish population

Silver perch fish exposed to Sb(III) had 100 % population extinction before the 14th day of exposure at all concentrations (Table 1). In contrast, most fish survived to 14 days with a few sporadic deaths in all the Sb(V) exposure concentrations (Table 1). There was an apparent trend in the exposure binomial data with Sb(III) showing greater toxicity than Sb(V). Only 2 fish deaths were observed in the untreated control group (Table 1).

3.2. DNA damage-SCGE assay

The SCGE DNA migration images of the exposures are shown in Fig. 1. Significant DNA migration was observed for Sb(III) treated groups at 2 d of exposure compared to the control ($P < 0.01$), but the migration was independent of Sb concentration, as there was no significant difference in DNA migration between exposure concentrations (Kruskal-Wallis, $P > 0.01$) (Table 2). Further exposure of the same fish population to Sb(III) to 6 d showed no significant increase in DNA migration compared with the control ($P > 0.01$), but all remaining fish died before 14 d (Tables 1 and 2). Antimony(V) concentrations at different times of exposure had no effect ($P > 0.05$) on DNA integrity of silver perch at all exposure concentrations and exposure periods (Table 2).

3.3. Micronucleus frequency and PCE:NCE ratio

There was no significant increase in MN frequency in silver perch exposed to different concentrations of Sb(III) and Sb(V) compared to the untreated control (Figs. 2a and b and 3). With exposure to Sb(III), a significantly greater PCE/NCE ratio ($F_{3,12} = 11.777$, $P = 0.001$) was observed only at 1.8 mg L^{-1} at 2 d exposure (Fig. 2c).

The immature erythroblasts in silver perch at 1.8 mg L^{-1} Sb(III) exposure was the only treatment that showed a significant increase ($1.92 \pm 0.07 \times 10^6$/ mm^{-3}) compared to the untreated control ($1.46 \pm 0.05 \times 10^6$/ mm^{-3}) ($P < 0.01$). Linear regression analysis indicated poor correlation between PCE/NCE ratios and MN frequency for exposure to 1.8 mg L^{-1} Sb(III) ($R^2 = 0.161$, $P = 0.599$). For Sb(V)

Table 1
Physical conditions in the 14 d exposure-dose-response treatments and cumulative mortality of Silver perch. Values are mean ± SD(Min-Max).

Antimonyspecies	Concentration(mg L^{-1})	Dissolved oxygen (mg L^{-1})	pH	Temperature (°C)	Fish mortality(cumulative number of fish (not sampled for bioassay) dead)		
					2 d	6 d	14 d
Sb(III)	Control	7.19±0.69 (6.00-7.80)	8.00±0.04 (7.95-8.07)	25.59±0.65 (24.80-26.50)	0	0	2
	0.4	7.50±0.41 (7.11-8.10)	7.94±0.07 (7.81-7.99)	25.34±0.53 (24.60-26.10)	2	5	8
	0.9	7.59±0.78 (6.19-8.30)	8.01±0.02 (7.98-8.03)	25.80±0.80 (24.70-27.0)	1	4	8
	1.8	7.62±0.65 (6.89-8.40)	7.88±0.03 (7.85-7.92)	25.39±0.55 (24.80-26.30)	4	7	8
Sb(V)	Control	7.19±0.41 (6.00-7.80)	8.00±0.04 (7.95-8.07)	25.59±0.65 (24.80-26.50)	0	0	2
	0.9	7.48±0.44 (6.81-7.90)	8.15±0.10 (8.02-8.23)	25.78±0.36 (25.10-26.20)	0	0	3
	2	7.18±0.67 (6.23-8.00)	8.21±0.02 (8.19-8.25)	25.12±0.59 (24.10-26.10)	1	3	3
	5	7.65±0.48 (7.12-8.43)	8.19±0.10 (8.06-8.31)	25.55±1.01 (24.10-26.80)	0	3	4

The water hardness varied between 125 to 143 mg L^{-1} in all exposure tanks.

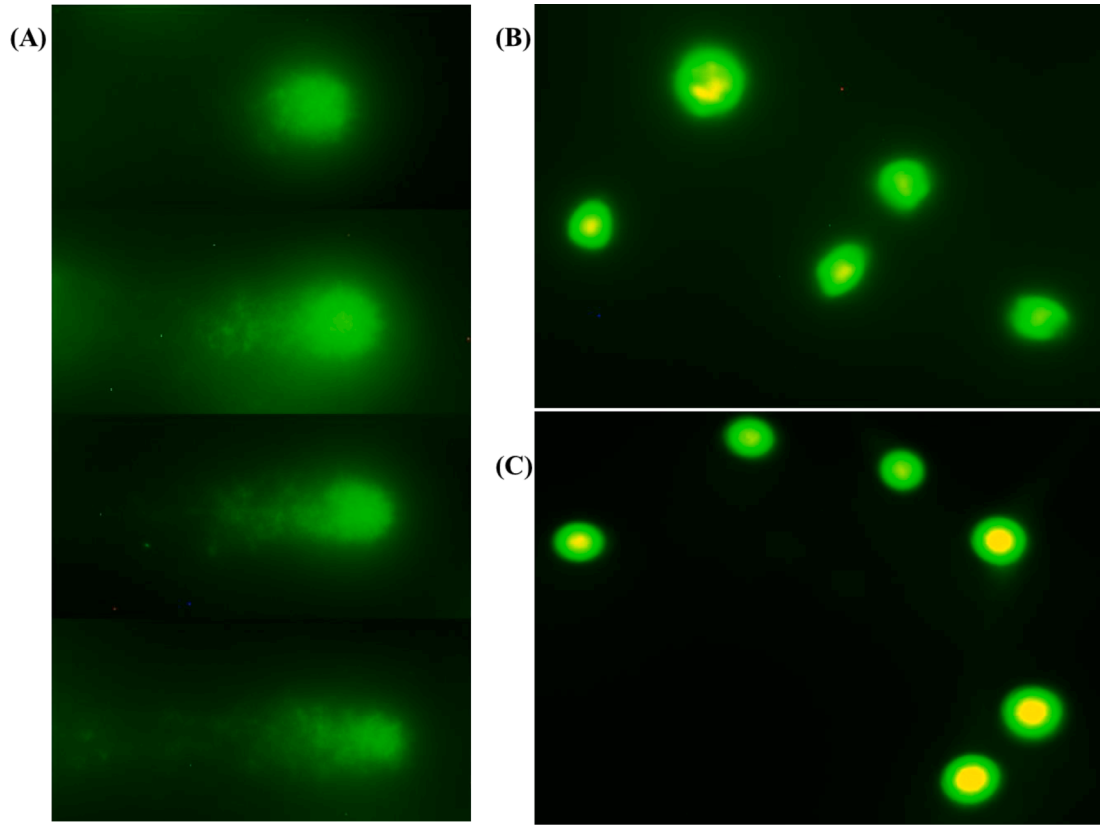

Fig. 1. SCGE assay. Erythrocyte cells of Silver perch showing: (A) DNA migration after exposure to Sb(III), (B) DNA images after exposure to Sb(V), and (C) Control DNA.

treatments, the PCE/NCE ratios for Sb exposed fish cells showed no statistical difference with those of the untreated control cells (Fig. 2d).

3.4. DNA migration-micronucleus correlation

There was no significant correlation between the SCGE and MN assay silver perch data for the Sb(III) exposures over the exposure time period of 14 d.

4. Discussion

4.1. Silver perch mortality in microcosms

In the present study, we tested the hypothesis that both Sb(III) and Sb (V) at sub-lethal and environmentally relevant concentrations would adversely affect the genomic integrity of a freshwater fish, silver perch. Exposure to Sb(III) also induced 100% mortality in test fish at all concentrations by 14 d. The greater mortality in silver perch exposed to Sb(III) (0.4–1.8 mg L^{-1}) over 14 d indicates that Sb(III) is more toxic compared to Sb(V), especially in light of the greater Sb(V) exposure concentrations used (0.9–5 mg L^{-1}). Other studies have also found Sb (III) to be more toxic than Sb(V) (reviewed in Obiakor et al. 2017a). The exposure concentrations used were considered sub-lethal at ≤ 1/10 of the 96 h LC50 value determined for silver perch (Connon et al., 2012; Gong et al., 2016; Sánchez-Bayo et al., 2016). These had been assumed to provide insufficient potency to cause mortality in test systems (Yang, 2014; Gong et al., 2016; Sánchez-Bayo et al., 2016; Parkinson et al., 2017). Nevertheless, mortality of all fish was observed in all Sb(III) treatments by 14 d and results indicated this was due to Sb(III) exposure, which implies that the range of concentrations used may be greater than the maximum tolerable dose for silver perch to Sb (III) over an extended period. For Sb(III), the three exposure concentrations were based on fractions of the 96 h LC50 (18 mg L^{-1}) estimated for silver perch exposed to Sb potassium tartrate in previous studies (Obiakor, 2017c), and

Table 2
Percent (%) tail DNA in erythrocytes of Silver perch after exposure to different concentrations of Sb(III) and Sb(V). All values in brackets are ±SD.

Sb(III) Exposure duration (day)	Concentration (mg L^{-1})	Mean	Median	75th	85th	95th	Sb(V) Concentration (mg L^{-1})	Mean	Median	75th	85th	95th
Day 2	Control	6.50 (1.07)	4.19 (2.49)	8.76 (0.89)	13.71 (4.65)	22.21 (9.38)	Control	6.50 (1.07)	4.19 (2.49)	8.76 (0.89)	13.71 (4.65)	22.21 (9.38)
	0.4	18.60 (1.54)*	14.87 (1.50)*	25.56 (5.0)**	31.77 (7.18)*	50.26 (16.95)*	0.9	10.28 (6.24)	8.88 (6.13)	13.61 (7.35)	16.41 (8.81)	22.28 (8.19)
	0.9	18.98 (2.05)*	15.74 (3.10)*	25.92 (3.84)**	31.68 (1.74)*	46.02 (4.23)*	2	9.88 (3.67)	7.74 (2.28)	13.51 (4.16)	17.98 (6.52)	28.78 (11.78)
	1.8	19.27 (2.25)*	14.49 (1.68)*	24.62 (1.52)**	31.61 (2.40)*	63.90 (13.62)*	5	10.49 (3.19)	8.35 (4.99)	13.95 (5.49)	18.35 (3.06)	35.05 (2.0)
Day 6	Control	9.42 (2.80)	5.68 (2.34)	13.21 (6.62)	19.87 (11.18)	33.22 (11.62)	Control	9.42 (2.80)	5.68 (2.34)	13.21 (6.62)	19.87 (11.18)	33.22 (11.62)
	0.4	11.77 (5.98)	8.36 (5.25)	14.97 (6.63)	20.51 (10.13)	34.03 (18.85)	0.9	10.03 (3.69)	7.30 (3.79)	14.50 (5.86)	19.78 (6.85)	30.13 (8.46)
	0.9	14.31 (5.53)	12.79 (5.99)	18.88 (5.43)	24.26 (6.38)	33.26 (7.60)	2	10.12 (3.47)	7.23 (3.58)	12.78 (4.86)	19.52 (7.14)	33.92 (9.63)
	1.8	15.55 (8.83)	13.39 (8.78)	21.26 (11.38)	26.34 (13.27)	36.21 (18.09)	5	9.69 (3.94)	6.86 (3.56)	14.16 (5.75)	17.99 (6.59)	32.57 (15.61)
Day 14	Control	10.99 (5.65)	8.42 (4.49)	15.02 (6.12)	20.69 (9.53)	31.52 (14.08)	Control	10.99 (5.65)	8.42 (4.49)	15.02 (6.12)	20.69 (9.53)	31.52 (14.08)
	0.4	-	-	-	-	-	0.9	10.86 (3.66)	8.94 (3.63)	14.13 (5.06)	18.03 (6.11)	30.29 (13.39)
	0.9	-	-	-	-	-	2	10.07 (3.97)	7.89 (4.16)	15.06 (6.76)	18.55 (7.67)	28.90 (8.49)
	1.8	-	-	-	-	-	5	11.23 (2.65)	10.26 (3.17)	16.52 (3.87)	19.20 (3.52)	26.29 (5.58)

* Significantly different from the untreated control at $P < 0.05$ analysed by Student t test.
** Significantly different from the untreated control at $P < 0.01$ as analysed by Mann-Whitney test.

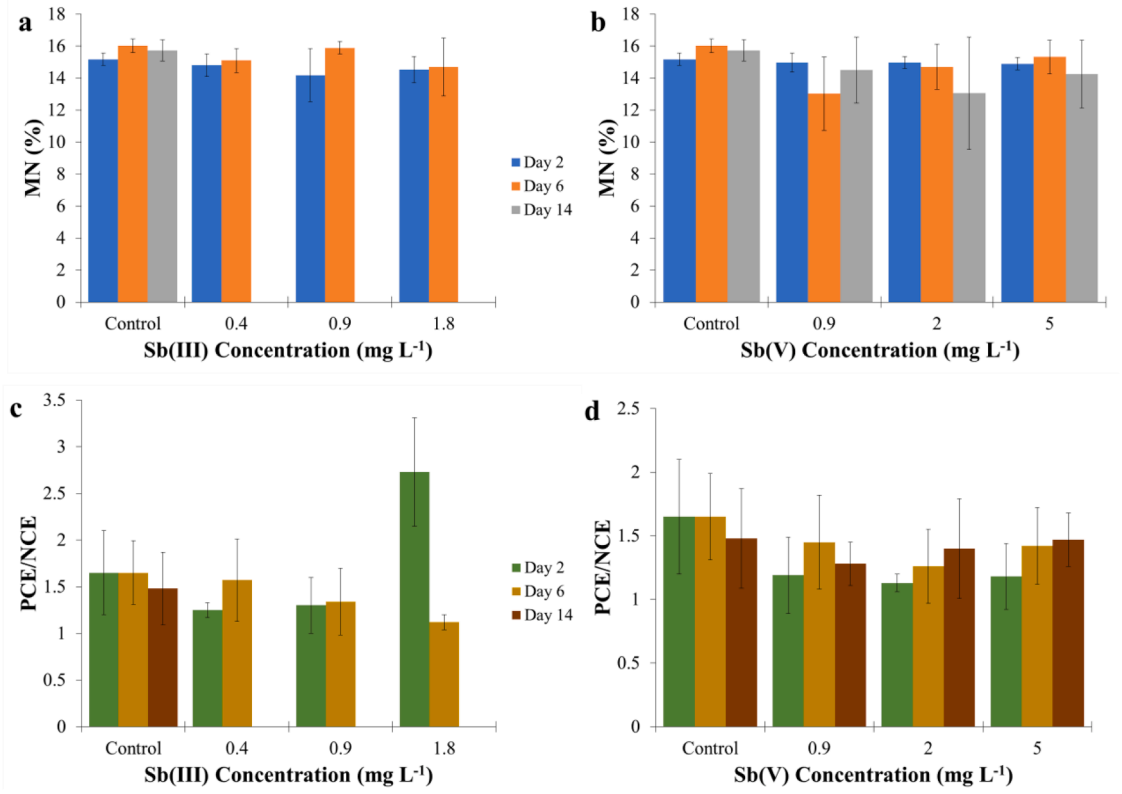

Fig. 2. Micronucleus (MN) frequency in erythrocytes (A and B), and polychromatic erythrocytes (PCE) to normochromatic erythrocytes (NCE) ratio (PCE/NCE) (C and D) of Silver perch exposed to different concentrations of Sb(III) and Sb(V) for 2, 6, and 14 d. Values are mean±SD.

included 0.4 mg L^{-1} (1/50 of 96 h LC50), 0.9 mg L^{-1} (1/20 of 96 h LC50) and 1.8 mg L^{-1} (1/10 of 96 h LC50). The 0.9 mg L^{-1} used for Sb(V) was reflective of the maximum Sb concentration detected in an Sb contaminated freshwater water in New South Wales Australia (Telford et al., 2009), while 2 mg L^{-1} and 5 mg L^{-1} Sb(V) were sub-lethal concentrations at 1/125 and 1/50, respectively of the approximate Sb(V) 96

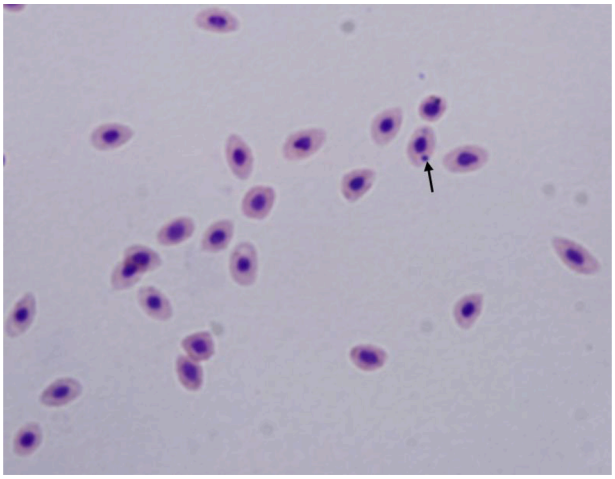

Fig. 3. MN assay. Erythrocytes of Silver perch showing micronucleated cell (arrow) in 1.8 mg L^{-1} Sb(III) on 2nd day of exposure.

h LC50 value (~ 259 mg L^{-1}) previously determined for silver perch (Obiakor, 2017c). Further, sensitivity of organisms, as well as assay conditions and exposure patterns, could have manifest in reduced survival over the extended microcosm exposure period (Ashauer et al., 2016). The only 2 fish deaths reported in the untreated control group could be explained by natural phenomenon as no effects other than lethal effects, such as different swimming behaviour, abnormal feeding, and or changes in fish appearance compared to the Sb treatment groups, were observed.

4.2. DNA damage and genotoxicity

The genotoxic effect of Sb as measured by DNA damage showed that Sb(III) induced a significant % tail DNA at 2 d of exposure. This was independent of Sb(III) concentration and continued exposure did not result in any additional significant DNA migration relative to the control. This may be explained by a DNA damage repair process occurring which prevented additional measurable damage during sample processing and/or electrophoresis (Burlinson et al., 2007; Vasquez, 2010). A shorter time (e.g., 6–24 h of the 14-day exposure) for sample collection may better elucidate the progression of DNA damage induced by exposure to Sb(III) at the concentrations used. Nevertheless, the SCGE assay indicated that exposure to Sb(III) induced genotoxicity. Our results are in accordance with observed positive DNA strand breaks and fragmentation induced by Sb(III) in both *in vitro* (Huang et al., 1998; Schaumloffel and Gebel, 1998) and *in vivo* (Cavallo et al., 2002) mammalian cell models.

In contrast, exposure to Sb(V) showed no significant alteration in DNA of silver perch for the total exposure period (2–14 days) under the SCGE assay conditions used. The SCGE assay conditions (Forchhammer et al., 2010; Azqueta et al., 2011; Ersson and Möller, 2011) and age of fish or sex (Ding et al., 2014) can strongly affect the extent of DNA migration detected in assays. Nevertheless, all preliminary investigations to optimise SCGE conditions also showed no DNA damage in the erythrocytes of silver perch with Sb(V) exposure (data not shown). Antimony(V) is reported in other studies to induce cellular toxicity through mutual interconversion with Sb(III) (Beyersmann and Hartwig, 2008). Under reductive conditions, Sb(V) may be converted to Sb(III), which then reacts with sulfhydryl groups of proteins in the cells (Gebel, 1997; Frézard et al., 2001; Beyersmann and Hartwig, 2008). *In vivo* reduction of Sb(V) to Sb(III) in human biological samples (whole blood, blood plasma, urine and hair) (Miekeley et al., 2002), macrophages (Hansen et al., 2011), and human blood (López et al., 2015) has been reported. The negative results in our study for DNA damage may indicate that Sb(V) was not reduced to Sb(III) after uptake. This may have been due to the pH of the silver perch erythrocyte microenvironments in the alkaline SCGE assay used in our study because in the phagolysosome of macrophages, Sb(V) was reduced to Sb(III) through non-enzymatic actions mediated by glutathione at a lower pH (pH 5) (Frézard et al., 2001).

4.3. Relationship between micronucleus induction and DNA damage

The nonsignificant incidence of MN indicated no clastogenic and/or aneugenic effects of Sb exposure from both Sb(III) and Sb(V) in silver perch. The observed DNA damage with Sb(III) exposure (SCGE assay) but absence of significant MN in silver perch treated with Sb(III) is not unexpected and could be related to many factors which moderated MN formation such as efficient detoxification of Sb and its reactive metabolites in the fish, modulation of xenobiotic-metabolising enzymes (Huuskonen et al., 1995), and destruction of micronucleated erythrocytes by the immune system and spleen (Baršienė et al., 2006; Udroiu, 2006; Bolognesi and Hayashi, 2011). Both SCGE and MN assays are considered complementary and measure different genotoxic endpoints with potential differences in response between the two tests (Vasquez, 2010; Zelazna et al., 2011; Gajski et al., 2019; Møller et al., 2020). Primary DNA damage observed by the SCGE assay is transient and could be readily repaired, compared to irreversible genome damage observed in MN test.

Further, there was no correlation between the % tail DNA migration and MN frequency at the different Sb(III) exposures. Other studies have shown that increased DNA damage, as determined from a SCGE assay, with exposure to potential genotoxicants has also resulted in other tissue and cell toxicities such as MN, necrosis, and histopathological damages (Huang et al., 1998; Sasaki et al., 1998; Burlinson et al., 2007; OECD, 2014). For example, Gebel et al. (1998) and Huang et al. (1998) showed both DNA damage and MN formation in Chinese hamster cells exposed to Sb(III). Similarly, Schaumloffel and Gebel (1998) demonstrated through SCGE that Sb(III) induced DNA strand breaks in human lymphocytes, and also found that sister chromatid exchanges were concurrently induced. Our results suggest, however, that under the assay conditions and number of samples used, MN incidence may not be a good marker of DNA damage triggered by Sb(III) in silver perch.

Our study demonstrates the importance of using a suite of genotoxic biomarker tests to fully understand the sub-lethal responses in organisms to contaminant exposure. The SCGE/MN assay protocol can provide an integrated approach to genotoxicity assessment (Recio et al., 2010; Bücker et al., 2012; Deutschmann et al., 2016), but our pilot study suggests that Sb(III) may produce DNA damage without MN formation. The use of a dye exclusion test for membrane integrity and metabolic competency (Strauss, 1991; Burlinson et al., 2007), tissue-specific plasma enzymes and proteins that are released early (1–2 h) from necrotic blood cells after apoptosis (Vasquez, 2010), or a low molecular weight DNA diffusion test (Tice et al., 2000), may better complement the SCGE assay than the MN assay, at least for Sb.

4.4. Cytotoxicity

The PCE/NCE ratio significantly increased at 2 d of fish exposure to 1.8 mg L^{-1} Sb(III) due to a decrease in the number of NCE and a corresponding increase in number of PCE. The concurrent significant increase in immature erythroblasts suggests rapid differentiation and multiplication of immature erythroblasts in silver perch to counteract the effect of Sb(III) on the cells (Witeska, 2013). There was no further change in the PCE/NCE ratio at 6 d exposure, a result possibly of a compensatory process such as efficient homeostatic fish haematopoiesis and release of matured erythrocytes from the spleen and kidney into the peripheral blood stream (Witeska et al., 2006; Witeska, 2013).

The significant DNA migration observed at 1.8 mg L^{-1} Sb(III) and the corresponding increased PCE/NCE ratio indicated that cyto-genotoxicity occurred in silver perch at this exposure treatment. In the

0.4 and 0.9 mg L^{-1} Sb(III) exposure treatments, however, significant DNA migration was not accompanied by a corresponding significant change in PCE/NCE ratio, suggesting that effects of Sb(III) at those concentrations under the conditions studied may be independent of cytotoxicity; however, further studies are needed to understand whether both genotoxic adverse outcomes follow different mechanistic pathways. Because no significant correlation was evident between MN frequency and PCE/NCE ratio, it appears that both endpoints are independent of each other for Sb exposure in silver perch. Based on PCE/NCE index, exposure to Sb(V) did not cause cytotoxicity for the erythropoietic cells examined.

Our study was limited by the fish samples available but, nevertheless, allowed for the systematic characterisation of Sb(III) and Sb(V) effects on DNA and cytological integrity in fish exposed using comet assay and micronucleus test. Further work required includes verifying number of scorable samples required for aquatic bioindicators as for mammalian cells (e.g., Tice et al. 2000) and evaluation of specific markers of exposure and effect.

5. Conclusion

This study provides the first evidence that at sub-lethal and environmentally relevant concentrations, Sb(III) may be genotoxic in the circulatory erythrocytes of the freshwater fish, silver perch, but not clastogenic and/or aneugenic effects under the experimental conditions used. Antimony(III) also appeared to be cytotoxic in silver perch erythrocytes. Silver perch exposed to Sb(V), at the similar concentrations, however, showed no DNA damage nor altered PCE/NCE cytotoxicity index. The study shows that the application of combined assays is essential to fully investigate the aquatic genotoxicity of Sb at sub-lethal concentrations. The SCGE assay seemed to be an effective early marker for Sb(III) genotoxicity, but our study showed that genotoxicity, as determined from DNA damage does not always manifest in other effects such as MN formation or cytotoxicity, at least with Sb(III) exposure in silver perch erythrocytes. Our findings suggest that the DNA damage and cytotoxicity in silver perch erythrocytes may translate into different biological processes leading to the two markers of Sb exposure and effects observed. These pilot data provide a background to advance understanding on mechanistic toxicity of Sb(III) and Sb(V) in aquatic organisms, but further investigation is needed to fully describe the multiple genotoxic markers of Sb eco-genotoxicity in freshwater ecosystems.

Ethics approval and consent to participate

The experimental protocol and animal handling procedures were approved by the Animal Ethics Committee of the University of New England in accordance with guidelines of the Australian Code for the Care and Use of Animals for Scientific Purposes (Authority No. AEC14-113).

Consent for publication

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Availability of data and materials

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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.

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