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Lethal concentration of methyl methanesulfonate in a new potential invertebrate model for ecotoxicology

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ABSTRACT

Sensitivity to different concentrations (0–200 mgL^{-1}) of the alkylating agent methyl methanesulfonate (MMS) was evaluated in the new potential invertebrate model, the amphipod *Quadrivisio lutzi* (Shoemaker, 1933) after 48 h of exposure in two separate bioassays. In the first bioassay (0, 50, 100 and 200 mgL^{-1} MMS), all amphipods at concentrations ≥ 50 mgL^{-1} MMS were dead before completing the 48 h of exposure, which was also observed in the second bioassay for concentrations ≥ 30 mgL^{-1} MMS. There was no significant effect of gender on average mortality. The median 48 h lethal concentration (LC_{50}) was 23.6 mgL^{-1} MMS for *Q. lutzi*. Therefore, 1/3 or less of this concentration may be used for positive control in sublethal conditions. The high sensitivity of *Q. lutzi* to MMS is discussed in comparison to other invertebrates.

KEY WORDS

Ecotoxicology, Amphipoda, *Quadrivisio lutzi*, Alkylating agent, Southeastern Brazil.

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Introduction

Amphipods have long been recognised as appropriate invertebrate models for ecotoxicological studies (Ingersol *et al.*, 1998; Schulz, 2003; Anderson *et al.*, 2004; Lee *et al.*, 2005; Melo and Nipper, 2007; Hindarti *et al.*, 2016; Artal *et al.*, 2017). They are among the most sensitive benthic organisms to anthropogenic disturbances such as pollution (Anderson *et al.*, 2004), including urban waste (de-la-Ossa-Carretero *et al.*, 2012), metals (Borgman *et al.*, 2005a; Roman *et al.*, 2007; Pérez-Landa *et al.*, 2008) and pesticides (Schulz, 2003). Amphipods bioaccumulate toxic substances that are naturally associated with sediments (Wang *et al.*, 2004; Borgman *et al.*, 2005b). Nonetheless, they may tolerate turbidity (Rowe *et al.*, 2002) and low levels of dissolved oxygen (Landman *et al.*, 2005). Amphipods and other aquatic organisms (Grosell *et al.*, 2007) may show variations in their sensitivity depending on their physiological conditions (Artal *et al.*, 2017), growth and reproductive stages (Pérez-Landa *et al.*, 2008) and degree of contact with sediments (de-la-Ossa-Carretero *et al.*, 2012). The freshwater/brackish amphipod *Quadrivisio lutzi* (Schoemaker, 1933) is found in coastal lagoons in the northern part of Rio de Janeiro state (Weber *et al.*, 2013). It inhabits the unstable environments of coastal lagoons, where it persists in part due to its high reproductive potential (Medeiros and Weber, 2016) and toleration to a wide range of salinities (freshwater to brackish). This amphipod have been used to study the genotoxic effects of petroleum (Weber *et al.*, 2013), gene expression (unpubl. data) and osmoregulatory responses (unpubl. data). The amphipod *Q. lutzi* has great potential as a local animal model for further studies, and efforts are being made to study its biology and to recognise its responses under controlled conditions to toxic compounds, such as methyl methanesulfonate (MMS). MMS ($C_2H_6O_3S$) is an alkylating agent commonly used as a reference substance or as a positive control in genotoxic studies (Bouffant *et al.*, 2008; Lewis and Galloway, 2008; Štambuk *et al.*, 2008; Lacaze *et al.*, 2010; Weber *et al.*, 2013). Ethyl methanesulfonate and MMS have been used as models to sudy responses to alkylating agents (Beranek, 1990). Alkylating agents may be endogenous (as a result of normal metabolisms) or exogenous, and their effects have been widely studied, either for inducing mutations and death or because of their use in human cancer treatment (Kleibl, 2002).

In the environment, alkylating agents such as methyl chloride, bromomethane, iodomethane, dimethyl sulphate and diethyl sulphate may come from the chemical industry, generated by plants and fungi or through the nitrosation of natural compounds (Kleibl, 2002; Štambuk *et al.*, 2008). Alkylating agents react with ring nitrogen (N) and extracyclic oxygen (O) atoms of DNA bases to generate a variety of covalent adducts ranging from simple methyl groups to complex alkyl additions (Fu *et al.*, 2012). MMS belongs to the monofunctional compound of the S_N2 mechanism, inducing mainly the formation of *N*7-methyl-guanine (N^7-meG) and *N*3-methyl-adenine (N^3-meA; Beranek, 1990).

Agents inducing predominantly *N*-methylations (S_N2 agents), such as MMS and DMS (dimethyl sulphates), exhibit a high clastogenic effect but low point mutagenic potency (Kaina, 2004). Therefore, our aim was to evaluate the susceptibility of the amphipod *Q. lutzi* to MMS and to establish it as a positive control for bioassays for organisms at this level.

Material and Methods

Amphipods of *Q. lutzi* (Fig. 1A) were found in the roots of aquatic macrophytes. Aquatic macrophytes with amphipods were transferred to the laboratory in plastic boxes filled with lagoon water, and then the amphipods were removed using plastic pipettes and transferred to flasks with filtered and Ultraviolet-sterilized lagoon water. Amphipods were obtained on 11 March and 8 April 2014 from Paulista Lagoon (22°13'52.6"S 41°32'14.2"W), Quissamã, in the north of Rio de Janeiro state, Brazil. Water parameters were measured with a multiparameter YSI-556 to determine salinity (0.6), dissolved oxygen (6.04 mgL^{-1}), conductivity (2.67 µS) and pH (8.4). Adult amphipods were separated by sex; males were identified by the size of the second pair of gnathopods and females by the brood pouch. Juveniles and ovigerous females were not used. Amphipods were transferred, in groups, to 125 mL finger bowls containing sterilized, filtered lagoon at 27 ± 0.5°C, changed daily during the 96 h acclimatisation period with 12/12 h (day/night) photoperiod. They were fed with 0.07 g vegetable sticks

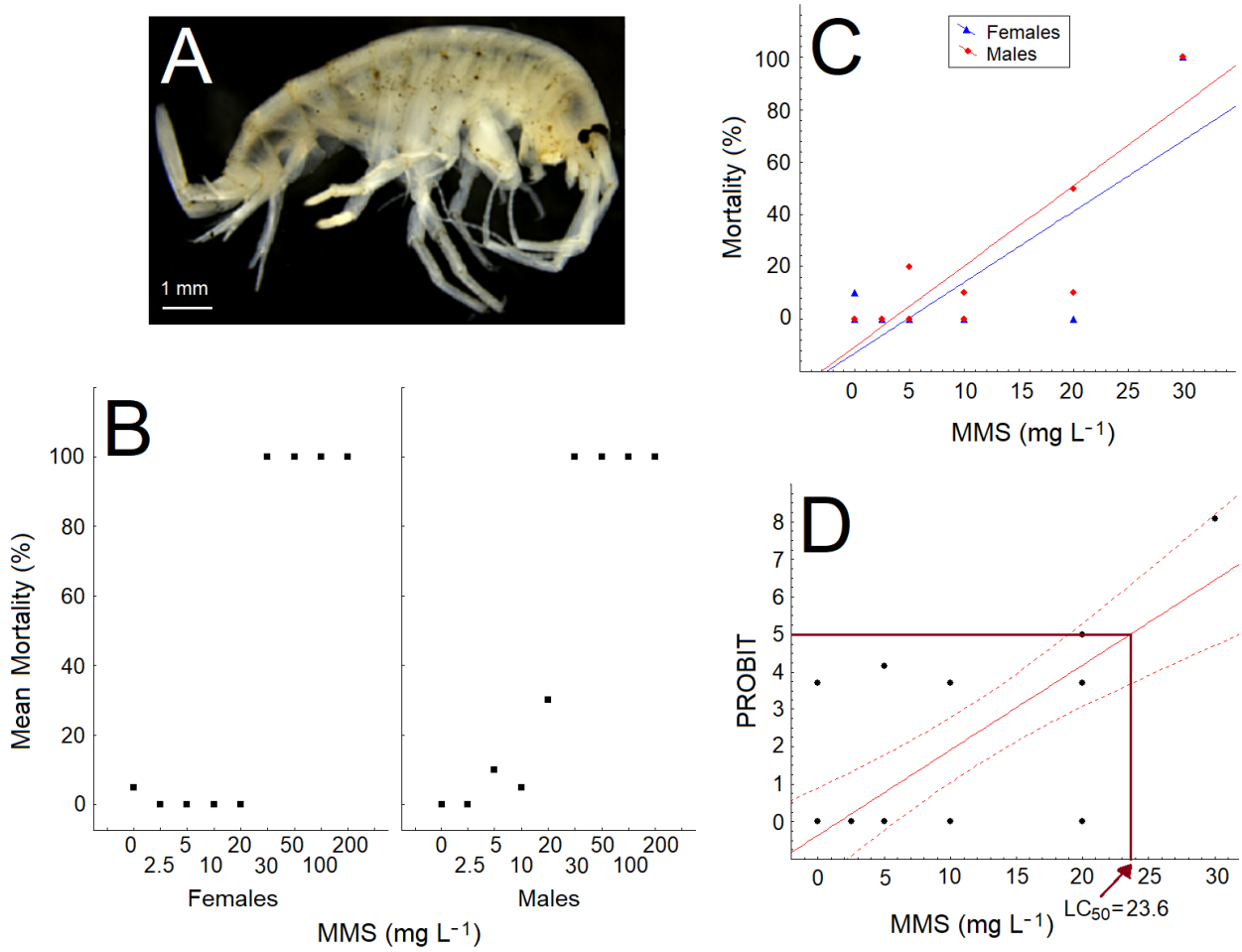

Figure 1. (A) The amphipod *Quadrivisio lutzi* (Schoemaker, 1933); (B) Average mortality of male and female amphipods *Q. lutzi* after 48 h of exposure to different MMS concentrations; (C) Regression lines that associate male and female average mortality with MMS concentrations; (D) Regression line that associates PROBIT values with MMS concentrations and extrapolation of the median lethal concentration for *Q. lutzi* from PROBIT 5 to MMS concentration.

from dried macrophytes obtained from the lagoon and with a commercial food supplement (Neon, MEP 200 complex, Alcon) added daily (1.24 mg/amphipod). A total of 400 amphipods were used for the bioassays. Exposure to all concentrations was performed in two replicates per gender, with 10 amphipods per replicate. Mortality was registered after 48 h. First, a broad range of MMS concentrations was tested: 0 (negative control), 50, 100 and 200 mgL^{-1}. After that, a narrow range of MMS concentrations (0, 2.5, 5, 10, 20 and 30 mgL^{-1}) was tested to determine the median lethal concentration (LC_{50}). Average mortality was obtained for each concentration and gender.

The effect of gender and all MMS concentrations on average mortality was evaluated by a factorial ANOVA. Mean comparisons were assessed using Tukey's test at 5% significance. Pearson linear regression was used to evaluate the association between average mortality and MMS concentration for males and females. The Univariate test of significance to compare slopes between gender regression lines was used. Median LC_{50} was determined by the PROBIT function, where mortality values of genders and replicates at different MMS concentrations were transformed to PROBIT values. The statistical package STATISTICA, v. 7 (Statsoft Inc.) was used for all analyses.

Results and Discussion

All of the amphipods (males and females) exposed to ≥ 30 mgL^{-1} of MMS were found dead within 48 h of exposure (Fig. 1B). No effect of gender was observed after factorial ANOVA ($F_{(1,22)} = 3.60$, $p = 0.071$), but, as was expected, a significant effect of MMS

concentrations on mortality was observed ($F_{(8,22)}$ = 210.37, $p < 0.001$). No effect of interaction gender-MMS concentration was observed ($F_{(8,22)} = 2.28$, p = 0.060). When evaluated by a non-protected Tukey's test of mean comparisons at each MMS concentration, was discovered that males showed significantly higher average mortality than females ($p = 0.031$) but only at a concentration of 20 mgL^{-1} of MMS (Fig. 1B; Tab. 1). At this concentration, males also differed significantly from the control (Tab. 1). Nonetheless, no significant differences were found between the controls and test groups at concentrations ≤ 20 mgL^{-1} of MMS after Tukey's test when comparisons were done for each concentration and for all amphipods (Tab. 1), nor when effects were evaluated for all treatments ≤ 20 mgL^{-1} of MMS together against controls (Treatment: $F_{(1,17)}$ = 0.226, $p = 0.640$; Gender: $F_{(1,17)} = 2.316$, $p = 0.146$).

Weber *et al.* (2013) found slight but significant differences in genotoxic response between genders (males were more sensitive) at the sublethal concentration of 10 mgL^{-1} of MMS. However, in the same article, females were found to be more sensitive than males to petroleum water-soluble fractions. Sornom *et al.* (2010) found that females of *Gammarus roeselii* (Gervais, 1835) were more sensitive than males to temperature and salinity stress. In the present study, where no significant effect of gender was observed, but at 20 mgL^{-1} of MMS males showed significantly higher mortality compared to females, we cannot rule out that high variation in males mortality could be related to their aggressive behaviour rather than the effect of MMS.

The results of the Pearson linear regressions to determine an association between average mortality of males and females and MMS concentrations are shown in Fig. 1C. A significant correlation was found between male and female average mortality and MMS concentrations (females: $F_{(1,10)} = 14.968$, $p = 0.003$, $R = 0.774$, $R^2 = 0.599$, $y = 12.954 + 2.707\,x$; males: $F_{(1,10)} = 38.006$, $p < 0.001$, $R = 0.890$, $R^2 = 0.792$, $y = 10.472 + 3.079\,x$). The univariate test of significance was used to compare the slopes of male and female regressions to evaluate the effect of gender on average mortality and indicated that there was no effect of gender on slopes ($F_{(1,20)} = 0.0349$; $p = 0.854$). The overall linear regression and the correlation that associates average mortality with MMS concentrations in *Q. lutzi* was significant ($F_{(1,22)} = 48.090$; $p < 0.001$; $R = 0.828$; $R^2 = 0.686$; $y = -11.7132 + 2.893\,x$). The PROBIT values that represent male and female mortalities showed a significant correlation with MMS concentration ($F_{(1,22)} = 48.090$; $p < 0.001$; $R = 0.777$; $R^2 = 0.603$; $y = -0.3608 + 0.2272\,x$; Fig. 1D). The extrapolation of PROBIT five over MMS concentration using this regression gave a median lethal mortality for the amphipod *Q. lutzi* of 23.6 mgL^{-1} of MMS after 48 h of exposure (48 h LC_{50} = 23.6 mg L^{-1} of MMS; Fig. 1D).

The concentration of 10 mgL^{-1} of MMS, in which mortality was not observed, was close to the value of 11 mgL^{-1} (0.1 mM) of MMS, at which Lacaze *et al.* (2010) found 9.5 times more DNA damage than controls in haemocytes of *Gammarus fossarum* (Koch, 1836) after five days of exposure. Similarly, Weber *et al.* (2013) found 2.5 times more DNA damage for *Q. lutzi* when treated with 10 mgL^{-1} MMS compared to the controls after two days of exposure.

At much higher concentrations of MMS (≥ 1,000 mgL^{-1}) than those used in our study (2.5 – 200 mgL^{-1}), severe damage has been found in the first stages of development of other invertebrates. Su (2010) found

Table 1. Results of Tukey's test of pair-wise mean comparisons between MMS-treated and control units, with the immediately anterior lower MMS concentration and between genders at each concentration (non-protected test).

Treatment (mgL^{-1})	Overall (n = 4)		*p* values after Tukey test				
			With lower	With Control			Males & Females (n = 8)
	Mean	SD		Males	Females	All	
0	2.5	4.6					0.999
2.5	0.0	0.0	0.999	1.000	0.999	0.999	1.000
5	5.0	10.0	0.994	0.991	0.999	0.999	0.991
10	2.5	5.0	0.499	0.999	0.999	1.000	0.999
20	15.0	23.8	0.499	0.008	0.999	0.314	0.031
30	100.0	0.0	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	1.000
50	100.0	0.0	1.000	*< 0.001*	*< 0.001*	*< 0.001*	1.000
100	100.0	0.0	1.000	*< 0.001*	*< 0.001*	*< 0.001*	1.000
200	100.0	0.0	1.000	*< 0.001*	*< 0.001*	*< 0.001*	1.000

that 9.1 mM (1,002.2 mgL^{-1}) of MMS slowed cleavage divisions by about 50% in embryos of the cnidarian *Hydractinia echinata* (Fleming, 1828); Bouffant *et al.* (2008) found that 10.0 mM (1,101.3 mgL^{-1}) of MMS completely inhibited the protein synthesis activity in early embryos of the sea urchin *Sphaerechinus granularis* (Lamarck, 1816). The high mortality of *Q. lutzi* at 30 mgL^{-1} (0.27 mM) of MMS observed in this study along with the high degree of DNA damage of *Q. lutzi* (Weber *et al.*, 2013) and *G. fossarum* (Lacaze *et al.*, 2010) at lower concentrations (10 – 11 mgL^{-1} of MMS) indicates a higher sensitivity of amphipods to MMS when compared with other invertebrate species, such as the aforementioned echinodermata and cnidaria species. Furthermore, the short range from sublethal to lethal concentrations of MMS (10 – 30 mgL^{-1}) in *Q. lutzi* may be explained by the drastic effects on protein synthesis and cell proliferation already observed in other invertebrates (Bouffant *et al.*, 2008; Su *et al.*, 2010).

The most frequent adduct on double-stranded DNA induced by MMS, N^7-meG, is considered relatively harmless because it does not block transcription and replication, but the adduct N^3-meA has caused death in bacteria and in mammals after generating formamidopyrimidine, which blocks DNA synthesis (Kleibl, 2002). The less-frequent adduct O^6-meG is the most dangerous, but MMS induces it in a lower proportion than the endogenous *N*-methyl-*N*-nitrosourea. DNA repair of the adduct O^6-meG mediated by base excision repair, present in all organisms, causes death by an uncertain mechanism; however, if repair does not occur, it leads to the point mutation A:T (Kleibl, 2002). The clastogenic effects of MMS may determine severe nuclear damage on haemocytes, which may lead cells to apoptosis. Formation and development of mature haemocytes in crustaceans involves proliferation and differentiation from undifferentiated haematopoietic cells, which are continuously and proportionally produced throughout the animal's lifetime (Lin and Söderhäll, 2011). The protein Astakine 1 (Ast1), found in crustaceans, is responsible for inducing the proliferation of undifferentiated haemocytes, which, under haematopenia, stimulates the rapid production and release of new cells from the haematopoietic tissue (Lin and Söderhäll, 2011). The time taken to produce new cells, which represents the recovery time, is after 24–48 h, as observed in crayfish after microbial infection (Söderhäll *et al.*, 2003). In the amphipod *Q. lutzi*, it is expected that new cells are already in the circulatory systems after 24 h of chemical damage based on the recovery of haemocytes after petroleum exposure (Weber *et al.*, 2013). In *Saccharomyces cerevisiae*, after MMS exposure, it was found that nuclear proteins associated with DNA repair, cell-cycle checkpoints and transcriptions were over-represented, suggesting events of DNA repair, apoptosis and induction of cell proliferation (Begley *et al.*, 2004). Nonetheless, in the few invertebrates that have been studied, the arrest of protein synthesis activity after exposure to MMS (Bouffant *et al.*, 2008) may also interrupt the synthesis of Ast1, which may accelerate death in amphipods.

We conclude that the amphipod *Q. lutzi* is highly sensitive to MMS. Therefore, MMS at concentrations of 1/3 of the 48 h LC_{50} (23.6 mgL^{-1}) may be used for positive control of bioassays with potentially genotoxic substances. To better understand the effects of MMS in amphipods, further studies are needed at the molecular and cytological levels.

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