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Humic substances and the water calcium content change the toxicity of malachite green

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Summary

Laboratory experiments were conducted to test interactive effects of calcium (Ca^{2+}) content and the presence of humic substance (HS) on malachite green (MAG)-induced toxicity in fish embryos and larvae by means of a semistatic 144-h-embryo-larval-test with zebrafish (*Danio rerio*). Two kinds of reconstituted water samples were used to produce the test media by mixing salts into deionized water resulting in either hard water (↑Ca − HS), or soft water (↓Ca − HS). By adding HS two additional test media were produced (↑Ca + HS, ↓Ca + HS). MAG was tested in concentrations of 0.05, 0.10, 0.15, 0.20, 0.25 mg L^{-1}. The toxicity ranking of MAG (mg L^{-1}) to embryos based on 96-h-LC_{50} in the different test water samples is: ↑Ca − HS (0.061) > ↑Ca + HS (0.123) = ↓Ca − HS (0.12) ≥ ↓Ca + HS (0.134) and on 144-h-LC_{50} to larvae is: ↑Ca − HS (0.038) > ↑Ca + HS (0.06) > ↓Ca − HS (0.077) = ↓Ca + HS (0.077). Mortality of all the groups was significantly different ($P < 0.05$). Increased Ca^{2+} concentrations did not protect zebrafish embryos and larvae from MAG-induced toxicity. At high Ca^{2+} conditions, the mortality of the embryos as well as of the larvae is reduced in the ↑Ca + HS group relative to the ↑Ca − HS group. Thus, at high Ca^{2+} conditions the HS does affect the MAG-induced mortality. The mechanism which causes the higher toxicity of MAG in the presence of higher Ca^{2+} concentrations is poorly understood. A probable explanation could be the stimulation of the calcium-binding protein calmodulin as well as the calmodulin kinase II in cell membranes in the presence of high Ca^{2+} concentrations.

Introduction

To control external fungi in ornamental fish breeding and aquaculture, the inexpensive therapeutic dye malachite green (MAG) is widely used. Owing to its cancerogenic properties, MAG application is therefore restricted to the very early developmental stages. The toxicity as well as successful use of chemicals frequently depends on dosage, abiotic parameters (e.g. temperature, hardness, organic load or pH (Gilbert et al., 1979; Alderman, 1985)), the species, and the developmental stages of fish (Okafor and Taege, 1996). Little, however, is known about the impact of humic substances (HS) interacting with other water ingredients on the toxicity of therapeutics. Humic substances are substances in 'sui generis' (Ziechmann, 1996) with a high structural variability and different molecular size. Nevertheless, some of their functional properties, such as binding of charged ions to the functional groups, are similar. Many studies have been conducted on the toxicity of metal ions in the presence of HS. Moreover, HS were found to alter the properties of pesticides and other xenobiotics in aquatic systems (McCarthy and Jimenez, 1985; Chin et al., 1997; Steinberg et al., 1997; Klaus et al., 1998a,b; Haitzer et al., 1999; Schulten and Leinweber, 2000; Kopinke et al., 2001). Oikari et al. (1992) found an increase in toxicity of some organic contaminants to *Daphnia magna*, which may be due to the altered bioconcentration or bioavailability of the pollutants. Meinelt et al. (2001b) describes the interaction between HS, cadmium and calcium (Ca^{2+}) changing the toxicity of cadmium to juvenile fish. HS are capable of binding hydrophilic contaminants on functional groups (Fent, 1998; Steinberg, 2003). Nevertheless, information concerning interaction of HS and organic therapeutic agents like MAG is scarce. Ca^{2+} is often described as an antidote reducing the toxicity of metals and xenobiotics to fish. That this is not necessarily true was shown by Meinelt et al. (2001a, 2002). However, no information is available on the interaction of MAG, HS and Ca^{2+}. But it is of general interest whether or not there is any interaction between MAG, HS and Ca^{2+} which possibly changes toxicity of the dye. If HS or Ca^{2+} change the toxicity of MAG, the presence of HS and varying Ca^{2+} concentrations would lead to alterations in the therapeutical index of this dye while using MAG as a treatment in fish breeding. Laboratory experiments were therefore conducted to examine potential effects of the Ca^{2+} content and the presence of HS on MAG-induced toxicity.

Materials and methods

We tested the toxicity of five MAG concentrations in four test media containing different concentrations of Ca^{2+} and HS. Two kinds of reconstituted water samples were used to produce the test media by mixing salts ($CaCl_2 \cdot 2\ H_2O$; $MgSO_4 \cdot 7\ H_2O$; $NaHCO_3$; KCl) into deionized water, resulting in either hard water (↑Ca − HS, 2 mmol L^{-1} Ca^{2+}) or soft water (↓Ca − HS, 0.2 mmol L^{-1} Ca^{2+}). By adding HS in concentrations of 5 mg L^{-1} [C], two additional test media were produced (↑Ca + HS, ↓Ca + HS). Finally, the test media were: (1) ↑Ca − HS; (2) ↑Ca + HS; (3) ↓Ca − HS and (4) ↓Ca + HS (Table 1). MAG was added to the test media in nominal concentrations of 0.05, 0.10, 0.15, 0.20, 0.25 mg L^{-1} MAG. Thus there was a total of 20 conditions tested (5 MAG concentrations × 4 Ca/HS combinations). The MAG was obtained from Sigma-Aldrich GmbH, Germany (97% dye content).

Table 1
Chemical parameters of the test water samples used to test the influence of Ca^{2+} and HS on MAG toxicity

Parameter	↑Ca − HS	↑Ca + HS	↓Ca − HS	↓Ca + HS
Calcium [mmol L^{-1}]	2	2	0.2	0.2
HS (dissolved organic carbon) [mg L^{-1} C]	0	5	0	5

Water conditions in all test water samples were: magnesium 0.5 mmol L^{-1}, potassium 0.077 mmol L^{-1}, sodium 0.77 mmol L^{-1}, temperature 26 ± 0.2°C, dissolved oxygen 7.0 ± 0.5 mg L^{-1}, pH 7.47–7.97.

The HS used in the test media was obtained by physical isolation. Water from Luther Marsh, Ontario, Canada, was centrifuged (Westphalia, 10 000 ***g***), filtered (glass-fibre filter, 1.0 μm pore-size) and then passed through a Pellicon 0.2 μm tangential-flow filtration unit (Millipore, Bedford, MA, USA). Potential metal concentrations from the HS were reduced by passage of the filtrate through a cation-exchange resin (H^+ form, AG50W-X8, BioRad). The Luther Marsh HS had a carbon : nitrogen ratio of 1 : 140 and a carbohydrate : protein ratio of 1 : 3820. Concentrations of dissolved organic matter were measured by high-temperature combustion (Beckman 915B total carbon analyser) after sample acidification with 30% phosphoric acid to remove inorganic carbonates.

Zebrafish (*Danio rerio*) were used as a test model, as this species is increasingly used in ecotoxicology because of their convenience for laboratory studies (Westerfield, 1995). Spawning zebrafish were kept at 26°C in modified glass aquaria with a 80 L volume of tap water. The bottom of each aquarium contained a 4-mm metal grid with a 4-mm mesh through which the spawned eggs passed, thus protecting them from predatory parents. Below the metal grid, a funnel-shaped base led into a tube through which the eggs were collected. After collection, the eggs were cleaned with 26°C test medium.

Zebrafish embryos reaching the four- to eight-cell stage were exposed to different concentrations of MAG for 144 h. The standardized 144-h-embryo-larval-test includes the most sensitive developmental stages of fish. Four controls were established. Twenty eggs were placed into each Petri dish. Each investigation was repeated three times consecutively. The respective test solutions were replaced every 24 h.

Toxic effects of MAG were determined by analysing the mortality of the embryos or larvae. Mortality was recorded every 24 h. Individuals were followed up to 144 h, after which all embryos and larvae were counted. For each population, the mortality was estimated by the Kaplan–Meier estimator (Kaplan and Meier, 1958). The LOG-RANK test (Rosner, 2000), a powerful and robust non-parametric procedure for testing for differences in mortality within populations, was used to detect significant differences among the test groups. Significance level was set at $P < 0.05$. To improve the statistical power, the observations from the three investigations were combined in an additional analysis. The 144-h-LC_{50} for zebrafish embryos and larvae for each test medium were calculated by probit analysis (Finney, 1971). The statistical analyses were performed using Statistical Package for the Social Sciences (SPSS, 1989–1997).

Results

All applied media caused mortalities among the zebrafish embryos and larvae. Both the 96-h-LC_{50} and 144-h-LC_{50} values are given in Table 2. The toxicity ranking of MAG to embryos based on 96-h-LC_{50} in the different test water samples is: ↑Ca − HS > ↑Ca + HS = ↓Ca − HS ≥ ↓Ca + HS, and to larvae based on 144-h-LC_{50} is: ↑Ca − HS > ↑Ca + HS > ↓Ca − HS = ↓Ca + HS. The mortality of all groups was significantly different ($P < 0.05$).

Discussion

According to the 144-h-LC_{50}, the ↓Ca + HS- and ↓Ca − HS groups revealed an identical mortality when exposed to MAG. This means that at low Ca^{2+} concentrations the HS does not affect the MAG-induced mortality to larvae. Elevated Ca^{2+} concentrations clearly did not protect zebrafish embryos and larvae from MAG. At high Ca^{2+} conditions the mortality of the embryos as well as of the larvae is reduced in the ↑Ca + HS group relative to the ↑Ca − HS group, indicating that at high Ca^{2+} conditions the HS does affect the MAG-induced mortality. The LC_{50} of the ↑Ca − HS group being half that of the other groups reflects the high potential of MAG-induced toxicity for embryos and larvae in the presence of high Ca^{2+} concentrations. As the MAG-induced toxicity is reduced in the ↑Ca + HS group, relative to the ↑Ca − HS group, some Ca^{2+} might have bound to the HS. In the three groups with lower MAG-induced toxicity (↑Ca + HS, ↓Ca − HS, ↓Ca + HS) the Ca^{2+} concentration is low as a result of low nominal concentrations or binding of Ca ions to HS. Therefore the high toxicity in the ↑Ca − HS group must be caused by the enhanced Ca^{2+} concentrations. This raises the question of how Ca^{2+} increases the MAG-induced toxicity. The mechanism which causes higher toxicity of MAG in the presence of higher Ca^{2+} concentrations is poorly understood. A probable explanation could be the stimulation of the calcium-binding protein calmodulin (CaM) as well as the calmodulin kinase II (CaMKII) in cell membranes (Yingst et al., 2001) in the presence of high Ca^{2+} concentrations. Saimi and Kung (1994) found that the Ca^{2+}–CaM binding activates ion channels. A Ca^{2+} binding to CaM leads to co-assembled complexes of pore-forming Ca^{2+}-activated potassium channels, alpha subunits and CaM (Keen et al., 1999). While stimulating Ca-binding proteins and forming pores in the presence of higher Ca^{2+} concentrations, the transport of the dye through the membranes by means of the CaM is conceivable. This scenario could explain our findings of the higher toxicity of MAG in the presence of higher Ca^{2+} concentrations. However, it was not in the context of our study and merits further research.

Our results indicate an influence of Ca ions and, indirectly, HS on MAG-induced toxicity. As HS and Ca^{2+} interactively alter the toxicity of MAG to embryos and larvae, the

Table 2
96-h-LC50 (embryos) and 144-h-LC50 (larvae) of MAG [mg L^{-1}] for each of the test water samples

	96-h-LC50	144-h-LC50
↑Ca − HS	0.061	0.038
↑Ca + HS	0.123	0.060
↓Ca − HS	0.120	0.077
↓Ca + HS	0.134	0.077

therapeutic index might also be affected by HS. If the toxicity of MAG to early developmental stages and juvenile fish changes with the Ca^{2+} content, the toxicity as well as the efficacy of the MAG treatment would presumably also change in the same direction. Ca^{2+} clearly does not protect early life stages of fish against MAG-induced toxicity. This should be taken into account while using MAG to treat particularly early developmental stages of fish.

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