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Unsuitable use of DMSO for assessing behavioral endpoints in aquatic model species

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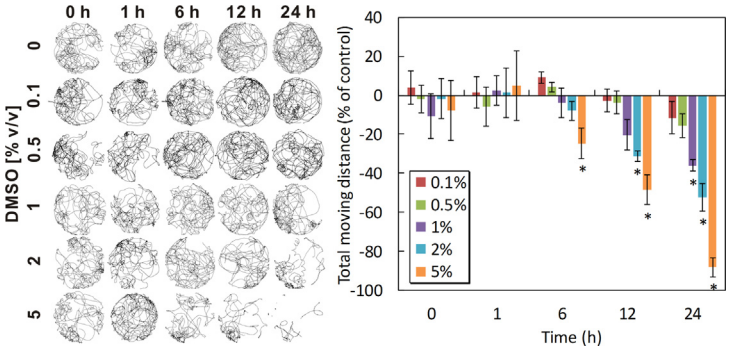
HIGHLIGHTS

- DMSO concentrations used to test highly hydrophobic chemicals affect sublethal behavioral endpoints.
- DMSO perturbs behavioral traits in a time-dependent manner.
- Locomotory responses of aquatic model species to carrier solvent are rapid.
- The effects are observed in both invertebrates and vertebrate aquatic models.
- Results of behavioral tests can be significantly influenced by carrier solvent.

GRAPHICAL ABSTRACT



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ABSTRACT

Dimethyl sulfoxide (DMSO) is a universally used aprotic solvent with the ability to permeate biological membranes and thus is commonly used to achieve appropriate biological availability of hydrophobic toxicants. While DMSO as a carrier medium has a reportedly low toxicity and is routinely employed in ecotoxicology, very little is known about its effect on dynamic behavioral parameters. This study presents a comparative analysis of the lethal and behavioral effects of exposures to DMSO concentrations of 0.1–10% on several test species such as: neonates of the freshwater crustacean *Daphnia magna*, nauplii of the marine crustacean *Artemia franciscana*, the marine crustacean *Allorchestes compressa*, embryos and larvae of the freshwater fish *Danio rerio*. The results demonstrated that DMSO did not cause statistically significant mortality even at concentrations close to 1% but induced clear and significant behavioral abnormalities in response to sublethal concentrations on all test species. These included hypoactivity syndrome in *A. franciscana*, *A. compressa*, *D. magna* and zebrafish larvae while a slight time-dependent hyperactivity response was observed in zebrafish embryos. For the majority of test species, behavioral changes such as moving distance, acceleration and burst movement were often observed during the first hours of exposure. These results indicate that caution should be exercised when using DMSO as a carrier solvent in experiments assessing behavioral endpoints.

1. Introduction

Hydrophobic toxicants and drugs exhibit notoriously low water solubility and this greatly affects the dilution of such chemicals in the test

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medium of diverse aquatic toxicity tests (Green and Wheeler, 2013; Hutchinson et al., 2006). Therefore, bioassays often require application of appropriate carrier solvents to solubilize many organic test compounds and allow permeation of biological membranes and thus achieve the chosen concentration gradient of hydrophobic test substances. The concentration of the carrier medium used for bioassays should not induce any biochemical or physiological alterations that can influence test endpoints (Green and Wheeler, 2013; Hutchinson et al., 2006). Dimethyl sulfoxide (DMSO) is a widely used aprotic organic solvent that was for many years perceived as the gold standard carrier vehicle due to its broad solubilizing capability. According to OECD recommendations, concentrations of DMSO employed for the dissolution of poorly water-soluble substances should not exceed a maximum of 100 mg/L (0.01% v/v) (OECD, 2002; OECD, 2012). Despite those recommendations, DMSO is commonly employed in many bioassays at concentrations of up to 10% v/v with apparent lack of toxic effects (Galvao et al., 2014; Kais et al., 2013). The main reasons for broad applications of DMSO are: (i) low toxicity, (ii) effectiveness to solubilize a wide range of polar and nonpolar compounds, (iii) excellent ability to permeate biological membranes without inducing structural integrity changes, and (iv) inhibition of bacterial growth in aquatic test samples (Kais et al., 2013; Sum and de Pablo, 2003).

Several recent reports demonstrated that at concentrations as low as 0.5–2.0% (v/v) DMSO can induce apoptosis in multiple in vitro models such as rat retinas, neuronal cell lines, murine lymphoid organs, hepatocytes and cochlear organotypic cultures (Aita et al., 2005; Banič et al., 2011; Galvao et al., 2014; Hanslick et al., 2009; Qi et al., 2008; Sumida et al., 2011). DMSO was also reported to induce apoptosis in vivo during the development of the central nervous system of C57Bl/6 mice embryos (Hanslick et al., 2009). A report by Nasrallah et al. (2008) demonstrated that DMSO could produce global gene expression changes in rat hepatocytes and even modulate brain metabolism in guinea pigs. It was further observed that, despite there being no apparent effects on survival, it can induce the occurrence of developmental abnormalities and stimulate the expression of Hsp70 stress proteins in zebrafish embryos (Chromcova et al., 2012; Hallare et al., 2006). Similarly, Hallare et al. (2004) demonstrated that responses of zebrafish embryos exposed to a drug diclofenac can be significantly altered by DMSO-triggered expression of Hsp 70 stress proteins. Low concentrations of DMSO were also shown to inhibit metabolism of ethoxyresorufin in zebrafish larvae (David et al., 2012). Moreover in particular relevance to the current study findings by Maes et al. (2012) showed reported brain necrosis and swim bladder effects after exposure of zebrafish larvae to concentrations of 2.5% DMSO. Such effects are of high relevance to behavioral alterations.

Despite ubiquitous usage of DMSO as a carrier medium and existing reports on its molecular and physiological effects very little is still known about its impact on behavioral responses. The latter are increasingly postulated as sub-lethal alternative endpoints to traditional mortality testing in ecotoxicology (Chevalier et al., 2015; Hellou, 2011; Melvin and Wilson, 2013; Zein et al., 2014). Behavioral bioassays are highly integrative and have distinct benefits for assessing aquatic toxicity because behavioral responses occur rapidly and can be used as an "early warning" signal of chemical stress (Chevalier et al., 2015; Hellou, 2011; Melvin and Wilson, 2013; Zein et al., 2014). In this regard, only a handful of studies have used behavioral endpoints to evaluate the sub-lethal effects of DMSO. To date, the majority of these studies evaluate the behavioral effects of DMSO on planarians species. Short-term exposure to DMSO (up to 1 h) had significant, but reversible, effects on locomotor activity of freshwater planarian *Dugesia japonica* (Yuan et al., 2012). Pagan et al. (2006) demonstrated that DMSO was toxic to planarians at concentrations above 5% when exposed for >2 min. Furthermore, sub-toxic concentrations of DMSO induced reversible inhibition of motility and several types of behavioral responses such as "corkscrew"-like movements, twitching and pharynx protrusion during exposures shorter than 10 min. In contrast to the above studies freshwater planarians *Schmidtea mediterranea* exhibited increased motility in response to sub-lethal concentrations of the solvent (Stevens et al., 2015).

Surprisingly, despite a widespread use of *Danio rerio* as a model organism in biomedicine, drug discovery and ecotoxicology only two studies investigated effects of DMSO on locomotor activity of zebrafish (Chen et al., 2011; Sackerman et al., 2010). Sackerman et al. (2010) observed anxiolytic effects in adult zebrafish exposed to 0.05% DMSO for 4 min. On the contrary, Chen et al. (2011) demonstrated increased swimming distance of zebrafish larvae following a 6-minutes exposure to a range of DMSO concentrations.

This study aims to provide a comparative analysis of the lethal and behavioral effects of exposures to DMSO concentrations of 0.1–10% on several test species such as: neonates of the freshwater crustacean *Daphnia magna*, nauplii of the marine crustacean *Artemia franciscana*, the marine crustacean *Allorchestes compressa*, embryos and larvae of the freshwater fish *Danio rerio*. The effects of exposure to sublethal concentrations of DMSO were evaluated against an array of behavioral responses characteristic for each species.

2. Materials and methods

2.1. Test organisms and maintenance conditions

Dormant cysts of the marine crustacean *A. franciscana* and ephippia of *D. magna* were hatched and cultured according to the Artoxkit-M and Daphtoxkit-F (MicroBioTests Inc., Belgium) standard operating protocols, respectively. *A. compressa* culture was established from specimens collected at Swan Bay in Victoria, Australia. *A. compressa* were held in an aerated aquatic system on a 12 h:12 h light: dark cycle at 18.0 ± 1.0 °C and pH of 8.1–8.3. Wild-type adults of zebrafish *D. rerio* (AB line) were held in a recirculating aquatic system on a 14 h:10 h light:dark cycle at temperature of 27.5 ± 0.5 °C and pH of 7.5–8.0. The culture conditions were monitored daily. Eggs were collected from a random pair mating, and embryos were pooled between the pairs. Fertilized eggs were collected within an hour of spawning and transferred to embryonic medium E3, consisting of 146 mg/L NaCl; 6.3 mg/L KCl; 24.3 mg/L $CaCl_2$; 40.7 mg/L $MgSO_4$. Embryos were incubated in E3 solution and held in a dark incubator at 28.0 ± 1.0 °C until use. Zebrafish stock was treated according to Monash University Animal Care regulations.

2.2. Lethal toxicity testing

For all lethal toxicity tests, at least three independent runs were performed in four replicates (each replicate consisting of an entire multiwell plate) for each concentration tested, unless otherwise indicated. Test standard solutions were prepared by diluting DMSO (Sigma-Aldrich) in corresponding culture mediums in order to yield six increasing concentrations, 0.1, 0.5, 1, 2, 5, and 10% (v/v) and applied to all toxicity tests except for zebrafish embryo test, where 0.1, 0.5, 1, 2, 4, 6, and 10% (v/v) concentrations were used. Negative control conditions for all tests were performed with medium alone.

Toxicity tests with *A. franciscana* and *D. magna* were performed according to Artoxkit-M and Daphtoxkit-F (MicroBioTests Inc., Belgium) standard operating protocols, respectively. Briefly, nauplii and neonates were exposed to selected concentrations of DMSO for 48 h in 24-well multiwell plates at 25.0 ± 0.5 and 20.0 ± 0.5 °C, respectively. Animals were exposed in groups of 5 per well and dead animals were excluded from the well. The number of dead organisms were counted manually every 24 h. Organisms were considered dead in absence of reaction for 10 s after gentle prodding. Maximum accepted mortality in negative controls did not exceed 5%.

A. compressa tests were performed as described previously (Cartlidge et al., 2017). Briefly, ten adult (~2 mm in length) intermolt amphipods were collected from the laboratory cultures, randomly loaded into 1 L sealed glass jars spiked with DMSO selected concentrations