Chemosphere 199 (2018) 372–381

Contents lists available at ScienceDirect

Chemosphere

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

# $^{1}$H NMR-based metabolomics reveals interactive effects between the carrier solvent methanol and a pharmaceutical mixture in an amphibian developmental bioassay with *Limnodynastes peronii*

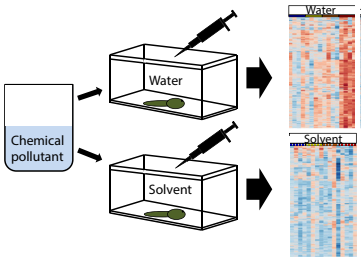
Steven D. Melvin [a, *], Oliver A.H. Jones [b], Anthony R. Carroll [c], Frederic D.L. Leusch [a, b]

[a] Australian Rivers Institute, Griffith University, Southport QLD, 4222, Australia
[b] Australian Centre for Research on Separation Science, School of Science, RMIT University, GPO Box 2476, Melbourne, VIC, 3001, Australia
[c] Griffith School of Environment, Griffith University, Southport QLD, 4222, Australia

## HIGHLIGHTS

- Carrier solvents are commonly used for chemical exposures in ecotoxicology.
- Test protocols overlook possible interactions between solvents and pollutants.
- We exposed tadpoles to a mixture of pharmaceuticals throughout development.
- The exposure was performed with and without the carrier solvent methanol.
- Metabolomics revealed considerable interactive toxicity caused by the solvent.

## GRAPHICAL ABSTRACT




## ARTICLE INFO

*Article history:*
Received 14 November 2017
Received in revised form
22 January 2018
Accepted 8 February 2018
Available online 10 February 2018

Handling Editor: Shane Snyder

*Keywords:*
Amphibian
Ecotoxicology
Interactive effect
Metabolomics
NMR spectroscopy

## ABSTRACT

Organic carrier solvents are used in aquatic toxicity testing to improve chemical solubility and facilitate the exploration of dose-response relationships. Both water- and solvent-control groups are normally included in these scenarios to ensure that the solvent itself has no effect on the test organism, but this fails to consider possible interactive effects between carrier solvents and contaminants of interest. We explored this topic by exposing *Limnodynastes peronii* tadpoles to a mixture of common water-soluble pharmaceuticals (diclofenac, metformin and valproic acid) in the presence and absence of the carrier solvent methanol, according to standard developmental bioassay methodology. Nuclear Magnetic Resonance (NMR) spectroscopy was applied as a platform for untargeted metabolomics, to compare broad sub-lethal hepatotoxicity in solvent- and solvent-free exposure scenarios. Considerable interactive effects were identified between the pharmaceutical mixture and a typical dose of methanol (0.003%). Specifically, pronounced differences were observed between the solvent- and solvent-free exposure groups for leucine, acetate, glutamine, citrate, glycogen, tyrosine, arginine, purine nucleotides and an unidentified metabolite at 6.53 ppm. Various other metabolites exhibited similar disparity related to the use of carrier solvent, but the interactions were non-significant. These results raise important questions about the use of carrier solvents for chemical exposures in aquatic ecotoxicology, and particularly for studies interested in sub-lethal mechanistic information and/or biomarker discovery.



* Corresponding author.
*E-mail address:* s.melvin@griffith.edu.au (S.D. Melvin).

https://doi.org/10.1016/j.chemosphere.2018.02.063
0045-6535/

## 1. Introduction

Reliable toxicity data is paramount to the effective identification and management of environmental threats from chemicals (EEA, 2010). At the forefront of global research efforts aimed at understanding chemical toxicity are controlled laboratory bioassays, which are extensively used to evaluate sub-lethal effects in non-target aquatic species (Amiard-Triquet et al., 2015). Standard exposure methods have been developed by international research organisations to establish guidelines for achieving robust toxicity information (OECD, 2012; ASTM, 2014). These and other resources describe situations where carrier solvents are necessary to improve chemical dispersion or increase solubility, for example to facilitate the exploration of dose-response relationships (Maes et al., 2012). In these scenarios, both water- and solvent-control groups are normally included to ensure that the solvent itself does not elicit a toxicological response in the test organism. However, recommended maximal solvent dosing has historically been based on adverse effects for apical endpoints, and the suitability of carrier solvents in the context of robust modern analytical techniques that can detect metabolic changes before major toxicity occurs warrants attention (Turner et al., 2012). Direct solvent toxicity is an important consideration, but another fundamental question exists with major consequence for ecotoxicology – are sub-lethal biochemical responses influenced by interactive effects between solvents and contaminants of interest (Melvin et al., 2017)?

Pharmaceutical contaminants are an emerging environmental concern and offer a particularly appropriate case for exploring this question. The potential for, and occurrence of, interactive effects between pharmaceuticals and alcohol is common knowledge, and humans are frequently advised to avoid mixing alcoholic drinks with therapeutic medicines (NIH, 2014; Weathermon and Crabb, 1999). The most common adverse side effect from alcohol-drug interactions is hepatic toxicity (Weathermon and Crabb, 1999; Adams, 2009). It is hypothesised that this may also hold true when using alcohols as carrier solvents in standardised toxicity bioassays. There has been surprisingly limited research on the topic; perhaps due to several studies indicating that solvent-drug interactions are of limited concern for apical endpoints (Chromcova et al., 2011; Barera and Adams, 1983). Surprisingly, methanol has even been deemed one of the least harmful solvents for vertebrate developmental bioassays and is commonly used in aquatic ecotoxicology (Maes et al., 2012), despite evidence that it may influence sub-lethal pathways involved with chemical detoxification (David et al., 2012). Considering the known influence of alcohol on drug metabolism and hepatotoxicity in humans and the widespread use of organic solvents like methanol in toxicity research, it seems prudent to evaluate the potential for interactive effects between solvents and chemical pollutants in sub-lethal ecotoxicology testing.

Metabolomics has been gaining recognition for its ability to provide a comprehensive picture of toxicity in living systems, including quantitative assessment of broad changes to the profiles of numerous small molecules in biological samples (Viant, 2007). The ability to simultaneously measure the end products from a wide range of cellular processes yields robust information about sub-lethal toxicological effects in response to environmental stressors (Spurgeon et al., 2010; Viant, 2008; Jones et al., 2008; Bouhifd et al., 2013). Somewhat paradoxically, the same sensitivity and comprehensive scope that makes metabolite profiling so attractive may make the approach susceptible to sources of confounding that have gone unnoticed using traditional test methods (Simmons et al., 2015). Understanding such factors is critical for the future successful application of metabolomics and other omics disciplines (e.g., genomics and proteomics) towards ecotoxicology research (Simmons et al., 2015). More broadly, this may reveal critical insights about the validity of established testing paradigms that could lead to improvements in future experimental strategies aimed at evaluating toxicological risks. The latter is vital for ensuring rigorous science and the provision of meaningful information, for example when attempting to identify biomarkers of exposure or integrate diverse toxicity information into an Adverse Outcome Pathway (Brockmeier et al., 2017; Ankley et al., 2010).

Here, we report alterations to hepatic metabolite profiles of larval amphibians exposed to a mixture of pharmaceutical contaminants in a standard developmental bioassay, using both solvent and solvent-free chemical dosing. Parallel exposures were performed in the presence and absence of methanol, to a concentration gradient of three representative water-soluble chemicals (i.e., diclofenac, metformin and valproic acid). We explored differences in response patterns triggered by solvent-drug interactions to assess the validity of using organic carrier solvents for omics research, including any implications for biomarker discovery or mechanistic research.

## 2. Methods

### 2.1. Chemical standards

The only way to conclusively assess the possibility for common carrier solvents to interact with pharmaceuticals (or other contaminants) is to compare responses to a concentration gradient in the presence and absence of solvent (Green and Wheeler, 2013). Importantly, this can only be achieved using water-soluble contaminants. Technical-grade standards (99.9% purity) of metformin, diclofenac and valproic acid were therefore purchased from Sigma-Aldrich (Castle Hill, Australia). These compounds were chosen since they are readily soluble in water, have been detected in sewage and environmental matrices in the low μg/L range, and have been flagged as potentially interacting with alcohol consumption in humans (Weathermon and Crabb, 1999). Concentrated working dilutions were prepared by dissolving each compound individually in Milli-Q water, with fresh stocks made every week throughout the experiment and maintained at −20 °C.

### 2.2. Experimental animals

A single fertilised foam egg nest of the Australian striped marsh frog (*Limnodynastes peronii*) was collected from an ephemeral pond located in Elanora, Queensland (Australia) and brought to the laboratory in a bucket filled with water from the collection site (QLD Government Collection Permit No. WISP16873916). The egg mass was held in the laboratory in a glass aquaria filled with 50 L moderately hard test water (USEPA, 1994) until hatching. Hatched larvae were fed Sera micron® fry food (Sera GmbH) *ad libitum* twice daily until most animals had reached Gosner developmental stage (Gs) 26 (Gosner, 1960). Temperature and photoperiod were maintained at 22.0 ± 0.2 °C and 12: 12 light: dark sequence, respectively, through holding and experimentation. This research was approved by the Griffith University Animal Ethics Committee in accordance with the principles in the Australian Code for the Care and Use of Animals for Scientific Purposes (Protocol No. ENV/03/16/AEC).

### 2.3. Developmental bioassays

Two exposures were performed in parallel, each comprising four replicate aquaria of control (nothing added) and 5, 50 and 500 μg/L of a mixture of metformin, diclofenac and valproic acid. Replicates contained 4 L of the appropriate mixture concentration and housed four *L. peronii* tadpoles (n = 16 per treatment), with