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Acute toxicity of aromatic and non-aromatic fractions of naphthenic acids extracted from oil sands process-affected water to larval zebrafish

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HIGHLIGHTS

- Toxicity of oil sands process water extract to fish determined (LC_{50} ~8 mg L^{-1}).
- Toxicity of esterifiable free naphthenic acids determined (LC_{50} 5.4 mg L^{-1}).
- Toxicity of ‘classical’ naphthenic acids determined (LC_{50} 13.1 mg L^{-1}).
- Toxicity of naphtheno-monoaromatic acids determined (LC_{50} 8.1 mg L^{-1}).
- Concentration range from no effect to 100% mortality < order of magnitude for all.

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ABSTRACT

The toxicity of oil sands process-affected water (OSPW) has regularly been attributed to naphthenic acids, which exist in complex mixtures. If on remediation treatment (e.g., ozonation) or on entering the environment, the mixtures of these acids all behave in the same way, then they can be studied as a whole. If, however, some acids are resistant to change, whilst others are not, or are less resistant, it is important to establish which sub-classes of acids are the most toxic.

In the present study we therefore assayed the acute toxicity to larval fish, of a whole acidified OSPW extract and an esterifiable naphthenic acids fraction, de-esterified with alkali: both fractions were toxic (LC_{50} ~5–8 mg L^{-1}). We then fractionated the acids by argentation solid phase extraction of the esters and examined the acute toxicity of two fractions: a de-esterified alicyclic acids fraction, which contained, for example, adamantane and diamantane carboxylic acids, and an aromatic acids fraction. The alicyclic acids were toxic (LC_{50} 13 mg L^{-1}) but the higher molecular weight aromatic acids fraction was somewhat more toxic, at least on a weight per volume basis (LC_{50} 8 mg L^{-1}; $P < 0.05$) (for comparison, the monoaromatic dehydroabietic acid had a LC_{50} of ~1 mg L^{-1}).

These results show how toxic naphthenic acids of OSPW are to these larval fish and that on a weight per volume basis, the aromatic acids are at least as toxic as the ‘classical’ alicyclic acids. The environmental fates and other toxic effects, if any, of the fractions remain to be established.

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1. Introduction

The oils sands operations of Canada produce large quantities of process affected water (OSPW), which is somewhat acutely and chronically toxic to a variety of organisms (e.g., Clemente and Fedorak, 2005; Kavanagh et al., 2011; Van den Heuvel et al., 2012 and references therein). The acute toxicity has been attributed to so-called naphthenic acids. However, the studies conducted so far have not usually been made on naphthenic acids fractions, but on unfractionated OSPW, which contains many other compounds which may or may not, be toxic (e.g., polycyclic aromatic hydrocarbons and metals (e.g., Allen, 2008; Gagné et al., 2011; Nero et al., 2006; Peters et al., 2007; Young et al., 2007 and others)). There is also apparently disagreement about the use of the term ‘naphthenic acids’ to describe the acids in OSPW (Grewer et al., 2010), but there has been a call for the addition of ‘naphthenic acids’ to the National Pollutant Release Inventory in Canada

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(Canada, 2012) so it would probably be useful if the toxicity of OSPW naphthenic acids, as well as whole OSPW, was also determined.

Furthermore, although many workers have assumed naphthenic acids are comprised only of alicyclic acids, in fact it is likely that aromatic acids, (first reported in the naphthenic acids of petroleum over 55 years ago (Knoterus, 1957)) are also present. Several acids have now been identified (some only tentatively) in OSPW (e.g., Rowland et al., 2011a–c) and the acute toxicities of the individual acids to a few endpoints, either measured or predicted by computer models (Jones et al., 2011; Scarlett et al., 2012; Tollefsen et al., 2012). The structures of these acids vary considerably: from those with diamondoid-type cages structures, to those apparently with planar aromatic rings. It is possible that these different acids may behave differently to remediation treatments, or in their partitioning behaviour in the environment. However, equally, their behaviour might be controlled only by the carboxylic acid group, assumed to be common to all the acids. Clearly, assays of the toxicity of different OSPW acids fractions might be useful.

To this end, Frank et al. (2008) used esterification of an acid OSPW extract with diazomethane, followed by Kugelrohr distillation, to isolate separate naphthenic acids fractions of varying molecular weight (boiling points), then de-esterified the esters and measured the toxicity of the fractions to the halo-tolerant bacterium, *Vibrio fischeri*. Toxicities (EC_{50}) of between about 40 and 65 mg L^{-1} were measured.

However, the separation effected by distillation was clearly based on boiling point, rather than on chemical structure or other properties. Also, whilst the *V. fischeri* assay is a useful screening method, whole organism assays are often more sensitive. (For example, using the same extraction method as Frank et al. (2008), Kavanagh et al. (2012) showed that reproduction of fathead minnows, *Pimephales promelas*, was impaired when exposed to 10 mg L^{-1} of a whole OSPW extract for 21 d).

To our knowledge, to date no whole organism assay of isolated fractions of OSPW shown to contain identifiable individual naphthenic acids, has been published.

In the present study we therefore assayed the acute toxicity of a crude acidified OSPW extract to larval zebrafish, *Danio rerio* and also determined the toxicity of an esterified ethyl acetate fraction (once de-esterified). This fraction has been shown to comprise overwhelmingly, a range of naphthenic monocarboxylic acids, both alicyclic and aromatic (Rowland et al., 2011a–c) and could be termed esterifiable naphthenic acids. We then fractionated the methyl esters of the OSPW by argentation (Ag^+) solid phase extraction (SPE) and examined the acute toxicity of the free acids of a non-aromatic 'classical' naphthenic acid fraction containing, for example, adamantane and diamantane carboxylic acids and of a naphtheno-monoaromatic acid fraction containing, for example, traces of dehydroabietic acid (DHAA) (Jones et al., 2012). DHAA was also tested as this provides a useful measure for the relative toxicity of the fractions reported herein and as a reference point for future toxicity tests.

2. Materials and methods

2.1. Fractionation of OSPW

All solvents were HPLC grade. Hexane and diethylether were purchased from Rathburn Chemicals Ltd. (Walkerburn, UK). Methanol was supplied by Fisher Scientific (Loughborough, UK). DHAA (purity > 99%) was supplied by Orchid Cellmark (New Westminster, Canada). OSPW naphthenic acid concentrated extract (stated NA concentration 2000 mg L^{-1} determined by Orbitrap mass spectrometry) was supplied by Environment Canada. This was a 3 L subsample of a concentrate of thousands of litres of oil sands tailings pond water collected from Syncrude Canada Ltd. West In pit settling basin in Fort McMurray, Alberta, Canada in spring 2009. The subsequent treatment to isolate a concentrated naphthenic acids (sodium salts) mixture was conducted in spring 2011 by Environment Canada by methods which have been described fully by Frank et al. (2008). Subsamples (3×1 L) of this concentrate as received (pH > 10) were acidified with hydrochloric acid to pH < 2 and extracted with ethyl acetate.

A pictorial representation of the methodology is provided as Supplementary Material (Fig. S1). Fractionation was essentially a scaled up process of the (SPE) method described by Jones et al. (2012); an additional hexane fraction was included to improve separation between the alicyclic and aromatic acids. In brief: the concentrated OSPW acidic extract extracted with ethyl acetate was dissolved in BF_3-methanol complex (Sigma–Aldrich, Poole, UK), heated at 70 °C for 3 h then extracted into hexane to produce methyl esters. Ag^+ SPE was conducted on the methylated OSPW extracts using 6 mL Discovery® Ag-Ion SPE cartridges (750 mg sorbent; Sigma-Aldrich, Poole, UK). The column was conditioned with hexane. OSPW extracts in hexane (300 mg) were then loaded onto the phase which was subsequently eluted using hexane (Fractions (F1-4), 95% hexane:5% diethyl ether (F5-7), 90% hexane:10% diethylether (F8), 100% diethyl ether (F9) and finally 100% methanol (F10). Fractions were collected, reduced to dryness under a steady stream of nitrogen at 40 °C and weighed. Of the recovered methyl esters, F3 and F6, each representing ca17% of the total (Fig. S1), were used for toxicity testing. System blanks were created for each fraction by eluting the sorbent with corresponding solvents in the absence of OSPW extract and were reduced in volume as described above.

2.2. Demethylation of methyl esters

Prior to toxicity testing, NA methyl esters were demethylated based on methods described by Frank et al. (2008). In brief: each of the dried methyl esters, F3 and F6, were rinsed into an amber jar using 9 mL methanol with 2.5 mL of 2M NaOH. The samples were incubated at 57 °C ± 1 °C until demethylation was complete as determined by Fourier Transform infrared spectroscopy. The samples were then rotary evaporated until a stable weight was achieved and then re-dissolved in HPLC-grade water to produce their sodium salts. Prior to zebrafish exposures, the test solutions were adjusted to pH 7.9 ± 0.1.

2.3. Zebrafish larvae exposure

Zebrafish (*D. rerio*) were maintained under routine approved animal welfare protocols. The photoperiod was 12 h and stock fish were fed three times daily with live brine shrimp nauplii, *Artemia* sp. or dry fish flake mix (equal proportions ZM Systems flake, brine shrimp, *Spirulina*, and TetraMin® stable flake). Larvae were routinely bred from bulk spawning of stock fish. Developing larvae were kept in plastic dishes (90 mm diameter, 50 mL) with daily water changes to remove unfertilized or un-developing eggs, and debris. Hatched embryos, 72 h post fertilisation, were used for acute larval exposures.

Larvae ($n = 10$ per glass beaker with 50 mL test solution, $n = 5$ beakers per treatment) were exposed (96 h) to an extract of OSPW, to an extract of OSPW that had been converted to methyl esters then demethylated and converted back to the sodium salts i.e. undergone the same processes as the fractions (OSPW-DM; esterifiable naphthenic acids), the major alicyclic fraction (F3, hexane eluate, classical naphthenic acids) and the major aromatic fraction (F6, 5% diethylether:95%hexane eluate, aromatic naphthenic acids)

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