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Adamantane carboxylic acids demonstrate mitochondrial toxicity consistent with oil sands-derived naphthenic acids

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ABSTRACT

Naphthenic acids (NAs) are thought to be a primary cause of toxicity of oil sands process-affected water (OSPW). The purpose of this study was to determine if commercially available adamantane carboxylic acids act by mitochondrial mechanisms similar to NAs found in OSPW. Mitochondria isolated from rainbow trout (*Oncorhynchus mykiss*) liver were exposed to commercially available adamantane acids, 3-hydroxyadamantane-1-carboxylic acid (CAS 42711-75-1) and 3,5-dimethyladamantane-1-carboxylic acid (CAS 14670-94-1), or to NAs extracted and purified from OSPW. The effects of these compounds on state 3 and 4 respiration, mitochondrial membrane potential, and mitochondrial reactive oxygen species (ROS; H_2O_2 production) were quantified. The compound 3-hydroxyadamantane-1-carboxylic acid only inhibited state 3 respiration at the highest concentration (2560 mg/L) and showed a concentration-dependent reduction in H_2O_2 production. Consistent with extracted OSPW NAs, 3,5-dimethyladamantane-1-carboxylic acid inhibited state 3 respiration and increased H_2O_2 production, but with a two-fold greater EC50 than the NA mixture extracted from OSPW. All three compounds uncoupled mitochondrial membrane potential and increased state 4 respiration. Based on these results, the adamantane 3,5-dimethyladamantane-1-carboxylic NAs may act via similar mitochondrial mechanisms as NAs extracted from OSPW and could be used to further explore toxic mechanisms.

Introduction

Most surface mined bitumen from oil sands is extracted using the Clarke hot water extraction method, resulting in the production of large volumes of wastewater referred to as oil sands process-affected water (OSPW). OSPW contains residual bitumen, dissolved salts, minerals, and a complex mixture of inorganic and organic compounds (Allen, 2008; Clemente and Fedorak, 2005). A mixture of alkyl-substituted acyclic and cycloaliphatic carboxylic acids, known as naphthenic acids (NAs), make up a large component of the organic tailings fraction and are thought to be responsible for much of the observed toxicity of OSPW (Allen, 2008; Clemente and Fedorak, 2005; Madill et al., 2001). Classical NAs refer to compounds that follow the general formula $C_nH_{2n+Z}O_2$ (Clemente and Fedorak, 2005). The $C_nH_{2n+Z}O_2$ formula only describes a portion of the total pool of acid extractable organics, for example O_3 (alcohols) and O_4 (alcohols and diacids) compounds are predominant carboxylic acids in the mixture and do not fit the general formula (LeClair et al., 2013; Frank et al., 2008).

The understanding of the specific toxic mechanisms and analytical chemistry of NAs has been limited to some extent by the unavailability of pure compounds. While commercial mixtures have been used, they have little similarity to the chemical profile of NAs in oil sands-affected material (Garcia-Garcia et al., 2011; MacDonald et al., 2013; Melvin et al., 2013). Some of the first individual NA isomers were identified in OSPW acid-extractable organic matter by Rowland et al. (2011) as tricyclic adamantane carboxylic acids. These acids fit within the general NA formula and have a diamondoid structure. Various adamantane acids have since been identified in OSPW from different industry OSPW samples (Bowman et al., 2014; Lengger et al., 2015; Rowland et al., 2012). It has been suggested that the cage structure of the adamantane naphthenic acid could impede degradation of these molecules, possibly contributing to their recalcitrance in weathered oil sands materials

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(Lengger et al., 2013). While the predominance of diamondoid structures in these complex mixtures remains relatively unexplored, the commercial availability of several relatively pure isomers in sufficient quantities at low cost and raises the potential of using those compounds as model compounds for environmental toxicology research.

Previous work has demonstrated that NAs may act through multiple mechanisms, including oxidative uncoupling of mitochondria and inhibiting electron transport system (ETS) complexes (Rundle et al., 2018). Exposure of isolated mitochondria from rainbow trout (*Oncorhynchus mykiss*) liver to NAs resulted in uncoupling of oxidative phosphorylation (OXPHOS), inhibition of the ETS, stimulation of H_2O_2 production (a measure of reactive oxygen species, or ROS), and increased mitochondrial oxidation state, indicating oxidative stress. When OXPHOS is uncoupled, the proton motive force is dissipated rather than being used to produce ATP (Kadenbach, 2003; Terada, 1990).

The hypothesis of this study is that an O_2 adamantane carboxylic acid found in oil sands mixtures will demonstrate similar mitochondrial mechanisms of to the bulk mixture of NAs found in OSPW. Furthermore, as oxidative uncoupling (decreased membrane potential and reduced respiration) depends to some extent on the amphipathic nature of the toxicant, it is hypothesized that O_3 NAs would not affect the ETS by the same mechanisms as O_2 NAs, or may not impact the ETS at all. To examine respiratory uncoupling, mitochondria isolated from rainbow trout (*Oncorhynchus mykiss*) livers were exposed to one of two commercially available model NAs: 3,5-dimethyladamantane-1-carboxylic acid and 3-hyroxyadamantane-1-carboxylic acid-these compounds represent O_2 and O_3 isomers, respectively. Several isomers of dimethyladamantane-1-carboxylic acid have been identified in oil sands waters (Rowland et al., 2011). Adamantanes have been observed to comprise the highest relative abundance of NA classes in aged tailings (Bowman et al., 2020). Hydroxy metabolites of those adamantanes have been shown to be microbial degradation products (Folwell et al., 2020), and O_3 NAs have been shown to comprise up to 10% of the acid extractable mixture (Leclair et al., 2013). Mitochondrial oxygen consumption, membrane potential, and hydrogen peroxide production were measured, and uncoupling potency of the substances examined were compared with that of oil sands-derived NAs extracted from 17 year-aged OSPW.

Methods

Chemicals and reagents

Adenosine diphosphate (ADP), aprotinin, bovine serum albumin (BSA), ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), L-malic acid, L-glutamic acid, sucrose and potassium phosphate were obtained from Sigma Aldrich (Oakville, Canada). Tris-hydrogen chloride was purchased from EMD Chemicals Inc. (Gibbstown, USA). Amplex Red and horseradish peroxidase (HRP) were purchased from Molecular Probes Inc. (Oregon, USA). The membrane potential indicator tetraphenylphosphonium chloride (TPP^+) was obtained from Alfa Aesar (Heysham, England). Adamantane compounds 3,5-dimethyladamantane-1-carboxylic acid (CAS 42711-75-1; Sigma; 97%), and 3-hydroxyadamantane-1-carboxylic acid (CAS 14670-94-1; Sigma; 97%) were acquired from Sigma-Aldrich (Oakville, Canda). Compound structures are illustrated in Fig. 1.

Mitochondria isolation

Rainbow trout (*Oncorhynchus mykiss*) were purchased from the Ocean Farms hatchery (Brookvale, Canada) and were 1+ year old. Trout were held in a 250-L tank at the Atlantic Veterinary College at 11 °C and fed a commercial pelleted diet at 1% of body weight daily. Mitochondria were isolated from rainbow trout (155 ± 96 g) livers using a previously published protocol for differential centrifugation (Sharaf et al., 2015).

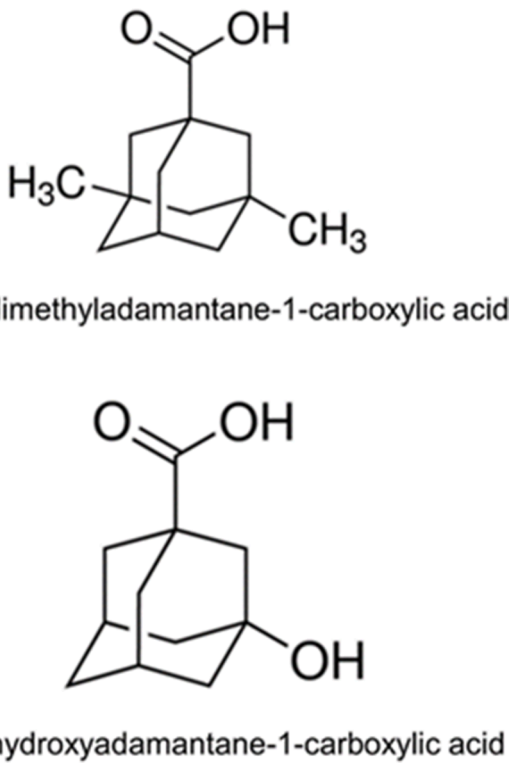

Fig. 1. Structure of two adamantane carboxylic acids chosen for the present study.

Isolated mitochondria were held on ice in mitochondrial isolation buffer until use (Sharaf et al., 2015). Protein concentration was measured using the Bradford protein assay (Bradford, 1976) against BSA.

Complex I respiration, H_2O_2 production, and mitochondrial membrane potential ($\Delta\Psi_{mt}$)

Respiration as oxygen consumption, mitochondrial membrane potential, and H_2O_2 emission were quantified simultaneously using the Oroboros (Innsbruck, Austria) Oxygraph-2k high resolution respirometry instrument using methods previously described (Sharaf et al., 2017; Rundle et al., 2018). Protein-normalized oxygen consumption was recorded in real time. The $\Delta\Psi_{mt}$ was derived by measuring TPP^+ uptake by mitochondria using a TPP^+ ion selective electrode. A calibration for TPP^+ was performed at the start of each experiment. The H_2O_2 production was measured using the Amplex Red-HRP assay. Amplex red reacts with H_2O_2 producing the fluorescent product resorufin that is measured by the Oroboros. A calibration curve was constructed using known concentration (0 - 0.45 μM) of H_2O_2 immediately prior to each trial.

NAs were extracted from OSPW using methods previously published (Frank et al., 2006; MacDonald et al., 2013; Rundle et al., 2018). Briefly, NAs were isolated from 17-year-old tailings from Syncrude Canada Ltd.'s experimental Pond 10. Tailings were acidified, the precipitate was concentrated via centrifugation and upon re-dissolution in 0.1 M NaOH. Redissolved NAs were filtered through diethylaminoethyl (DEAE) cellulose to remove humic material, and then extracted with dicholoromethane (DCM) liquid-liquid extraction to remove neutral compounds. The sample was again acidified, washed, and then freeze dried to produce a solid NA extract. This extract was characterized by negative mode direct injection HRMS and found to be 99% carboxylic acids with approximately 90% fitting the classical NA formula, $C_nH_{2n+Z}O_2$ with the majority of the remainder being O_3 carboxylic acids (Leclair et al., 2013; Rundle et al., 2018). The profile of NAs in the mixture conforming to the $C_nH_{2n+Z}O_2$ formula are shown in Fig. 2 and are described according to

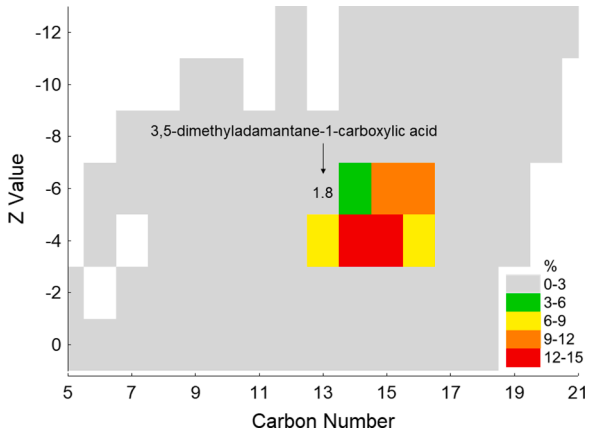

Fig. 2. Carbon number and *Z* value distributions of the NA mixture. Values are shown as percentage of total ion abundances in the mixture of all compounds conforming to the formula $C_nH_{2n+Z}O_2$.

number of carbons and *Z* value (hydrogen deficiency indicating number of rings or double bonds; modified from Rundle et al. (2018). The relative position of 3,5-dimethyladamantane-1-carboxylic acid in the NA profile is shown according to its carbon number and *Z* value.

NAs were added successively to mitochondrial preparations during each run to achieve target concentrations (Rundle et al., 2018). A total of nine concentrations were added by direct injection into the Oroboros cell and a logarithmic series of concentrations (two-fold dilutions) ranged from 20 to 2560 mg/L (Fig. 3). Concentrations were chosen based on previous research (Rundle et al., 2018) to encompass a range from no effect, to maximal effect to facilitate calculation of endpoint estimates (EC50, IC50). Sequential additions were required to complete a full concentration-effect curve within the 4 h viability of mitochondria and to limit the number of live fish required. A minimum of three replicates were performed for each endpoint for each compound. A concentration-effect curve for the NA mixture and each model NA was performed during both state 3 and 4 respiration. Concentration-effect curves were prepared using a four-parameter logistic nonlinear regression as described in Rundle et al. (2018). Differences in EC_{50} and IC_{50} values between treatments were evaluated using ANOVA, with a level of significance set at $p < 0.05$. The assumption of normality was examined using normal probability plots and heteroscedasticity using Levene's test. Dunnett post-hoc test was used to examine individual treatment differences using the NA mixture as the control. All statistics were performed using Statistica v.13 (Tibco Software Inc., USA)

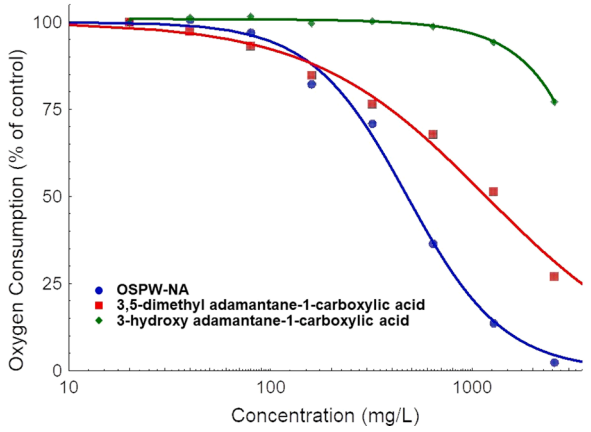

Fig. 3. Example of concentration-effect relationships for state 3 respiration for OSPW-NA, 3,5-dimethyl adamantane-1-carboxylic acid, and 3-hydroxy adamantane-1-carboxylic acid. Individual IC50s are 476, 1160, and > 2560 mg/L, respectively.

Results and discussion

The NA mixture and adamantane compounds examined all reduced state 3 respiration (Table 1) and an example of the concentration-effect relationship of typical individual replicates is shown in Fig. 3. The NA mixture showed significantly greater potency (2.5-fold) in the inhibition of state 3 respiration than 3,5-dimethyl adamantane-1-carboxylic acid. The NA mixture caused almost total cessation of oxygen consumption in state 3 respiration at the highest concentration (2,560 mg/L) with a mean of 7% of control values as compared to the methyl-substituted adamantane that showed a mean oxygen consumption of 26% at the highest concentration. The alcohol substituted adamantane only showed declines in state 3 oxygen consumption at the highest concentration and the IC_{50} could not be calculated.

State 4 oxygen consumption for all substances showed a bimodal pattern with an initial increase in oxygen consumption at the lower concentrations, followed by a decrease in oxygen consumption at higher concentrations (data not shown). The potency of each compound/mixture was evaluated by using only the concentrations at which oxygen consumption was increasing and decreasing points were removed (Table 1). The relative potency with regards to the three substances was in the same order to what was found for state 3 oxygen consumption, however the EC_{50} values were lower that than those found for state 3 oxygen consumption. We speculate that the increase in oxygen consumption is a compensatory response, consistent with lower EC_{50} values, to alterations in the upstream ETS. A compensatory response would be expected to be more apparent in state 4 for which oxygen consumption is low, as opposed to state 3 when oxygen consumption reaches a maximum. At higher concentrations, the ETS is impacted, and this compensatory response is eliminated.

Only the NA-mixture and 3-hydroxy adamantane-1-carboxylic acid were observed to consistently reduce state 3 membrane potential with the NA-mixture being two-fold more potent (Table 1). Unlike state 3 oxygen consumption, the change in membrane potential is generally modest and has the highest variability between replicates. For example, at the highest concentration of the NA-mixture that nearly eliminated oxygen consumption in state 3 while the mean reduction in membrane potential is only 16%. The absence of the dissipation of membrane potential for 3,5-dimethyl adamantane-1-carboxylic acid given its ability to reduce state 3 oxygen may be due to an inability to detect the modest response and suggests that the reduction in respiration is not due to changing membrane potential. However, membrane potential is at its lowest during state 3 respiration, and thus the effects on this endpoint in

Table 1
Mean IC or EC_{50} values mg/L (standard error, *n*) calculated for each parameter based on Oroboros measurements. Curve fits were conducted on individual replicates and parameters were averaged. The mean variance explained by the curve fits for each parameter are indicated. Parameters for which concentration-effect curves could not be fitted for all replicates are indicated as no effect. Asterisks indicate a statistically differences between model NAs and OSPW-NAs, $p < 0.05$.

Parameter	OSPW-NAEC/IC_{50} (SEM,n)	3,5-dimethyl adamantane-1-carboxylic acidEC/IC_{50} (SEM,n)	3-hydroxy adamantane-1-carboxylic acidEC/IC_{50} (SEM,n)
State 3 oxygen consumption	↓ 648 (176, 6)	↓ 1545 (135, 5)*	IC_{50} > 2560
State 4 oxygen consumption	↑ 94 (12, 6)	↑ 543 (25. 3)*	IC_{50} > 2560
State 3 $\Delta\Psi_{mt}$	↓ 721 (347, 4)	No response	↓ 1450 (408, 3)*
State 4 $\Delta\Psi_{mt}$	↓ 473 (171, 3)	↓ 1703 (118, 3)*	↓ 1750 (68, 3)*
State 3 H_2O_2 emission	↑ EC_{50} 198 (51, 4)	↑ EC_{50} 535 (290, 3)	↓ IC_{50} 1519 (151, 3)
State 4 H_2O_2 emission	↑ EC_{50} 212 (73, 4)	↑ EC_{50} 559 (228, 3)	↓ IC_{50} 724 (78, 3)

state 3 may are harder to detect.

All three compounds tested reduced membrane potential in state 4. Consistent with other endpoints, the NA-mixture had a 3-fold lower IC_{50} while the two adamantane carboxylic acids did not differ in efficacy for state 4 membrane potential. Despite membrane potential being at its highest in state 4 respiration, reduction of this potential by the chosen treatment was still modest. At the highest concentration, reduction from control values was 38, 11, and 18% for the NA-mixture, 3,5-dimethyl adamantane-1-carboxylic acid, and 3-hydroxy adamantane-1-carboxylic acid, respectively. These results suggest that passive uncoupling, where compounds transport protons across the mitochondrial membrane decreasing electromotive force, may not be the key mechanism in causing mitochondrial dysfunction. The expectation also would be that an alcohol-substituted adamantane carboxylic acid would be less amphipathic than a methyl-substituted adamantane carboxylic acid, yet the two substances did not show potencies for reducing membrane potential consistent with their structure, again suggesting that reduction in membrane potential is not the primary driver of reduced oxygen consumption.

Only the NA-mixture and 3,5-dimethyl adamantane-1-carboxylic acid increased net H_2O_2 production with the NA-mixture observed to be approximately 2.5-fold higher in its potency regarding this endpoint ($p < 0.05$, Table 1). In contrast, 3-hydroxy adamantane-1-carboxylic acid resulted in a concentration-dependent reduction in H_2O_2 production during both state 3 and state 4 respiration. However, the IC_{50} was two-fold lower in state 4 than in state 3. The compound showed a mean reduction in H_2O_2 production of 41% and 13% for state 3 and state 4, respectively. This can be explained as distal effect which is defined as impairment on any of the steps responsible for providing reducing equivalents to the complexes (e.g. NADH; Fato et al. 2009). This will reduce the supply of electrons, decreasing the potential for H_2O_2 emission. Conversely, any direct impairment of the complexes will cause reduced electron flow, increasing the possibility of leakage to increase H_2O_2 emission.

The relevance of these mechanistic observations, and the ability to use these compounds for future work is predicated on their presence in the environment. Adamantane carboxylic acids have been identified in multiple sources of OSPW (Lengger et al., 2015; Rowland et al., 2011; Rowland et al., 2012). Unknown structural variants of dimethyl 1-admantane carboxylic acid are among the identified compounds (Rowland et al., 2011). Tricyclic carboxylic acids have been considered major constituents of NAs in oil sands (Clemente and Fedorak, 2005; Grewer et al., 2010) and it has been shown that they and bicyclic carboxylic acids also make up a major component of OSPW-NAs (Rowland et al., 2011). Tricyclic diamondoid or adamantane NAs identified by Rowland et al. (2011) are thought to be biotransformation products of adamantane hydrocarbons (Rowland et al., 2011; Wilde and Rowland, 2018).

The distribution of individual adamantane NAs have proven useful for profiling OSPW and differentiating between industry samples (Rowland et al., 2012; Lengger et al., 2015). It has been found that aged tailings samples have a higher proportion of adamantane NAs (Lengger et al., 2015). Based on this, adamantane NAs may be useful in assessing biodegradation and aging of OSPW as well as identifying sources of environmental contamination with NAs (Rowland et al., 2012; Lengger et al., 2015; Wilde and Rowland, 2018).

The potential for use of adamantane compound as surrogate toxicants for oil sands NAs is also dependent on the relative potency. However, the compounds chosen herein had less potency for the inhibition of oxidative phosphorylation than the extracted NA mixture. The most abundant NAs in the mixture had either 15 or 16 carbons (30 and 28%, respectively; Rundle et al. 2018). The compounds 3,5-dimethyl adamantane-1-carboxylic acid and 3-hydroxy adamantane-1-carboxylic acid have 13 and 11 carbons, respectively. The majority of the NA mixture (greater than 3% relative abundance) has 13–16 carbons, and a *Z* value of -4 or -6 (Fig. 2). The carbon number and Z value corresponding to 3,5-dimethyl adamantane-1-carboxylic acid represents 1.8% of the mixture. Thus, it is expected that the model compounds have lower K_{ow} than the average of the NAs in the mixture. As per substances exhibiting a narcotic mode of action (McCarty and Mackay 1993), hydrophobicity is an important quantitative structure-activity relationship (QSAR) in terms of the ability to cross membranes, and the ability to interact with hydrophobic regions of proteins such as enzymes and membrane channels. It is fully expected that the inhibition of oxidative phosphorylation would correlate with hydrophobicity, just as it does for narcotic modes of action due to an increased ability to cross lipid bilayers and interact with proteins within the membrane which is the prevalent school of thought for the action of narcotic compounds as well.

Some diamondoid adamantane NAs were found to act via the same mitochondrial pattern of mechanism as the OSPW-NA mixture previously studied (Rundle et al., 2018). It was predicted that O_2 NAs would be more potent inhibitors of oxidative phosphorylation than O_3 NAs. This effect was observed for the oxygen consumption and H_2O_2 generation endpoints as the O_2 compound, 3,5-dimethyl adamantane-1-carboxylic acid was much more potent than the O_3 compound, 3-hydroxy adamantane-1-carboxylic acid. However, the exception to this was observed in the disruption of membrane potential where the relative potency was similar. This is an important finding as it further emphasizes the conclusions made in Rundle et al. (2018) that multiple mechanisms may be at play with regards to the effects of the NA-mixture. It seems the reduction of membrane potential may not be entirely responsible for the nearly complete inhibition of oxidative phosphorylation based on several lines of evidence. Firstly, as mentioned, the relative potencies for membrane potential reduction are different here than the other endpoints. Secondly, despite almost complete inhibition of oxygen consumption, membrane potential is still largely maintained. The production of H_2O_2 by the NA mixture and 3,5-dimethyl adamantane-1-carboxylic acid suggest that this is a proximal mechanism. Thus, direct inhibition of mitochondrial complexes for these substances is indicated while the effect of 3-hydroxy adamantane-1-carboxylic acid could be an entirely different distal mechanism perhaps involving the provision of NADH to the complexes.

Complexes I and III are the largest producers of ROS along the ETS (Turrens, 1997; Turrens, 2003) and inhibition of either one of these complexes often results in increased production of ROS (Barrientos and Moraes, 1999; Koopman et al., 2005; Raha et al., 2000). However, inhibition of complex I can result in either an increase or decrease in ROS production depending on the site of inhibition (Brand et al., 2004; Fato et al., 2009). Complex I inhibitors have been broadly classified into two main categories based on their effects on ROS production, class A inhibitors that induce ROS production and class B inhibitors that prevent ROS production (Fato et al., 2009; Lenaz et al., 2006). Thus, the possibility remains that 3-hydroxy adamantane-1-carboxylic acid could be a class *B* complex I inhibitor. The compound 3,5-dimethyl adamantane-1-carboxylic acid could potentially be a class A inhibitor though it may inhibit other complexes as well.

It is concluded that a commercially available adamantane carboxylic acid, 3,5-dimethyl adamantane-1-carboxylic acid, may be suitable toxicant surrogates for studying the mitochondrial mechanisms of action of OSPW-NAs. Individual adamantane carboxylic acids have been previously identified in different OSPW samples making them environmentally relevant (Lengger et al., 2015; Rowland et al., 2011). The present study has shown that 3,5-dimethyl adamantane-1-carboxylic acid and the isolated OSPW-NA mixtures share the effect of ETS inhibition as indicated by decreased state 3 respiration, and H_2O_2 production as a measure of ROS in isolated mitochondria. Taken together this suggests that passive uncoupling of the proton gradient, while it does occur inconsistently, may not be the main mechanism of NA disruption of oxidative phosphorylation. Understanding the precise site on the complexes where these effects are taking place is required to confirm that mechanisms are identical. Further work is needed with different

adamantane carboxylic acids and on the other electron transport complexes to make more concrete conclusions on the full effect on mitochondrial respiration. If adamantane carboxylic acids can be used as mechanism of action surrogates for OSPW-NAs, this mechanism could be explored in greater detail in mitochondria or on a cellular level. Increasing the knowledge of the toxicity and mechanisms of action of adamantane-type NAs will provide more insight into the biological properties and toxicity of OSPW containing these acids and their harmful levels in the environment.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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