Accepted by *Chemosphere*

Aquatic Hazard Assessment of a Commercial Naphthenic Acids Mixture

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Accepted by *Chemosphere*

ABSTRACT

This paper presents chemical composition and aquatic toxicity characteristics of a commercial naphthenic acids mixture. Naphthenic acids (NAs) are derived from the refining of petroleum middle distillates and can contribute to refinery effluent toxicity. NAs are also present in oil sands process-affected water (OSPW), but differences in the NA compositions from these sources precludes using a common aquatic toxicity dataset to represent the aquatic hazards of NAs from both origins. Our chemical characterization of a commercial NAs mixture showed it to contain in the order of abundance, 1-ring > 2-ring > acyclic > 3-ring acids (~84%). Also present were monoaromatic acids (7%) and non-acids (9%, polyaromatic hydrocarbons and sulfur heterocyclic compounds). While the acyclic acids were only the third most abundant group, the five most abundant individual compounds were identified as C_{10}-$_{14}$ n-acids (n-decanoic acid to n-tetradecanoic acid). Aquatic toxicity testing of fish (*Pimephales promelas*), invertebrate (*Daphnia magna*), algae (*Pseudokirchneriella subcapitata*), and bacteria (*Vibrio fischeri*) showed *P. promelas* to be the most sensitive species with 96-h LL50 = 9.0 mg/L (LC50 = 5.6 mg/L). Acute EL50 values for the other species ranged from 24-43 mg/L (EC50 values ranged from 20-30 mg/L). Biomimetic extraction via solid-phase-microextraction (BE-SPME) suggested a nonpolar narcosis mode of toxic action for *D. magna*, *P. subcapitata*, and *V. fischeri*. The BE analysis under-predicted fish toxicity, which indicates that a specific mode of action, besides narcosis, may be a factor for fish.

Key words: Naphthenic acids, Aquatic toxicity, Biomimetic extraction

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1. INTRODUCTION

Naphthenic acids (NAs) are constituents in crude oil usually present at 0% to 3% (Brient, 1995; CEATAG, 1998). They are complex and composed predominantly of alkyl-substituted cycloaliphatic carboxylic and acyclic (paraffinic) acids. Rings may be fused or bridged, and the acyclic carboxylic acids may be straight-chain or highly branched (Rowland et al., 2011a,b,c). NAs are assigned the general formula $C_nH_{2n+Z}O_2$ (Clemente and Fedorak, 2005), where the value n represents the number of carbon atoms, and Z may be zero or a negative even integer, reflecting the hydrogen deficiency resulting from ring formation (Clemente and Fedorak, 2005).

NAs have boiling points that fall within the range of middle distillate petroleum streams (e.g., kerosene, diesel) (Brient, 1995). They cause corrosion problems in distillation units (Brient, 1995; CEATAG, 1998) and can contribute to the aquatic toxicity of refinery wastewater (Dorn, 1992). Owing to their corrosive nature, NAs are removed from middle distillates to improve burning qualities, storage stability, and odor of the finished fuels (Brient, 1995; Suarez, 1996). This removal process then provides a source of crude NAs that can be further refined for use in emulsifying agents, wetting agents, anti-fungal agents, gelling agents, antioxidants, and dryers in oil-based paints (Brient, 1995; CEATAG, 1998). The extraction of bitumen from oil sands deposits generates oil sands process water (OSPW) which can also contain 20-120 mg NAs/L (Holowenko et al., 2002; Headley and McMartin, 2004; NRC, 2010).

NAs are considered the key constituents responsible for the aquatic toxicity of OSPW and some refinery effluents (MacKinnon and Boerger, 1986; Verbeek et al., 1994; Dorn, 1992). Due to their ready availability, commercial NAs have at times been used as a surrogate for evaluating NAs from OSPW extracts. However, significant compositional variability exists in NAs from different sources and the use of commercial NAs as surrogates for OSPW NA has been questioned (West et al., 2011). These include differences in i) the ranges of molecular weights of constituent NAs (Clemente et al., 2003; Lo et al., 2003), ii) principal ring structure groups (Clemente at al., 2004; Grewer at al., 2010; Rowland et al., 2011a,b,c), and iii) the presence of oxy-NAs in OSPW extracts (Grewer et al., 2010). Commercial NAs and OSPW extracts also demonstrated differences in aquatic toxicity. Nero et al. (2006) reported that at equal NA concentrations, commercial NAs were more toxic to yellow perch than OSPW extracts, and Peters et al. (2007) demonstrated in two fish species that commercial NAs showed lower thresholds for body deformities and growth reductions than OSPW extracts.

Since commercial NAs are transported throughout the world, an accurate hazard assessment of these substances and their potential effects on the aquatic environment is important. Early studies of the aquatic hazard of commercial NAs have limited value due to incomplete compositional and exposure concentration analysis. Additionally, compositional differences between OSPW extracts and commercial NAs may prevent a single aquatic hazard dataset from representing NA

from all sources (West et al., 2011). Therefore, the Petroleum HPV Testing Group conducted testing under the US EPA (2000) High Production Volume (HPV) Challenge Program to evaluate aquatic hazard of a commercial NA with a comprehensive compositional analysis. The present study provides aquatic toxicity data for a commercial NA to four trophic levels of aquatic organisms. Various tools were used to assess the composition of the commercial NA and to characterize the mode of toxic action of these NA substances. A detailed chemical analysis of the NA test substance helped progress the knowledge of specific NA structures in commercial NAs. Biomimetic extraction – solid phase microextraction (BE-SPME) analyses were used to evaluate mode of action and those measurements served as a surrogate for critical body burdens of these substances. These new data are anticipated to provide an aquatic hazard assessment that may be used in a regulatory context for risk assessment purposes.

2. METHODS AND MATERIALS

2.1 Test Substance

The sample of commercial naphthenic acids (CAS# 1338-24-5) was supplied by Merichem Company (Houston, TX). Manufacturer's specification data for the test sample provided the following: acid number, 235 mg KOH/gm; total unsaponifiables, 4.9 %; viscosity @ 40°C, 32 cst; specific gravity @20°C, 0.969 g/mL; color (Garner), 4.5 GI; water content, 0.07%; phenolic content (acid), 0.31 %; total sulfur, 0.34%?; CP – flash point (COC), 343 °F.

The test substance was used to prepare all test solutions, matrix spiking solutions and analytical standards. Quantification of the carbon numbers (C-num) and Z-family groups were performed by the Department of Biological Sciences, University of Alberta (Alberta, Saskatchewan) using published methods (Holowenko et al., 2002).

2.2 Test Organisms and Test Design

Toxicity tests were conducted using a freshwater fish (fathead minnow, *Pimephales promelas*), an invertebrate (Cladoceran, *Daphnia magna*), an alga (*Pseudokirchneriella subcapitata*), and a bacterium, (*Vibrio fischeri*). Fish, invertebrate, and alga tests were performed following standard regulatory guidelines (US EPA, 1996; OECD, 2009) and additional guidance (OECD, 2000) for testing mixtures using water accommodated fractions (WAF). The Microtox bacteria toxicity test was based on the general principles described by ISO (2007). Bacterial luminescence readings were measured using a Microtox Model 500 Analyzer (Modern Water Inc.). A summary of the test conditions are shown in Table 1. Fish, invertebrates, and algae were obtained from in-house cultures maintained by the testing facility. Freeze-dried *V. fischeri* were purchased (Modern Waters, Inc). Dilution water used in the fish and *Daphnia* tests was laboratory freshwater prepared to yield a total hardness of 130 to 160 mg/L as CaCO3. Dilution water used in the alga

test was freshwater algal nutrient medium (ASTM, 1997), and Microtox diluent was used for the bacterial toxicity test.

2.3 Analytical Characterization of Naphthenic Acids Test Substance

The test substance was characterized for Z-family and carbon number distributions using the gas chromatography-mass spectrometry (GC-MS) method described by Young et al. (2008). An aliquot of the test substance was derivitized with N-methyl-N-(t-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) to create t-butyldimethylsilyl esters of the constituent NAs. The total ion current mass spectra was collected from the GC-MS and the data were put into a Microsoft Excel spreadsheet (Holowenko et al., 2002) to create a table of the relative abundances of each ion corresponding to the general formula, $C_nH_{2n+z}O_2$.

The extracted ion current chromatograms of selected M-57 ions for different carbon number (n) and z number NA were integrated and the data put into a Microsoft Excel spreadsheet (Holowenko et al., 2002) to create a table of the relative abundances of each NA corresponding to the general formula, CnH2n+zO2. This then defined, approximately, the relative proportions of acyclic, 1-, 2-, 3-ring etc and aromatic NA classes (Figure 1). (It is known that this method may overestimate some NA classes if oxy-NA (e.g. hydroxy 'O3'acids (Bataineh et al., 2006) or 'O4' diacids (Lengger et al., 2013)) which may yield bis-derivatives, are present- as is common in OSPW NA. However the proportion of such O3 and O4 species in commercial NA from Merichem is generally low (Grewer et al., 2010), so the present method is adequate for purpose. Methods now commonly used for estimating n and Z based on electrospray ionization (ESI) and ultra high resolution MS have rarely been calibrated for the variable ESI responses or matrix effects of the different O2-4 NA species).

Identification of selected NA compounds in the test substance employed comprehensive two-dimensional gas chromatography/time-of-flight mass spectrometry (2D GC/ToF-MS) (Rowland et al., 2011c). Naphthenic acids were derivitized to methyl esters and analyzed using an Agilent 7890A gas chromatograph (Agilent Technologies, Wilmington, DE, USA) fitted with a Zoex ZX2 GC×GC cryogenic modulator (Houston, TX, USA) interfaced with a Almsco BenchToF dx™ time-of-flight mass spectrometer (Almsco International, Lantrisant, UK).

Isolation of the mono-aromatic acids as a group from the total NAs was achieved using argentation solid-phase extraction (SPE) of the methyl esters on silica with hexane and hexane:diethyl ether eluants (Jones et al., 2012). Separation of the *n*-paraffinic acids (*n*-acids) from the highly branched *iso*-paraffinic (*iso*-acids) and cyclic acids employed the method of urea adduction (Speight, 2007). Each experiment was repeated three times.

2.4 Quantitation of Total Dissolved Naphthenic Acids in Water Samples and Distribution of Z-family and Carbon Number Groups

Concentrations of dissolved naphthenic acids in the exposure solutions used in fish, *Daphnia*, and algal toxicity tests were measured using Fourier transform infrared spectroscopy (FTIR) (Jivraj et al., 1991). Exposure solutions used in the Microtox test were not analyzed.

Analyses of the proportional abundance of the different Z-family and C-number distributions were made of each initial WAF used in the fish, *Daphnia*, and algal toxicity tests (Merlin et al., 2007). Derivatization and analysis was followed as described for the NA test substance.

2.5 Biomimetic Extraction-Solid Phase Microextraction Analysis (BE-SPME)

A linear relationship between NA loading rate and SPME was established at NA loading rates of 0 (control), 2.1, 4.7, 10.3, 22.7, and 50 mg/L. Except for the control, samples were acidified to pH $\leq$ 2 with concentrated HCl. An additional sample of the 50 mg/L treatment was similarly taken in VOA vials but not acidified. A 30 µm PDMS (0.132 µL) SPME fiber (Supelco) was equilibrated with each sample for 100 minutes at 30°C with orbital agitation at 250 rpm. A single SPME fiber was used for all automated sample analyses. The SPME fiber along with hydrocarbon calibration standards diluted in solvent were analyzed on a Perkin-Elmer Autosystem XL gas chromatograph with a flame ionization detector and 15 m x 0.53 mm id capillary column with 1.5 µm Rtx-1 stationary phase (Restek). The molar response factor of 2,3-dimethylnaphthalene was used for converting the observed GC-FID response to nanomoles of organic constituents on the PDMS fiber. The three levels of 2,3-dimethylnaphthalene standards corresponded to approximately 0.064, 0.32 and 0.95 nanomoles on-column. Fiber results were normalized to the volume of PDMS and reported as micromoles (µmol) as 2,3-dimethylnaphthalene/milliliter (mL) PDMS. The quantitation limit was approximately 0.5 µmol as 2,3-dimethylnaphthalene/mL.

2.6 Statistical Analyses

All statistical analyses were performed using SAS statistical software (SAS, 2004). Results of the fish, *Daphnia*, and algal toxicity tests were reported on the basis of WAF loading rates (LL/EL50) and as measured dissolved NA concentrations (LC/EC50). Point estimates of the 50% effect levels (LL/EL50 and LC/EC50) in the fish and *Daphnia* tests were determined using either the probit or trimmed Spearman-Karber methods. When the P value for Goodness of Fit was >0.05 the probit method was selected for reporting. Alternatively, the Trimmed Spearman-Karber method was selected. The no-observed-effect concentrations (NOEC) and loading rates (NOELR) were determined either by using a Fisher's exact test or by the absence of lethality or abnormal effects. A Hochberg adjustment was used in the Fisher's test to control the experimental error rate at 0.05.

The algal test endpoints (E_rC50/E_rL50 values) were determined based on the inhibition of the average specific growth rate for a 96-h exposure. Growth data were evaluated for normality and homogeneity of variance using Shapiro-Wilk's and Levene's tests, respectively. Determination of the NOELR and NOEC values was made using a non-parametric analysis of variance (SAS Proc GLM) when transformation of the growth data failed to correct for non-normality.

3. RESULTS AND DISCUSSION

3.1 Test Substance Compositional Characteristics

3.1.1 GC-MS Analysis

GC-MS analysis of Z-family and carbon number groups (n) revealed the test sample to be dominated by one (39%) and two (30%) ring NAs (Figure 1). Acyclic NAs were the third most abundant group (24%). NAs containing 3, 4, or 5 rings accounted for only 5%, 2%, and <1%, respectively. Among the Z-family groups the frequency distributions of NA compounds were approximately bell-shaped with peak NA abundances occurring for compounds having 12 or 13 carbon atoms.

This pattern of carbon number and Z-family distributions is similar to that reported for other commercial NAs from this supplier (Grewer et al., 2010). However, commercial NAs from other suppliers may not have the same distribution of constituents. Grewer et al. (2010) showed that NA samples from two other commercial sources contained 80% and 15% acyclic, 8% and 46% 1-ring, and 8% and 33% 2-ring constituents, respectively. Scott et al. (2005) obtained unreliable analytical measurements using standards made from one commercial source when measuring aqueous concentrations of NAs of other commercial substances. The principal similarity among all the commercial NAs was the few NAs with three or more rings. This contrasts with that of OSPW extracts, which were shown to contain NAs with a wider range of molecular weight constituents and higher proportions having 3 or more rings (Holowenko et al., 2002; Scott et al., 2005; Grewer et al., 2010).

3.1.2 2D GC/ToF-MS Analysis

The GC-MS method employed to construct the Z-family and carbon number distributions does not discriminate individual NAs. Shown in Figure 2A is a 2D GC/ToF-MS total ion current chromatogram of the test sample . Unlike conventional GC-MS in which individual NA are rarely resolved and instead displaty as an unresolved complex mixture or 'hump', the 2D GC method employing an ionic liquid stationary phase in the first GC column, produced a chromatogram in which the presence of approximately 2000 components composed of non-aromatic (e.g. acyclic and alicyclic) NA, aromatic NA and non-acids were revealed. Peaks labeled 1-5 in Figure 2A are the five most abundant components in the NA test substance as

measured by peak height. These were tentatively identified as methyl esters of C10 through C14 *n*-acids (*n*-decanoic to *n*-tetradecanoic) from interpretation of the mass spectra and comparison with National Institute of Standards and Technology (NIST) library mass spectra. The existence of *n*-acids in these substances in commercial NAs has only been reported once previously (Rowland et al., 2011c). These and similar acyclic (Z=0) acids constituted part of the 24% of the acids derivatized by MTBSTFA and measured by the GC-MS method (Figures 1 and 2B).

Combining all sources of analytical data (i.e., GC-MS, argentation SPE, and urea adduction) made it possible to assign approximate percentages to the abundance of various groups of compounds in the NAs(Fig. 2B). GC-MS of the urea-adducted fraction, as expected, showed a predominance of *n*-acids ranging from C_9 nonanoic to C_{18} octadecanoic acids, with minor amounts of methyl and ethyl-branched acids. This fraction was estimated to be 12% of the total (i.e. half of the 24%) Z=0 acyclic acids (Figure 2B). The non-adducted fraction (72%) consisted of polymethyl-branched acyclic acids plus the 1-, 2-, and 3-ringed acids (Figure 2B). The GC-MS analysis of Z-family and carbon numbered groups revealed 24% acyclic structures; therefore, 12% existed as simply branched and *n*-acids, 12% as polymethyl-branched acids, while all ringed acids totaled 60%. Argentation SPE determined the monoaromatic acids totaled 7%. The remaining portion (9%) comprised a variety of non-acids and uncharacterized material. Manufacturer's data showed that small amounts of phenols acids were present (0.31 %) as identified in other commercial NAs (West et al., 2011). GC-MS analysis of this fraction showed it also contained unknown proportions of polyaromatic hydrocarbons and heterocyclic compounds.

In addition to the five most abundant acids, 15 additional NAs representing z=0, -2, -3, -4, -6, and -8 classes of acids were similarly identified against library matches, commercially available standards, or laboratory-prepared compounds. Figure 3 shows representatives of highly branched acyclic, cyclohexyl, decalin, and adamantine acids and the standard matches tentatively identified in the NA sample. No tetracyclic NAs were identified

3.2 Aquatic Toxicity Tests

Table 2 presents the concentration-response data for each toxicity test and the test endpoints determined on the basis of loading rates (LL/EL50) and mean measured NA concentrations (LC/EC50). All tests met the acceptability criteria specified in the test guidelines.

Few aquatic toxicity studies of commercial NAs have reported simultaneous quantitative measurements of the dissolved NAs in the test solutions. Previous reports of aquatic toxicity tests of commercial NAs are difficult to compare to our data owing to the lack of details of the experimental methods or descriptions of the chemical composition of the test substances (Cairns et al., 1965; Dokholyan and Magomedov, 1984). In general, exposure solutions were prepared on a nominal basis using dilutions of a stock solution. In the few studies that reported measured concentrations of NAs in solution, relatively low analytical recoveries were reported (Peters et

al., 2007; Young et al., 2011). Our studies show that exposure solutions prepared using the WAF technique produced consistent concentrations that were stable over time. The coefficient of variability (CV %) of measured NAs within the WAFs all tests ranged from 1% to 14%.

The fish acute toxicity endpoint reported here (fathead minnow 96-h LL50 = 9.0 mg/L, LC50 = 5.6 mg/L) is lower than the range reported by Dokholyan and Magomedov (1984) for fish exposed to sodium naphthenate (LC50s ranged 25 to 75 mg/L). Those authors did not measure the exposure concentrations and differences in toxicity may be due to biological (fish species, age/size factors) or chemical factors (water pH, NA composition). Of studies based on measured NA concentrations, Nero et al. (2006) reported for perch a 96-h LC100 of 3.6 mg/L using a commercial NA, and Scarlett et al. (2013) reported LC50s for zebrafish larvae of 13.1 mg/L and 8.1 mg/L, respectively, for non-aromatic and aromatic NA fractions of OSPW extracts. Both studies support an LC50 substantially lower than those reported by Dokholyan and Magomedov (1984). The toxicity of commercial NAs has not been reported previously for *D. magna* and *P. subcaptitata*, and test endpoints reflected a similar sensitivity for these two species. Test results reported in this study are new data for *D. magna* and *P. subcapitata*. Rapid toxicity screening using Microtox has increasingly been used to assess the relative toxicity of OSPW extracts (Holowenko et al., 2002; Frank et al., 2008) and individual NA compounds (Frank et al., 2009; Jones et al., 2011). However, the application of this assay to a commercial NA sample has not been reported previously.

Polar narcosis has been suggested as the likely mode of action (MOA) for acute toxicity of NAs based on their suspected structures and surfactant nature (Frank et al., 2009). Because the narcotic effect of a molecule is correlated with its size and lipophilicity (van Wezel and Opperhuizen, 1995), higher molecular weight fractions of NA mixtures should demonstrate a greater toxic potency. This direct relationship of increasing toxicity with increasing lipophilicity (generally correlated to log Kow) is observed until the solubility of the chemical limits its bioavailability. At this solubility cut-off, there is insufficient chemical in the dissolved phase to cause toxicity. Jones et al. (2011) demonstrated this effect for the toxicity of a variety of NA structures (e.g., acyclic, 1-, 2-, 3-ring, monoaromatic). Solubility cut-offs for toxicity to *V. fischeri* generally occurred in the range of C12 to C14, depending on the NA group.

The NAs responsible for toxicity in these mixtures and their MOA are uncertain, and require further investigation. A few researchers proposed a mechanism of action based on the spectrum of observed histopathological effects (Nero et al., 2006; Peters et al., 2007; Melvin et al., 2013). Biodegradation studies of OSPW and their extracts showing reduced toxicity with biodegradation of the low molecular weight NAs constituents imply toxicity is attributed to the low molecular weight fractions (MacKinnon and Boerger, 1986; Lai et al., 1996; Clemente et al., 2004). These earlier studies correlating biodegradation of low molecular weight NAs with reduced toxicity might appear contrary to the narcosis mode of action, but such observations would result if the non-degraded fraction consisted predominantly of NAs with molecular

weights over the solubility cut-offs. Toxicity assessments of OSPW extracts fractionated into molecular weight groups also seemed to align more with the correlative studies ascribing toxicity to the lower molecular weight NAs (Frank et al., 2008).

3.3 Biomimetic Extraction – Solid Phase Microextraction

Figure 4 presents the BE-SPME analyses of acidified water samples prepared at loading rates of 2.1, 4.7, 10.3, 22.7, and 50 mg NA/L. The relationship between loading and the measured BE-SPME was linear within the range of loading rates used in the toxicity tests ($y = 2.1173x - 2.553$; $r^2 = 0.9976$). Comparison of the BE-SPME results for the 50 mg/L sample, with and without acidification showed the importance of the neutral form of the naphthenic acids to maximize extraction. The acidified 50 mg/L sample yielded a mean BE of 103 µmol/mL PDMS while the corresponding non-acidified sample resulted in a BE concentration of 0.40 µmol/mL PDMS, which was slightly below the detection limit (0.5 µmol/mL PDMS).

The mathematical relationship fitting BE concentrations to the toxicity test loading rates and the predicted BE concentrations corresponding to the LL/EL50 endpoints are reported in Table 3. Based on the results of the mathematical regression, the BE concentrations equivalent to the LL/EL50 endpoints for *P. promelas, D. magna, P. subcapitata*, and *V. fischeri* are 16.5, 48.3, 88.5, and 63.1 umol/mL PDMS, respectively.

The BE-SPME technique was used as a means to compare toxicity test results on the basis of system exposure (i.e., lipid uptake) rather than on external water concentrations. BE-SPME fiber concentrations in the range of approximately 40-80 umol/mL PDMS correspond to a 50% acute effect in aquatic organisms attributable to non-polar narcosis for petroleum hydrocarbon mixtures (Parkerton et al., 2000). In this study, the BE analyses fit reasonably well for *D. magna*, *P.subcapitata*, and *V. fischeri* in that the critical C_{fiber} concentrations were 48.3, 88.5, and 93.9 umol/mL PDMS, suggesting a narcosis mode of action. The LL50 value for fish was 9.0 mg/L, with a correlating C_{fiber} concentration of 16.5 umol/mL PDMS, and indicates that a specific MOA besides narcosis may be a factor in fish toxicity. Nero et al. (2006) identified reduced gill surface area in fish exposed to acutely toxic NA concentrations, and this may also contribute to NA toxicity to fish.

4. CONCLUSIONS

The chemical composition of the classes and of the individual acids of a commercial sample of NA test sample obtained by a combination of GC-MS and 2D GC-MS methods using an ionic liquid GC stationary phase. The data revealed a predominance of 1- and 2-ringed acids (x%). Acyclic acids constituted the third most abundant group (x%), and C10 through C14 *n*-acids were the five most abundant individual acids in the sample. Examples of individual acids identified included acyclic, 1- to 3-ring and monoaromatic acids as well as some non-acids. The

test sample was most toxic to fish, while to invertebrates (*D. magna*), algae (*P. subcapitata*), and bacteria (*V. fischeri*) the NA showed similar, lower toxicity. Assessing acute aquatic toxicity using BE-SPME suggested a polar narcosis MOA for the invertebrate, algae, and bacteria, while that for fish may also include NA interaction with gill tissue.

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Acknowledgements

This project was sponsored and funded by the Petroleum HPV Testing Group (PHPVTG), an unincorporated group of manufacturers affiliated by contractual obligation to fund a voluntary data disclosure and toxicity testing program on certain petroleum-related chemical substances in response to EPA's HPV Challenge Program. The American Petroleum Institute (API) manages the PHPVTG's activities. We acknowledge the assistance of Paula Podhasky, API; Dr. Phillip Fedorak, University of Alberta, (GC-MS analyses); and Dan Letinski and Tricia Knarr-Cousineau, ExxonMobil Biomedical Sciences, Inc., (BE-SPME analyses and Microtox testing, respectively). Funding of the GCxGC-MS instrumentation used in this research was provided by an Advanced Investigators Grant (no. 228149) awarded to SJR for project OUTREACH, by the European Research Council, to whom we are also extremely grateful.

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TABLES

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Table 1.
Test parameters

Test design parameter	Fish	algae	daphnia	microtox
Test Type	96-h semi-static, 24-h renewal interval	96-h static	48-h semi-static 24-h renewal interval	15-min static
system	3.8L jars 2.0 L of WAF	250-mL flasks 100mL WAF	250-mL glass jars 200 mL of WAF	Microtox cuvettes 0.9 mL solution
Replicates/no of organisms	1/7 juvenile fish	$7/10^4$ cells/mL	4/5, <24hr old	1/approximately $1x10^6$ cells
Loading rates mg/L naphthenic acid	0 (control), 1.3, 2.5, 5.0, 10, 20	0, 2.5, 5.0, 10, 20, 40, 80	0, 5.0, 10, 20, 40, 80	0, 6.3, 13, 25, 50, and 100 mg/L [a]
Other test conditions	22±1°C under a 16 h light/8 h dark photoperiod	Shaken at 100 rpm, 24±2°C, 4300 lux continual illumination	20±2°C under a 16 h light/8 h dark photoperiod	15°C
Test Endpoint	mortality, %	inhibition of growth rate, %[b]	immobility, %	inhibition of luminescence, %

[a] Exposure solutions used for Microtox were dilutions of a stock WAF. Concentrations assume total dissolution of the NA test substance.
[b] Calculations of average specific growth rates, percent inhibition, and $E_rL/C50$ values followed OECD 201 guidelines.

Table 2.
Exposure-response data and toxicity test endpoints.

P. promelas			*D. magna*			*P. subcapitata*			*V. fischeri*	
Loading Rate (mg/L)	Mean Measured NA (mg/L)	Percent Response[1]	Loading Rate (mg/L)	Mean Measured NA (mg/L)	Percent Response[2]	Loading Rate (mg/L)	Mean Measured NA (mg/L)	Percent Response[3]	Nominal Concentration	Percent Response[4]
0 (control)	ND	14	0 (control)	ND	5	0 (control)	ND	--	0 (control)	
1.3	0.90	0	5.0	3.9	0	2.5	1.6	1	6.3	12
2.5	2.1	0	10	7.7	0	5.0	3.5	1	13	18
5.0	3.2	14	20	17	25	10	7.4	1	25	32
10	6.0	57	40	33	100	20	15	7	50	48
20	14	100	80	69	100	40	28	39	100	74
						80	45	97		
96-h LL50 = 9.0 mg/L (6.6-12 mg/L)			48-h EL50 = 24 mg/L (21 – 27 mg/L)			96-h E_rL50 = 43 mg/L (42 – 45 mg/L)			15-min EC50 = 46 mg/L (33 – 63 mg/L)	
96-h LC50 = 5.6 mg/L (2.5 – 10 mg/L)			48-h EC50 = 20 mg/L (17 – 23 mg/L)			96-h E_rC50 = 30 mg/L (29 – 31 mg/L)				

[1] mortality
[2] immobilization
[3] inhibition of growth rate
[4] photoluminescence

pH? Remove LL and EL?

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Table 3.
BE-SPME concentrations for toxicity test loading rates and predicted critical fiber (C_{fiber}) concentrations for test endpoints.

P. promelas		*D. magna*		*P. subcapitata*		*V. fischeri*	
Nominal Loading (mg/L)	Calculated BE-SPME umol/mL PDMS	Nominal Loading (mg/L)	Calculated BE-SPME umol/mL PDMS	Nominal Loading (mg/L)	Calculated BE-SPME umol/mL PDMS	Nominal Loading (mg/L)	Calculated BE-SPME umol/mL PDMS
1.3	0.20	5	8.03	2.5	2.74	6.3	10.79
2.5	2.74	10	18.62	5	8.03	12.5	23.91
5	8.03	20	39.79	10	18.62	25	50.38
10	18.62	40	82.14	20	39.79	50	103.31
20	39.79	80	166.83	40	82.14	100	209.18
				80	166.83		
96-hr LL50=9.0	C_{fiber}[1]= 16.5	48-h EL50=24	C_{fiber}= 48.3	96-h E_rL50=43	C_{fiber}= 88.5	15-min EC50=46	C_{fiber}= 93.9

[1] The C_{fiber} concentration represents the predicted concentration of organic constituents normalized to 2,3-dimethylnaphthalene that partitioned to the PDMS fiber at the toxicity endpoint.

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Figures

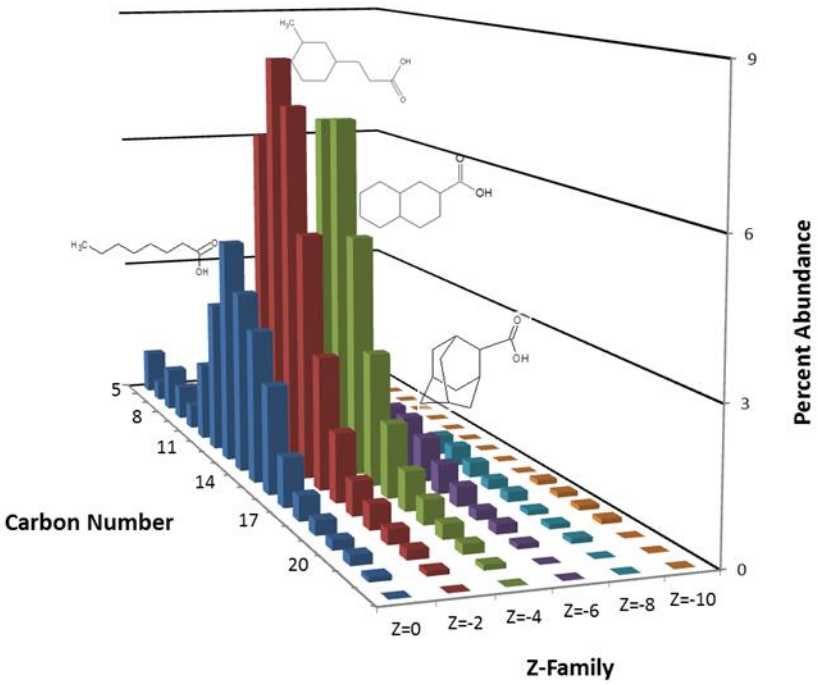

Fig. 1. GC-MS analysis of the distribution of carbon numbers and Z families of NAs in the commercial NA sample. The columns represent the percentage (by number of ions) of NAs in the sample that account for a given carbon number of a given Z family (corresponding to a specific *m/z*). The sum of all columns equals 100%.The structures shown are specific examples only of individual members of each class which were identified by 2D GC-MS as shown in Figures 2 and 3.

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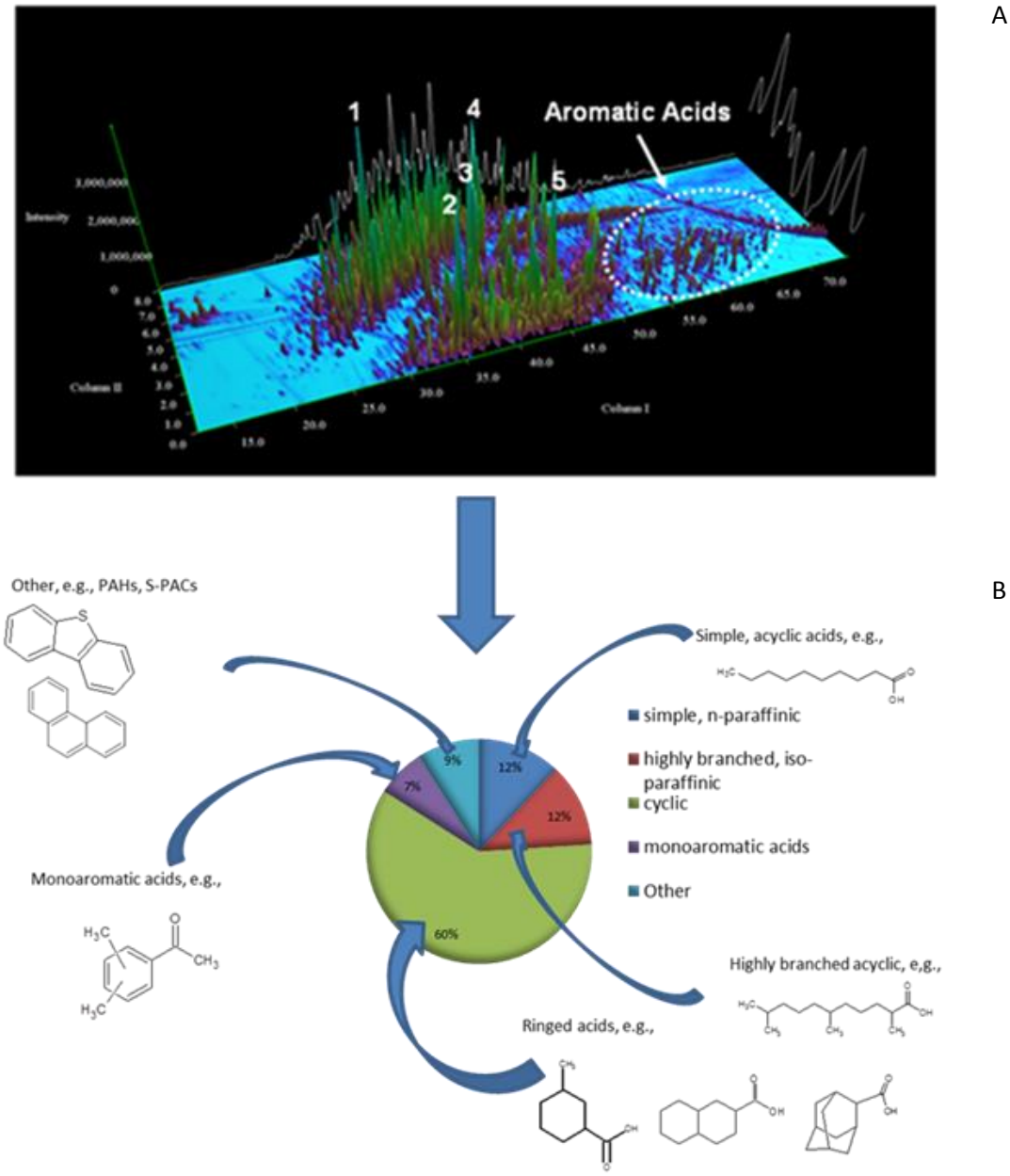

Fig. 2. Total ion current chromatogram showing the 5 most abundant acids in the NA test sample (A). A breakdown of the test sample shows component groups and their estimated proportional abundance (B). Subdivide 60% into 1-,2- 3- ring from fig 1?

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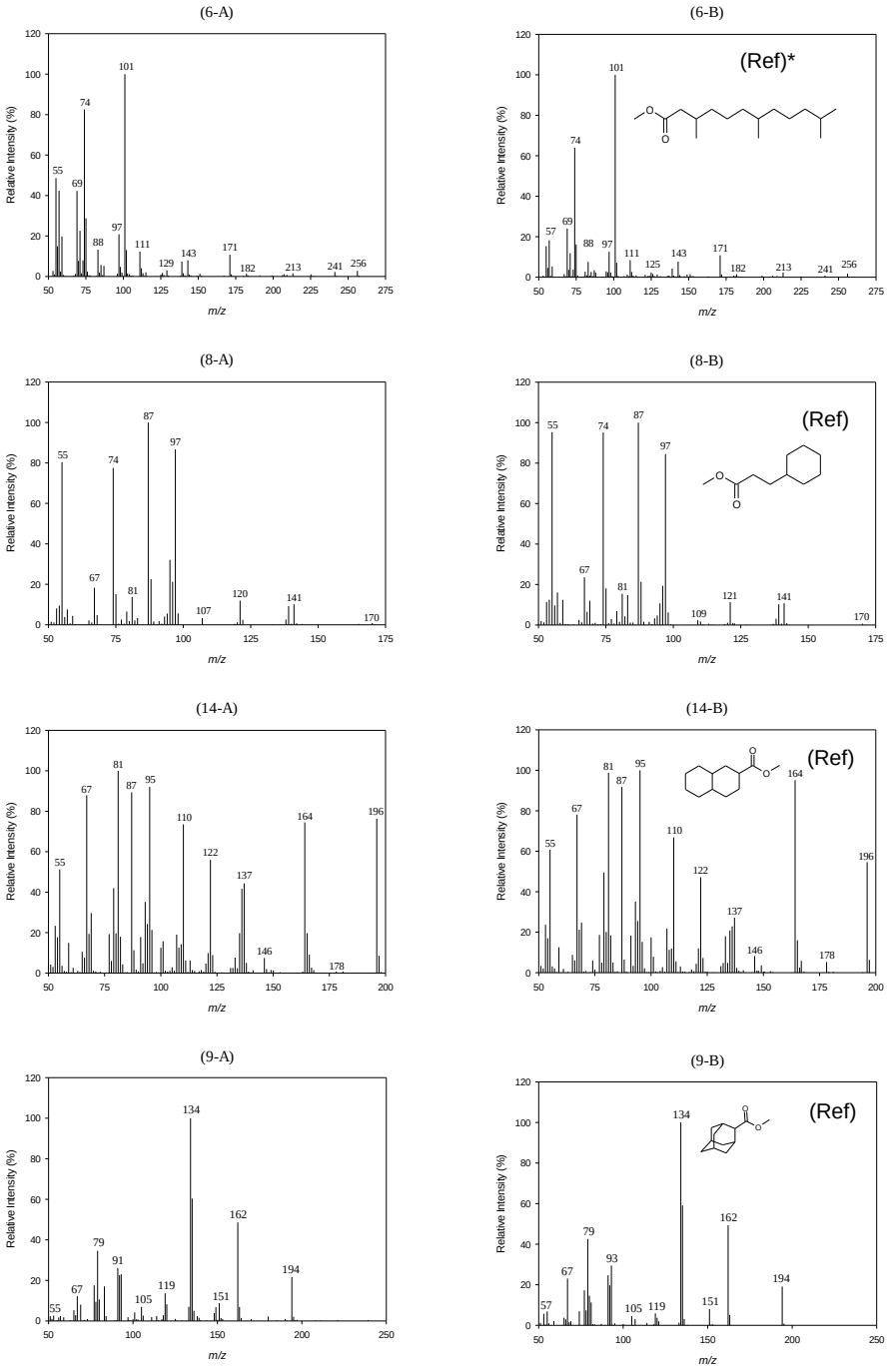

Fig. 3. 2D GC-MS mass spectra of methyl esters of unknowns and spectra of reference compounds showing identifications for 3,7,11-trimethyldodecanoic acid, cyclohexyl-3-propanoic acid, decalin-2-carboxylic acid, and adamantane-2-carboxylic acid as further examples of acyclic (24%) and mono- to tricyclic (60%) acids. Further spectra can be found in the Supplementary Information.

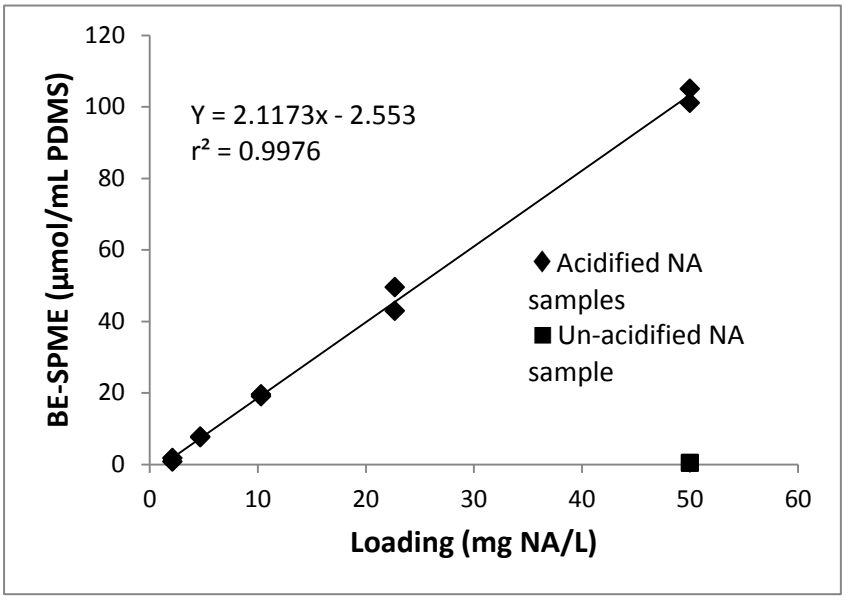

Fig. 4. Relationship between NA loading and BE-SPME of acidified water. Results of un-acidified duplicate samples at 50 mg/L are included.