Memorandum

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Re 13TP2 Final Report 1219328
"Microtox Test"

[redacted]

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Date July 12, 2013

This memo serves as the final report for Study 1219328 and describes the test methods and results of the Microtox analysis of the samples described below.

Approval Signatures

[redacted]

12 Jul 13
Date

12 Jul 13
Date

Sponsor American Petroleum Institute
1220 L Street, NW
Washington, DC 20005-4070

Test Substances:

EMBSI ID	Compound Name
MRD-12-193	Naphthenic Acid (API – HPV-1203) (received in 2012)
MRD-13-383	Naphthenic Acid (API – HPV-1203) – BE-SPME analysis only) (received in January 2013)

Summary:

The objective of this study was to provide predictive and measured bacterial toxicity of a commercial naphthenic acid (NA) mixture. Bacterial toxicity of the test substance was determined utilizing a Microtox Model 500 Analyzer with the testing based on the general principles described in EN ISO11348-3 (International Organization for Standardization (2007) Water qualiy – Determination of the inhibitory effect of water samples on the light emission of Vibrio fischeri (Luminescent bacteria test) – Part 3: Method using freeze-dried bacteria.)

Testing also included analyzing separate acidified samples of the test substance in water by automated BE-SPME at 50 mg/L. Additionally, a non-acidified sample from the 50 mg/L treatment loading was also analyzed.

Solution Preparation and Microtox Analysis

A stock solution was prepared in Microtox diluent at approximately 110 mg/L. The solution was initially shaken by hand for 10 minutes. When it was determined that the test substance was not going into solution, the volumetric flask was placed on a stir plate and left stirring vigorously overnight. Visible test substance was removed from the surface of the solution prior to a sample being taken for the range-finding and definitive portions of the study.

A range-finding test consisting of nine (9) concentrations and a control was initially run to determine the correct levels at which to run the definitive test.

The test protocol followed for both the range-finding and definitive testing, was the 81.9% basic test from the Microtox Omni Software. The test consisted of the following preparation and analysis steps:

- Cuvettes were added to the appropriate incubator wells.
- The 110 mg/L stock solution was added to a cuvette and 1:1 serial dilutions were prepared using Microtox diluent to achieve the desired test concentrations in a stepwise manner.
- Microtox Acute Toxicity Reagent was reconstituted and diluted to the appropriate concentration for the test.
- The diluted reagent was added to the cuvettes intended for bacteria and blank reagent readings were taken using the Microtox Model 500 Analyzer.
- 900µL of the prepared serial dilution samples and control were transferred into the cuvettes containing the bacteria.
- Readings were taken at 5 and 15 minutes after the addition of the samples to the bacteria.

Concentrations tested in the range-finding portion of the test can be found in Table 1.

Table 1.

Compound Name	Concentrations tested (mg/L)*
Naphthenic Acid (API – HPV-1203)	100, 50, 25, 12.5, 6.3, 3.1, 1.6, 0.78, 0.39

*As the stock solution was prepared in Microtox diluent, the dilution of the stock in the test system was 10%, rather than the 18.1% prescribed in the methodology

Concentrations tested in the definitive portion of the test can be found in Table 2.

Table 2.

Compound Name	Concentrations tested (mg/L)*
Naphthenic Acid (API – HPV-1203)	100, 50, 25, 12.5, 6.3

*As the stock solution was prepared in Microtox diluent, the dilution of the stock in the test system was 10%, rather than the 18.1% prescribed in the methodology

Automated Biomimetic Extraction using SPME (BE-SPME) Analysis:

Separate mixtures of the test substances were prepared in moderately hard recon water at 50 mg/L for both the MRD-12-195 and MRD-13-383 test materials. Water samples for BE-SPME analysis were acidified and portions transferred from VOA vials to ca. 20 mL glass autosampler vials, with no headspace, and capped with Teflon® faced septum lids. The vials were placed on a LEAP Technologies (CTC Analytics) Combi PAL autosampler configured for automated SPME injections. 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 BE method was calibrated by making 0.5 µL liquid injections of a series of aromatic hydrocarbon standard solutions. 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 is approximately 0.5 µmol as 2,3-dimethylnaphthalene / mL.

Results

Microtox Analysis

Bacterial toxicity results, 95% confidence ranges and coefficients of determination are listed in Tables 3 (range-finding study) and 4 (definitive study).

Table 3. Range-Finding Study Results

Compound Name	**5 minute EC50 (mg/L)**	**95% Confidence Range (mg/L)**	**Coefficient of Determination**
Naphthenic Acid (API – HPV-1203)	36.36	21.75 – 60.06	0.9300
Compound Name	**15 minute EC50 (mg/L)**	**95% Confidence Range (mg/L)**	**Coefficient of Determination**
Naphthenic Acid (API – HPV-1203)	30.76	18.64 – 50.78	0.9398

Table 4.

Compound Name	5 minute EC50 (mg/L)	95% Confidence Range (mg/L)	Coefficient of Determination
Naphthenic Acid (API – HPV-1203)	51.44	36.70 – 72.10	0.9774
Compound Name	**15 minute EC50 (mg/L)**	**95% Confidence Range (mg/L)**	**Coefficient of Determination**
Naphthenic Acid (API – HPV-1203)	45.57	32.76 – 63.40	0.9757

BE-SPME Results

In order to demonstrate mixtures of the test substance used for determination of Microtox bacterial toxicity were similar to those previously analyzed in establishing the relationship between naphthenic acid loading and acidified BE-SPME (12TP 26, EMBSI study 1219390), additional mixtures of both MRD-12-193 and MRD-13-383 were prepared at 50 mg/L in moderately hard recon water.

BE-SPME results for MRD-12-193 and MRD-13-383 are listed in Table 5. The mean BE-SPME values for acidified 50 mg/L water samples was 108 µmol as 2,3-dimethylnaphthalene / mL PDMS and is in close agreement with previous BE-SPME determination of 103 µmol as 2,3-dimethylnaphthalene / mL PDMS for a 50 mg/L acidified water sample. These results demonstrate BE-SPME equivalency across the two studies and permit correlation of bacterial toxicity (Microtox) to acidified BE-SPME.

It has been previously demonstrated that BE-SPME fiber concentrations in the range of approximately 40-80 µmol/mL PDMS correspond to a 50% acute effect in representative aquatic organisms (e.g. fish, algae, daphnia) attributable to non-polar narcosis for petroleum hydrocarbon mixtures. See attachment 12TP 26 (1219390 Att) for a summary of the SPME analysis of API NA mixture and interpretation of results.

Table 5. BE-SPME results

Sample Treatment	Rep	Auto BE Conc µmol as 2,3-DiMeNphthln/ mL of PDMS	Mean
control acidified	1	0.700	0.700
MRD-12-193 50 mg/L (acidified)	1	109	113
	2	116	
MRD-12-193 50 mg/L (no acid)	1	2.57	2.09
	2	1.60	
MRD-13-383 50 mg/L (acidified)	1	102	103
	2	104	
MRD-13-383 50 mg/L (no acid)	1	1.68	1.85
	2	2.03	

nd not detected quantitation limit ~0.5 µmol/mL PDMS

Biomimetic Extraction using Solid Phase Microextraction (BE-SPME) analysis of an API commercial Naphthenic Acid (NA)

Summary: Characterization of the commercial naphthenic acid substance using BE- SPME was conducted to provide a basis by which to compare the bioavailability of this complex substance with API aquatic toxicity test results. SPME is an analytical technique that is used as a surrogate for critical body burdens and provides a means to compare results on the basis of systemic exposure (i.e., lipid uptake) rather than on external water concentrations. SPME extraction and analysis of NA-WAFs is performed by converting the GC-FID response to molar units of test material that accumulates on the PDMS resin coating the SPME fibers (e.g., umol/ml PDMS).

Fiber concentrations are used to analyze the results of toxicity studies in fathead minnows, algae and daphnids). These results were also compared to historical critical fiber concentrations derived from No 2 Fuel WAFs. Results of BE-SPME analysis show reliable estimation of aquatic toxicity of complex substances.

Background: The BE work is an extension of previous work showing passive samplers (SPME) can be used to integrate and normalize toxic responses following exposures to complex substances. The BE technique provides an estimate of critical effects levels for complex substances for chemicals which have similar modes of action.

Principle: CBBs provide a method for evaluating the toxicity of chemicals that have different physic-chemical properties (e.g., BCF, solubility, $\log K_{ow}$, etc.) but have similar toxic modes of action, such as hydrocarbons (McGrath and Di Toro 2009). Further, the CBB concept provides a way for evaluating the toxicity of complex mixtures (Redman et al 2012). CBB is a constant for different chemicals which act by a common mode of action. Furthermore, the Critical Body Burden (CBB) concept allows ranking species by their relative sensitivities, which is very useful for deriving Water Quality Guidelines (WQG) by extrapolating down to protective levels using accepted statistical

The Target Lipid Model (TLM) uses a CBB framework, which has been validated for petroleum hydrocarbons (McGRath and Di Toro 2009), and application to other chemical classes such as naphthenic acids is being evaluated (Redman et al 2012b). The organism's internal concentrations ($C_{organism}$) are compared to the key metric (CBB), under the assumption that metabolites contribute to the overall toxicity. This relationship is illustrated in Figure 1.

In this work the SPME fiber is assumed to be a surrogate for the CBB.

The advantage of this approach is that the total accumulation of all material on the fiber can be easily measured using standard analytical methods (Figure 2). It provides a sampling method for determining the integrated exposure from complex substances. This kind of work is becoming widely accepted and has been validated in our lab for application to oils, field samples and more recently is being adapted for use with NA. For the NA work, aqueous samples are acidified to so that the neutral form of the NA are partitioning to the fiber.

The CBB concept is also very useful for addressing mixtures as long as all chemicals have a similar toxic mode of action, and also implicitly accounts for the contribution of metabolites to toxicity. This is relevant for NA since they exist as mixtures of various functionalities (C#, Z-families, etc). For hydrocarbons this is straight forward, but is a bit more complex for ionizable organics, NAs in particular, which ionize at pH~4.5. There are a number of key technical issues that require attention as this modeling approach is adapted to NAs. Chiefly that, unlike hydrocarbons, NA are ionizable which can affect the bioconcentration and toxicity of this class of compounds, and it is commonly accepted that they behave as polar narcotics (Frank et al 2009). Several datasets have been compiled to address this issue , as well as the development of new data that will also be used to train these models further and to evaluate the application of the SPME method for estimating bioavailability of complex mixtures.

Results and Discussion: Biomimetic extraction via BE-SPME and quantification of an acidified WAF of the API NA mixture was performed and is reported in EMBSI report …. In this study, the aquatic effects from API contracted studies versus NA aqueous concentration (loading) and aquatic effects versus BE-SPME concentration were graphically evaluated (Figures 3 and 4). A linear relationship between loading (0, 2.1, 4.7, 10.3, 22.7, 50 mg/L) and measured BE in acidified samples was established (Figure 5). A mathematical relationship fitting BE concentrations to LL50 or EL50 values for the NA mixture was determined in respective fathead minnow, algal, daphnia acute tests and the predicted BE values are reported in Table 1. It has been previously demonstrated that BE-SPME fiber concentrations in the range of approximately 40-80 µmol/mL PDMS correspond to a 50% acute effect in aquatic organisms attributable to non-polar narcosis for petroleum hydrocarbon mixtures. Based on the results of the mathematical regression, the equivalent EC50 or LC50 approximately corresponds to the 22.7 mg/L treatment analyzed in this study. However, although this 22.7 mg/L treatment value would be protective of algae and invertebrates, where EC50 values were 23.8 and 24 mg/l, with an estimated BE of 47.8 and 48.3 µmol /ml of PDMS , respectively, the BE analysis under predicts fish toxicity. The LC50 value for fish at 96 hr. was 9.0 mg/L of NA (nominal) based on the results of the API testing, with a correlating BE value of 16.5 µmol/mL PDMS, which indicates that a specific mode of action besides narcosis may be a factor in fish toxicity, specifically gill tissue interaction.

Conclusion: Single naphthenic acids testing (Redman et al 2012b) at EMBSI indicates that toxicity increases as the carbon number and $\log K_{ow}$ increase. As the carbon number increases further a toxicity cut-off will be reached. There is a reasonable correlation between critical fiber concentrations and the modeled CBBs. Based on the results reported in EMBSI lab report for this study, the QSAR model parameters were extended to the more complex compositions of the commercial API NA sample and provides a reliable method for estimating toxicity of NA. However, fish toxicity results were underestimated by BE-SPME determinations, since the predicted NA mixture LC50 value was 16 mg/L, whereas test data showed an LC50 of 9 mg/L.

References:

Veith G. and Broderius S. 1990. Rules for distinguishing toxicants that cause type I and type II narcosis syndromes.Environ Health Persp87: 207-211.

Kavanagh R., Frank, R.A, Burnison, B.K, Young, R., Fedorak, P.M., Solomon, K.R. and Van Der Kraak, G. 2012. Fathead minnow (Pimephales promelas) reproduction is impaired when exposed to a naphthenic acid extract. Aquatic Toxicology. 116-117. (34-42)

Redman, Lampi, Parkerton, 2012. A proposed framework to derive water quality criteria for naphthenic acids. Env. Recl. and Reveg Group meeting, Edmonton, AB Jan 30 2012

Figure 1. Illustration of Critical Body Burden and Log Kow as Surrogate

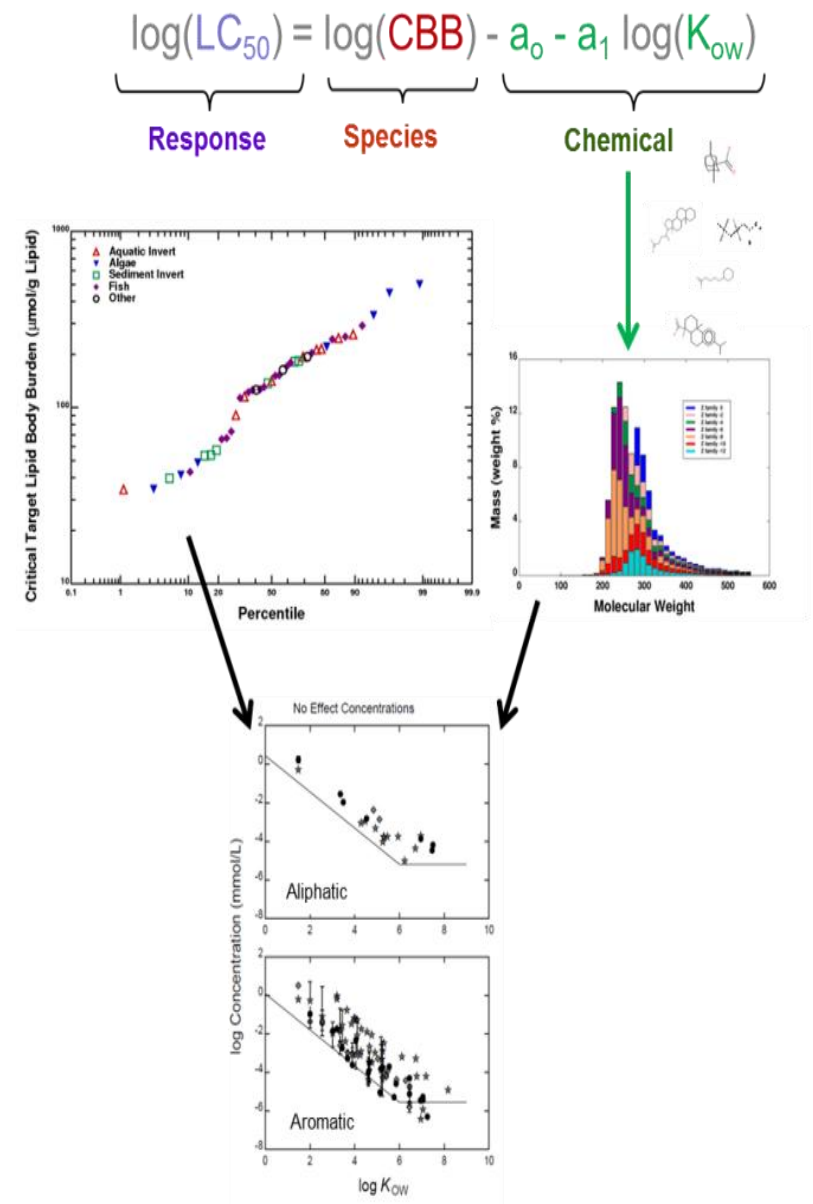

Figure 2: BE-SPME analysis and application to NA mixtures

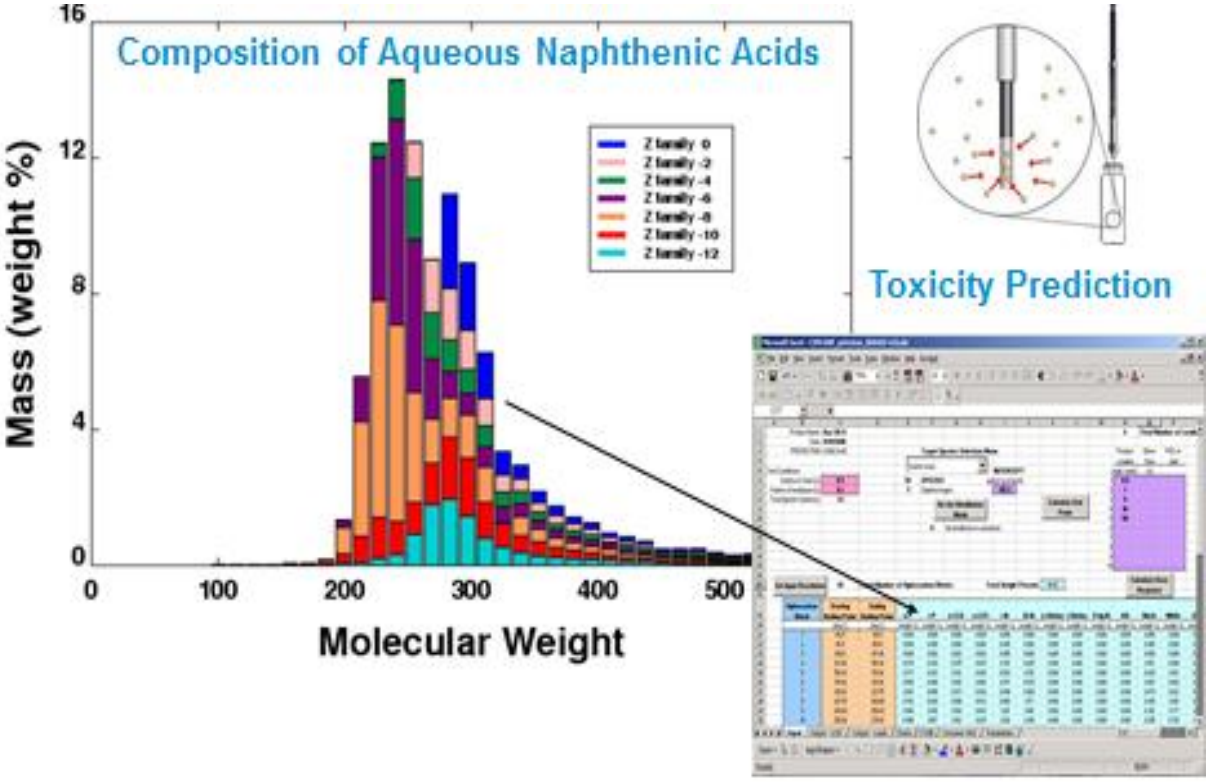

Figure 3. Effects versus NA aqueous concentration (loading)

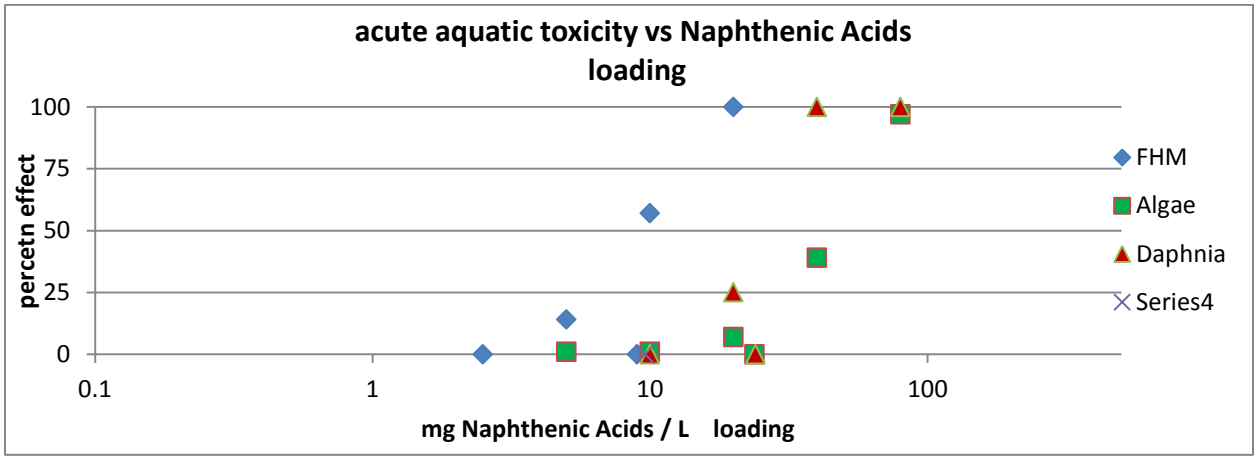

Figure 4. Effects versus BE-SPME concentrations (of NAs in aqueous solution).

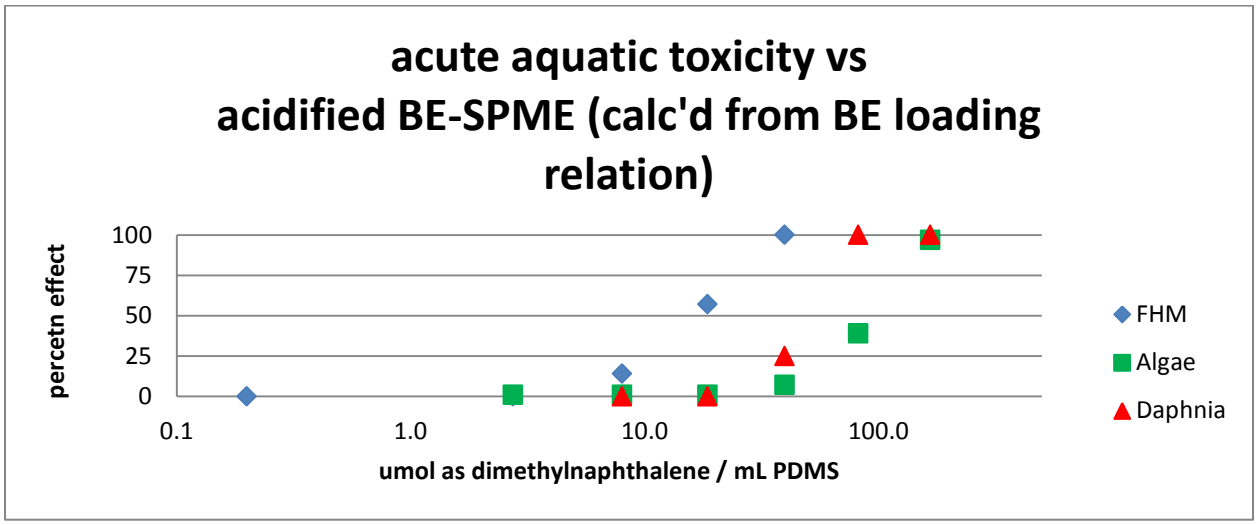

Figure 5 Linear relationship between BE-SPME concentrations and NA aqueous loading

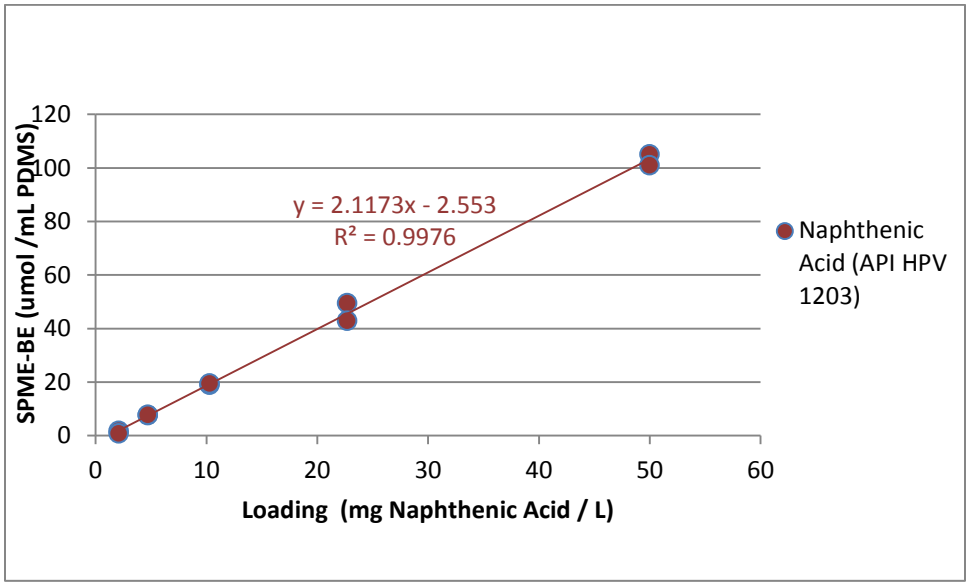

TABLE 1

FHM			Algae			Daphnia		
nominal loading (mg/L)	mortality (%)	calculated BE-SPME umol/mL PDMS	nominal loading (mg/L)	inhibition (%)	calculated BE-SPME umol/mL PDMS	nominal loading (mg/L)	immobilization (%)	calculated BE-SPME umol/mL PDMS
0	14		0	0		0	5	
1.3	0	0.20	2.5	1	2.74	5	0	8.03
2.5	0	2.74	5	1	8.03	10	0	18.62
5	14	8.03	10	1	18.62	20	25	39.79
10	57	18.62	20	7	39.79	40	100	82.14
20	100	39.79	40	39	82.14	80	100	166.83
			80	97	166.83			
9.0	***96hr LL_{50}***	***16.5***	***23.8***	***72hr EyL50***	***47.8***	***24***	***48hr EL_{50}***	***48.3***
		C_{fiber}			***C_{fiber}***			***C_{fiber}***