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Development of Automated Methods of Identifying Toxicants in the Environment

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Many NPDES permits today require the development of Toxicity Reduction Evaluation (TRE) programs designed to reduce toxicity to acceptable levels when effluent toxicity is detected. The first of two approaches commonly used in the abatement of effluent toxicity is treatment without the specific identification and confirmation of the causative toxicants. The second method involves specific identification and confirmation of the causative toxicants with the goal of locating and eliminating the problem at its source. Specific methods for characterizing, identifying and confirming causative toxicants were developed by USEPA in the late 1980's (Mount and Anderson-Carnahan 1988 a-c). These procedures typically entail manual physical/chemical manipulation of a toxic sample followed by toxicity assessment and analytical analysis in three separate steps. The need for more rapid, cost-effective, and analytical methods of identifying environmental toxicants warranted the development of automated procedures using commercially available equipment and techniques. In this report, the development and preliminary validation of an automated identification methodology for water-borne toxicants using an Ion Chromatograph/High Pressure Liquid Chromatograph (IC/HPLC) is presented. Development of the methodology included testing the system with synthetic media blanks to ensure that the equipment was not introducing toxicity, as well as evaluating the purity of the samples prepared. Ammonia, dissolved solids, copper, pentachlorophenol, and diazinon toxicant standards, as well as a composited mixture of the individual toxicants, were also used to develop and fine-tune the operation of the automated system. Preliminary validation of the automated methodology was performed by comparing the results obtained from toxicity identification studies with an industrial wastewater effluent using conventional methods to studies employing the newly developed automated methods. Preliminary results indicated that the automated approach will provide the scientific community with a rapid, cost-effective, automated method for performing standard toxicity identification studies.

MATERIALS AND METHODS

Automation of conventional methods designed to isolate and identify water-borne environmental toxicants employed an integrated system capable of performing analytical ion chromatography while simultaneously executing modified toxicity identification procedures. A simplified diagram of the IC/HPLC system is presented in Figure 1. The ion chromatography system

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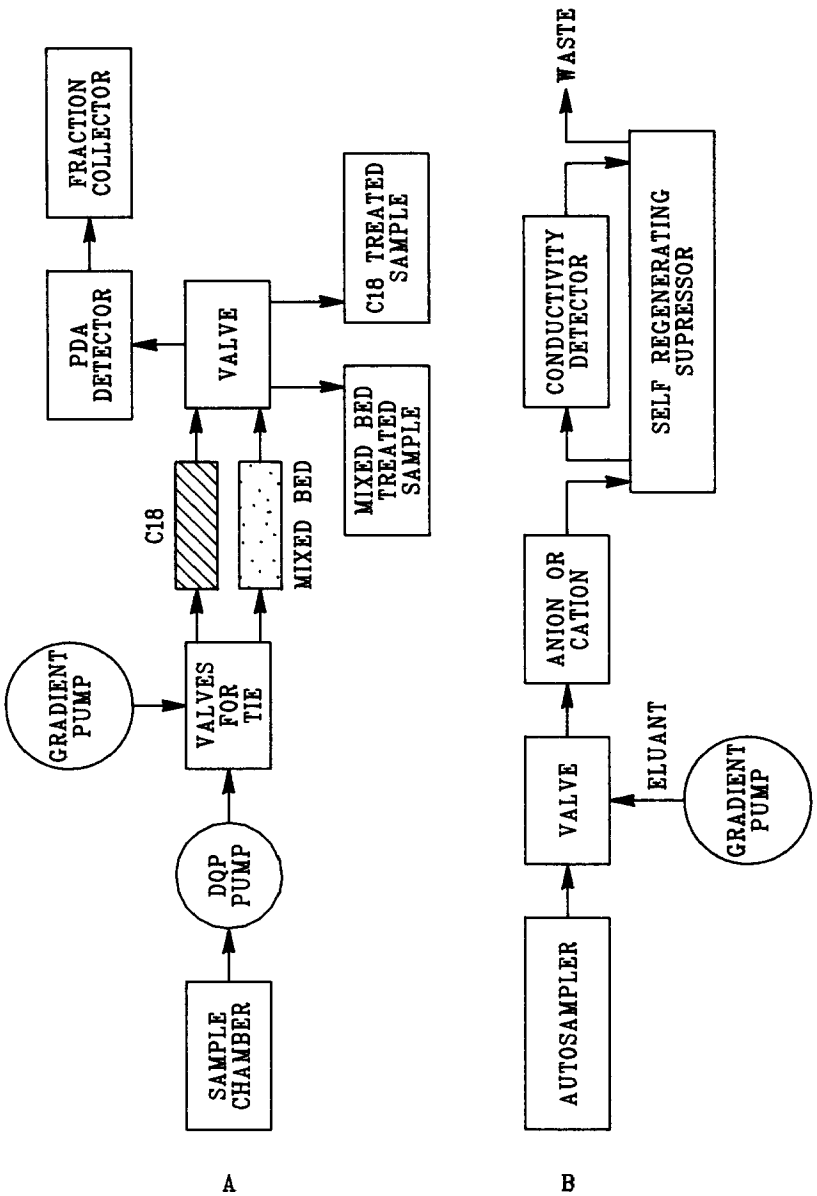

Figure 1. A. Simplified schematic of TIE module of IC/HPLC system.
B. Simplified schematic diagram of analytical module.

consisted of a Dionex Model DX-300 IC/HPLC with two gradient pumps, an electrochemical conductivity detector, and a pulsed electrochemical detector. The IC module was outfitted with an Ion Pac AS4A anion separator column with an AG4A guard column, an Ion Pac CS12 cation separator column with a CG12 guard column, and an automatic self-regulating suppressor system for both anion and cation analyses. A 9 mm x 25 cm ID column packed with a Barnstead D-8902 mixed-bed ion exchange resin having a total ion capacity of 1200 grains (as NaCl) was used for concentrating ionic contaminants. The HPLC module utilized a Groton Technologies Solonet B ultraviolet-visible diode array detector with a wavelength range of 190-700 nm and a Foxy 200 fraction collector. A Zorbax C_{18} column (4 mm x 12.5 mm ID) was used for concentrating organic contaminants in the sample.

Prior to loading samples, the C_{18} column was conditioned as follows: 100% HPLC-grade methanol for 5 min at a flow rate of 2 mL/min; a linear water methanol gradient over a 10 min period eventually concluding with pure deionized water conditioning for an additional 8 min. A Dionex DQP single piston pump was used to load samples onto the appropriate column at a flow rate of 5 mL/min. Following the C_{18} column loading, flow was switched to allow the sample to be loaded onto the mixed-bed ion exchange column. Eluate samples (100 mL) from each column were collected for toxicity evaluation. Concurrently with mixed-bed ion exchange column loading, the HPLC module was washed with 25% methanol:water for ten min to prepare for column elution and chemical-specific data collection. Compounds trapped on the C_{18} column were then eluted with a 25 to 100% methanol step gradient at a rate of 1 mL/min. Spectral (254 and 280 nm) data were collected during the gradient elution step and the elutriate fractions (25, 50, 75, 80, 85, 90 and 100% methanol) were automatically collected by the fraction collector throughout the run and used for further toxicity evaluations. At the conclusion of the methanol:water column elution, the C_{18} column was cleaned with 100% HPLC-grade acetonitrile (2 mL/min for 5 min). Spectral data were acquired during acetonitrile cleaning to detect potential contaminants in the elutriate which were not eluted during the methanol gradient. At the conclusion of the C_{18} column elution the column was stored in 100% methanol to prepare for a subsequent conditioning/sampling cycle.

Development and validation of the automated methods were performed using standard toxicant solutions including ammonia [25 mg/L, as nitrogen], conductive dissolved solids (sodium chloride) [9.3 mmhos/cm and 18.1 mmhos/cm], copper [0.2 mg/L], pentachlorophenol [5 mg/L], and diazinon (1 mg/L). Reagent-grade ammonium chloride, sodium chloride, and copper were obtained from Sigma Chemical Company (St. Louis, MO). Pentachlorophenol and diazinon were obtained from Fluka Chemical Corporation (Ronkonkoma, NY) and Ultra Scientific (Houston, TX). All stock solutions were adjusted to pH 7.0 prior to evaluation. A complex composite mixture of the five reference toxicants was prepared. This mixture contained: 4.0 mg/L ammonia-nitrogen, 1.0 g/L sodium chloride, 0.05 mg/L copper, 1.0 mg/L pentachlorophenol, and 0.2 mg/L diazinon. An industrial effluent sample was also evaluated using the automated technology and compared to results obtained via conventional methods.

Forty-eight hr static toxicity tests were conducted with the C_{18} and mixed bed ion exchange eluate samples, as well as the C_{18} methanol elutriate fractions according to the methods described by Weber (1991). For each test performed

with the C_{18} and mixed-bed ion exchange eluate, six to eight concentrations were tested. Moderately hard reconstituted water (Weber et al. 1989), a medium suitable for the culture of *Ceriodaphnia dubia* and *Pimephales promelas*, was used to prepare the concentrations tested. For each test, reconstituted water and reconstituted water treated by the IC/HPLC were tested for toxicity and designated as the negative and treatment controls, respectively. Five <24-hr old *C. dubia* neonates and five <24-hr old *P. promelas* larvae were placed in each of two separate replicates per concentration. Appropriate methanol elutriate fractions were screened for toxicity by diluting 150 μL of the 3 mL of elutriate collected into 10 mL of moderately hard reconstituted water. Five *C. dubia* or *P. promelas* were placed in each single replicate cup. *C. dubia* and *P. promelas* tests were conducted in 20-mL plastic souffle cups containing 15 mL of total solution. Adult *C. dubia* and *P. promelas* were cultured as described by (Weber 1991). Mortality was recorded at 24-hr intervals. Forty-eight hr median lethal effect concentrations (LC50) and 95% confidence intervals were calculated for each toxicity test by the method of Spearman-Karber (Hamilton et al. 1977). Chemical-specific data were acquired from the IC/HPLC. Pentachlorophenol and diazinon concentrations of the stock solutions, treated samples, and appropriate methanol ellutriate fractions were also verified with gas chromatography-mass spectrometry.

RESULTS AND DISCUSSION

Analytical and toxicological results from sample blank fractions (reconstituted water) collected from the IC/HPLC system suggested that the automated system produced a clean baseline and did not impart toxicity to either *C. dubia* or *P. promelas*. An analytically clean baseline devoid of artifactual contaminants is not usually obtained using conventional methods. In fact, phthalate esters are common artifactual contaminants of conventional C_{18} preparations possibly due to leaching from the plastic solid phase extraction syringe (Fort et al. unpublished). Since the IC/HPLC system utilizes a stainless steel column and telflon-lined tubing, phthalate ester contamination is reduced. However, further work will be required to determine if phthalate esters prove to be problematic following continued use of the system. Results of toxicity and analytical characterization of the negative and treatment controls and the untreated toxicant standards of ammonia, sodium chloride, copper, pentachlorophenol, and diazinon, the toxicant standards, and the composited mixture using the automated IC/HPLC system are provided in Tables 1 and 2. Negative control mortality rates were <10% in all toxicity studies reported. Mixed-bed ion exchange treatment reduced the ammonia levels in the ammonia standard approximately 3.0-fold. The toxicity of the ammonia standard to *P. promelas* was also reduced following mixed-bed ion exchange treatment. C_{18} treatment failed to reduce the concentration of ammonia in the standard solution, as well as the toxicity to *P. promelas*. Since ammonia acts as a weak base, it was primarily ionized at pH 7.0 (NH_4^+ form), and thus was removed by ion exchange treatment. However, as the pH increased, the concentration of the primary toxic species increased (NH_3 [unionized]) which was not effectively removed by ion exchange treatment.

Mixed-bed ion exchange reduced the conductive dissolved solids levels of the 9.3 and 18.1 mmhos/cm sodium chloride standard 4-fold and 3.4-fold, respectively. Mixed-bed ion exchange also decreased the toxicity of the sodium chloride standards to *C. dubia* and *P. promelas*. As expected, C_{18} treatment failed to alter the toxicity of either the sodium chloride standard solution or the

Table 1. Effect of control treatments and untreated standards produced by IC/HPLC on *C. dubia* and *P. promelas* toxicity.

Toxicant Standards[1]	Negative Control			Treatment Control[2]			Untreated Standard		
	Toxicity[3]		Concentration[4]	Toxicity[3]		Concentration[4]	Toxicity[5]		Concentration[4]
	P. promelas	*C.dubia*		*P. promelas*	*C.dubia*		*P. promelas*	*C.dubia*	
Ammonia[6]	0.0	-	0.02	0.0/0.0	-	0.5/0.6	62.5 (50.0-75.0)	-	17.1
Sodium Chloride[7]	0.0	0.0	0.6	10.0/0.0	10.0/0.0	0.6/0.003	73.5 (66.7-80.1)	52.4 (46.7-58.6)	9.3 18.1
Copper	-	0.0	<0.02	-	10.0/10.0	<0.02/<0.02	-	37.5 (25.0-50.0)	0.16
Pentachlorophenol	10.0	10.0	<0.02	0.0/0.0	0.0/0.0	<0.02	12.5 (1.0-25.0)	12.5 (1.0-25.0)	5.0
Diazinon	0.0	10.0	<0.02	-	0.0/0.0	<0.02	-	12.5 (1.0-25.0)	0.7
Composite Mixture[8]	0.0	0.0	-	0.0/0.0	0.0/0.0	-	37.5 (32.5-43.5)	12.5 (1.0-25.0)	-

[1] pH 7.0.
[2] C_{18} treatment/ion exchange treatments.
[3] Expressed as percent mortality.
[4] Expressed as mg/L which exception of sodium chloride expressed as mmhos/cm.
[5] Forty-eight hr LC50 with respective 95% confidence intervals. Expressed as percent sample.
[6] Due to *P. promelas* sensitivity to ammonia, studies with *C. dubia* were not conducted.
[7] 9.3 mmhos/cm standard used for toxicity studies with *C. dubia*; 18.1 mmhos/cm standard used with *P. promelas* studies.
[8] Mixture contained 4.0 mg/L ammonia, 1.0 g/L sodium chloride, 0.05 mg/L copper, 1.0 mg/L pentachlorophenol, and 0.2 mg/L diazinon.

Table 2. Effect of automated C_{18} and mixed bed ion exchange treatments prepared with IC/HPLC on toxicant standard toxicity.

Toxicant Standards[1]	C_{18}			Ion Exchange		
	Toxicity[2]		Concentration[3]	Toxicity[2]		Concentration[3]
	P. promelas	*C.dubia*		*P. promelas*	*C.dubia*	
Ammonia[4]	68.5 (53.0-78.5)	-	17.7	>100.0	-	5.5
Sodium Chloride[5]	58.6 (50.2-65.7)	52.8 (44.8-60.3)	9.3 18.2	>100.0	98.3 (94.6-100.0)	2.3 5.4
Copper	-	58.6 (42.6-66.5)	0.12	-	>100.0	<0.02
Pentachlorophenol	12.5 (1.0-25.0)	18.4 (4.2-28.6)	1.6	>100.0	>100.0	<0.02
Diazinon	-	>100.0	<0.02	-	12.5 (1.0-25.0)	0.6
Composite Mixture[6]	47.5 (42.5-53.5)	87.5 (75.0-100.0)	-	>100.0	16.5 (12.5-20.5)	-

1 pH 7.0.
2 48-hr LC50 with respective 95% confidence intervals. Expressed as percent sample.
3 Expressed as mg/L with exception of sodium chloride expressed as mmhos/cm.
4 Due to *P. promelas* sensitivity to ammonia, studies with *C. dubia* were not conducted.
5 9.3 mmhos/cm standard used for toxicity studies with *C. dubia*; 18.1 mmhos/cm standard used for *P. promelas* studies.
6 Mixture contained 4.0 mg/L ammonia, 1.0 g/L sodium chloride, 0.05 mg/L copper, 1.0 mg/L pentachlorophenol, and 0.2 mg/L diazinon.

levels of conductive dissolved solids. Mixed-bed ion exchange significantly reduced the copper concentration and reduced toxicity to *C. dubia* from the copper standard. C_{18}-SPE removed only a small fraction of copper in the standard and did not significantly reduce the toxicity of the copper standard to *C. dubia*.

Since many inorganic water-borne toxicants are ionic at neutral pH, it is not surprising that mixed-bed ion exchange removed the contaminants from the sample, and thus reduced toxicity. However, because the speciation of many organic aquatic toxicants is also dependent on pH, pentachlorophenol was also chosen for study with the automated system. Results from studies conducted at pH 7.0 suggested that mixed-bed ion exchange was capable of substantially reducing the toxicity of the pentachlorophenol standard solution to *C. dubia* and to *P. promelas*. At pH 7.0, mixed-bed ion exchange treatment completely removed pentachlorophenol from the sample, whereas C_{18} treatment reduced the pentachlorophenol concentration 3.1-fold, but did not alter the toxicity of the standard. These results suggested that at pH 7.0, C_{18} was unable to reduce pentachlorophenol levels below the threshold toxic level to *C. dubia* and *P. promelas*. Results from toxicity tests with the methanol gradient eluates suggested that some pentachlorophenol was trapped on the C_{18} column, but that elution was achieved primarily in 25% methanol (0.25 mg/L pentachlorophenol). Studies are currently being conducted at an acidic pH. Preliminary results indicate that at pH 3.0, mixed-bed ion exchange treatment was slightly less effective in trapping pentachlorophenol and reducing toxicity. However, C_{18} was significantly more effective in removing pentachlorophenol and reducing toxicity to *C. dubia* and *P. promelas*. In addition, 75%-85% methanol was required to elute the pentachlorophenol from the column. These results suggested that at an acidic pH ($<pK_a$), pentachlorophenol exists as a more non-polar species, whereas at neutral and alkaline pH ($>pK_a$), pentachlorophenol possessed ionic properties.

Mixed-bed ion exchange did not reduce the toxicity of the diazinon standard to *C. dubia*, whereas C_{18} treatment reduced toxicity to *C. dubia* 8-fold. Results from the toxicity tests with the methanol elutriate fractions indicated that the diazinon was concentrated in the 75% methanol fraction, which was confirmed by GC/MS analysis.

Mixed-bed ion exchange treatment reduced the toxicity of the composited toxicant mixture to *P. promelas* 2.7-fold, and 1.3-fold to *C. dubia*. C_{18} treatment of the composited mixture reduced the toxicity to both *C. dubia* (7-fold) and *P. promelas* (13-fold). Further evaluation of the mixed-bed ion exchange elutriate indicated that ammonia and ionized pentachlorophenol were removed by this treatment. Since mixed-bed ion exchange was more effective in reducing toxicity to *P. promelas* than *C. dubia*, pentachlorophenol and ammonia appeared to be responsible for toxicity induced to *P. promelas*. Results from toxicity and analytical analyses conducted with the C_{18} methanol elutriate fractions indicated that toxicity to *C. dubia* was concentrated in the 75% fraction. Toxicity to *P. promelas* was induced by the 25% fraction only. Pentachlorophenol was detected in the 25% methanol fraction and diazinon in the 75% fraction. Because C_{18} reduced toxicity to both species, these results confirmed that pentachlorophenol was responsible for toxicity induced to *P. promelas*, whereas a diazinon was responsible for the effects observed with *C. dubia*.

The automated methodology was also used to evaluate potential causes of toxicity to *C. dubia* in a complex industrial effluent. Preliminary conventional studies suggested that conductive dissolved solids, ammonia, and non-polar organic compounds were primarily responsible for toxicity. Both conventional and automated mixed-bed ion exchange treatment increased the *C. dubia* 48-hr LC50 values from 6.5% to 83.4% and 79.2% effluent, respectively. Both systems reduced the concentration of ammonia and conductive dissolved solids to nominal levels. Mixed-bed ion exchange elutriate contaminants collected from the IC/HPLC included ammonia and an assortment of conductive dissolved solids, including toxic levels of nitrite (130 mg/L) and potentially toxic levels of nickel (170 μg/L) and zinc (280 μg/L). C_{18} was virtually ineffective in removing toxicity from this sample, increasing the *C. dubia* 48-hr LC50 values from 16.5% to 28.5% and 32.1% effluent, respectively. Trace organic compounds identified in the 85 and 90% methanol elutriate fractions including acetone, acetophenone, 2, 6-dimethylisocyanate, quinoline, benzothiozole, and 2-mercaptothiazole may have been responsible for a small portion of the toxicity induced. Characterization studies using the automated methods were completed in less than two hrs, whereas conventional characterization methods required at least 8 h (not including the toxicological evalutions). Based on the results obtained in this study, the automated methods developed to identify water-borne contaminants in the environment should provide the scientific community with a more rapid, cost-effective, and versatile alternative to the conventional methods.

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REFERENCES

Hamilton MA, Russo RC, and Thurston RV (1977) Trimmed Spearman-Karber method of estimating median lethal concentration in toxicity bioassays. Environ Sci Technol 11: 714-719

Mount DI and Anderson-Carnahan L (1988a) Methods for aquatic toxicity identification evaluations: phase I toxicity characterization procedures. EPA/600/3-88/034, Duluth, MN

Mount DI and Anderson-Carnahan L (1988b) Methods for aquatic toxicity identifications: phase II toxicity identification methods. EPA/600/3-88/035, Duluth, MN

Mount DI and Anderson-Carnahan L (1988c) Methods for aquatic toxicity identifications: phase III toxicity confirmation methods. EPA/600/3-88/036, Duluth, MN

Weber CI (1991) Methods for testing the acute toxicity of effluents and receiving waters to freshwater and marine organisms. EPA/600/4-90/027, Cincinnati, OH

Weber CI, Peltier WH, Norberg-King TJ, Horning WB, Kessler FA, Merkedick JK, Neiheisel TW, Lewis PA, Klemm DJ, Pickering QH, Robinson EL, Lazorchak JM, Wymer LJ, and Freyberg RW (1989) Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms-second edition. EPA/600/4-89/001, Cincinnati, OH