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AN EXAMPLE OF THE IDENTIFICATION OF DIAZINON AS A PRIMARY TOXICANT IN AN EFFLUENT

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Abstract—A toxicity identification evaluation (TIE) conducted on a municipal wastewater discharge from the southeast United States was part of a research project aimed at developing U.S. Environmental Protection Agency (EPA) TIE methods for acutely toxic effluents. The effluent consistently exhibited acute toxicity to *Ceriodaphnia dubia* but not to fathead minnows (*Pimephales promelas*). Toxicity characterization procedures revealed that the primary toxicant was a nonpolar organic. Toxicity was recovered through C_{18} solid-phase extraction and concentration steps. Gas chromatography–mass spectroscopy of these concentrates revealed the presence of diazinon (*O,O*-diethyl *O*-[6-methyl-2-(1-methylethyl)-4-pyrimidinyl] phosphorothioate). Diazinon concentrations in whole effluent, determined by GC analyses, correlated well with the toxicity measurements of each sample. Relative species sensitivity also implicated diazinon as the primary toxicant. This study illustrates the successful application of EPA TIE methodologies for identifying a nonpolar organic toxicant in a complex effluent. The significance of detecting diazinon at acutely toxic concentrations in municipal wastewater may indicate a more widespread problem in this region of the United States. This toxicity problem may be attributed to the chemical characteristics of diazinon and its applications.

Keywords—Diazinon Effluent TIE/TRE *Ceriodaphnia*

INTRODUCTION

The National Pollution Discharge Elimination System (NPDES) is the major mechanism for regulating point source discharge of toxic effluents in the United States [1]. Initially, the NPDES permitting program sought to control effluent toxicity exclusively through chemical-specific analyses, emphasizing 129 "priority pollutants" [1]. However, it rapidly became apparent that chemical-specific approaches for controlling toxicity were of limited value, primarily because many effluents, whether from municipal or industrial sources, contain thousands of potentially toxic chemicals that may or may not be detected by routine chemical analyses. It is difficult to predict the effects of factors such as pH, hardness, or dissolved organic carbon on the toxicity of chemicals in effluents; for example, although measured concentrations of a chemical may be high, the bioavailability of the chemical may be low. In 1984, in an attempt to remedy these problems, the U.S. Environmental Protection Agency (EPA) issued a statement recommending an integrated approach to NPDES permitting that featured the use of whole effluent toxicity testing to complement chemical-specific analyses [2]. Many NPDES permits are now being written to include specific limits on whole effluent toxicity.

In order to make use of toxicity limits in the NPDES program, methods to reduce effluent toxicity to an acceptable level are needed. Two approaches for the abatement of effluent toxicity are reduction of complex effluent toxicity by treatment without identifying specific chemicals, and identification of causative toxicants, which enables strategies aimed at reducing toxicity such as source control to be implemented [3]. Since 1985, the EPA has worked to develop a set of procedures designed to characterize, identify, and confirm the causes of toxicity in acutely toxic complex effluents [4–6].

The basis for the EPA toxicant identification procedures is to track toxicity through various sample manipulations and fractionations. Fractionation of the sample greatly simplifies toxicant

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identification by reducing the number of nontoxic components associated with the toxicants. This, in turn, enables direct relationships to be more easily established between toxicants and measured analytical data, thereby avoiding the problems inherent with chemical-specific approaches to limiting toxicity.

Toxicity-based fractionation procedures have been used successfully in studies concerning the separation and identification of mutagens from complex mixtures of chemicals [7–9]. Toxicity fractionation schemes with higher organisms and responses such as acute toxicity have been attempted but, for a number of reasons, have had limited success [10–13]. Some of the problems inherent with these approaches include (a) certain manipulations (e.g., solvent extraction) could result in such a high degree of artifactual toxicity that toxicity due to actual toxicants was confounded, (b) fractionation steps often were not specific enough to effectively separate compounds, and (c) some procedures (e.g., solvent exchanges) resulted in the loss of certain classes of toxicants before analytical procedures could be initiated. These problems are largely avoided in the toxicity identification procedures developed by the EPA.

The toxicity identification evaluation (TIE) process is divided into three phases. Phase I [4] consists of methods to identify the physical and chemical nature of the constituents that cause acute toxicity. Phase I results are intended as a first step in identifying toxicants; however, the data generated can be used to develop specific treatment methods to remove toxicity without identifying specific toxicants. Phase II [5] describes procedures such as fractionation schemes and associated analytical methods to identify the toxicants. Phase III [6] describes procedures to confirm the presence of the suspected toxicants.

In this paper, we describe the application of these three procedures to an acutely toxic effluent from a publicly owned treatment works (POTW) in the southeastern United States.

METHODS

Sampling

Thirteen effluent samples were collected from August 23, 1986, through January 5, 1988. Sampling over an extended period should reveal the temporal consistency of the effluent toxicant(s). Twenty-four-hour continuous composite samples were collected through initial phases of the study to increase the likelihood of obtaining representative toxic samples. When the toxicant had been consistently identified, a series of grab samples were collected. Grab samples are more likely to vary in toxicity and in suspected toxicant concentrations. This variability would strengthen the correlation/regression step used in Phase III confirmation.

Toxicity tests

Toxicity test methods [4] are briefly described below. *Ceriodaphnia dubia*, ≤48 h old, were obtained from cultures at the National Effluent Toxicity Assessment Center (NETAC) in Duluth, Minnesota [14]. Fathead minnow larvae, ≤24 h old, were reared at the Environmental Research Laboratory–Duluth. Test chambers were 30-ml polystyrene beakers. *Ceriodaphnia* test volumes were 5 or 10 ml, depending on sample availability. Fathead minnows were exposed in 15-ml volumes. Lengths of *Ceriodaphnia* and fathead minnow toxicity tests were 48 and 96 h, respectively. All toxicity tests were conducted at 25 ± 1°C with a 16:8-h light:dark photoperiod.

Tests were conducted with one or two replicates, depending on the TIE phase. All Phase I and most Phase II toxicity tests used one replicate. These tests are designed to provide a general indication of toxicity not requiring a high measure of accuracy. Phase II tests for determining the toxicity of single suspect toxicants were performed with two replicates to obtain an LC50 value possessing a greater degree of accuracy. Phase III confirmation testing requires definitive data, and therefore all Phase III tests used two replicates.

Dilute mineral water (DMW) was used as dilution water and consisted of a 1:9 dilution of mineral water (Perrier®) and high-quality organic-free water from a Millipore® Super Q System [14]. In general, DMW characteristics were pH 8.0, dissolved oxygen 8.2 mg/L, hardness 39.0 mg/L as $CaCO_3$, and alkalinity 29.0 mg/L as $CaCO_3$. Dilute mineral water characteristics were determined after each batch was prepared. New batches were prepared every 14 d.

TIE methods

The TIE performed on this POTW effluent was used, in part, to develop the EPA's standardized TIE methods. All of the present Phase I treatments (Fig. 1) that determine the physical and chemical characteristics of the toxicant were not performed on this effluent because they had not yet been developed. During this development period, sodium thiosulfate and EDTA additions, aeration at pH 3.0, initial pH (pHi), and pH 11.0 and C_{18} solid-

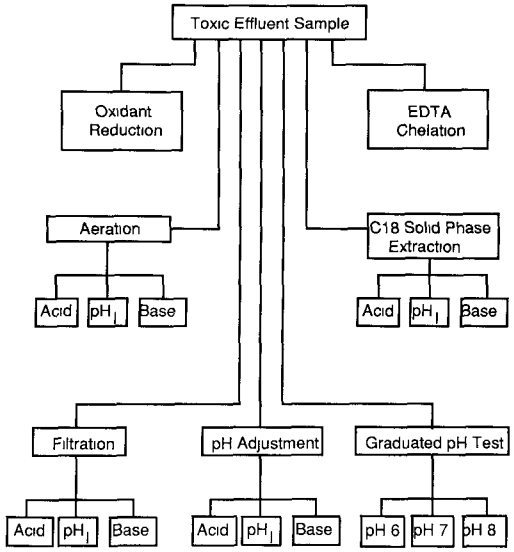

Fig. 1. Flow chart of Phase I manipulations (as described by Mount and Anderson-Carnahan, 1989) [5].

phase extraction (SPE) treatment at pHi were the only manipulations that had been refined for application. The characteristics of the toxicant were determined by observing the alteration of the toxicity of the effluent via each treatment. A reduction of effluent toxicity by additions of sodium thiosulfate would indicate the presence of oxidants at toxic concentrations. Presence of cationic metals (i.e., zinc, copper, nickel, etc.) would be suspected if effluent toxicity was removed by adding EDTA.

Reductions in toxicity caused by aeration at the three pHs may be due to compounds that are oxidizable, spargeable or removable by sublation. If toxicity is pH sensitive, then one can infer that the toxicants are changed in physical or chemical state by pH. Removal of toxicity by the C_{18} SPE column would suggest that the toxicant is a nonpolar organic compound.

Phase II toxicity identification procedures consisted of SPE fractionation, concentration of toxic fractions, and, ultimately, identification by GC-MS. Initially, 500 ml of effluent at pHi was passed through a 3-ml SPE column. Specific details concerning how columns were conditioned prior to extraction and chromatography procedures are given elsewhere [15]. The column was then eluted sequentially with 2 ml each of a series of methanol/water solutions. Initially, the solutions were 25, 50, 75, 80, 85, 90, 95, and 100% methanol/water (v/v). Acute 48-h *Ceriodaphnia* toxicity tests were then conducted with these samples to determine which fractions contained toxicity. Assuming a 100% recovery of nonpolar organic compounds, each fraction theoretically contained 250 times the whole effluent concentration of toxicants. One-hundred microliters of each fraction was diluted with 5 ml DMW to yield a final concentration of five times the whole effluent for the highest concentration in the toxicity tests. The highest methanol concentration used in any toxicity test solution was 2% (v/v), which is below the 48-h LC50 for *Ceriodaphnia* [5]. If methanol present at percent levels is contributing to toxicity, it will be discovered in the Phase III confirmation steps.

SPE fractions identified as acutely toxic were back-diluted to 100 ml with Millipore water. That solution was then passed through a 1-ml J.T. Baker (Phillipsburg, NJ) SPE column. The column was then eluted with 200 μl of 100% methanol. The purpose of this step is to concentrate the sample and to obtain a sample that can be analyzed directly by GC-MS and tested for toxicity. This process produced a fraction that was theoretically at 2,500 times the concentration of the whole effluent. Toxicity tests were performed with the concentrated fractions to ensure that a measurable amount of toxicity had been retained.

The toxic concentrates were injected onto a GC-MS for identification of suspect toxicants. Mass spectral analyses were performed on a Finnigan-Mat (San Jose, CA) ion trap detector 700 (ITD) interfaced to a Hewlett Packard (Avondale, PA) 5790 GC. The GC parameters were column of 30 m DB5, 0.25 mm i.d., 0.25-μm film (J&W Scientific, Folsom, CA); helium carrier with a linear velocity of 40 cm/s at 100°C, splitless injection of 2 μl, injection port temperature 250°C, GC oven temperature programmed from 50° to 250°C at 10°C/min with a 15-min hold at 250°C; transfer line temperature of 270°C with an open split interface. The ITD acquisition was used in a full scan mode from 50 to 550 m/z with 1 s per scan. Identifications were based on a comparison of sample spectra to EPA/NBS/NIH library spectra (37,000 compounds). When standards were available, GC retention times were also compared. Literature searches were performed on identified compounds to obtain available toxicity information [16–20]. Suspected compounds—those with toxicity data or estimates within 1,000 times of the effluent concentration—were then tested under NETAC laboratory conditions to determine their toxicity to *Ceriodaphnia* [4,5]. Upon identification of diazinon as a likely toxicant, routine quantitative anal-

ysis of whole effluent samples was performed by GC. To accomplish this, the 75, 80, and 85% SPE fractions were combined and back-diluted to 200 ml with Millipore water; this solution was then vortex-extracted with 10 ml of hexane in a 250-ml volumetric flask on a magnetic stirrer. After 1 h, the hexane layer was transferred to a 15-ml graduated centrifuge tube and the exact volume of the transferred hexane was recorded. The hexane was evaporated to 200 μl under a stream of dried N_2. (The exact volume of hexane was measured with a 250-μl syringe.) The hexane concentrate was analyzed by a Hewlett Packard 5880 GC with a flame photometric detector.

One Phase III confirmation step compared toxicity of the effluent to predicted toxicity by using regression techniques performed with a standard statistical package [21]. The approach is to show whether a consistent relationship exists between the concentration of the suspect toxicant and the effluent toxicity [6]. The experimental line of regression from this comparison is then compared to the expected line of regression to determine if the suspect toxicant concentration accounts for the observed effluent toxicity. Another portion of the Phase III process involved a relative species sensitivity comparison. This step required the comparison of responses to the effluent of two test species with different sensitivities to diazinon. Acute LC50 values for the toxicity tests were calculated with the trimmed Spearman-Karber method [22].

Table 1. Toxicity of the test effluent to *Ceriodaphnia*

Sample	48-h LC50	Toxic units
8/23/86–I[a]	71	1.41
3/09/87–I	87	1.15
5/02/87–I	35	2.86
5/03/87–I	65	1.54
5/04/87–I	71	1.41
6/27/87–I	25	4.00
6/27/87–II	41	2.44
6/27/87–III	18	5.56
9/22/87–I	71	1.41
12/18/87–IA[b]	>100	<1.00
12/18/87–IB	87	1.15
1/05/88–I	66	1.52
1/05/88–II	63	1.59

LC50 values are given in percent effluent.
Toxic units = 100/LC50.
[a]Sample designation is equivalent to date on which sample was received. Roman numeral suffix represents discrete samples arriving on the same day.
[b]The letter following the sample number indicates aliquots in different containers.

RESULTS AND DISCUSSION

Effluent LC50s for *Ceriodaphnia* ranged from 18 to >100% (Table 1). Phase I toxicity characterization results revealed that effluent toxicity was completely and consistently removed by the SPE treatment. Other Phase I treatments did not alter the toxicity of the effluent samples.

Phase II fractionation revealed that toxicity consistently eluted from the SPE column in the 80% methanol/water fraction, in one-fourth of the 75% fractions, and in one-half of the 85% fractions (Table 2). The toxic units for each fraction were calculated by the equation [100/*Ceriodaphnia* 48-h LC50]/[fraction concentration tested]. During the course of experimentation with this effluent, the elution sequence of the SPE column was altered by adding a 70% fraction and removing the 95% fraction. This simplified subsequent chemical analyses by attaining greater separation around the 75% fraction.

Toxic fractions were concentrated by using 1-ml SPE columns, as described above. Toxicity testing showed that toxicity was retained in the concentrates. These concentrates were injected onto the

Table 2. Toxicity of SPE fractions to *Ceriodaphnia*, expressed in toxic units[a]

	SPE fractions		
Sample	75%	80%	85%
8/23/86–I	<0.20	>0.20	>0.20
3/09/87–I	<0.20	0.36	0.57
5/02/87–I[b]	–	–	–
5/03/87–I[b]	–	–	–
5/04/87–I[b]	–	–	–
5/23/87–I	<0.20	0.57	0.28
5/23/87–II	<0.20	0.57	0.28
5/23/87–III	<0.20	0.57	0.28
6/27/87–I	<0.20	0.57	0.31
6/27/87–II	<0.20	1.25	0.71
6/27/87–III	<0.20	2.22	0.53
9/22/87–I	0.56	0.56	0.28
12/18/87–IA	0.23	0.36	<0.20
12/18/87–IB	<0.20	0.33	<0.20
12/18/87–IC	<0.20	0.25	<0.20
1/05/88–IA	<0.20	0.51	<0.20
1/05/88–IB	0.23	0.51	<0.20
1/05/88–IIA	>1.67	0.74	<0.20
1/05/88–IIB	<0.20	0.57	<0.20

[a]All tests with *Ceriodaphnia*. Toxic units are expressed on a whole effluent basis and were calculated by (100/LC50)/5.
[b]Fraction toxicity not tested for these samples.

Table 3. Compounds identified by GC-MS in the 80% methanol/water SPE fraction obtained from the 3/09/87—I sample and their estimated or literature toxicity values for *Daphnia magna*

CAS no.[a]	Chemical	Estimated concentration (μg/L)	*Daphnia magna* 48-h LC50 (μg/L)
109013	Piperazine, 1-methyl	0.201	1.58×10^{6} [b]
100414	Ethylbenzene	0.122	7.50×10^{4} [d]
61142072	Cyclopentene, 1-ethenyl-3-methylene	0.086	8.50×10^{3} [b]
623370	3-Hexanol	0.537	9.70×10^{5} [c]
106467	*p*-Dichlorobenzene	0.191	1.10×10^{4} [d]
2461156	Oxirane [[(2-ethylhexyl)oxy]methyl]-	64.373	— [e]
111875	1-Octanol	0.103	4.70×10^{4} [c]
585342	Phenol, 3-(1,1-dimethylethyl)	0.221	4.80×10^{3} [b]
615225	Benzothiazole, 2-(methylthio)	0.193	1.98×10^{4} [b]
131179	1,2-Benzenedicarboxylic acid, di-2-propenyl ester	1.499	2.00×10^{4} [c]
333415	Diazinon	0.171	0.96 [f]
17851535	1,2-Benzenedicarboxylic acid, butyl-2-methylpropyl ester	0.253	— [e]
27554263	1,2-Benzenedicarboxylic acid, diisooctyl ester	2.493	— [e]
56052803	Heptane, 1-(1-butenyloxy)-	1.084	— [e]

[a]Chemical abstract registry numbers.
[b]Toxicity estimates obtained from quantitative structure-activity relationships (QSAR).
[c]See Bringmann and Kuhn [17].
[d]See LeBlanc [18].
[e]No experimental toxicity values or QSAR toxicity estimates available.
[f]See AQUIRE [19].

Table 4. Compounds identified by GC-MS in the 85% methanol/water SPE fraction obtained from the 3/09/87—I sample and their estimated or literature toxicity values for *Daphnia magna*

CAS no.[a]	Chemical	Estimated concentration (μg/L)	*Daphnia magna* 48-h LC50 (μg/L)
108907	Benzene, chloro	0.197	8.60×10^{4} [b]
61142072	Cyclopentene-1-ethenyl, 3-methylene	0.733	5.40×10^{3} [c]
622968	Benzene,1-ethyl-4-methyl-	0.138	1.22×10^{3} [c]
620144	Benzene,1-ethyl-3-methyl-	0.141	1.22×10^{3} [c]
54986446	Benzene,(1,3,3-trimethylnonyl)-	0.165	— [d]
108678	Benzene,1,3,5-trimethyl-	0.111	1.06×10^{3} [c]
95636	Benzene,1,2,4-trimethyl-	0.111	1.06×10^{3} [c]
62108263	Decane,2,6,8-trimethyl-	0.096	— [d]
62108230	Decane,2,5,6-trimethyl-	0.353	— [d]
74381401	Propanoic acid, 2-methyl-,1-(1,1-dimethylethyl)-2-methyl-1,3-propanediyl ester	0.628	— [d]
140669	Phenol,4-(1,1,3,3-tetramethylbutyl)-	0.253	— [d]
104405	Phenol,4-nonyl-	0.343	— [d]
131179	1,2-Benzenedicarboxylic acid, di-2-propenyl ester	0.866	2.00×10^{4} [e]
333415	Diazinon	0.343	0.96 [f]
27554263	1,2-Benzenedicarboxylic acid, diisooctyl ester	4.344	— [d]

[a]Chemical abstract registry numbers.
[b]See Cowgill et al. [20].
[c]Toxicity estimates obtained from QSAR.
[d]No experimental toxicity values or QSAR toxicity estimates available.
[e]See Bringmann and Kuhn [17].
[f]See AQUIRE [19].

GC-MS system; 14 chemicals were tentatively identified in the toxic concentrates derived from the original 80% methanol/water fraction from the SPE column, and 15 chemicals were tentatively identified in the 85% fraction (Tables 3 and 4).

Diazinon, an organophosphate pesticide, was identified in both the 80 and the 85% fractions and was selected as the most likely candidate causing acute toxicity based on estimated effluent concentrations and historical toxicity data for cladocerans. Determination of the toxicity of diazinon to *Ceriodaphnia* revealed an LC50 of 0.35 μg/L. Subsequent measurement of diazinon in toxic effluent samples routinely revealed concentrations in excess of 0.35 μg/L (Table 5).

Relative species sensitivity is one procedure that can be used to collect evidence to confirm that the suspect toxicant is the cause of toxicity. In this case, fathead minnows were selected as test animals. Fathead minnows are readily available and can be tested in small volumes. The literature also contains information concerning the toxicity of diazinon to fathead minnows. Because fathead minnows are over 1,000 times less sensitive to diazinon than *Ceriodaphnia* [23], it would not be expected that the effluent samples would be acutely toxic to fathead minnows if diazinon was the only toxicant present. The fathead minnow LC50 values for all samples were >100%, which is consistent with the selection of diazinon as being a major toxicant present in these samples.

Another confirmation step was to compare effluent toxicity to predicted toxicity, based on measured concentrations of the suspected toxicant. In order to plot the data on a linear scale, the LC50 and concentration values were transformed to toxic units. Effluent toxic units were determined with the equation [100%]/[effluent LC50 (%) for *Ceriodaphnia*], and diazinon toxic units were calculated with the equation [effluent diazinon concentration (μg/L)]/[0.35 μg/L]. The expected line of regression, based on predicted vs. observed toxic units, should have a slope of 1.0 and a *y*-intercept of zero. Figure 2 indicates the observed regression line. The *y*-intercept of 0.08 ± 0.45 and the slope of 1.15 ± 0.22 show that the observed line is not significantly different from the predicted line. The *r* value of 0.86 also shows that a strong relationship exists between the concentration of diazinon and whole effluent toxicity.

Table 5. Measured diazinon concentrations (μg/L) for the POTW effluent

Sample	Diazinon concentration	Diazinon toxic units[a]
8/23/86–I	0.51	1.46
3/09/87–I	0.27	0.77
5/02/87–I	0.81	2.31
5/03/87–I	0.67	1.91
5/04/87–I	0.60	1.71
6/27/87–I	1.31	3.74
6/27/87–II	0.99	2.83
6/27/87–III	1.13	3.23
9/22/87–I	0.41	1.17
12/18/87–IB	0.21	0.60
1/05/88–I	0.34	0.97
1/05/88–II	0.40	1 14

[a]Toxic units = [effluent diazinon concentration (μg/L)]/[0.35 μg/L].

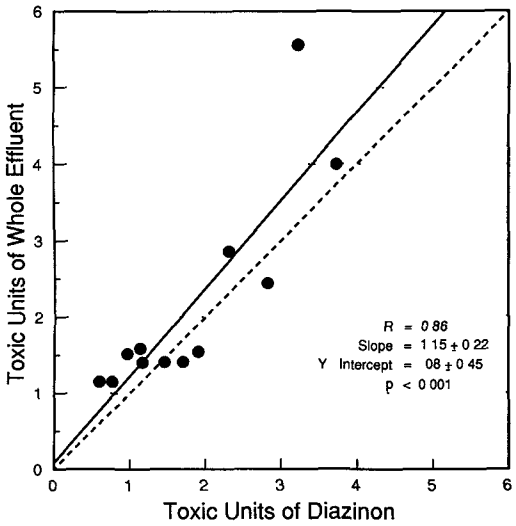

Fig. 2. Toxic units of diazinon vs. toxic units of whole effluent.

$$\text{Toxic units of diazinon} = \frac{\text{effluent concentration } (\mu g/L)}{\text{diazinon LC50 } (\mu g/L) \text{ for } \textit{Ceriodaphnia}}$$

$$\text{Toxic units of whole effluent} = \frac{100\%}{\text{effluent LC50 } (\%) \text{ for } \textit{Ceriodaphnia}}$$

The dotted line represents the expected regression line if the diazinon concentration completely predicts whole effluent toxicity. The solid line represents the experimental regression, comparing measured effluent diazinon concentrations to effluent toxicity.

In summation, the evidence pointing to diazinon as the major effluent toxicant is

1. Phase I treatment for removal of nonpolar organic compounds was consistently effective in removing toxicity
2. Toxicity of the whole effluent compared favorably with toxicity contained in the C_{18} SPE fractions
3. Diazinon was present at toxic concentrations in the 80 and 85% SPE fractions
4. Fathead minnows were not sensitive to the effluent
5. Regression of whole effluent toxic units to diazinon concentration toxic units fits well with the expected model, assuming diazinon to be the major toxicant

Although diazinon concentrations accounted for most of the toxicity, it appears that other nonpolar organic compounds were sometimes present at low and occasionally toxic amounts

Identifying diazinon as a major toxicant in a municipal wastewater treatment plant effluent from the southeastern United States is not startling, considering the uses and persistence of this pesticide Applications of diazinon are aimed at controlling pests such as cockroaches, ants, silverfish, beetles, fleas, ticks, grubs, and nematodes Uses include widespread indoor applications in commercial and residential structures and outdoor treatments of building perimeters and turf [24] Therefore, the contribution of diazinon to the influent of a POTW probably cannot be attributed to any particular point source

A survey of diazinon concentrations in municipal wastewaters from different regions of the United States was triggered by results of this study, along with results of other TIE studies Preliminary survey results indicate that this organophosphate is a relatively common contaminant in POTW discharges Overall, the present study shows that the TIE methods developed by the EPA can be successfully applied to identify nonpolar organic compounds causing acute toxicity in complex municipal wastewaters

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