Final Report

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Title: Real-time Fluorometric Assay for Sewage Presence: A Cost-effective Method to Determine Potential Water Quality Threats to Swimmers and Ecosystem Health

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Summary:

The detection and quantification of two common fluorescent whitening agents (FWA) have been investigated in laboratory-prepared solutions, Lake Michigan water samples and secondary treated wastewater samples (from the Portage Indiana Water Reclamation Facility) using HPLC (High Performance Liquid Chromatography) equipped with fluorescence detection. Additional verification of the presence of FWAs is provided by high through put fluorescence spectrophotometry and excitation-emission matrix techniques. These water samples have also been analyzed for the presence of *E coli* using the membrane filtration method. The Lake Michigan water samples were collected from 3 different sites on the southern shore of the lake in Northwest Indiana: Burns Ditch and two nearby beach locations (Ogden Dunes Beach). Burns Ditch is the outfall of the Little Calumet River into southern Lake Michigan and directly impacts the Ogden Dunes Beach particularly during north to northeast winds. **The presence of these FWAs in the lake water samples do not show a correlation with the presence of *E. coli.***

INTRODUCTION

An increase in heavy precipitation events is an expected consequence of climate change (Parry et al., 2007). Global climate models project more future extreme rain events around the Great Lakes, which translates into a rise in frequency of combined sewer overflows of 50-120% by century's end (Patz et al., 2008). Many municipalities along or near Lake Michigan discharge treated wastewater into the lake, rendering the lake susceptible to combined sewage overflows, which affect the quality of the lake water. The average rain volume that overwhelms typical wastewater containment capacities is 2 inches or greater, and these events lead to the release of contaminants from combined sewer overflows. (McLellan et al., 2007). Heavy precipitation events lead to an increased risk of waterborne disease

outbreaks from drinking water or recreational water contamination (Patz et al., 2005; St Louis and Hess, 2008).

The majority of wastewater treatment plants (~70%) in the United States utilize primary and secondary treatment facilities to process wastewater, the minimum requirement established by the Clean Water Act (2004a). While an effective primary/secondary treatment plant can result in the removal of up to 90% of dissolved organics, the system relies on nature to remediate the remaining contaminants. In many cases the treated water is discharged into a natural body of water. A 2004 U.S. EPA report states that an estimated 850 billion gallons of sewage water from 770 treatment plants is released to natural bodies of water each year (2004b). The treated wastewater from most municipal facilities still contains, at minimum, 10% of the dissolved organics, with a significant amount originating from household laundry practices.

Fluorescent whitening agents (FWAs) are part of laundry detergent formulations. Commercial whitening agents are generally of two types: either the diaminostilbene (DAS1) or the distrylbiphenyl (DSBP) variety. Several commercial variants of the two structures are used. The structures of DAS1 and a commercially used DSBP compound, Tinopal CBS-X, are shown in Figure 1. An estimated 90% of all whitening agents contain either the DAS1 or DSBP structures (Hagedorn et al., 2005).

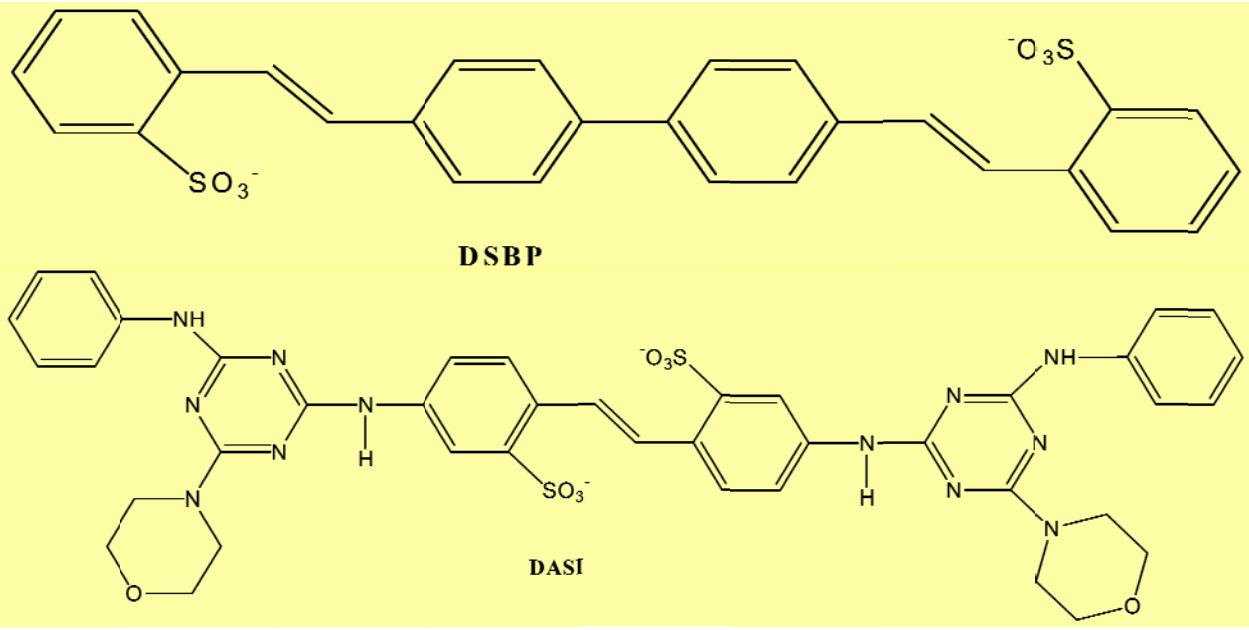

Figure 1:Structure of the most common fluorescent whitening agents, DAS11 and DSBP.

The presence of these FWAs in natural bodies of water, i.e. Lake Michigan, may be linked to human bacterial contamination originating predominantly from wastewater treatment plant discharges. In the typical testing process for bacterial contamination, biological assays are utilized and require at least 18 hours for cultures of *Escherichia coli* (*E. coli*) or *enterococci* to develop. A proposed, faster method for the detection of human contamination involves the utilization of chemical markers, such as fluorescent whitening agents, which are part of sewage discharge (Hartel et al., 2007). These FWAs can also be used to track and monitor wastewater effluent dispersion in watersheds. A variety of studies in watersheds across the globe, ranging from the River Glatt in Switzerland to Tokyo Bay in Japan and watersheds in Rhode Island and Mobile, Alabama, have documented the dispersion of these FWAs in natural water systems (Poiger et al., 1999; Hayashi et al., 2002; Boving et al., 2004; Close et al., 1998). In most cases, determination of the FWAs has been made using solid phase extraction (SPE) cartridges followed by either fluorescence spectrophotometry and/or HPLC/fluorescence detection. In the Rhode Island study, the authors did carry out direct fluorescence measurements of water samples, but due to instrumental constraints were unable to distinguish between interfering natural organic matter and FWAs (Boving et al., 2004). A number of groups have used EEM methods to discriminate between fluorescent species in natural waters (Baker 2001, 2002; Chen et al., 2003) albeit no quantification of FWAs were attempted.

We have used HPLC with fluorescence detection to track the distribution of fluorescent whitening agents along a southern portion of the Lake Michigan shoreline. Fluorescence measurements were also performed on the filtered water samples. Solid phase extraction (SPE) of standards and natural water samples followed by HPLC analysis was used to quantitatively determine the presence of confirm the presence of DAS1 and DSBP. Also, the potential link between these FWAs and E. coli contamination has been examined. The data are reported for normal weather conditions and following a heavy rainfall event from the month of June through August 2009.

Materials and Methods

Water Samples: All lake water and treated wastewater samples were vacuum filtered using 0.2 µm filter paper and utilized within three hours of the collection. All water samples analyzed for fluorescent whitening agents were prepared and/or stored in the dark to avoid extraneous photochemical reactions.

Chemicals: Both DSBP (Trade name: Keyfluor White CBS-X) and DAS1 (Trade name: Photine CBUS) were generously donated by Keystone Chemicals, USA and Lambson Fine Chemicals, UK. Both whitening agents

were used as supplied. Suwannee River Fulvic Acid Standard II and NOM were purchased from IHSS (International Humic Substances Society).

Solid Phase Extraction (SPE): Solid phase extraction disks, 47 mm ENVI-Disk™ (C18 bonded) were purchased from Supelco and used with a vacuum filtration assembly. The disks were utilized as stated in the supplied procedure: 5mL of methanol were poured through the filter followed by 5mL of water and then the solution of interest (usually 2L). Finally, 10mL of methanol (in two successive 5mL aliquots) was used to recover the membrane extracted solute. If we assume 100% extraction efficiency and utilize 2 L of solution, the concentration of the extracted solute in methanol should be 200 times that of the original solution. In order to determine the SPE efficiencies of the FWAs, standard solutions were prepared and tested. One liter of solutions of DSBP at different concentrations ranging from 0.01 to .1 ppb was extracted and concentrated 200-fold using the SPE method. The percent FWA extracted ranged from 90-95% and was linear over the concentration range.

FWA Analyses: A Beckman System Gold HPLC, equipped with a Supelco Discovery® C18 column (5 µm, 250 mm x 4.6 mm) and a JASCO FP 1520 fluorescence detector, was used to separate, detect and quantify the fluorescent compounds. The excitation/emission wavelengths for all samples were 350 nm and 430 nm. The isocratic mobile phase was 0.4 % tetrabutylammonium hydrogen sulfate in a 50% acetonitrile/water mixture adjusted to pH 8.0. A replicate analysis was always performed on each sample and methanol blanks and FWA standards were run at an interval of every five samples.

Direct fluorescence measurements were performed on both filtered and extracted samples using a Jobin-Yvon fluorimeter (Fluormax4). In all fluorescence measurements, the scans were recorded in steps of 1nm, with integration time of 0.1s and 2 nm slit widths for both excitation and emission monochromators. Excitation for the fluorescence measurements was at 350 nm, while the emission was monitored from 390-600 nm. A deionized water sample (solvent blank) free of any fluorescent impurity was recorded prior to every ten sample measurements. All fluorescence spectra from the natural water samples shown have been corrected for the water blank. Methanol was used as the blank for the SPE extracted samples and was similarly subtracted. For the excitation-emission matrix (EEM) measurements, the excitation wavelengths were incremented from 230-400 nm in 5 nm steps, while the emission was scanned from 350-600 nm in 1 nm increments. Slit widths and integration times were the same as in the fluorescence measurements. Fluorescence quantum yield measurements were made using quinine sulfate in 0.1 M H_2SO_4 as the standard.

TOC Analyses: Total dissolved organic carbon was measured using a Shimadzu Total Organic Carbon Analyzer, model TOC-5050 equipped with an ASI-500A autosampler. All water samples were acidified and sparged gently with nitrogen to remove the inorganic carbon. Measurements were carried out in triplicate. The reported values are within the experimental error of 5%.

E. coli measurements: Determinations of the *E. coli* levels were performed at the USGS Ecological Research Station in Porter, IN. *E. coli* was analyzed by the defined substrate technology (2005), using Colilert-18 and Quanti-Tray-2000 method, as previously described (Byappanahalli et al., 2003). All *E. coli* analyses included appropriate controls: *E. coli* ATCC 25922 (positive-control) and phosphate-buffered water, PBW (negative control). *E. coli* counts were expressed as most probable number (MPN)/100 ml. These values are made public online: https://extranet.idem.in.gov/beachguard/public/default.aspx.

RESULTS AND DISCUSSION

The concentrations of FWAs in the southern Lake Michigan watershed (northwest corner of Indiana) were determined by HPLC measurements of water samples obtained from five different sites: 2 beach sites on the southern shore of Lake Michigan in Northwest Indiana (Portage Beach and Ogden Dunes) and 3 upstream sites along the East and West branches of the Calumet River (labeled as LCE and LCW) which discharge via Burns Ditch (BD) into Lake Michigan. The river, which dominates the Lake Michigan watershed, drains three counties that include five wastewater treatment plants with occasional sewer overflows, industrial discharges and a wide variety of non-point sources. The LCW and LCE sites chosen to collect samples were approximately 200 meters upstream from the confluence of the two river branches where Burns Ditch begins. In general, *E. coli* levels in Burns Ditch are higher (with counts often reaching 10,000 CFU/100 ml) than the adjacent lake (beach). The outflow from BD directly impacts the two beach locations mentioned above particularly on days where north to northeast winds predominate.

A complete map of the five sites in relation to Lake Michigan is given in Figures 2-4. The water samples were collected typically about 6-10 inches below the surface. For control purposes, secondary treated and UV-light treated wastewater samples were also obtained from the Portage Reclamation Facility (labeled PRF) in Portage, IN, which discharges into Burns Ditch via the West branch of the Little Calumet River. An additional control sample of lake water was obtained from the City of East Chicago Water District which gets its water from a location five miles offshore into Lake Michigan.

Figure 2: Aerial photograph of Burns Ditch emptying into Lake Michigan in Portage, Indiana

All collected water samples were filtered and, if necessary, concentrated using SPE disks (solid phase extraction) and analyzed using a) HPLC with fluorescence detection, b) direct fluorimetry and c) TOC analyzer.

Figure 3: Aerial photograph of the Little Calumet watershed emptying into Burns Ditch in Portage, Indiana

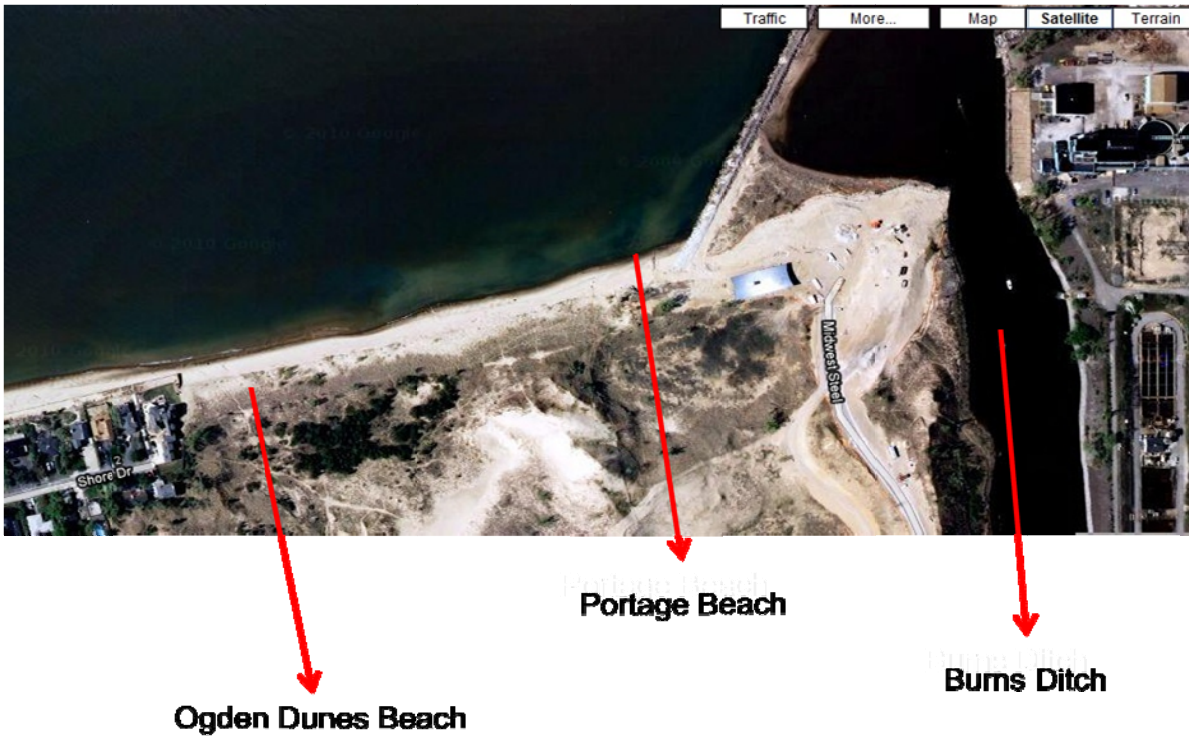

Figure 4: Aerial photograph of the two Lake Michigan beach sites impacted by Burns Ditch.

Direct Fluorimetry

Figure 5 shows representative fluorescence spectra for direct filtered water samples collected from Burns Ditch and from the neighboring Ogden Dunes beach site labeled OD1, as well spectra recorded for standard samples of DSBP and DAS1 at 1ppb concentrations. The observed fluorescence spectra from the lake water samples are generally broader than the standards and the emission maxima are red-shifted by about 10 nm (λ_{max} = 450nm) as compared to the standards. The likely explanation for this red-shift is the presence of other fluorophores in aquatic systems, found in natural dissolved organic matter (NOM) such as fulvic acid-type substances. The fluorescence maxima for fulvic acids are typically around 455 nm upon 350 nm excitation. The fluorescence spectra of 10 ppm Suwannee River fulvic acid and Suwannee River natural organic matter (NOM) solutions are shown in Figure 6. We used excitation-emission matrix (EEM) characterization techniques to determine the nature of the fluorescence as a function of excitation wavelengths. Also, a representative 3D EEM spectra of a BD sample is shown in Figure 7. The notable similarities in the contour plots of the filtered natural water samples (ie. BD) and the natural organic matter standards make the task of quantifying the FWAs based on simple direct fluorimetry extremely difficult.

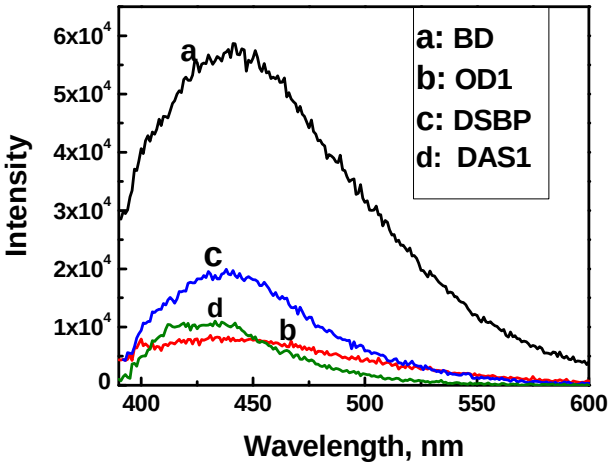

Figure 5: Fluorescence spectra of filtered, field water samples from a) BD and b) OD1. Also shown are spectra obtained from standard samples of c) DSBP, 1 ppb concentration and d) DAS1, 10 ppb concentration. All spectra were obtained under identical conditions: 350 nm excitation, 2 nm slitwidths for both emission and excitation monochromators and 0.1s integration times. All spectra have been corrected for water background.

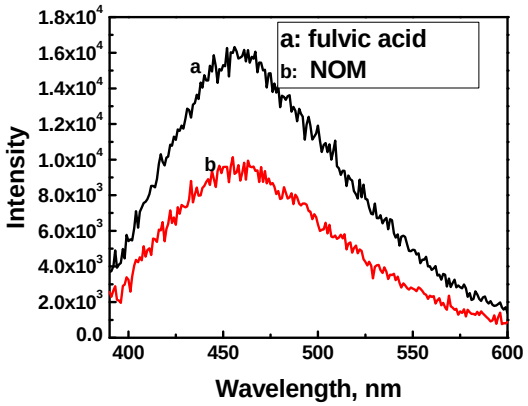

Figure 6: Fluorescence spectra of a) 20 ppm Suwanee River fulvic acid standard and b) 20 ppm NOM standard. Both spectra were obtained under identical conditions: 350 nm excitation, 2 nm slitwidths for both emission and excitation monochromators and 0.1s integration times. Both spectra have been corrected for water background.

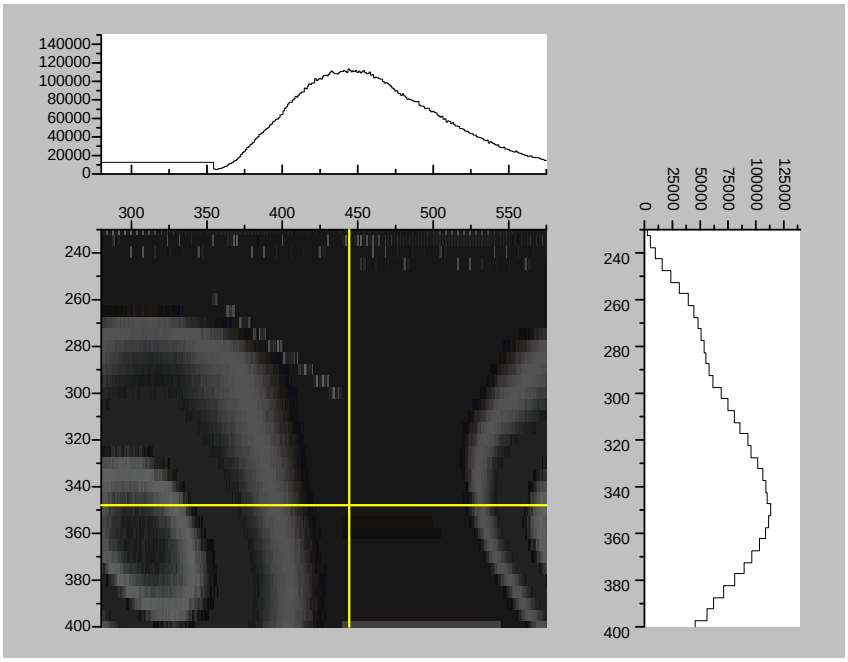

Figure 7: 3D-Excitation emission matrix spectra of a filtered BD sample. The spectra are subtracted for water background.

HPLC Analysis Using Fluorescence Detection

While water samples were collected during the first summer of the project (2008), these samples were utilized in the method development for the separation, detection and quantification of the FWAs with the HPLC system. The sensitivity associated with fluorescence detection exposed the very small percentage of impurities in a few of the chemicals used in the system, namely the ion pairing reagents required in the HPLC solvent. The separation of the FWAs also required a specific HPLC solvent pH for optimal ion pairing.

Several studies ranging from measurements in Tokyo Bay to septic tank measurements in New Zealand have concluded that DAS and DSBP are the most prevalent among the FWAs in sewage discharges. Figure 8 shows representative chromatograms obtained following the extraction of 2 L of solution into 10 ml methanol of i) a standard sample of DSBP, (ii) a treated wastewater sample from the Portage Reclamation Facility (PRF) and (iii) a control sample of Lake Michigan water (drinking water sample from the City of East Chicago).

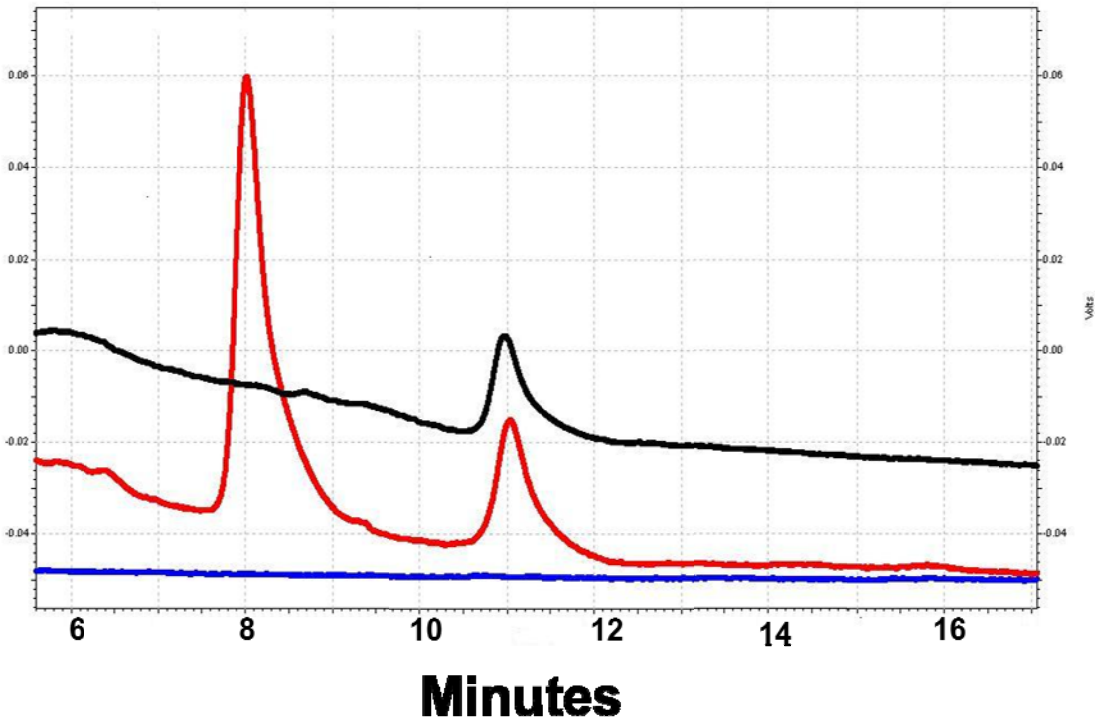

Figure 8: HPLC–fluorescence detected chromatogram of methanol extracts of a) control sample from Lake Michigan which should not show any FWA, b) an effluent sample from the Portage treatment facility collected 14th July, 2009 and c) standard solution of 1ppb DSBP. Injection volumes in both cases were 100 μL with a flow rate of 1ml/min.

Chromatograms of DSBP standards in methanol at varying concentrations were obtained to generate a calibration plot. The chromatogram of the wastewater sample exhibits two later eluting peaks, one of which corresponds very well with the DSBP standard. The earlier peak does not correspond to DAS1 but is likely a close analog of it. At this point we are not sure about the identity of this FWA. The drinking water control from Lake Michigan shows no peaks under the same conditions. We have carried out repeated extraction and chromatographic analyses of PRF samples obtained on different days and in each case we observe the presence of DSBP, albeit in varying amounts. The consistent presence of DSBP in the PRF sample indicates that this FWA is not removed completely during wastewater treatment and hence is a reliable marker for tracing sewage discharge routes into Lake Michigan.

Water samples were collected once every week for 8 weeks beginning the week of June 18th. HPLC analyses of the methanol extracts were carried out and the amount of DSBP in them was determined by calibration using standards. Table 1 shows the amounts of DSBP (in ppb) in each of the 40 samples collected, arranged by sampling site and collection date. The levels of the DSBP from the beach samples are lower, reflecting the dilution of the treated sewage stream as it enters Lake Michigan. While the June 18th *E. coli* counts were high for both the waterways and beaches, and consequently corresponded to a heavy rain event the previous day, the levels of DSBP were low or not detectable.

Figure 9 show representative chromatograms of extracted water samples obtained from the Little Calumet West sampling site. The chromatogram from July 14th corresponds to normal dry weather conditions while the one from July 29th corresponds to a sample obtained after a major rain event**. The data are clearly anomalous and suggest that the levels of FWAs in the river waters are a function of dilution. While heavy storms do indeed result in sewer overflows and high bacteria counts, the FWA levels do not show a correlation.**

Conclusions and Future Prospects:

While our data do not indicate correlation between FWA and *E. coli*, this might well be due to insufficient data. The summer of 2009 was remarkably dry so that more data gathered during heavy storms might give a different picture although dilution is still expected to play a major part. What the data show is that the FWAs along with other anthropogenic chemicals commonly present in sewage

(such as caffeine) could be used to study the dispersal of sewage in a watershed. We are using the knowledge gained in sampling methods to extend this to pharmaceuticals present in natural waters.

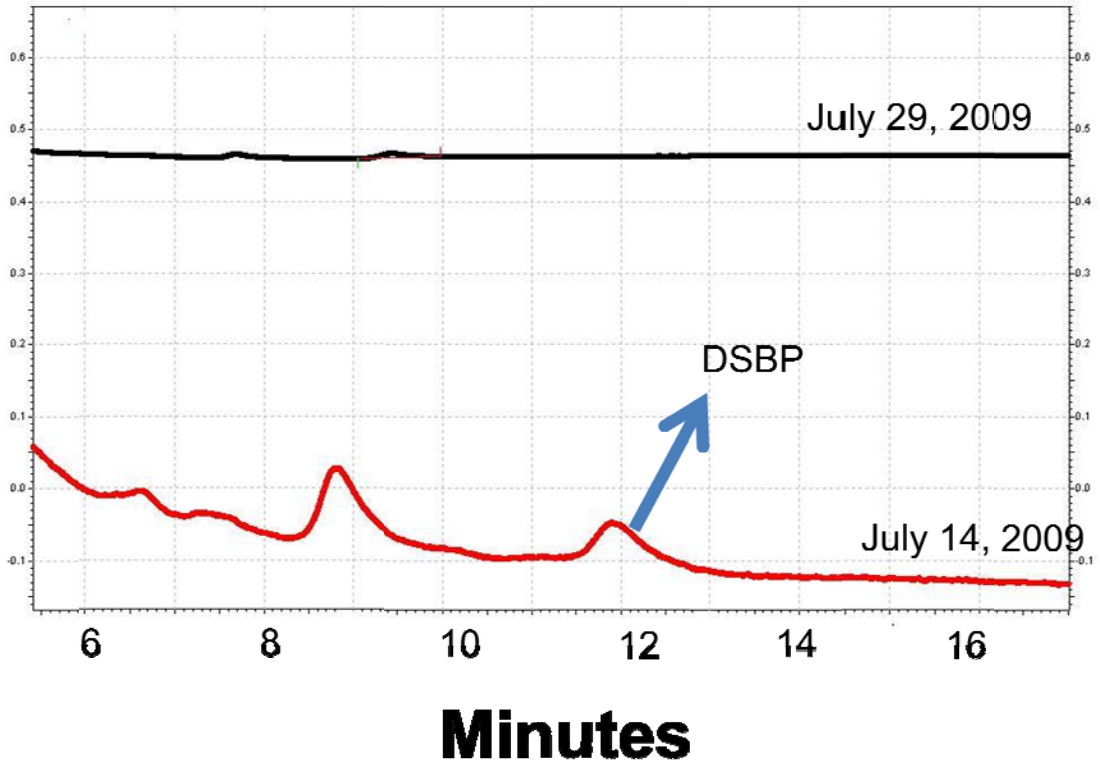

Figure 9: HPLC–fluorescence detected chromatogram of methanol extracts of samples from Little Calumet West collected 14th July, 2009 and July 29th 2009. Injection volumes in both cases were 100 µL with a flow rate of 1ml/min.

ACKNOWLEDGEMENTS:

We would like to acknowledge and thank the Portage Water Reclamation Facility for the treated wastewater samples.

Table 1 Measured values of DSBP and E. coli from five different designated natural water sources

Date		LCE	LCW	BD	PB	OD
18-June	DSBP (ppb)	0	0	4.1	0	0
	E*coli* counts	398	731	551	142	261
22-June	DSBP (ppb)	0	1.87	0	0	0
	E*coli* counts	202	192	731	111	147
29-June	DSBP (ppb)	0	3.59	2.5	0	0
	E*coli* counts	204	98	129	80	79
7-July	DSBP (ppb)	5.99	8.72	17	2.2	1.35
	E*coli* counts	197	119	177	56	
14-July	DSBP (ppb)	32.6	76.6	193.4	6.06	36.55
	E*coli* counts	159	62	149	59	30
20-July	DSBP (ppb)	5.78	0.6	4.02	1.04	12.9
	E*coli* counts	203	139	125	33	11
29-July	DSBP (ppb)	0.24	0.65	7.55	14.3	20.6
	E*coli* counts	3066	1538	1827	153	144
11-August	DSBP (ppb)	0	23.1	3.42	0	0
	E*coli* counts	108	76	45	6	2

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Participating undergraduates:

08-09 Keith Murphy, Rebecca Turpin.
09-10 Fiyin Obajuluwa, Joe Marciniak.

Conference presentations:

Poster presentation at the Water Unifies International Conference at UC, Irvine, December 10, 2008.

EPA National Beach Conference, Huntington Beach, CA April 20-22, 2009

Great Lakes regional Meeting, ACS, Chicago, June 2009

IUN undergraduate research conference in the Sciences and Health Professions, April 2009 and 2010.

Post-project outcomes:

Research projects involving FWA detection that will involve high school teachers in the summer months. Project funding from the Indiana Department of Natural Resources Lake Michigan Coastal Grant Program: "Awareness, Education and Action: Students and Educators Taking Ownership of the Lake Michigan Watershed through Integrated Curriculum"

Submitted proposal for GLRI grant money entitled "The Impact of Pharmaceutical Collections on S. Lake Michigan Watershed"