

**OCCURRENCE AND ECOLOGICAL EFFECTS OF AMPHETAMINE TYPE
STIMULANTS IN WASTEWATER EFFLUENT**

A Final Report of the Tibor T. Polgar Fellowship Program

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ABSTRACT

The presence of illicit drugs and their metabolites has become an increasingly important topic worldwide, and their potential ecological effects are essentially unknown. Some of the most interesting of these illicit compounds are the amphetamine type stimulants (ATSs). Very little research has been conducted in the Hudson River Valley (HRV) to determine the presence of various pharmaceuticals, let alone illicit drugs. This report provides information from the summer of 2013 as to the presence of ATSs and several other pharmaceutical compounds at six different sites in the HRV. Amphetamine (AMPH) was detected at one site, the outflow of Kingston's wastewater treatment plant (WWTP). The detection at a combined sewer overflow (CSO) point indicates the release of ATSs into the HRV, but lack of detects suggests these compounds are subjected to high dilution and therefore persist at concentrations below current technologies limit of detection. The ability to derivatize and quantify dopamine and other catecholamines in algae is discussed in this report. This is in response to possible biological effects ATSs have on the presence of catecholamines such as dopamine within algae. Detection by high performance liquid chromatography (HPLC) coupled with fluorescence was first evaluated, but determined to lack the sensitivity desired for this research. Currently a method utilizing derivatization with N-Methyl-N-trimethylsilyl Trifluoroacetamide (MSTFA) and detection using gas chromatography mass spectrometry (GC-MS) is being developed. This research lays the framework for artificial stream experiments that will be conducted in the spring of 2014 in which chronic exposure of biofilms will be monitored for potential biological effects.

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INTRODUCTION

The use of pharmaceuticals to combat against commonly known diseases has been on a steady climb over the years. Until recently, there has been no sign that the use of pharmaceuticals has had anything but a positive impact on the world; however, there is mounting evidence indicating that pharmaceuticals excreted from human waste, improperly disposed, or released through manufacturing processes are occurring in combined sewer overflows (CSOs) and wastewater treatment plant (WWTP) effluent (Ternes 1998; Zuccato et al. 2000; Heberer 2002; Castiglioni et al. 2006a; Zuccato et al. 2008). Though there has been extensive research indicating the presence or absence of various pharmaceuticals present in aquatic systems, the effects of these compounds on aquatic organisms such as algae and invertebrates are presently unknown (Pal et al. 2012).

Currently most research in wastewater effluent has focused on evaluating the use and misuse of illicit amphetamines, such as methamphetamine (METH) and AMPH, through the occurrence of these compounds in aquatic systems. A majority of these studies have been conducted in various areas of Europe, though several rivers in the United States have been investigated (Zuccato et al. 2008; Huerta-Fontela et al. 2008; Castiglioni et al. 2006b; Jones-Lepp et al. 2004). Few studies in the Hudson River Valley (HRV) have examined the presence of pharmaceuticals (Kolpin et al. 2002; Yu et al. 2006; Palmer et al. 2008; Cantwell et al. 2010; Li and Brownawell 2010; Reiner and Kanna 2011; Liao et al. 2012). None have focused on the presence of illicit ATS.

Stimulant drugs affect dopamine, a chemical associated with pleasure, and other catecholamine levels in the brain of higher-level organisms. Other pharmacological

effects include loss of appetite as well as increased wakefulness and attentiveness. These desired biological effects have led to the increased abuse of stimulant medications in the treatment of diseases such as attention deficit hyperactivity disorder (ADHD) and narcolepsy. Since these medications are comprised of similar chemicals as many illicit substances, they are now on a list of abused substances worldwide (CDC 2005; Kessler et al. 2006; Biederman and Faraone 2005). An example is AdderallTM; this ADHD medication is a 3:1 mixture of d- and l-enantiomers of amphetamine salts (Cody et al. 2003). Based upon the increases in both medicinal and illicit uses, there is cause to speculate that the metabolism and excretion of them and related medications, contribute to the increased release of stimulants to various aquatic environments across the globe.

Previous work on illicit amphetamines has focused on the origin of these contaminants in wastewater and their persistence in surface waters (Ternes 1998; Zuccato et al. 2000; Heberer 2002; Jones-Lepp et al. 2004; Castiglioni et al. 2006a; Castiglioni et al. 2006b; Huerta-Fontela et al. 2008; Zuccato et al. 2008). To date, there has been very little research directed to the understanding of potential long-term effects that may occur in organisms exposed to WWTP effluent and how much can be contributed to occurrence of stimulants. A recent study demonstrated that effluent-influenced concentrations of a specific benzodiazepine anxiolytic drug alter the behavior of European Perch (*Perca fluviatilis*) (Brodin et al. 2013).

While the effects of stimulants on aquatic organisms are not currently understood (Pal et al. 2012), the literature does provide background to support expected effects. Catecholamines have been found in 44 plant families in which they normally serve a protective role against predators, injuries, and detoxification of nitrogen (Kuklin and

Conger 1995). Recent studies have revealed that naturally produced dopamine in the marine green algae *Ulvaria obscura*, comprising 4.4% of the algae's dry weight, was related to decreased grazing by sea urchins (*Strongylocentrotus droebachiensis*) (Van Alstyne et al. 2006). Dopamine is a monoamine neurotransmitter that many believe to be regulated by amine stimulants in higher organisms. If exposure to trace amounts of stimulants results in a change of natural dopamine production in aquatic organisms (similar to what occurs in mammals) this suggests potential pathways toward altering microorganism metabolism and productivity, as well as ecosystem function. Exposure of algae to these dopamine-regulating substances may alter their natural production of dopamine, if it is present.

The first major goal of this study was to measure the concentrations of stimulants in the HRV. It is hypothesized that ATSS will be present in the watershed due to the high population density surrounding the sampling sites and large number of combined sewer overflows (CSOs) that occur in the area. The second goal was to collect and process algae samples to quantify dopamine and other catecholamine concentrations. These samples will test the hypothesis that chronic exposure to amphetamines increases the presence of dopamine and other catecholamines in algae. This fieldwork will lay the framework for later artificial stream experiments that will determine whether these stimulants influence algae and algal consumers (e.g., stream invertebrates).

METHODS

INVESTIGATED COMPOUNDS

Pharmaceuticals

The compounds researched in this study have been reported in previous research studies monitoring effluent at several sites across both North America and Europe (Jones-Lepp et al. 2004; Castiglioni et al 2006a; Castiglioni et al 2006b; Huerta-Fontela et al. 2008; Kasprzyk-Hordern et al. 2008; Postigo et al. 2008; Bartelt-Hunt et al. 2009; Bijlsma et al. 2009). This study focused on substituted amphetamines such as AMPH, METH, 3,4-Methylenedioxyamphetamine (MDMA), 3,4-Methylenedioxyamphetamine (MDA) and others (Figure 1).

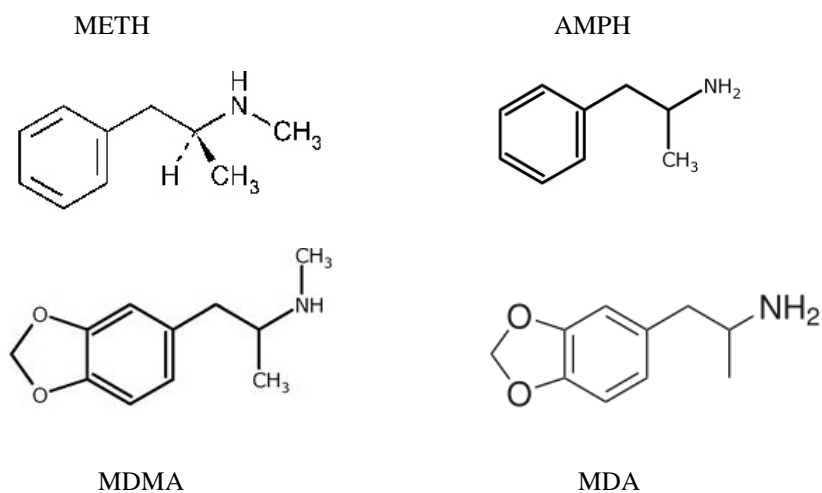


Figure 1. Structures of amphetamine type stimulants being researched in this study.

Water samples were also processed for several compounds that are not within the amphetamine stimulant family. These compounds include: acetaminophen (a common pain reliever), caffeine, 1,7-dimethylxanthine (a metabolite of caffeine), carbamazepine

(used to treat seizures), cimetidine (used in the treatment of ulcers), cotinine (a metabolite of nicotine), diphenhydramine (DPH) (Benadryl), morphine (a common pain reliever), sulfadimethoxine (a sulfonamide antibiotic), sulfamethazine (a sulfonamide antibacterial), sulfamethoxazole (a sulfonamide bacteriostatic antibiotic) and thiabendazole (a fungicide and parasiticide).

Catecholamines

Three catecholamines were measured in algae: dopamine, epinephrine, and norepinephrine (Figure 2). The expected levels of catecholamines in algae are virtually unknown, but Van Alstyne et al. (2006) extracted dopamine from the green algae *Ulvaria obscura* suggesting the potential of occurrence in other species. Catecholamine salts used for calibrations and standards were purchased from Sigma.

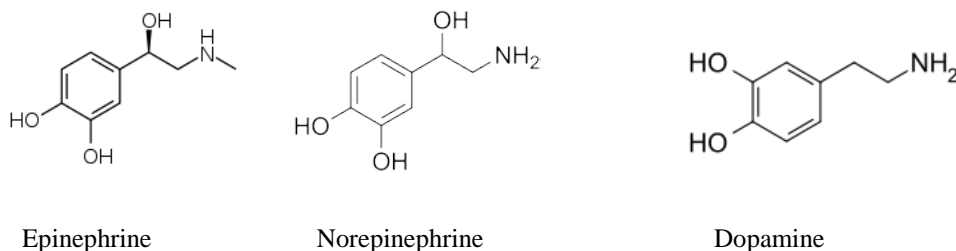


Figure 2. Structures of catecholamines.

STUDY SITES

The main stem of the Hudson River was sampled at three sites June 4, 2013 (Figure 3). These sites included Albany, Castleton and Coxsackie. The goal was to assess the levels of pharmaceuticals in the Hudson where effluent occurs and collect algae samples for dopamine analysis.

Originally three additional sites were to be tested (Hudson, Kingston, Poughkeepsie), but due to weather and equipment issues, samples were only collected

from the three northern sites. All six sites and an additional two sites, Fort Montgomery and Haverstraw Bay, are a part of a long term research project directed by the Cary Institute of Ecosystem Studies. This group has water quality data dating back over twenty years from a study that has focused on the impacts of the zebra muscle *Dreissena polymorpha* (Strayer et al. 2004), making their sites perfect locations for the study of pharmaceutical analysis.

Three other sites in surrounding tributaries of the Hudson River were sampled in June 2013 (Figure 3 D-F). These sites were chosen based upon collaboration with the

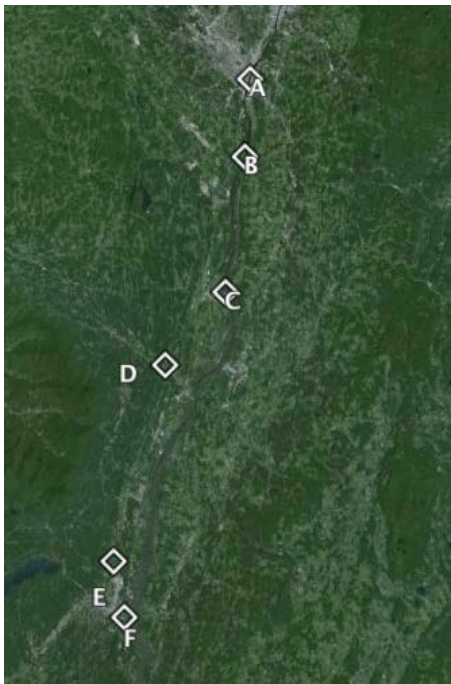


Figure 3. Locations of the six sampling sites visited within the HRV:
A - Albany, B – Castleton,
C – Coxsackie,
D – Catskill Creek,
E – Esopus Creek,
F – Kingston WWTP

Hudson River Keeper, and coordinated with several sampling sites they visit on a regular basis to monitor the prevalence of the sewage indicating bacteria *Enterococci*. One location on the Esopus, Catskill, and Rondout Creeks was visited with the goal of again determining the extent to which these areas are polluted with amphetamines and to collect algal samples.

SAMPLING

Study sites: Hudson River

At each sampling site, a single water sample and six algae samples were collected. Water samples were filtered in

the field using a Geopump™ (Figure 4). The water was collected directly off the boat and

then placed in an amber glass jar for analysis at a later date. At these sites, algae was sampled directly from the water column. In the case of the Cocksackie site, water and algae samples were collected directly from shore due to equipment issues concerning the boat that was being used. Algal samples were filtered from the water column onto 0.47 μ m glass fiber filter paper using the Geo-pump. Water was filtered directly from the river into a graduated cylinder to ensure accurate filtrate measurements.



Figure 4. Water sample collection off the side of the Cary Institute boat at the Albany sampling site.

Study sites: Tributaries

Water samples were collected from the three tributary sites in a similar fashion to that described above for the Cocksackie site; however, at these three sites benthic algae was collected instead of algae within the water column. Six rocks at each site were bagged and put on ice for transport to the Cary Institute of Ecosystem Studies, where each rock was processed. Each rock

was individually cleaned by rinsing with deionized water and carefully scrubbed in order to remove biofilms. The water used to rinse and wash the rocks was measured and recorded. A fixed amount of this water was filtered through 0.47 μ m glass fiber filter paper to determine chlorophyll *a*, ash free dry mass, and potentially other pigments. Algae were then filtered onto three additional filters for later determination of potential catecholamine concentrations.

PROCESSING WATER SAMPLES

Collected water samples were put on ice and transported back to the Cary Institute where they were frozen upon arrival. To quantify ATs and other pharmaceutical compounds solid phase extraction (SPE) was conducted using HLB 6cc cartridges. Each cartridge was initially primed with deionized water and acetone. Cartridges with sample extracts were then transported to the University of Nebraska-Lincoln, where they were eluted with 6 ml of Optima grade acetone. Samples were then blown down to dryness under vacuum and constant stream of nitrogen gas. For each sample, 200 µl of methanol/water (50:50) was added, as well as 100 ng of internal standard. Samples were analyzed using liquid chromatography tandem mass spectrometry (LC/MS), a technique that has been proven to provide best analytical results for illicit drugs and metabolites (Castiglioni et al. 2006a; Castiglioni et al. 2006b; Pal et al. 2012).

After initial analysis, it was determined that acetone alone did not provide quantitative recovery for some compounds. Cartridges were re-eluted with 6 ml of Optima grade methanol. This was combined with the original extracts and the steps above were repeated for re-analysis. This additional step improved recovery of compounds in a method limit detection test.

MEASURING DOPAMINE

Algae samples were collected on glass fiber filters as stated above and then kept frozen until analysis. Dopamine and other catecholamine levels in algae were extracted using methods described by Van Alstyne et al (2006) combined with a derivatization method described by Suzuki et al. (2003). Approximately 0.1g of algae was frozen for 36 hours in 80% aqueous methanol (MEOH) at -16°C. Then, 2 ml of each extract was

filtered using a GF/A glass fiber filter followed by a 0.2 µm filter following Van Alstyne et al. (2006). Extracts were then blown down to dryness under vacuum and a steady stream of nitrogen gas. Dried samples had 50µl MSTFA with 1% TCMS and 50µl pyridine added before being capped and placed on a vortex for approximately 10 seconds (Suzuki et al. 2003). This mixture was then incubated at 40°C for 30 minutes and a 1 µl sample analyzed via GC-MS.

RESULTS

PRESENCE OF CONTAMINANTS

Initial analysis of water samples from each field site resulted in no detects of ATs; however, there were consistent detects of 1,7-dimethylxanthine, acetaminophen, caffeine, and DPH in a majority of locations sampled. Highest detects were seen in tributary sites, which is likely a result from sampling of smaller streams. Re-elution of the HLB cartridges with methanol was able to improve the detection of amphetamine, carbamazepine and cotinine.

Figure 5 (A and B) refers to the compounds that were detected at the Hudson River sites and tributaries, respectively. Amphetamine was only detected at the Kingston WWTP site (Figure 6). Fewer detects of ATs potentially resulted from high dilution of the contaminants being released within the HRV. A more complete overview of the amounts of each compound detected is presented in Table 1.

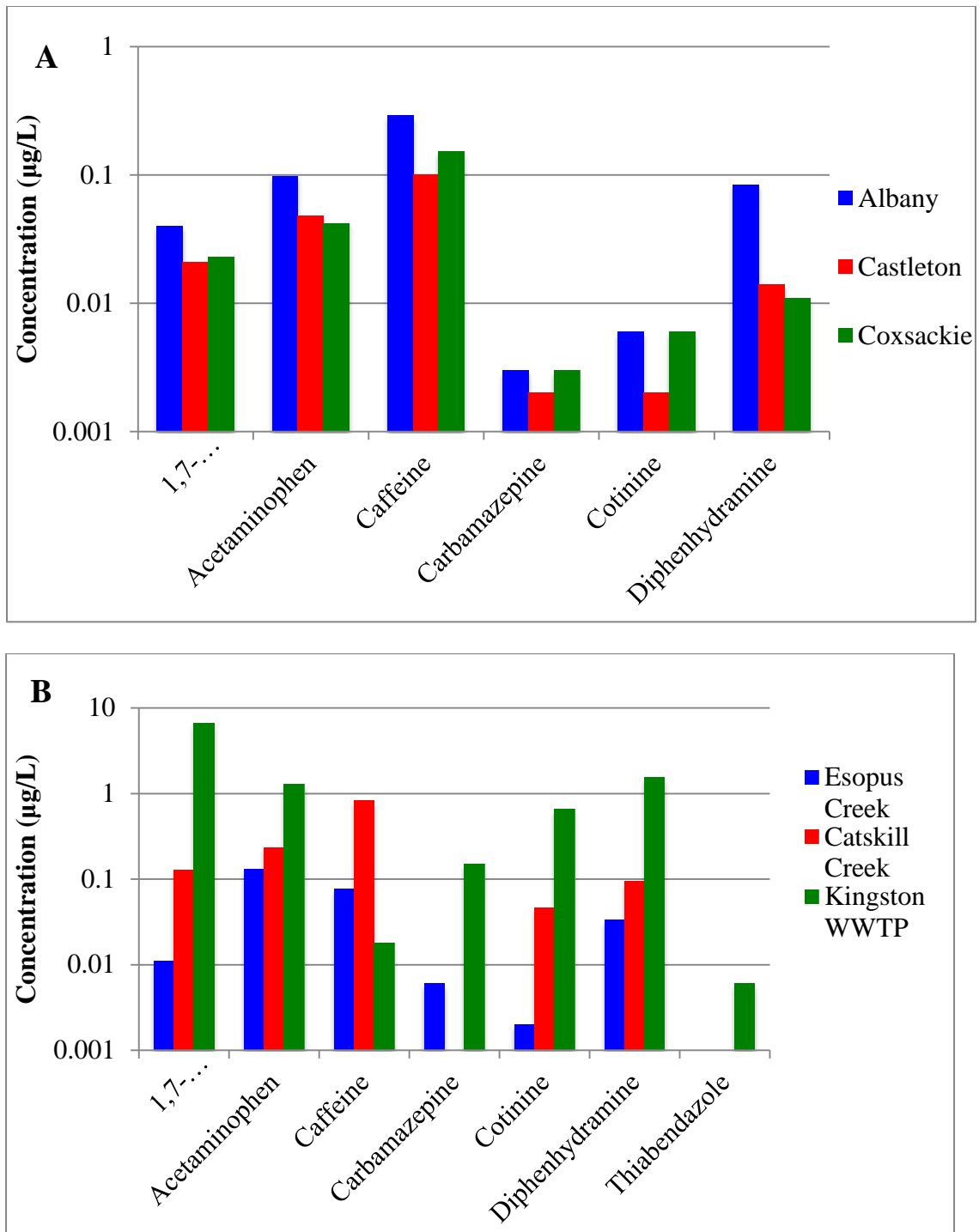


Figure 5. (A) Concentrations of pharmaceutical compounds detected at the three sampling sites on the main stem of the Hudson River. (B) Concentrations of pharmaceutical compounds detected at three tributary sites within the HRV.



Figure 6. Sample collection at the Kingston WWTP CSO that empties into Rondout Creek.

	Albany	Castleton	Coxsackie	Esopus Creek	Catskill Creek	Kingston WWTP
1,7-Dimethylxanthine	0.04	0.021	0.023	0.011	0.126	6.533
Acetaminophen	0.098	0.048	0.048	0.131	0.233	1.292
AMPH	**	**	**	**	**	0.018
Caffeine	0.291	0.101	0.153	0.077	0.833	14.563
Carbamazepine	0.003	0.002	0.003	0.006	**	0.15
Cotinine	0.006	0.002	0.006	0.002	0.046	0.66
Diphenhydramine	0.084	0.014	0.011	0.033	0.094	1.552
Thiabendazole	**	**	**	**	**	0.006

Table 1. Concentrations of pharmaceutical compounds found at each sample site ($\mu\text{g L}^{-1}$). Spaces marked with (**) showed no detections at this site.

IDENTIFICATION OF CATECHOLAMINES

Catecholamines were identified after extraction from algae and derivatization.

Figure 7 shows the ion chromatograms of the three catecholamines being analyzed. 3,4-Dihydroxybenzylamine hydrobromide was purchased from Arcos Organics to be used as

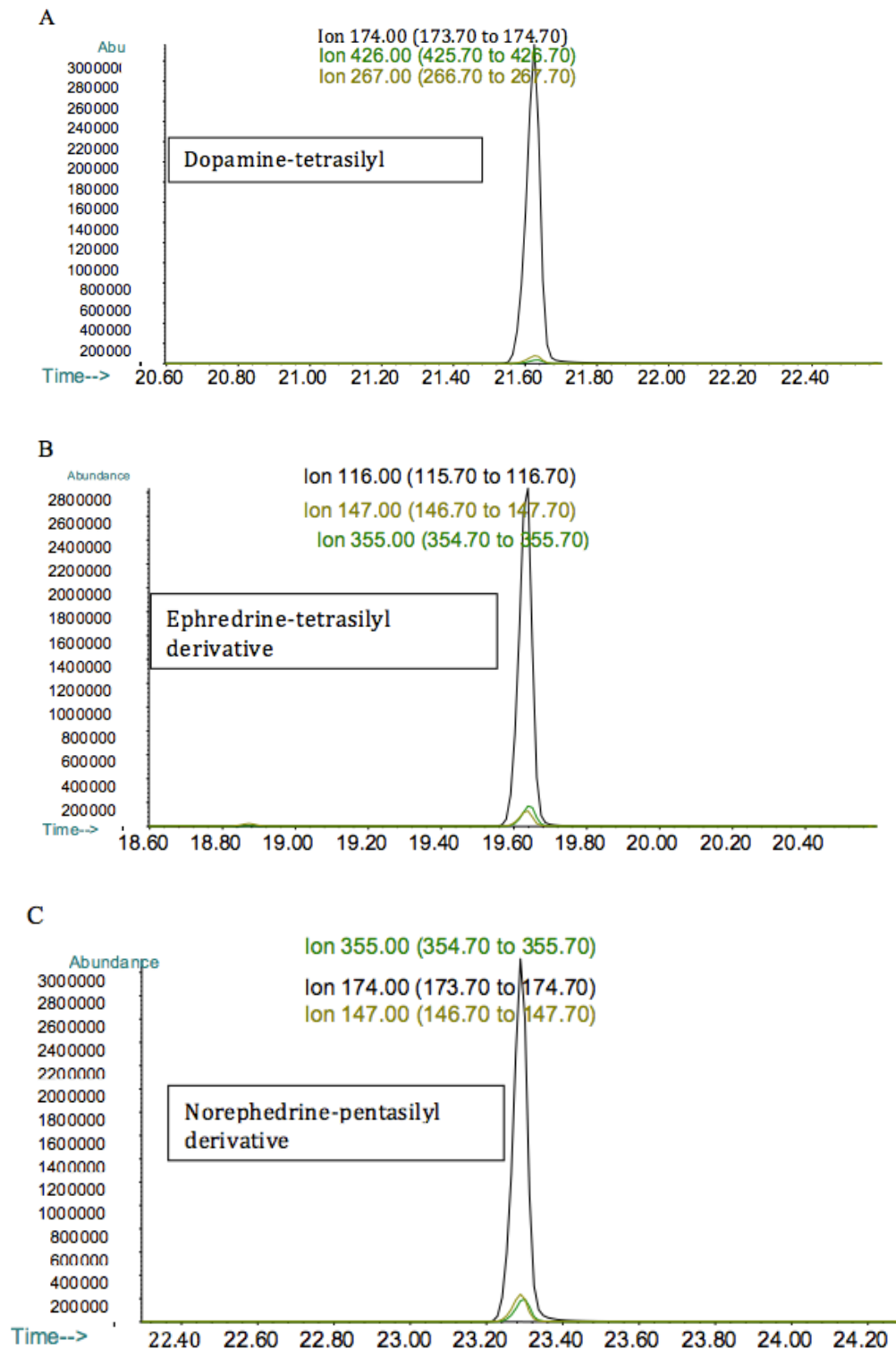


Figure 7. Ion chromatograms of silyl derivatives for (A) Dopamine, (B) Ephedrine and (C) Norepinephrine.

the internal standard. The m/z ratio for each compound is as follows: m/z 116 epinephrine, m/z 174 norepinephrine, m/z 174 dopaomine, and m/z 179 3,4-Dihydroxybenzylamine. M/z of 355 and 147 for both norepinephrine and epinephrine were also monitored since initial tests indicated multiple derivatives were produced for these compounds.

DISCUSSION

The results of this study did not demonstrate large amounts of amphetamines within effluent and receiving water in the HRV; however, detection of amphetamine at Kingston's WWTP effluent outflow does indicate the potential for the occurrence of these stimulants. Limited detections of target compounds are most likely due to high dilution factors. Due to the high cost of analysis, few water samples could be processed. This limited the number of sampling sites visited (Figure 3).

A majority of sampling was done after large rain events. The relevance of this is due to the use of CSOs along the Hudson River. CSOs are designed to collect rainwater, domestic sewage and industrial wastewater within the same pipe and route it all to various WWTPs. Several issues arise during or immediately following large rain events as pipes become overloaded and raw sewage bypasses WWTPs, to be released directly into streams and rivers (EPA 1994). Therefore, the pharmaceutical information presented in this paper is a good indication of what can be expected to be present after large rain events. Further research could potentially lead to detection of compounds not analyzed in this study, as well as detection of compounds that were not seen at these six sampling sites.

Of the compounds that were consistently detected, there is cause for some concern regarding the persistence of DPH and carbamazepine (Table 1). DPH has been known to cause behavioral changes in fathead minnows (*Pimephales promelas*) (Berninger et al. 2011). Carbamazepine has been identified as a compound that alters feeding behavior (Nassef et al. 2010), impairs growth (Van den Brandhof and Monforts 2010), and induces stress responses in several different fish species (Li and Brownawell 2010). The detection of these two compounds as well as amphetamine, acetaminophen, and thiabendazole provides new information in addition to what has been previously identified (Kolpin et al. 2002; Yu et al. 2006; Palmer et al. 2008; Cantwell et al. 2010; Li and Brownawell 2010; Reiner and Kanna 2011; Liao et al. 2012).

Determination of catecholamines in algae has not been completed at this point in time. Initially, catecholamines were to be derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl (AQC) and then measured with HPLC-fluorescence. Initial tests yielded broad peaks and relatively low sensitivity. To improve the derivatization of catecholamines, new AQC was synthesized following Cohen and Michaud (1993) and a new C-18 column was purchased from Thermo Scientific. When results did not improve, a borate buffer with ascorbate and EDTA was used to aid in the derivatization. Boughton et al. (2011) indicated that this small change should improve the recovery of target catecholamines and slow the breakdown of derivatives; however, these changes did not result in improved peaks or sensitivity, so it was determined that HPLC was not sensitive enough.

It was decided that GC-MS analysis similar to methods in Suzuki et al. (2003) was a more practical way to measure dopamine and other compounds within algae. Initial

analysis of algae collected at Esopus Creek, Catskill Creek and the Kingston WWTP showed no detects at the 0.25 ng/ μ l detection limit. Samples from Albany, Castleton and Coxsackie have not been processed at this time due to the small algal samples collected from these sites. The lack of detection of catecholamines from the samples that were processed may have resulted from small sample sizes (~0.1g), or, alternatively, the specific algal species collected does not possess the ability to create these catecholamines. The algae processed by Van Alstyne et al. (2006) was *Ulvaria obscura*, a marine green algae. A pure culture experiment will be run in the future using a species similar to *U. obscura*.

The information presented in this study is a precursor to experiments that will be conducted within artificial streams. Stream experiments were originally planned for fall 2013, but since this work involves the use of controlled substances a special permit was needed before any work could begin. Though the permit has now been obtained, these experiments will be postponed until spring 2014. This will allow for determination of the best possible method to extract catecholamines from algae, as well as determine concentrations of amphetamine to spike artificial streams.

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