♻EPA Stability of Pharmaceuticals, Personal Care Products, Steroids, and Hormones in Aqueous Samples, POTW Effluents, and Biosolids

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ACKNOWLEDGMENT AND DISCLAIMER

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EXECUTIVE SUMMARY

EPA Methods 1694 and 1698 cover pharmaceuticals and personal care products (PPCPs), steroids and hormones in sewage influent, effluent and sludge. Although researchers have published several holding time and preservation studies, the studies have been limited in the number and variety of matrices, chemicals, and preservation techniques tested. Because these studies were also conducted with a range of different methods, comparing data between studies is difficult. In this work, EPA describes a study conducted to revise the holding times and preservation conditions for EPA Methods 1694 and 1698. This study tested a broad number and variety of chemicals, matrices and preservation techniques under conditions expected in samples collected in support of Clean Water Act programs.

EPA has tested 89 chemicals that included PPCPs and hormones in reagent water, sewage treatment plant effluent and biosolids samples. EPA evaluated the effects of chlorine, dechlorinating agents, container types and temperature on the target chemicals, which included macrolide antibiotics, quinoline antibiotics, beta-lactam antibiotics, sulfonamide antibiotics, tetracycline antibiotics, synthetic hormones and other PPCPs. Samples were tested at 0, 7, 14 and 28 days to identify the most promising holding times and preservation conditions. EPA selected conditions from this initial work to test a second set of samples at 0, 4, 7 and 14 days.

The choice of the most suitable sample bottle varies between individual PPCPs. Higher doses of chlorine (2 ppm) significantly decreased the concentration of or completely destroyed the chemicals. The combination of chlorine and dechlorinating agents (sodium thiosulfate or ascorbic acid) affected many PPCPs including: antibiotics and various over the counter and prescription medications. However the combination of chlorine and sodium thiosulfate resulted in more frequent destruction of all classes of antibiotics studied. In the absence of chlorine, the addition of sodium thiosulfate resulted in nondetects for clinafloxacin, sarafloxacin, fluoxetine, diphenhydramine. In contrast, the use of ascorbic acid in the absence of chlorine, did not significantly affect these compounds. The combined effects of lower doses of chlorine (0.5 ppm) and ascorbic acid in effluent samples resulted in nondetects for tetracyclines, chlorotetracyclines and tetracycline degradates as well as several other PPCPs including: 17 alpha dihydroequilin, desogesterol, carbadox, sulfadimethoxine, sulfathiazole, cefotaxime and linomycin. In contrast, effluent samples not containing chlorine or ascorbic acid had less frequent nondetects.

EPA has used the results of this study to revise the holding times and preservation conditions in EPA Methods 1694 and 1698 as follows:

- Bottles - amber high density polyethylene or glass containers.
- Dechlorinating Reagent - ascorbic acid.
- Shipping and Storage Conditions - in the dark at <6°C (optional freezing for biosolids).
- Holding Times - extract samples within seven days; analyze extracts as soon as possible, not to exceed 30 days (10 days in the case of tetracyclines).

These holding times are precautionary, as they protect the most sensitive compounds. They are not universal holding times for PPCPs or steroids and hormones. It is also important to point out that these holding times apply only to wastewater samples and not to drinking water samples. Due to the diverse nature of these chemicals, more holding time studies may be warranted when new chemicals are targeted.

INTRODUCTION

Contaminants of emerging concern (CECs) originate from many different sources. The chemical classes that comprise CECs include but are not limited to: flame-retardants, fluorinated alkyl phenol surfactants, phthalates bis-phenol A, steroids, hormones, pharmaceuticals, and various personal care products (PPCPs). Over the last ten years, researchers have identified many CECs in POTW influents, effluents, biosolids and other environmental samples.

There are many published reviews[1] covering general topic knowledge and analytical methods as well as many reports of the occurrence of PPCPs in various types of samples.[2] Some of the earliest reports detailed the occurrence of a variety of PPCPs in surface waters, and in publicly owned treatment works (POTWs).[3,4,5] These include pollutants, such as 17 alpha-ethynyl estradiol (EE2) which is designed to affect the human endocrine system.

To support occurrence surveys of CECs, researchers have been developing new, and refining existing analytical methods. These environmental monitoring tools need to work in a wide variety of sample types, and measure a wide variety of chemicals. An important part of method refinement is establishing sample collection, preservation, storage and holding time protocols.

Improving our understanding of the stability of CECs in environmental samples is important for several reasons: researchers are not collecting and handling CEC samples in a uniform way; few studies have been conducted in sewage samples; and the majority of PPCPs are bioactive and hence likely susceptible to breakdown by bacteria or other transformation reactions. Some biologically active compounds, such as illicit substances, may be actively degraded and/or metabolized by the bacteria found in sewage matrices.[6] Other CECs, such as synthetic and naturally occurring estrogens, can undergo a variety of transformations in the environment and exist in a number of conjugated forms. For example, 17 alpha-ethynyl estradiol (EE2), 17 beta-estradiol, estrone and testosterone are susceptible to biodegradation and transformation by various types of bacteria.[7] Humans can metabolize synthetic and naturally occurring estrogens to form estrogen sulfates and glucuronides which are excreted in urine[8] and potentially hydrolyzed back to their unconjugated forms in wastewaters.[9,10]

Although researchers have published several holding time and preservation studies, the studies have been limited in the number and variety of matrices, chemicals, and preservation techniques tested.[11, 12] There are several studies in surface waters; fewer in sewage matrices. In general, these studies have covered a small subset of CECs compared to the larger set monitored in published occurrence studies. These studies have examined a relatively narrow range of sample handling and preservation conditions. For example, the role of containers in sample preservation has not been reported. Our study tested a broader number and variety of PPCPs, steroids, hormones, matrices and preservation techniques, and under conditions expected in samples collected in support of Clean Water Act (CWA) programs.

This report describes the experimental design, results, and findings from a comprehensive investigation of sample collection and handling procedures for 89 chemicals in reagent water, POTW effluent and sewage samples. The conclusions from this study will be used to revise sampling, storage and holding times in EPA Methods 1694 and 1698.

EXPERIMENTAL

The study consisted of four phases, all of which used the same analytical conditions (Table 1) and the same target list of analytes (Table 2). In Phase 1, storage containers were tested. In Phase 2, several combinations of preservation techniques were tested to identify the most promising holding times and preservation conditions in aqueous samples. In Phase 3, several temperatures were tested for the preservation of biosolids samples. In the first three phases, samples were tested at 0, 7, 17 and 28 days. In Phase 4, EPA selected "best combination" conditions from Phase 1 to test a second set of samples at 0, 4, 7 and 14 days. The study design and the distribution of replicate samples are described in Table 3.

Analytes and Analytical Methods

There is no uniform or consensus list of PPCPs, steroids or hormones to include in occurrence surveys of POTW or environmental samples. As our understanding of the occurrence, persistence and toxicity of these compounds increases, the target lists will be refined both by elimination of some compounds that are not commonly found, and by addition of others.

In 2007, EPA published an LC/MS/MS method (Method 1694), which was used in this study to test 72 PPCPs.[13a] This method was modified to include 17 of the steroids and hormones in EPA Method 1698. Together these methods defined the target list of 89 chemicals, comprised of six subsets of analytical run conditions (Tables 1 and 2.)

Table 1.   Summary of Method/Analyte Categories

Category	No. of Compounds	Extraction	LC/MS/MS Mode
Method 1694 List 1	48	Acidic	ESI +
Method 1694 List 2	14	Acidic	ESI +
Method 1694 List 3	6	Acidic	ESI -
Method 1694 List 4	4	Basic	ESI +
Hormones List 1	10	Acidic	ESI +
Hormones List 2	7	Acidic	ESI -

Solids were extracted by sonication in buffered acetonitrile and exchanged into water. Solid extracts and water samples were extracted by solid phase extraction and analyzed by LC/MS/MS according to the scheme in Table 1. Three to four bottles of water, effluent, or biosolid were prepared for each sample. Therefore, the multiple extractions required for analysis of each sample were performed on individual sample aliquots. The target compounds are listed in Table 2 according to a compound classification system that is used in the discussion of results.

Table 2. Analyte Categories Used During This Study

Macrolide Antibiotics - Acidic Extraction ESI+		Misc PPCPs List 3 - Acidic Extraction ESI+	
Compound	Compound Classification	Compound	Compound Classification
Azithromycin	Macrolide Antibiotic	1,7-Dimethylxanthine	Antispasmodic, caffeine metabolite
Clarithromycin	Macrolide antibiotic	Acetaminophen	Antipyretic, Analgesic
Erythromycin-H2O	Macrolide antibiotic	Caffeine	Stimulant
Roxithromycin	Macrolide antibiotic	Carbamazepine	Anticonvulsant
Tylosin	Macrolide antibiotic	Ormetoprim	Diaminopyrimidine
Virginiamycin	Macrolide antibiotic	Codeine	Opiate
Quinoline Type Antibiotics - Acidic Extraction ESI+		Cotinine	Nicotine metabolite
Compound	Compound Classification	Dehydronifedipine	Nifedipine metabolite
Ciprofloxacin	Quinoline antibiotic	Digoxigenin	Immunohistochemical Marker Steroid
Clinafloxacin	Quinoline antibiotic	Digoxin	Cardiac glycoside
Lomefloxacin	Quinoline antibiotic	Diltiazem	Antihypertensive
Norfloxacin	Quinoline antibiotic	Diphenhydramine	Antihistamine
Ofloxacin	Quinoline antibiotic	Fluoxetine	SSRI Antidepressant
Enrofloxacin	Quinolone antibiotic	Miconazole	Antifungal agent
Flumequine	Quinolone antibiotic	Norgestimate	Hormonal contraceptives
Oxolinic Acid	Quinolone antibiotic	Thiabendazole	Fungicide and parasiticide
Carbadox	Quinoxaline Antibiotic	**Tetracyclines, Chlorotetracyclines & Degradates - Acidic Extraction ESI+**	
Sarafloxacin	Fluoroquinolone antibiotic	Compound	Compound Classification
beta-Lactam & Misc Antibiotics - Acidic Extraction ESI+		4-Epianhydrochlortetracycline (EACTC)	Chlorotetracycline degradate
Compound	Compound Classification	4-Epianhydrotetracycline (EATC)	Chlorotetracycline degradate
Trimethoprim	Pyrimidine antibiotic	4-Epichlortetracycline (ECTC)	Chlorotetracycline degradate
Lincomycin	Lincosamide antibiotic	4-Epioxytetracycline (EOTC)	Oxytetracycline degradate
Cefotaxime	Cephalosporin antibiotic	4-Epitetracycline (ETC)	Tetracycline degradate
Cloxacillin	ß-Lactam antibiotics	Anhydrochlortetracycline (ACTC)	Chlorotetracycline degradate
Oxacillin	ß-lactam antibiotics	Anhydrotetracycline (ATC)	Chlorotetracycline degradate
Penicillin G	ß-Lactam antibiotics	Chlortetracycline (CTC)	Tetracycline antibiotic
Penicillin V	ß-Lactam antibiotics	Demeclocycline	Tetracycline antibiotic
Sulfonamide Antibiotics - Acidic Extraction ESI+		Doxycycline	Tetracycline antibiotic
Compound	Compound Classification	Isochlortetracycline (ICTC)	Chlorotetracycline degradate
Sulfachloropyridazine	Sulfonamide antibiotic	Minocycline	Tetracycline antibiotic
Sulfadiazine	Sulfonamide antibiotic	Oxytetracyclin (OTC)	Tetracycline antibiotic
Sulfadimethoxine	Sulfonamide antibiotic	Tetracycline (TC)	Tetracycline antibiotic
Sulfamerazine	Sulfonamide antibiotic	**Steroids & Hormones - Acidic Extraction ESI+ and ESI-**	
Sulfamethazine	Sulfonamide antibiotic	Compound	Compound Classification
Sulfamethizole	Sulfonamide antibiotic	Allyl Trenbolone	Sex Hormone
Sulfamethoxazole	Sulfonamide antibiotic	Androstenedione	Anabolic agent
Sulfanilamide	Sulfonamide antibiotic	Androsterone	Hormone metabolite
Sulfathiazole	Sulfonamide antibiotic	Desogestrel	Ovulation inhibitor
Misc PPCPs List 1 - Acidic Extraction ESI-		Mestranol	Ovulation inhibitor
Compound	Compound Classification	Norethindrone	Ovulation inhibitor
Gemfibrozil	Antilipemic	Norgestrel	Ovulation inhibitor
Ibuprofen	Analgesic	Progesterone	Sex Hormone
Naproxen	Non-steroidal anti-inflammatory drug	Testosterone	Sex Hormone
Triclocarban	Antimicrobial, disinfectant	Estriol	Sex Hormone
Triclosan	Antimicrobial, disinfectant	17 alpha-Dihydroequilin	Sterol
Warfarin	Anticoagulant	17 alpha-Estradiol	Sex Hormone
Misc PPCPs List 2 - Basic Extraction ESI+		17 alpha-Ethynyl-Estradiol (EE2)	Ovulation inhibitor
Compound	Compound Classification	17 beta-Estradiol	Sex Hormone
Albuterol	Antiasthmatic	Equilenin	Hormone replacement
Cimetidine	H2-receptor antagonist	Equilin	Hormone replacement
Metformin	Anti-diabetic drug	Estrone	Sex Hormone
Ranitidine	Anti-acid reflux		

Holding Time and Preservation Conditions

This study was designed to investigate the stability of PPCPs, steroids and hormones in reagent water and POTW effluent and biosolid matrices. The parameters investigated included sample containers (glass, silanized glass and high density polyethylene [HDPE] bottles), storage conditions (-20 ºC or 4 ºC), the presence of residual chlorine, addition of dechlorinating agent (sodium thiosulfate or ascorbic acid) and time (from Day 0 to up to 28 days after preparation). Statistical relevance of the results was evaluated by the use of replicate samples for each set of conditions. All samples were spiked with known amounts of the target analytes at the beginning of the study to provide concentrations of all target compounds that could be reliably quantified by the analytical methods.

Table 3.   Holding Time Study Treatments, Conditions, and Parameters

Treatment and Condition				Number of Replicates					
Sample	Bottle	Temp	Treatment	Day 0	Day 2	Day 4	Day 7	Day 14	Day 28
Phase I: Container Type									
Reagent Water	HDPE	4 ℃	-	3	0	0	3	3	3
Reagent Water	Unsilanized Glass	4 ℃	-	3	0	0	3	3	3
Reagent Water (Control)	Silanized Glass	4 ℃	-	3	0	0	3	3	3
Phase II: Preservation Techniques (Dechlorination)									
Reagent Water	Silanized Glass	4 ℃	2 mg/L Cl$_2$	2	0	0	2	2	2
Reagent Water	Silanized Glass	4 ℃	2 mg/L Cl$_2$ + 80 mg/L Na$_2$S$_2$O$_3$	2	0	0	2	2	0
Reagent Water	Silanized Glass	4 ℃	2 mg/L Cl$_2$ + 50 mg/L ascorbic acid	2	0	0	2	2	0
Phase II: Preservation Techniques (Dechlorination follow up samples)									
Reagent Water	HDPE	4 ℃	80 mg/L Na$_2$S$_2$O$_3$	1	1	0	0	0	0
Reagent Water	HDPE	4 ℃	50 mg/L ascorbic acid	1	1	0	0	0	0
Phase II: Preservation Techniques (Temperature)									
Unspiked Effluent	Silanized Glass	4 ℃	-	3	0	0	0	0	0
Spiked Effluent	Silanized Glass	4 ℃	-	3	0	0	3	3	3
Spiked Effluent	Silanized Glass	-20 ℃	-	3	0	0	3	3	3
Spiked Effluent	Silanized Glass	4 ℃	pH=2.0	2	0	0	2	2	0
Phase III: Biosolids									
Unspiked Biosolid	Polypropylene	4 C	-	3	0	0	0	0	0
Spiked Biosolid	Polypropylene	4 C	-	3	0	0	3	3	3
Spiked Biosolid	Polypropylene	-20 ℃	-	3	0	0	3	3	3
Phase IV: POTW Effluent Best Techniques									
Spiked Effluent	HDPE	4 ℃	0.5 mg/L Cl$_2$ + 50 mg/L ascorbic acid	4	0	4	4	4	0

Phase 1 samples were analyzed at 0, 7, 14, and 28 days; and Phase 2 samples were analyzed at 0, 4, 7, and 14 days.

Preparation of Samples for the Holding Time Study

Spiking Solution

All samples were spiked at the mid range of the calibration curve in the corresponding method used (see Table 4). Each sample was spiked with all 89 chemicals. This produced samples containing the same amount of target analytes as the on-going precision and recovery sample (OPR) used for analytical method quality control. The spiking solution was a concentrated methanol solution so that only 80 µL was used for each 1-L sample.

Table 4. Spiking Concentration for PPCPs, Steroids, and Hormones

Compound	80 µL spike gives (ng)	Compound	80 µL spike gives (ng)
Mestranol	500	Penicillin G	144
Estrone	100	Penicillin V	144
17α-Dihydroequilin	100	Roxithromycin	14.5
17α-Ethinyl Estradiol	500	Sulfachloropyridazine	72.1
17α-Estradiol	100	Sulfadiazine	71.9
17ß-Estradiol	100	Sulfadimethoxine	14.4
Desogestrel	3000	Sulfamerazine	28.9
Norethindrone	100	Sulfamethazine	28.8
Equilin	200	Sulfamethizole	28.8
Norgestrel	100	Sulfanilamide	721
Testosterone	20	Sulfathiazole	71.9
Progesterone	20	Thiabendazole	72
Androstenedione	50	Trimethoprim	71.9
Estriol	1200	Tylosin	288
Allyl Trenbolone	20	Virginiamycin	143
cis-Androsterone	500	1,7-Dimethylxanthine	7507
Equilenin	20	Ciprofloxacin	262
Acetaminophen	2882	Clinafloxacin	300
Azithromycin	99.2	Enrofloxacin	150
Caffeine	720	Lomefloxacin	150
Carbadox	72.2	Norfloxacin	749
Carbamazepine	71.9	Ofloxacin	749
Cefotaxime	288	Sarafloxacin	749
Clarithromycin	71.9	Gemfibrozil	74.7
Cloxacillin	144	Ibuprofen	748
Codeine	144	Naproxen	150
Cotinine	71.9	Triclocarban	150
Dehydronifedipine	28.7	Triclosan	2992
Diphenhydramine	28.8	Warfarin	74.7
Diltiazem	14.4	Sulfamethoxazole	29.9
Digoxin	720	Tetracycline (TC)	101
Digoxigenin	288	Oxytetracycline (OTC)	102
Erythromycin	14.4	Doxycycline	102
Flumequine	71.9	Chlorotetracycline (CTC)	101
Fluoxetine	72	Anhydrochlortetracycline (ACTC)	253
Lincomycin	144	Anhydrotetracycline (ATC)	254
Miconazole	71.9	4-Epianhydrochlorotetracycline (EACTC)	1008
Norgestimate	143.9	4-Epianhydrotetracycline (EATC)	252
Ormetoprim	28.9	4-Epichlortetracycline (ECTC)	253
Oxacillin	144	4-Epioxytetracycline (EOTC)	101
Oxolinic acid	28.8	4-Epitetracycline (ETC)	101
Cimetidine	30.4	Isochlortetracycline (ICTC)	101
Metformin	1498	Demeclocycline	253
Ranitidine	30	Minocycline	1011
Albuterol	15		

Aqueous Samples

Samples were not preserved with sodium thiosulfate as specified in EPA Method 1694 unless sodium thiosulfate was specifically being tested in that sample. Samples were dechlorinated with 50 mg/L of ascorbic acid instead of the 80 mg/L of sodium thiosulfate in Method 1694, and stored in the dark at 4 °C.[11c,14] Samples preserved with sulfuric acid were treated immediately after collection by adjusting to pH 2 with concentrated sulfuric acid.

Chlorinated Samples

Chlorinated effluent and reagent water (MilliQ water) samples were prepared by adding sodium hypochlorite solution to reagent water or effluent samples immediately after they had been spiked with target compounds. Chlorinated samples were chlorinated at either 0.5 or 2 mg/L which was verified using HACH procedure 8167 for Total Chlorine. Note that for all chlorinated samples, each sample in this study was prepared (i.e., spiked and chlorinated) in its own bottle, not in a bulk container and then aliquoted into 1-L sample bottles. Because of this, adding the quenching agents to the bottles prior to sample collection was not possible.

Biosolid Samples

A bulk biosolid sample was collected from a POTW process employing screening, followed by treatment with bacteria in a settling pond and then discharge of the aqueous material as final effluent. To ensure adequate time for homogenization, subsampling and moisture determination of biosolid samples, these procedures were carried out the day before the study began. Samples were collected in two 500-mL jars using a large plastic scoop. The contents of the two jars were emptied into a large, solvent-rinsed stainless steel bowl and, using forceps, the large pieces of non-sediment material (vegetable skin, paint chips, etc.) were removed and the sample was well mixed using a disposable teaspoon. The resulting homogenous mixture was portioned out into ten 125-mL jars for ease of handling. Four separate subsamples were taken from this mixture for percent solid determination. The sample was determined to be 27.7% solids; therefore 0.5 g dry weight was equivalent to 1.8 g wet.

Effluent Samples

Effluent samples were obtained from the same POTW as for the biosolids samples. Effluent was collected using a peristaltic pump from a final discharge pipe containing flowing effluent. Ten five gallon pails were filled over the course of less than ten minutes. They were not combined prior to filling the sample bottles. The effluent was collected and immediately taken to the lab for dispensing into individual sample bottles, treatment and storage. The effluent was analyzed unspiked as part of this project and tested for total chlorine before use and found to be chlorine-free. pH or ammonia concentration was not characterized in POTW effluent. Therefore, an estimate the proportions of free and combined chlorine, or chloramines present in the chlorinated effluent samples cannot be know. However, by measuring "total chlorine" via Hach Procedure 8167, all of these forms were included in the measurement.

Sample Containers

All sample bottles were silanized amber glass except those indicated as unsilanized or HDPE. The HDPE bottles had polypropylene lids. The frozen effluents were stored in 2.5-L bottles to prevent breakage. All other containers for aqueous samples were 1 L. Silanized glass bottles (1-L and 2.5-L size) were purchased from ESS Environmental Sampling Supply (Oakland, CA).

Procedure for Aqueous Sample Preparation

For each "sample," three individual 1-L bottles were prepared: one each for acid and base extraction for PPCPs and one for steroids and hormones extraction. Pre-labeled sample bottles were filled and spiked in random order. For reagent water controls, sample bottles were filled with 900 mL of reagent water and spiked with target compounds and mixed by shaking. Then 100 mL of reagent water was added to fill the bottle and again mixed. For chlorinated water samples bottles were filled with 100 mL of reagent water, spiked with target compounds and mixed by shaking. Then 900 mL of chlorinated water was added and the sample was mixed again. For sodium thiosulfate samples, 100 mL of reagent water was added and the bottles were spiked with target compounds and mixed by shaking. 800 mL of chlorinated water was added and samples were mixed by shaking. 80 mg of sodium thiosulfate was added to the bottles and samples were again mixed by shaking. Finally, chlorinated water was added to fill the bottle to 1 L and samples were mixed. For ascorbic acid samples, bottles were filled with 100 mL of reagent water, samples were spiked with target compounds and mixed, then 800 mL of chlorinated water was added to each bottle, mixed, then 50 mg ascorbic acid was added and samples were mixed one final time. Bottles were filled to 1 L with reagent water. For reagent water samples, the bottles were filled with 900 mL of reagent water, spiked with all target compounds and mixed. Reagent water was added to fill the bottle to 1 L. For effluent samples, bottles were filled with 900 mL of POTW effluent, spiked with target compounds and mixed. Effluent was added to fill the bottle to 1 L. For frozen effluent samples, 2.5-L bottles were filled with 1 L of effluent, spiked with target compounds and mixed. For effluent samples preserved with sulfuric acid, bottles were filled with 900 mL of effluent, spiked with target compounds and mixed. The samples were adjusted to pH 2.0 with 1:1 H_2SO_4 and then mixed. The bottles were filled with effluent up to 1 L. The pH was tested and adjusted as necessary and mixed. For aqueous samples with sodium thiosulfate and ascorbic acid, but without chlorine, samples were prepared as described for other samples containing sodium thiosulfate or ascorbic acid, but were stored in 1 L HDPE bottles with polypropylene lids. For best evaluated techniques, each HDPE bottle was filled with 950 mL of effluent, and an aliquot of target analyte spiking solution was added. The bottle was capped and shaken to mix. Dilute sodium hypochlorite solution (1.9 mL of a 0.15% NaOCl) was added to give a total chlorine of 0.5 mg/L. The bottle was capped and shaken to mix. Exactly one minute after addition of the chlorine solution 50 mg of ascorbic acid was added. The bottle was capped and again shaken to mix. Each sample was then topped up to 1 L with effluent.

Procedure for Biosolids Sample Preparation

For each "sample," three individual containers were prepared: one each for acid and base extraction of PPCPs and one the extraction of steroids and hormones. Between 1.8 and 1.9 g of wet material was subsampled into each of 111 50-mL polypropylene tubes that would eventually be used as extraction containers. This provided the 0.5 g of dry biosolids specified in EPA

Method 1694 for each aliquot. The sample tubes were filled and stored overnight at 4 °C. On Day 0, 96 of the replicate samples were spiked in random order with the analytes of interest. The other 15 tubes were "controls" and were not spiked. Then 3.0 mL of reagent water was added to each sample aliquot (all 111) and the biosolids, water, and spiking solution (80 µL) were mixed on a vortex mixer. The resulting replicate samples contained approximately 10% solids, and were ready for storage or extraction and subsequent analysis on each day of the study.

Storage and Analysis of Samples

Samples were prepared on Day 0 of each study. Analysis of Day 0 samples commenced within three hours of preparation. All other samples were immediately placed into the designated storage condition until analyzed. Samples that were stored at 4 ºC were kept in the refrigerator until the morning of their analysis. Samples that were stored at -20 ºC were removed from the freezer the evening before analysis and allowed to thaw overnight in the refrigerator.

RESULTS

Analytical Results

Each sample in Table 3 was analyzed for the 89 target compounds and the results were statistically analyzed (Tables 5-11). The individual compounds were organized into groups or classifications to simplify the data analysis. These classifications are listed in Table 2 and described below. A comprehensive statistical analysis of the results was conducted to determine which set of conditions caused changes in the target compound concentrations.

Each analysis batch consisted of up to 15 test samples plus a procedural blank and an on-going precision and recovery (OPR) sample, as specified in EPA Method 1694. Instrument quality control included initial instrument calibrations consisting of a minimum of five concentrations of all target compounds and on-going calibration verifications as described in Method 1694. All data was subjected to two levels of validation. Specifically, a primary validator reviewed data focusing on instrument performance, batch QC, correctness of calculations and further work (dilutions, extra cleanup, repeat analysis, etc.). A secondary validator spot checked the primary validator's work and focused on overall data set quality, flagging of results, reasonableness, completeness of documentation, accuracy and completeness of the electronic data deliverable (EDD) and final report. A final check was performed by the laboratory project manager prior to reporting the data. The percent recoveries for target analytes from 20 randomly selected OPR samples from each study phase are shown in Appendix 1.

Replicate samples were prepared and analyzed (see Table 3) for each sample treatment to provide data that would be amenable to statistical analysis. Replicate results are therefore an indication of overall precision, including both analytical and sampling variance. Of the over 10,000 results collected for aqueous samples, less than 1% were associated with analyte recoveries being outside the specified range for OPR samples; 2.5% were associated with labeled internal standard recoveries being outside the method specified range; and less than 1.2 % were associated with blank contamination. Of the over 2,700 results collected for biosolid samples, none was associated with analyte recoveries being outside the specified range for OPR samples; 1.8 % were associated with labeled internal standard recoveries being outside the method specified range; and less than 1.0 % was associated with blank contamination.

Statistical Analysis

Classification of Compounds for Statistical Analysis

Given that this work utilized screening analytical methods and evaluated a total of 89 analytes with multiple replicate analyses for the majority of tests being conducted, we realized that to accurately and efficiently examine the large amount of data produced, and to identify statistically significant changes in concentration, a robust statistical approach would be needed. Some sort of system for classifying these compounds into distinct groupings was required to facilitate statistical analysis.

One limitation in performing a statistical analysis for any group of chemicals is that in some cases, each subset of compounds represents a different class or classes of chemicals that may behave very differently under similar conditions, due to differences in their physicochemical properties. Specifically for this work, various chemicals may be prone to different reaction pathways in environmental samples than others. For this reason, the chemicals in this study were examined both by statistical analysis of different subsets (see Table 2) and, when appropriate, on a compound-by-compound basis. Appendix 2 provides additional data on the degradation of assorted compounds in this study which may not fit well within subgroups identified for the purposes of statistical analysis.

PPCPs were divided into groups (Table 2) for the purposes of statistical analysis. Different antibiotic classes, namely, macrolides, quinolines, sulfonamides, and beta lactam antibiotics, all refer to classes of antibiotics that were grouped individually to make statistical analysis more meaningful. Even though most of these chemicals may be amenable to extraction by similar SPE conditions and analysis by similar instrumental conditions (in this case acidic extraction followed by ESI+ LC/MS/MS analysis), they may react very different under environmental conditions. For similar reasons, tetracyclines, chlorotetracyclines and degradates, all of which were extracted under acidic conditions followed by ESI+ LC/MS/MS analysis, were designated as one group of chemicals for the purposes of statistical analysis because of chemical and structural similarities. Several steroids and hormones also were grouped together for the purposes of statistical analysis.

There were a wide variety of miscellaneous PPCPs analyzed in this work which did not fit well into groups with chemical similarities. For this reason, they were grouped into four groups, based on the Table 1 conditions of analysis. Specifically miscellaneous (Misc.) PPCP list 1 (Table 1) refers to chemicals extracted under acidic conditions and analyzed by ESI-LC/MS/MS, Misc. PPCPs list 2 refers to chemicals extracted under basic conditions and analyzed by ESI+ LC/MS/MS and Misc. PPCP list 3 refers to compounds that were extracted under acidic conditions and analyzed by ESI+ LC/MS/MS.

Statistical Tests

Statistical analyses were performed on the analytical results for each compound to determine which set of conditions resulted in a significant change in the concentration of target analytes. Identification of statistical differences for each compound was performed using a multi-step processes that involved a one-way Analysis of Variance (ANOVA) and a Dunnett's test. The results from the holding time study were stratified by analyte and treatment. For each

stratification, an ANOVA model was fit to assess the effect of holding time on the analyte recoveries, in a two-step process.

In the first step, an overall F-test in the ANOVA model was used to determine whether there was an overall difference between holding times (i.e., whether recovery at one holding time was significantly different from recovery for at least one other holding time). The ANOVA asks the question: "Does the mean recovery at any one holding time differ significantly from the mean recovery at any other holding time?" Phrased differently, the question is: "Based on the observed variability between replicate analyses, can we rule out the possibility that there are no differences in analyte recovery between any of the different holding times?" If the ANOVA determines that the answer is "No, there is not a difference," then we can stop evaluating the results for that analyte and treatment.

If the ANOVA gives a "yes" answer, we concluded that at least one holding time had a mean recovery significantly different from at least one other holding time. Therefore, the second step involved pairwise comparisons between Day 0 and each of the other holding times, using Dunnett's test. Dunnett's test was used to specifically compare results from one reference time (Day 0) to all other holding times, without directly comparing the individual non-zero times to each other.

Because fewer comparisons are run using Dunnett's test than for other pairwise comparison procedures, Dunnett's test yields more statistical power for the given set of analyses. In addition, because the Dunnett's test compared all of the results back to the Day 0 result, it ensured that small changes over time were not overlooked (e.g., a gradual downward trend might not be apparent when comparing results from adjacent days).

Based on the ANOVA and the Dunnett's test, we determined whether or not there was a statistically significant difference in the mean result for each analyte on each day of the study (e.g., 7, 14, and 28), compared to the results for the analyte on Day 0. The differences for each analyte were classified as significant (Y) or not significant (N).

Magnitude of Statistical Differences: If a statistical difference was observed using the tests described above, the magnitude of the difference for each analyte, in terms of percent difference in mass compared to the mass on Day 0 was also calculated.

Using a 20% difference (positive or negative) in mass as an indication of a change that could affect a decision about the data in a real-world sample, we counted the number of analytes for which there was a statistically significant change (i.e., Y) and the magnitude of the change was greater than 20%. We tabulated the number of analytes in each family that had significant differences greater than 20% and those that did not. If there was a statistically significant change with a magnitude less than 20%, we did not count that change in this evaluation of holding time (e.g., a change of 5% in mass, while perhaps statistically significant, is not sufficient reason to establish a holding time).

The use of the 20% threshold provided additional protection against a high frequency of false positive decisions being made due to the large number of matrices, test conditions and analytes for which holding time comparisons were performed. Considering the variability observed in the analysis of biosolids and some other sewage matrices, we believe that this threshold provides

added protection against counting variations in recovery as significant concentration increases or decreases.

While other approaches to holding times determinations are available, the ANOVA along with the use of a Dunnett's test offered a number of benefits. For instance, in ASTM D4841,[15] the study data quality objective is to be able to detect a 15% difference between holding times. Ultimately, under ASTM D4841, holding time study data is evaluated using a separate t-test for each holding time. The ANOVA used in conjunction with a Dunnett's test is more statistically powerful than using a separate t test for each compound.

We performed our statistical analysis at a 95% confidence level rather than the 99% confidence level used in ASTM D4841, to increase the statistical power (the ability to identify a holding time difference). Even using a Dunnett's test, the statistical power would have been rather low if we had run the test at the 99% confidence level given the practical resource limitations in the study (the number matrices which needed to be covered and the number of replicate analysis which could be reasonably performed). A result of using this approach is that it introduces a 5% risk of concluding there is a holding time effect when there is not (rather than 1% as it would be if the tests were run at the 99% confidence level).

In cases where the variability between replicate analyses in this study is low, utilizing the 20% cutoff mentioned above decreases the 5% risk of concluding there is an effect when there is not.

Ultimately, the choice to identify both statistically significant increases and decreases was based on previous work[10], and on the observation that, for some of these analytes, a temporal effect, or change in concentration might not result only in a decrease in concentration. For example, some of the analytes in this study are breakdown products of other analytes being assessed. A significant decrease for one analyte could result in a significant increase for another analyte. In other cases, increases in concentration might be the result of conjugated forms of analytes breaking down into unconjugated forms.

A tally was made of the numbers of analytes that "survived" on each day of the study (i.e., the mean did not differ by more than 20% from the mean on Day 0). Each total was divided by the total number of analytes in that chemical group, to arrive at the "percent survival" for each treatment/family/day combination. We also calculated a survival rate for all 89 analytes in each treatment.

Treatment of Non-Detects

Some target analytes did not survive under some conditions (e.g. chlorination) and therefore were not detected even at Day 0 of the study. For the analytes that were not detected (ND) on Day 0 and never observed on later days, we subtracted those analytes from the total in calculating the survival rate. For example, if two analytes were ND on Day 0 and one other analyte had a statistically significant difference greater than 20% on Day 7, all three analytes were treated as not surviving on Day 7. If there were only four analytes in that family to start with, then the survival rate was only 25% (1/4) on Day 7. On Day 14, analytes which were initially ND, were also treated as not surviving.

Results of Statistical Analysis

Container Types

Although silanized, unsilanized and HDPE containers were evaluated in this study, silanized glass containers were chosen for control samples, POTW effluent and biosolids samples primarily because many of the studies reported in the literature used this type of bottle.

Results of the statistical analysis of the effect of sample container on target compound stability are presented in Table 5. A comparison of the percentage of analytes at Day 7 without a statistically significant change greater than 20% for reagent water samples stored in silanized bottles with those stored in plain glass or HDPE suggests that for some compounds there may be little benefit when using silanized glass bottles. The potential benefit of the silanized glass bottles exists primarily for quinoline antibiotics. Data indicated that for days 14 the "advantage" of the silanized glass bottles is less evident for quinoline antibiotics and by Day 28, increases in concentration are seen for some compounds, possibly due to a desorption effect. For quinoline antibiotics, HDPE and unsilanized glass containers had more statistically significant changes on days 7, 14 and 28. Sulfonamide antibiotics showed the least statistically significant changes in concentration when stored in silanized glass and HDPE. Analyte-specific results for these compounds indicate that it is difficult to determine which of the evaluated containers is the most beneficial overall and container selection should be project specific and based on the compounds of greatest interest.

Table 5.   Percentage of Analytes at Day 7 without a Statistically Significant Change Greater than 20%, by Bottle Type

Analytical Family	Total Number of Analytes	Bottle Type		
		Silanized Glass	Plain Glass	HDPE
Tetracyclines	14	57.1	92.9	85.7
beta-Lactam + misc antibiotics	7	71.4	71.4	57.1
Macrolide antibiotics	6	50	28.6	57.1
Sulfonamide antibiotics	9	100	88.9	100
Quinolone type antibiotics	10	100	50	50
Steroids/Hormones	17	94.1	100	100
Misc PPCPs List 3	16	75	100	87.5
Misc PPCPs List 1	6	83.3	100	83.3
Misc PPCPs List 2	4	50	100	100

Samples stored in unsilanized containers had fewer statistically significant changes in concentration for tetracyclines, however, this category of compounds showed changes after 7 days regardless of container. In addition, 4-epianhydrochlorotetracycline, 4-epianhydro-tetracycline and anhydrochlorotetracycline either decreased significantly or were not recovered past Day 0 when stored in silanized glass containers. Decreases for these compounds in HDPE containers were less dramatic than in silanized containers. Ultimately, it may be the stability of these compounds that plays a role in these decreases rather than the container selected.

Macrolide antibiotics had statistically significant changes when stored in HDPE. One compound, azithromycin, was not recovered when stored in HDPE. A number of β-lactam antibiotics had statistically significant increases in concentration on Days 7, 14 and 28 when being stored in

HDPE but had statistically significant decreases in unsilanized and silanized glass containers. For sulfonamide antibiotics, both HDPE and silanized containers yielded results with fewer statistically significant decreases. Steroids and hormones showed few statistically significant changes in concentration at Day 7 in all containers tested.

The benefits obtained from using silanized glass containers are evident primarily for quinoline and sulfonamide antibiotics. In contrast, unsilanized glass containers appear to yield fewer statistically significant changes for a number of miscellaneous PPCPs, steroids, and hormones. Although the cost of using silanized containers is not prohibitive, there is added complexity in sampling procedures using different types of glass containers for different analytes. Although our results suggest that the limited benefit observed in this study from silanization may not warrant the use of silanized glass bottles in all situations, we recommend that this be evaluated, when appropriate, on a case-by-case basis for the specific compounds being tested.

It is possible that adsorption to container surfaces would be reduced in more complex sewage matrices relative to the reagent water samples studied in the container component of this work. This may mean that choice of container type may be more critical for some types of samples than others. We note that our data may not be relevant to analysis in drinking water samples where the concentrations of PPCPs are expected to be considerably lower than those being spiked into samples in this study. In those cases, slight concentration decreases from adsorption could mean the difference between presence and absence.

Chlorination and Dechlorinating Agents

The effect of various preservatives and dechlorinating agents has been investigated for specific chemicals. Glassmeyer and Shoemaker (See Reference 12) reported degradation of PPCPs in chlorinated samples Vanderford, et al. (See References 11 and 12) , tested sodium thiosulfate, ammonium chloride and ascorbic acid to dechlorinated surface water samples. They reported that sodium thiosulfate degraded trimethoprim, erythromycin, fluoxetine, atrazine, diazepam, progesterone and diclofenac, while ascorbic acid only partially degraded erythromycin. Ammonium chloride did not affect compound recoveries.

The chlorine concentrations used in this study (2 mg/L or 0.5 mg/L) were selected to reflect a range of concentrations that might be found in NPDES permits, drinking water, and POTW effluents. The effects of the chlorine on the PPCPs studied in this work were dramatic. This is shown in Table 6, which compares the numbers of study analytes that were spiked into each sample, but not detected on Day 0, the same day that the spiked samples were prepared. The majority of antibiotics, PPCPs, steroids and hormones were not detected in chlorinated samples that had not been dechlorinated. This is consistent with literature reports that chlorine destroys a number of PPCPs (See Reference 12).

Table 6.   Numbers of Analytes Not Detected on Day 0 in Samples Chlorinated to 2 mg/L

Analytical Family	Total Number of Analytes	Chlorinated Water	With Ascorbic Acid	With Sodium Thiosulfate
Tetracyclines	14	14	12	14
beta-Lactam + misc antibiotics	7	7	3	7
Macrolide antibiotics	6	3	0	6
Sulfonamide antibiotics	9	9	0	9
Quinolone type antibiotics	10	10	0	10

Table 6. Numbers of Analytes Not Detected on Day 0 in Samples Chlorinated to 2 mg/L

Analytical Family	Total Number of Analytes	Chlorinated Water	With Ascorbic Acid	With Sodium Thiosulfate
Steroids/Hormones	17	13	1	1
Misc PPCPs List 3	16	13	0	12
Misc PPCPs List 1	6	4	1	0
Misc PPCPs List 2	4	2	2	2

This study examined the effect of two different reagents that are often used to remove residual chlorine from samples: ascorbic acid and sodium thiosulfate.[16] In general, for antibiotics, the use of sodium thiosulfate (80 mg/L) was least effective in the protection of analytes in the presence of 2 mg/L of chlorine (Table 6). In contrast, there were many fewer non-detects in the chlorinated samples treated with ascorbic acid (50 mg/L). Both dechlorinating agents protected the majority of steroids and hormones in this study, however 17α-dihydroequilin was not recovered using either dechlorinating agent. In the miscellaneous PPCP category, sodium thiosulfate appeared to protect warfarin, while ascorbic acid did not, however, in general, samples dechlorinated with sodium thiosulfate showed more non-detects. With the exception of a few PPCPs, steroids, and hormones (data not shown), the majority of compounds were found to be better preserved when samples were dechlorinated with ascorbic acid. Dechlorination with sodium thiosulfate in the presence of 2 ppm chlorine had low or no recovery of a number of β-lactam, tetracycline and quinoline antibiotics. It is evident that some of the compounds in this study are simply too labile to withstand the effects of chlorine or dechlorination agents. Specifically, 17 α-dihydroequilin, cimetidine, ranitidine and many of the tetracycline, chlorotetracyclines and their degradates were not detected in chlorinated or dechlorinated samples.

The direct effect of each dechlorinating agent was evaluated in reagent water samples without any added chlorine. Four reagent water samples were prepared and spiked with all of the analytes. Ascorbic acid was added to two of the samples, and sodium thiosulfate to the other two samples. One sample from each treatment was analyzed on Day 0 and the remaining two samples were analyzed on Day 2. All of the analytes were present on Day 0 in the samples treated with ascorbic acid. However, clinafloxacin, diphenhydramine, fluoxetine, and sarafloxacin, were not detected on Day 0 or Day 2 in the samples treated with sodium thiosulfate. That only one sample of each treatment was analyzed on each day makes statistical comparisons impossible, however this data may be used to better assess the direct effects of these dechlorinating agents on PPCPs.

For almost all tetracycline, chlorotetracycline, and degradates, there were notable decreases by Day 2 in the presence of both dechlorinating agents. Decreases in concentration for sulfonamide antibiotics, steroids, and hormones in these samples appeared to be minimal in the presence of either sodium thiosulfate or ascorbic acid.

This data, in combination with that from the earlier tests which evaluated chlorine and dechlorinating agents, suggests that ascorbic acid is a more suitable dechlorinating agent for PPCPs than sodium thiosulfate and that sodium thiosulfate destroys some of the PPCPs evaluated in this study.

Temperature and Acid Preservation Effects on Effluent Samples

Snyder et al. (See Reference 11) reported rapid decreases in the concentrations of androstenedione, testosterone, progesterone, trimethoprim, acetaminophen, and fluoxetine in unpreserved surface waters (See Reference 11). Snyder hypothesized that this was due to microbial degradation and subsequently tested some biocide techniques. The addition of formaldehyde (1%, v/v) to freshly collected samples stabilized these compounds, while other compounds, including acetaminophen, meprobamate, dilantin, TCEP, and isopromide, showed signs of degradation by the formaldehyde. Sulfuric acid preservation prevented degradation of the compounds affected by formaldehyde and did not adversely affect the recoveries of the other compounds.

While there are a variety of antimicrobial agents such as $CuSO_4$ and diazolidinyl urea[17] that have been used to prevent microbial degradation in drinking water samples, their use has seldom been extended to sewage samples. On the other hand, EPA has used acid to prevent the bacterial degradation of some aromatic compounds in wastewater samples. The most notable of these are benzene, toluene, and ethyl benzene which are susceptible to rapid biological degradation under certain environmental conditions.[18] Strong mineral acids such as HCl or H_2SO_4 can be used to reduce the pH of a sample to less than 2, thereby causing many bacterial cells to lyse and minimize the biological activity present in the sample. Acid preservation in conjunction with reduced temperatures is not uncommon for preservation of samples.

Sewage treatment plant effluent samples were stored at either 4 ºC (cold) or -20 ºC (frozen). The goal of both temperature treatments was to reduce the biological activity of the samples, as well as reduce the rates of any chemical reactions in the effluent that might affect the analytes of interest. Acidification of the sample is a treatment that also might reduce biological activity in the samples.

The results of the statistical analysis of the storage temperature and acidification data are summarized in Table 7. For macrolide antibiotics, sulfonamide antibiotics and steroids and hormones, freezing the effluent samples did not increase survival on Day 7. For quinoline and β-lactam antibiotics, little to no differences were observed between samples which were frozen and those which were stored at 4 ºC for a number of compounds (data not shown).

Table 7. Percentage of Analytes in Effluents at Day 7 without a Statistically Significant Change Greater than 20%, by Storage Condition

Analytical Family	Total Number of Analytes	Storage Condition		
		Cold	Cold and pH < 2	Frozen
Tetracyclines	14	21.4	7.1	64.3
beta-Lactam + misc antibiotics	7	71.4	28.6	57.1
Macrolide antibiotics	6	83.3	66.6	50
Sulfonamide antibiotics	9	88.9	0	66.7
Quinolone type antibiotics	10	60	70	90
Steroids/Hormones	17	88.2	82.4	47.1
Misc PPCPs List 3	16	62.5	87.5	75
Misc PPCPs List 1	6	83.3	83.3	100
Misc PPCPs List 2	4	100	75	100

For some miscellaneous PPCPs, tetracycline antibiotics, and quinolone antibiotics which were initially protected by freezing, concentrations for some these compounds in frozen samples dropped off dramatically in the later days of the study. These results suggest that freezing may not provide substantial benefits to effluent samples which are expected to be much less biologically active than biosolids. And samples collected in glass and then frozen can break if not filled and stored correctly.

Increased concentrations of certain analytes, such as desogesterol, digoxin, equilenin, virginiamycin, roxithromycin, and sulfanilamide, were observed in some samples after storage (See Figure 1). It is possible that the increases in concentration over the duration of the study may reflect the presence of conjugated forms of some of these analytes or interactions between the spiked analytes and the sample matrix that vary with time.

Figure 1.   Macrolide Antibiotics in Frozen POTW Effluent
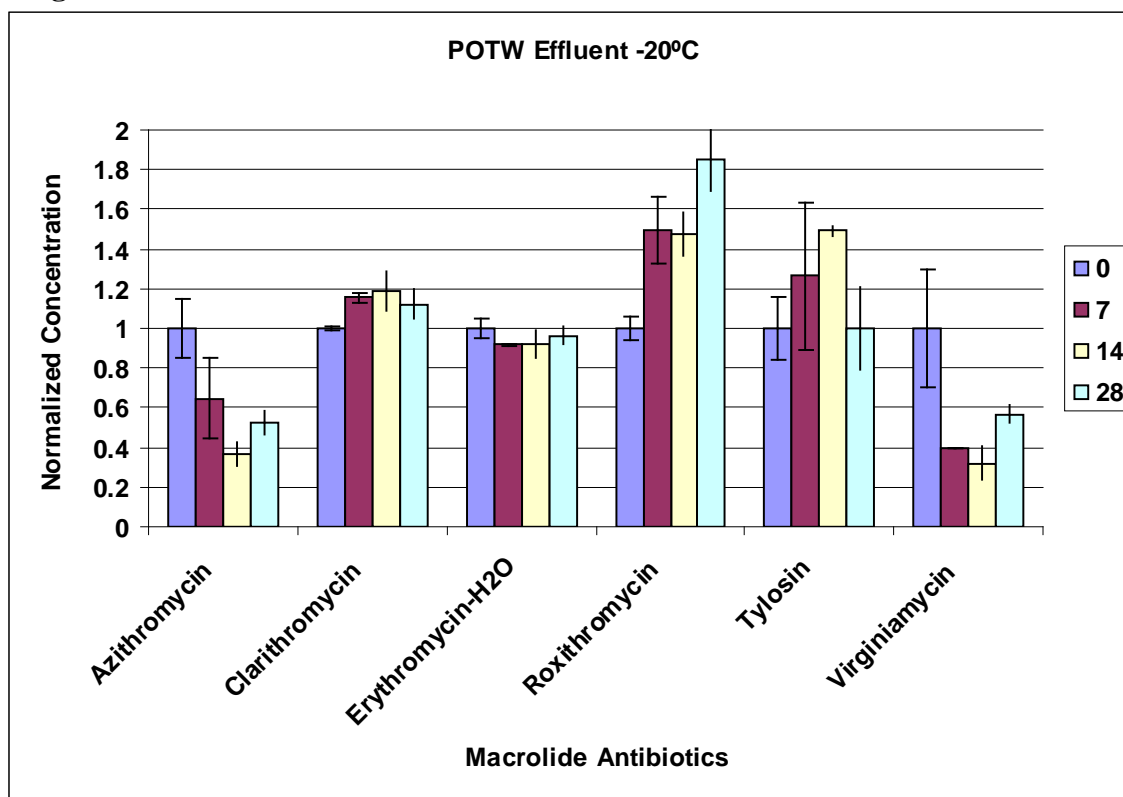
The combination of acidification and cold storage in this study achieved only mixed success relative to cold storage or freezing alone. Our studies revealed that samples preserved with sulfuric acid had marked decreases in recoveries for β-lactam, sulfonamide, tetracycline antibiotics. When comparing samples preserved with acid to those simply stored at 4 ºC for a series of other PPCPs (Table 7), preservation with acid provided few benefits and actually destroyed a number of PPCPs on Day 0, including norgestimate and ranitidine.

For steroids and hormones, treatment with acid actually produced increases in the concentrations of a number of compounds. Deconjugation of estrogen conjugates, matrix enhancement, or other reactions in POTW samples may have contributed to this increase, however further analysis would be required to prove this hypothesis.

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Combination of Best Aqueous and Effluent Treatments

The goal of Phase IV of this study was to test the "best combination" of storage and preservation conditions that had been observed in Phase 1 and 2. These "best combination" conditions were: chlorinated effluent samples, held in HDPE bottles, dechlorinated with ascorbic acid, and stored at 4 ºC, with no pH adjustment. HDPE containers were selected because earlier results did not reveal that one type of container was significantly better than another for all of the PPCPs and hormones in this study, and because these bottles had been used in previous EPA studies of sewage samples. Ascorbic acid was selected because it proved to be the best dechlorinating agent tested. Storage at 4 ºC was selected as opposed to freezing because freezing provided minimal benefit and presented the additional problem of breakage. Four replicate samples were analyzed on each of Day 0, Day 4, Day 7, and Day 14 (16 samples total). Analyses on Day 4 were included to see if any changes over the first 7 days would be apparent prior to Day 7. Effluent samples used for this portion of the study had no detectable levels of chlorine, so they needed to be chlorinated. The spiking levels of chlorine were decreased from 2 mg/L to 0.5 mg/L to better reflect what might be more commonly found in typical POTW samples. In addition, given the dramatic losses of analytes in the chlorinated reagent water samples earlier in the study, this reduction in the concentration could offer more useful information. The use of four replicate samples on each day added statistical power to the study, compared to the three replicates used for earlier effluent samples.

The results for the best combination analyses are compared to those for unchlorinated Phase 2 effluent samples in Table 8 to illustrate comparison of chlorinated POTW effluent treated with ascorbic acid and unchlorinated POTW effluent not treated with ascorbic acid. The third and fifth columns in Table 8 illustrate the differences in the number of non-detects at the start of each experiment (Day 0) and the fourth and sixth columns illustrate the percentage of analytes in each family that survived to Day 7 (e.g., those without a statistically significant difference greater than 20%).

Table 8. Comparison of Results for the Best Combination Treatment of Effluents and Simple Cold Storage

Analytical Family	Total Number of Analytes	Best Combination (n=4)		Cold Storage only (n=3)	
		# NDs Day 0	% Survival on Day 7	# NDs Day 0	% Survival on Day 7
Tetracyclines	14	9	21.4	0	21.4
beta-Lactam + misc antibiotics	7	2	28.6	0	71.4
Macrolide antibiotics	6	0	83.3	0	83.3
Sulfonamide antibiotics	9	2	66.7	0	88.9
Quinolone type antibiotics	10	1	30	0	60
Steroids/Hormones	17	0	64.7	0	88.2
Misc PPCPs List 3	16	0	81.3	0	62.5
Misc PPCPs List 1	6	0	83.3	0	83.3
Misc PPCPs List 2	4	0	75	0	100

Note: % Survival indicates those analytes without a statistically significant difference greater than 20%

Even under these "best" conditions several compounds were not detected at Day 0. Specifically, nine tetracycline compounds (4-epaanhydrochlorotetracycline, 4-epianydrotetracycline, 4-

epichlorotetracycline, 4-epioxytetracycline, anhydrochlorotetracycline, anhydrotetracycline, chlorotetracycline, isochlorotetracycline and oxytetracycline), two β-lactam compounds (cefotaxime and lincomycin), two sulfonamide (sulfadimethoxine and sulfathiazole) and one quinolone antibiotic (carbadox) were ND in these samples (data shown in appendix 2). As this extent of loss was not previously observed in POTW effluents stored cold without treatment (Table 8), we concluded that the selected treatment combination affected the survival of a number of analytes on Day 0. The results from earlier chlorinated reagent water analyses show that all of the tetracycline, chlorotetracycline and degradates, and a number of antibiotics were lost on Day 0 in those samples, supporting the conclusion that it may be more likely that the chlorine, rather than the ascorbic acid, destroyed most of these compounds.

The number of non-detects on Day 0 and the percentage of analytes that survived on Day 7 for both treatments may illustrate the combined effects of chlorine and ascorbic acid. With the exception of macrolide antibiotics, fewer antibiotics, steroids and hormones survived in the presence of chlorine and ascorbic acid in these samples. The survival rates for tetracyclines and several miscellaneous PPCPs were not affected, although the analytes that survived in best combination samples and simple cold storage samples differed to some degree. Only for the Misc. PPCPs in List 3, was survival higher in the best combination treatments.

Table 9. Comparison of Results for the Best Combination Treatment of Effluents on Days 4, 7, and 14

Analytical Family	Total Number of Analytes	# NDs on Day 0	% of Analytes Surviving		
			Day 4	Day 7	Day 14
Tetracyclines	14	9	35.7	21.4	21.4
beta-Lactam + misc antibiotics	7	2	57.1	28.6	42.9
Macrolide antibiotics	6	0	83.3	83.3	83.3
Sulfonamide antibiotics	9	2	66.7	66.7	77.8
Quinolone type antibiotics	10	1	50	30	70
Steroids/Hormones	17	0	88.2	64.7	64.7
Misc PPCPs List 3	16	0	75	81.3	75
Misc PPCPs List 1	6	0	100	83.3	83.3
Misc PPCPs List 2	4	0	50	75	100

Comprehensive data for the best combination of samples are shown in Table 9. These results illustrate many of the same time trends observed in Phase 1 of this study. Some of the statistically significant differences in these results reflect increases in analyte concentrations over time. For example, the survival rates for quinolines, sulfonamides and PPCPs lists 2 and 3 (cimetidine, diphenhydramine, ranitidine and warfarin) on Day 7 are lower than the survival on later Days. Figure 2 shows the results for Quinoline and several other antibiotics. These results suggest that the background compounds present in the effluent may be undergoing changes as the samples age.

Figure 2. Quinoline and Several Other Antibiotics in POTW Effluent with 0.5 mg/L Cl₂, Preserved with Ascorbic Acid and Stored at 4 ºC in HDPE
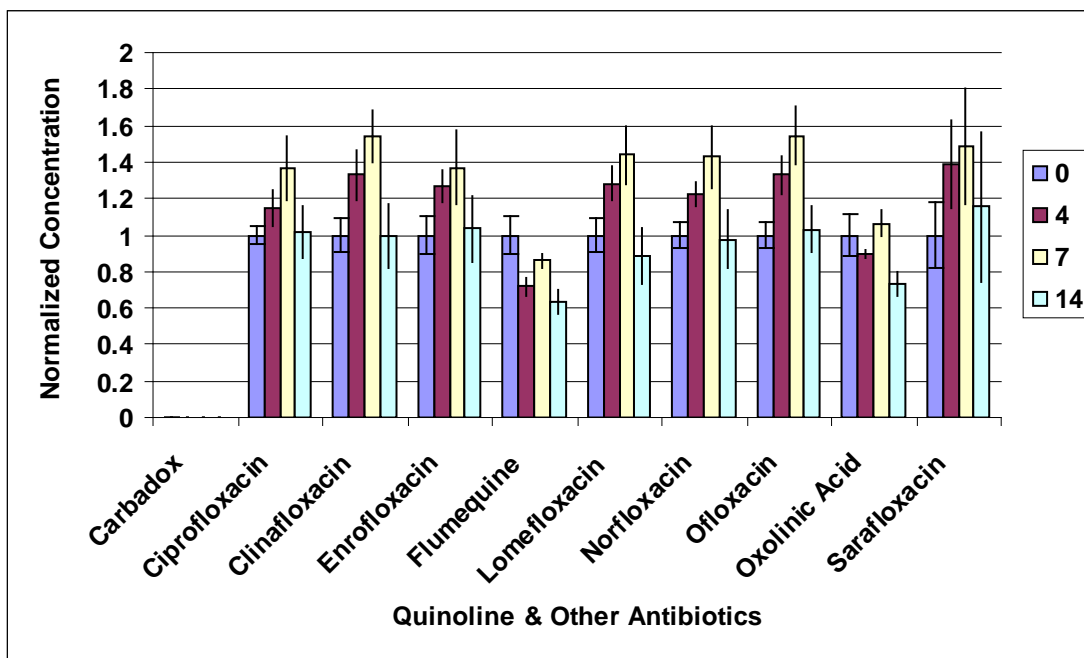
The results for steroids and hormones reflect decreases in the concentrations for 17 alpha-dihydroequilin, androsterone and desogesterol. Mestranol had a statistically significant loss on Day 7 that was not apparent on Day 14. In fact, the mean result for this analyte on Day 14 was within 0.5% of the Day 0 mean result. Examination of the Day 7 data for mestranol revealed that there is no evidence of an outlier among the four replicate results that would explain the magnitude of the loss of this analyte.

Effect of Time and Temperature on Storage of Biosolids

The increased biological activity of biosolids may account for changes in the concentration of PPCPs in biosolids. It is generally not practical to add chemical preservatives to solid samples at the time of collection. Therefore, most methods for solid matrices rely solely on reduced storage temperatures to minimize biological activity and preserve the analytes of interest. Two temperatures were investigated in this holding time study: half of the samples were stored at 4 ºC, and half at -20 ºC. The results are summarized in Table 10, in terms of the percentage of analytes surviving on each day without a statistically significant difference greater than 20%.

There were several analytes that were not detected in either set of biosolids samples on Day 0, despite being spiked into all of the sample aliquots. Both cefotaxime and desogestrel were non-detects on Day 0, and on all subsequent days in the biosolids samples stored at 4 ºC and those stored -20 ºC. Although sarafloxacin was not detected on Day 0 in all four of the frozen biosolids samples, it was detected on all subsequent days in the frozen samples, at levels that represent 60 to 75% of the initial spike level. Examination of the analysis QC data associated with the Day 0 analyses did not identify any analytical problems for sarafloxacin, however matrix enhancement and/or suppression cannot be ruled out as contributing factors.

Table 10. Percentage of Analytes in Biosolids Surviving without a Statistically Significant Change Greater than 20%, by Storage Temperature

Analytical Family	Total Number of Analytes	% Survival					
		Day 7		Day 14		Day 28	
		Cold	Frozen	Cold	Frozen	Cold	Frozen
Tetracyclines	14	64.3	64.3	78.6	78.6	50	50
beta-Lactam + misc antibiotics	7	42.9	42.9	0	42.9	20	42.9
Macrolide antibiotics	6	83.3	66.6	83.3	100	66.6	100
Sulfonamide antibiotics	9	22.2	88.9	33.3	88.9	22.2	55.6
Quinolone type antibiotics	10	90	40	70	60	80	90
Steroids/Hormones	17	29.4	52.9	41.2	53.0	47.1	70.6
Misc PPCPs List 3	16	68.8	68.8	56.3	68.8	50	68.8
Misc PPCPs List 1	6	83.3	83.3	100	100	66.7	100
Misc PPCPs List 2	4	75	75	75	75	75	75

Comparing the data for Day 7 in Table 10, freezing biosolids samples does not protect more of the analytes than simple cold storage, except for sulfonamide antibiotics and perhaps some steroids and hormones. When examining data past Day 7, the benefit for freezing seems more apparent for beta-lactam, macrolide antibiotics and steroids and hormones. However, as seen in results for effluent samples, the results for the later days in the study show statistically significant increases in concentration, rather than decreases, for some analytes when compared to Day 7 results. For example, all five of the statistically significant differences for tetracyclines on Day 7 were increases greater than 20% (See Figures 3a and 3b). The only statistically significant decreases in tetracycline, chlorotetracycline, and several of their degradates appeared on Day 28. The data for tetracyclines in cold biosolids samples suggests that concentrations peak at Day 14, but in frozen samples, the results continue to increase to Day 28. The lag between the peaks in the cold and frozen samples may reflect a temperature dependence of any reactions occurring in the stored sample.

Figures 3a and 3b.   Tetracycline and Tetracycline Degradates in Biosolids Samples at 4 ºC and -20 ºC

Biosolids 4ºC

Biosolids -20ºC
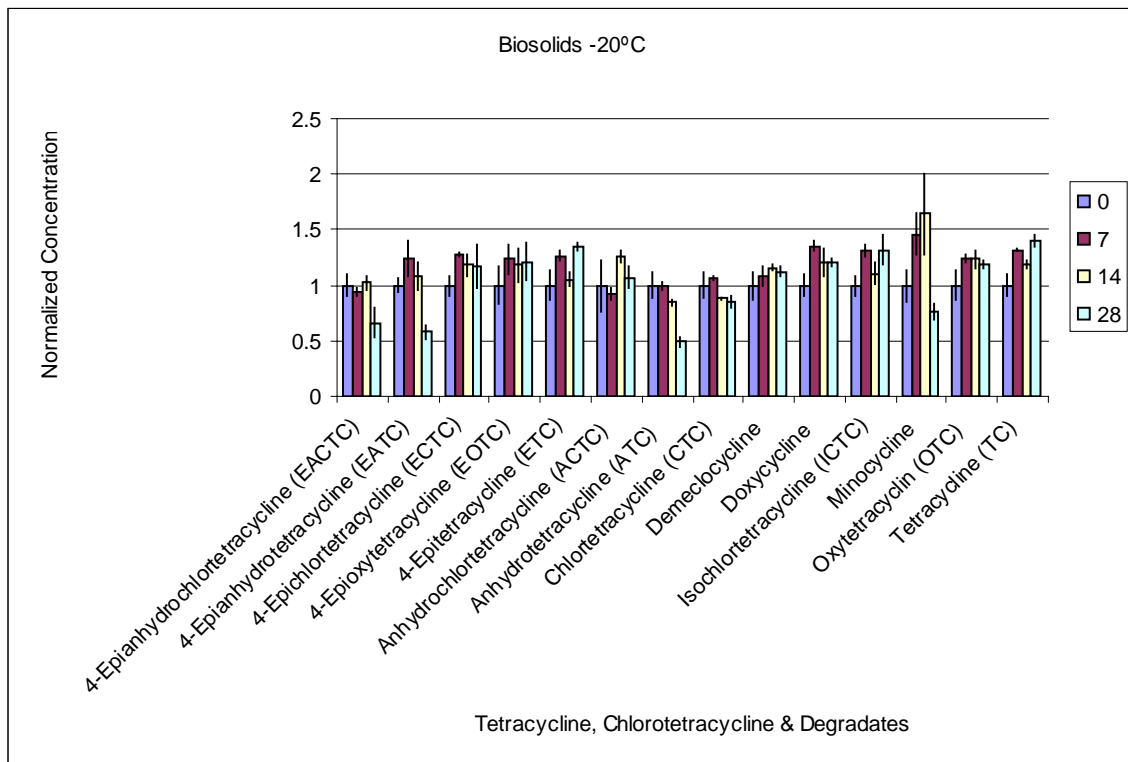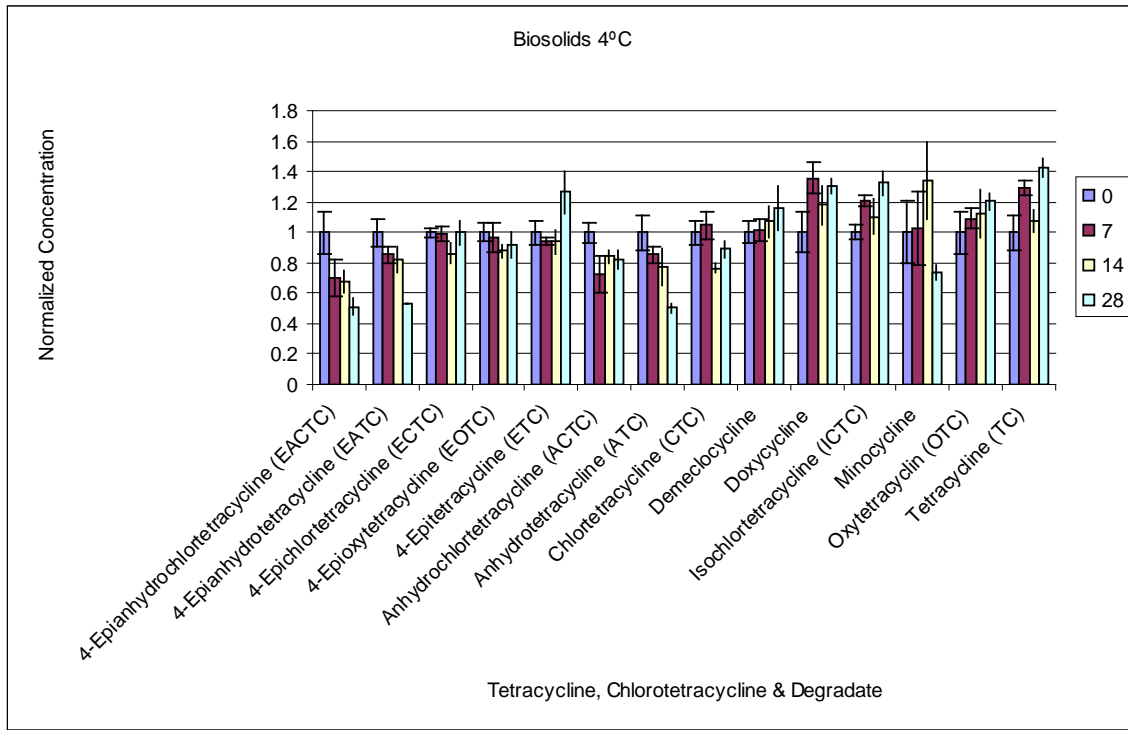
For samples that were frozen, 7 out of 33 antibiotics exhibited statistically significant differences on Day 7 that were due to increases rather than decreases in concentration. Cloxacillin, lincomycin, oxacillin, penicillin G and penicillin V all exhibited dramatic decreases in

concentration in biosolids samples which were not frozen. Decreases in concentration for these compounds were less substantial in biosolids samples that were frozen. Cefotaxime was not recovered in frozen biosolids samples. For quinoline, sulfonamide and macrolide antibiotics, a combination of statistical analysis (Table 10) and examination of data for individual compounds (data not shown) indicates there may only be moderate benefits to freezing.

For the steroids and hormones examined, there were marked increases in concentrations for 17 alpha-estradiol, equilenin, equilin and estrone on Day 7 in both the cold and frozen samples. For a few analytes, the increases continued through the later days in the study even in samples stored at -20 ℃. Table 11 provides the mean results for these analytes in the frozen biosolids samples over time.

Table 11. Mean Results for Several Hormones in the Frozen Biosolids Samples over Time

Analyte	Mean Day 0	Mean Day 7	Sig Dif?	Mean Day 14	Sig Dif?	Mean Day 28	Sig Dif?
17 alpha-dihydroequilin	69.0	80.9	N	86.3	N	81.5	N
17 alpha-Estradiol	32.5	203.3	Y	248.3	Y	80.1	N
17 beta-Estradiol	76.5	100.3	N	102.4	N	71.2	N
Equilenin	16.6	71.6	Y	46.4	Y	21.4	N
Equilin	149.0	208.7	N	424.7	Y	124.1	N
Estrone	64.7	389.7	Y	453.7	Y	162.0	N

There are different trends for some of the steroids and hormones over time. For example, for both the frozen and cold biosolids samples, the results for estrone and equilin appear to peak at Day 14, and then decrease dramatically by Day 28. The increases to Day 14 are statistically significant for both of these analytes in the frozen and cold samples.

Extract Stability

Extracts of effluent and OPR samples stored at 4 ℃ were re-analyzed at various time intervals after initial analysis to determine their stability over time. A minimal number of replicates were included in this portion of the study and therefore rigorous statistical analysis was not possible. Most PPCPs (with the exception of tetracyclines, Figures 5a and 5b) and hormones in this work appeared to be stable in extracts stored for at least one month (data not shown). While there were some compounds that were stable beyond one month, this limited data set suggests that for most compounds (with the exception of tetracyclines) precautionary extract holding time of up to 30 days may be sufficient to preclude extensive degradation of some compounds in extracts. It is likely that for other compounds (e.g. tetracyclines, an extract holding time of less than 30 days may be required.

Figures 4a and 4b. 4a. PPCPs extract stored in methanol/formic acid buffer solution and analyzed at Day 0 and day 29. Samples were reagent water samples. 4b. Steroids and Hormones extract stored in methanol/formic acid buffer solution and analyzed at Day 0 and Day 38. Samples were reagent water samples. Concentration is normalized to Day 0.
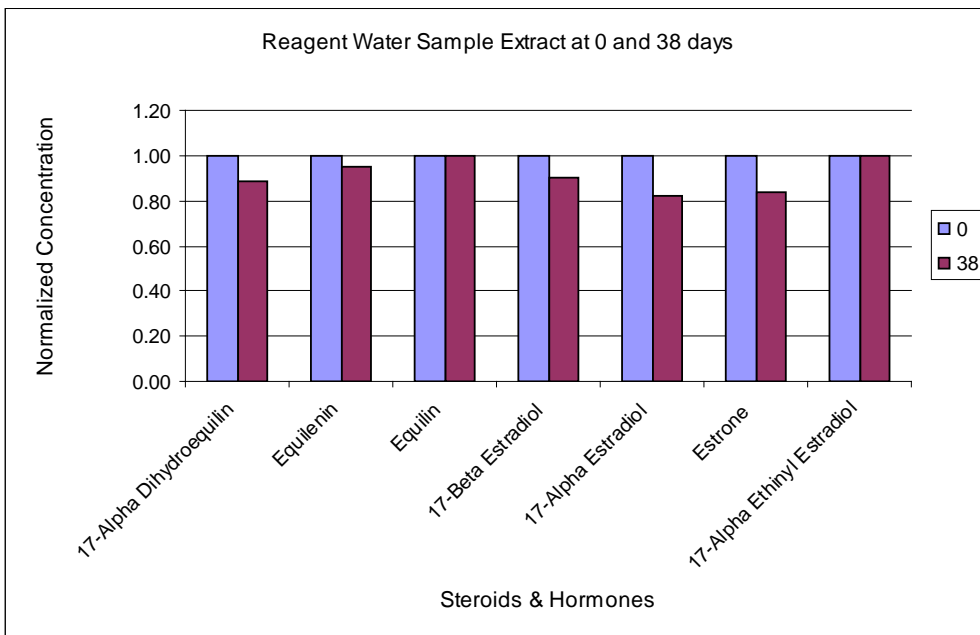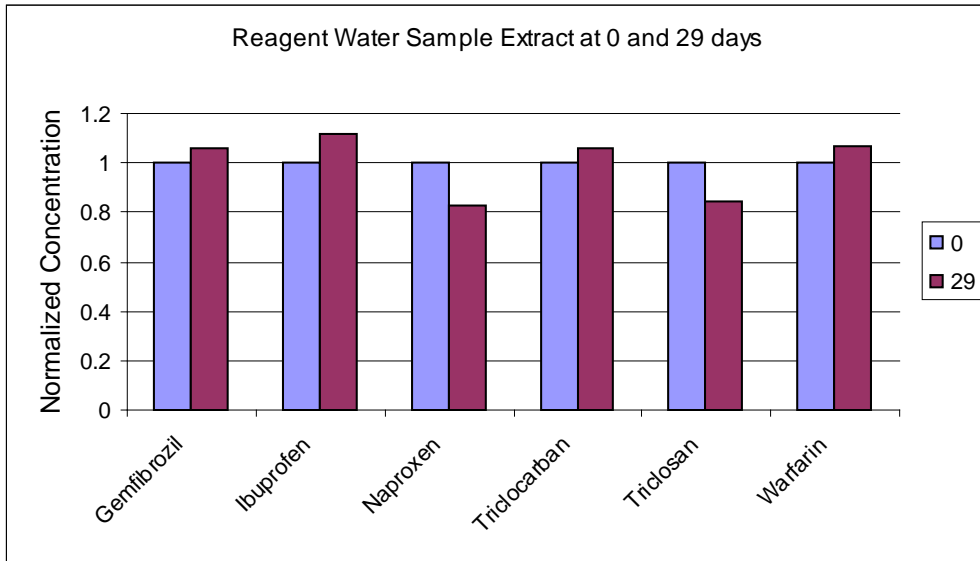
Figure 5a and 5b. **5a. Tetracycline, chlorotetracyclines and degradates data from two separate extracts stored in methanol/formic acid buffer solution and analyzed at 0 and 24 days. Samples were reagent water treated with ascorbic acid in the presence of 0.5 mg/L chlorine.**
5b. Data for miscellaneous PPCPs for extracts stored in methanol/ formic acid buffer solution and analyzed at 0 and 130 days. Samples were reagent water samples. Concentration is normalized to Day 0.
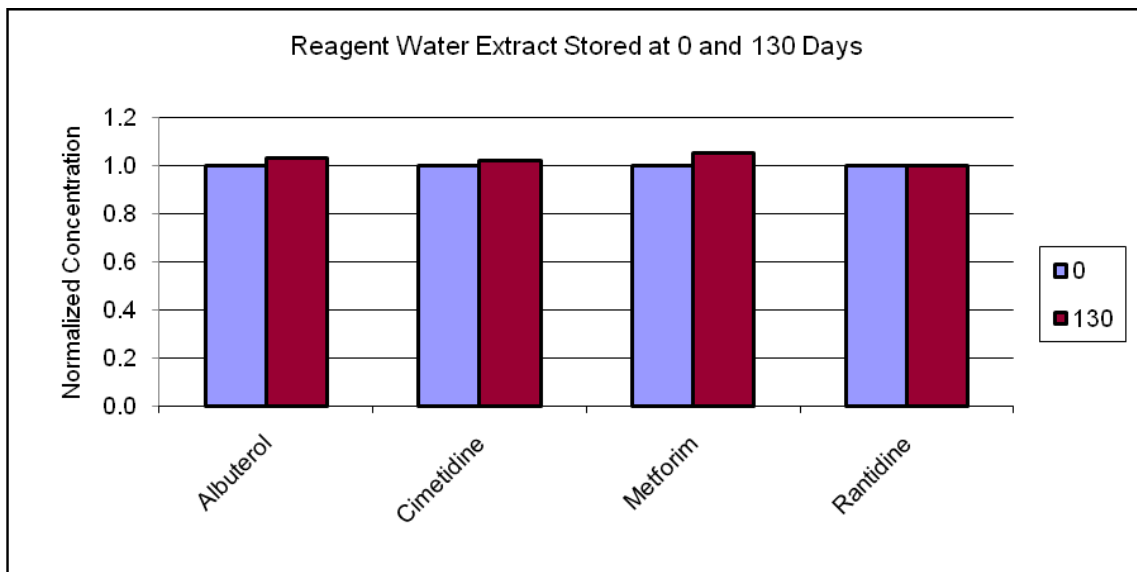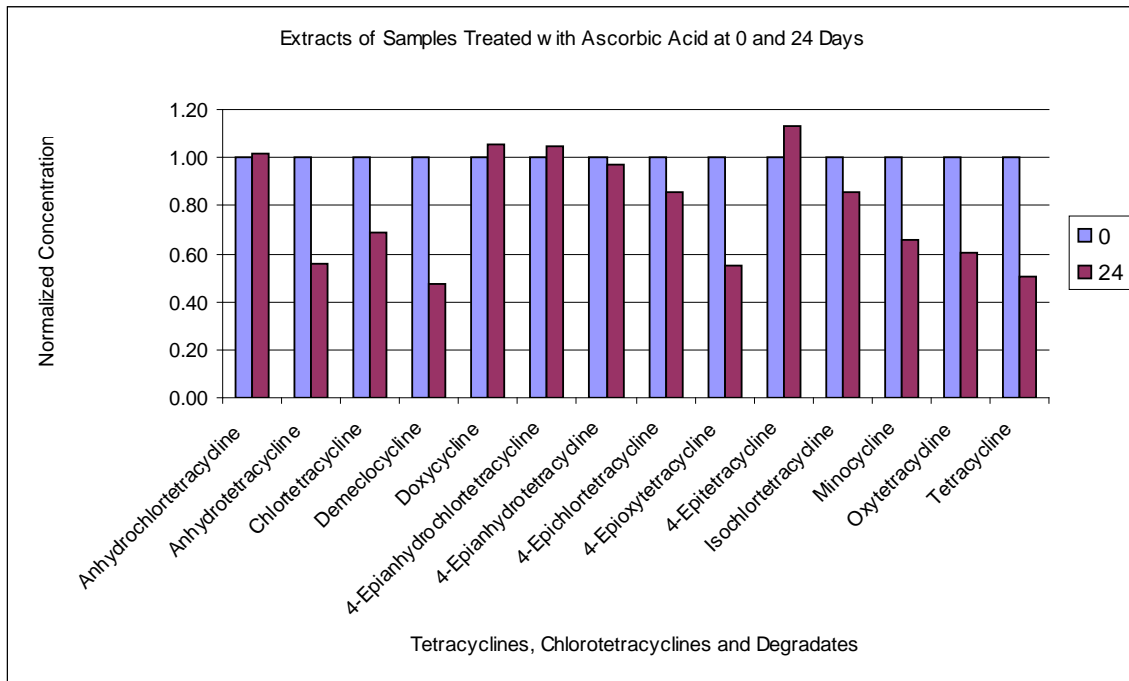
DISCUSSION

It is not surprising that there is no clear-cut answer to the question "What should the holding times and preservation conditions for PPCPs, steroids and hormones be?" For instance, for some samples and analytes, different containers may be appropriate. The results of this study can be used to recommend some sample preservation and treatment conditions that are more useful than others, but ultimately, for some parameters tested, there is no one best choice for all chemicals. For example, HDPE containers may be adequate for some classes of analytes but silanized glass containers may be more appropriate for others. Containers may need to be selected based on the analytes being studied.

Likewise, chlorinated samples treated with ascorbic acid exhibited fewer nondetects than those treated with sodium thiosulfate. The data for various samples which did not contain chlorine indicate that some classes of analytes will survive for 7 days without a statistically significant change greater than 20%. We observed that some analytes exhibit statistically significant changes in concentration in as few as seven days, even in the absence of chlorine (Table 7). Thus, for aqueous samples not containing chlorine, a maximum holding time of 7 days until time of analysis may be advisable.

The addition of sulfuric acid as a preservative reduced the recovery of a number of compounds while freezing was only marginally successful at preventing the degradation of analytes and may lead to problems with sample breakage. Thus, based on this study, we recommend storing sewage treatment plant effluent samples at 4 ℃ and without sulfuric acid. Even in best combination samples the concentrations of tetracyclines, chlorotetracyclines and tetracycline degradates as well as several other PPCPs and hormones including: 17 alpha dihydroequilin, desogesterol, carbadox, sulfadimethoxine, sulfathiazole, cefotaxime and linomycin were shown to decrease quickly.

For biosolids samples, we observed that the concentrations of some analytes increase significantly in samples held up to 14 days, possibly as a result of the release of conjugate forms of some of the analytes from the biosolids. Cold storage, at 4 ℃ appears to be sufficient to preserve many of the analytes in biosolids for this period, however, for antibiotics such as beta lactams, freezing samples at -20 ℃ may be more effectively prevent degradation. For these reasons, a holding time of 7 days cold or frozen for biosolids samples is likely reasonable.

There are several limitations to conclusions to draw from the data in this study. Because the methods used in this work cover a wide range of matrices and analytes, the results include a subset of non-quantitative results, specifically for those compounds not measured by isotope dilution. It is not likely that our findings may be extended to matrices such as drinking water, where small differences in concentration can mean the difference between presence and absence. We relied on statistical analysis, and in some cases, presence/absence to make our holding time determinations. Studies using more precise analytical methods and focusing on fewer target compounds may reveal more subtle differences than those observed in this study. Ultimately, holding times arising from this study are protective of analytes which degraded more quickly than others and are more suited to Methods 1694 and 1698 than to analytical methods targeting a subset of the chemicals in EPA Methods 1694 and 1698.

CONCLUSIONS

Most CEC analytical methods cover a large number of chemicals from different classes of chemicals. Thus, uniform holding times and preservation conditions may not apply across all of these classes. More relevant data might be obtained when using holding times and storage conditions specific to the class of compounds and samples being tested with an analytical method designed specifically for the target CECs. The results of this 2008 study may help researchers design stability studies to identify holding times and preservation conditions appropriate to their samples and target analytes.

The results of this study have been used to revise the holding times and preservation conditions for PPCPs in EPA Method 1694 and steroids and hormones in Method 1698. Previous holding time and preservation conditions in these methods specified the use of amber glass containers (optionally Method 1698 allowed the use of amber plastic), sodium thiosulfate for dechlorination, storage at less than 4 ºC for samples (with freezing as an option), storage of sample extracts in the dark at less than -20 ºC, start of sample extraction within 7 days of collection (within 48 hours is strongly encouraged), analysis of sample extracts within 40 days of extraction. and pH adjustment to 5.0 - 9.0 for samples not extracted within 48 hours of collection.

Based on the results of this study, these conditions have been revised as follows:

- Bottles - amber high density polyethylene or glass containers.
- Dechlorinating Reagent - ascorbic acid.
- Shipping and Storage Conditions - in the dark at <6 °C (optional freezing for biosolids).
- Holding Times - extract samples within seven days; analyze extracts as soon as possible, not to exceed 30 days (10 in the case of tetracyclines).

It is essential to point out that the above holding times are not universal, but are protective due to the large number of compounds found in Method 1694.

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18. See 40 CFR, Part 136: a) EPA Method 624. Volatile Organic Compounds by GCMS.
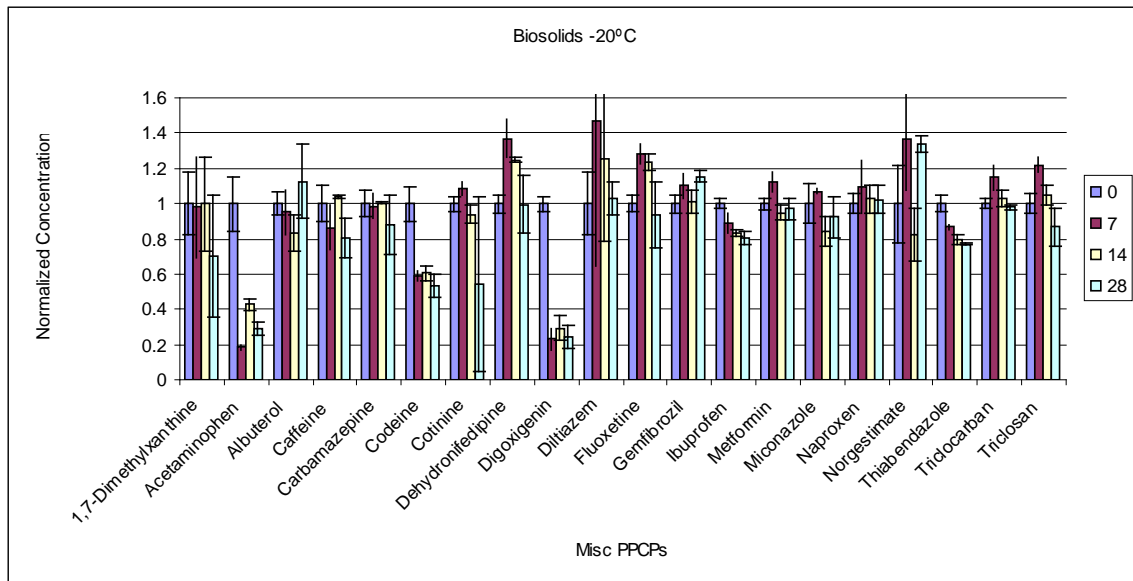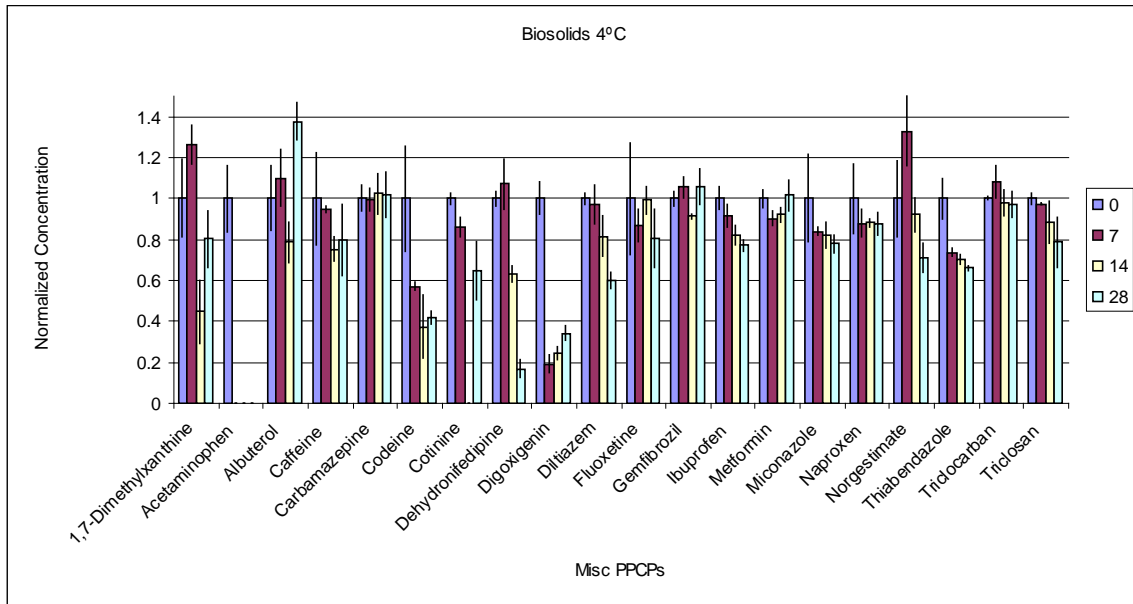b) EPA Method 1624b. Volatile Organic Compounds by Isotope Dilution GCMS.
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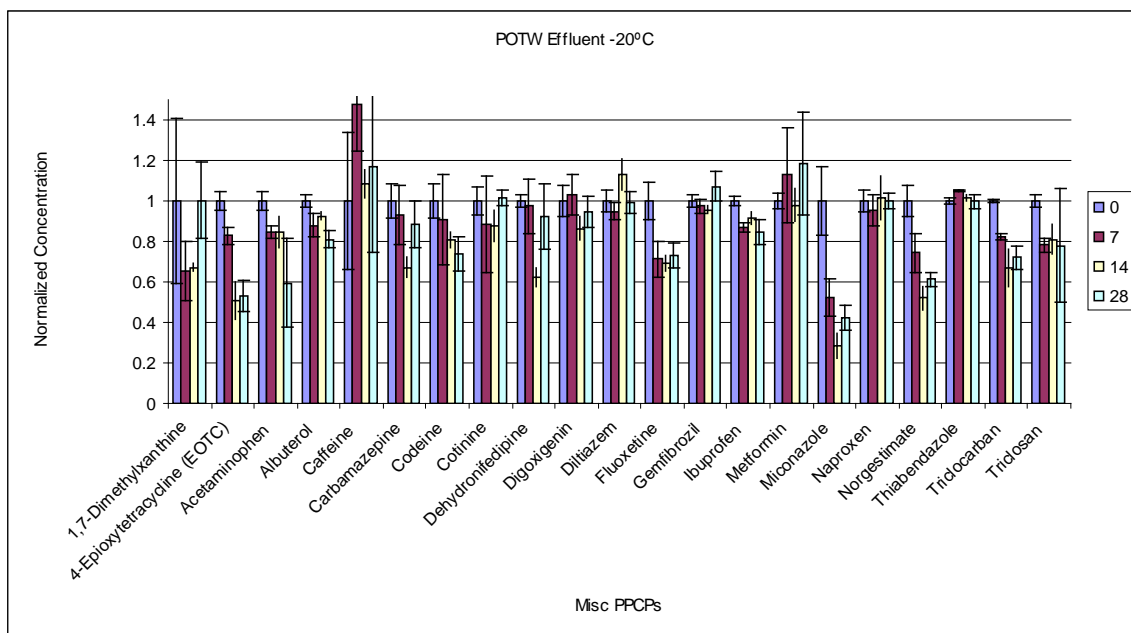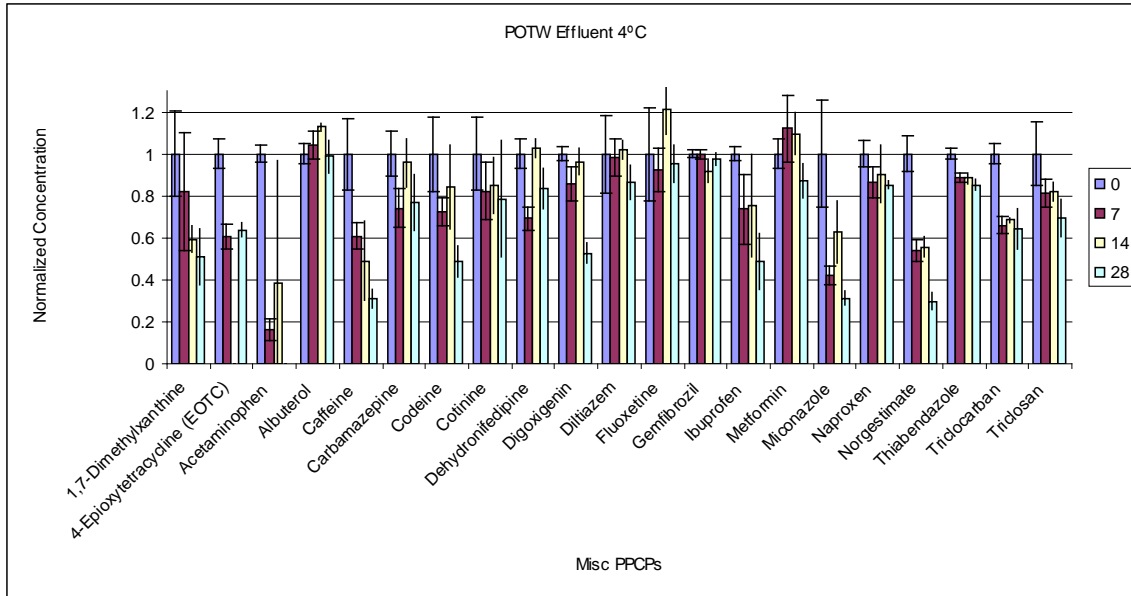
APPENDIX 1. % Recoveries for Target Analytes from 20 Randomly Selected OPR Samples by Study Phase
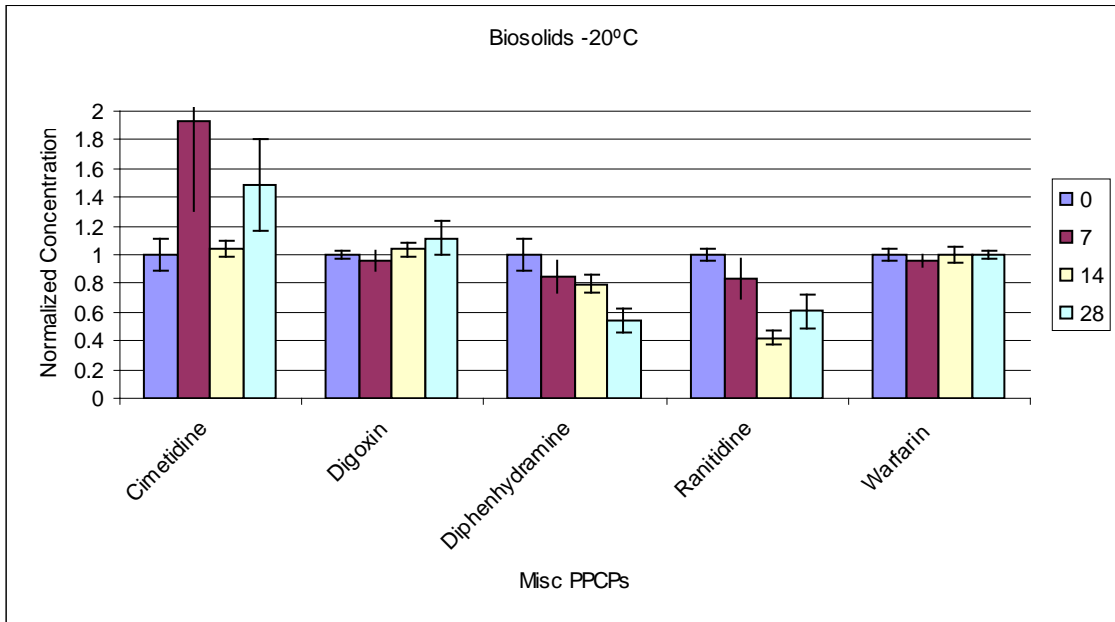
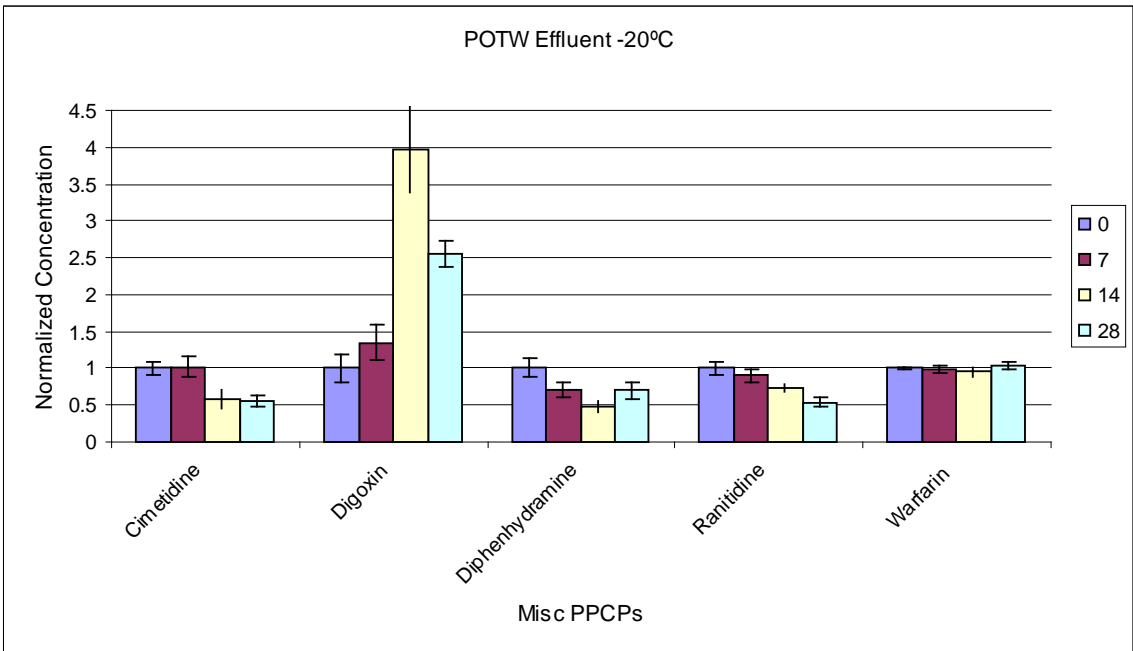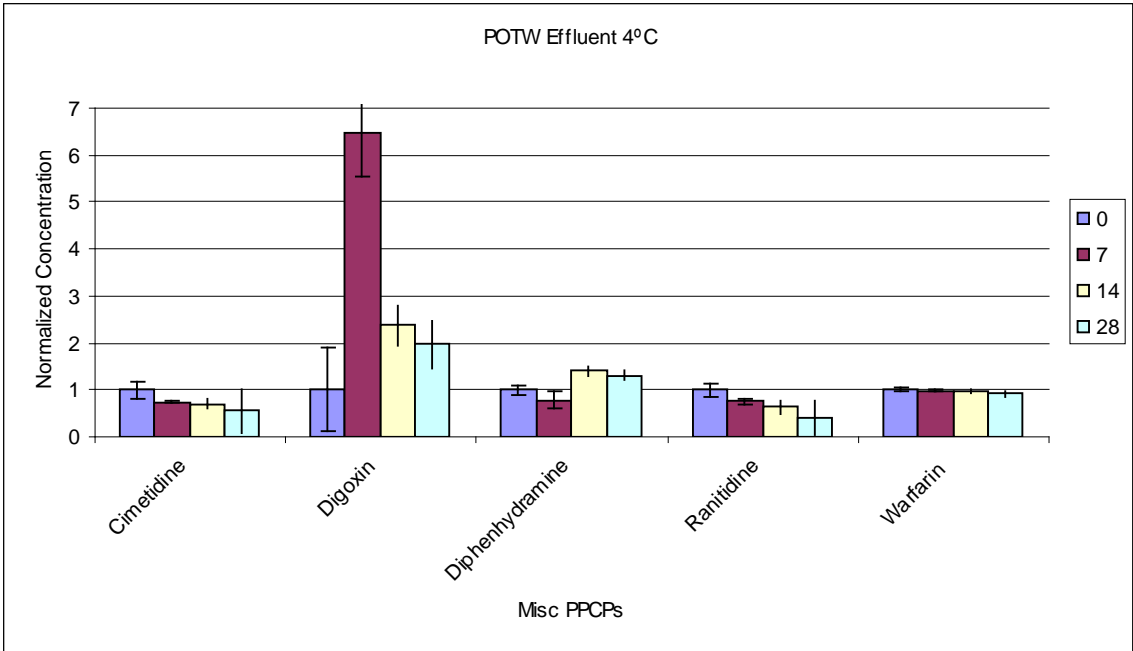| Phase 1 and 2 OPRs		Phase 3 OPRs	
Compound	% Recovery	Compound	% Recovery
Gemfibrozil	95	Sulfamethoxazole	94
Cimetidine	70.5	Erythromycin-H_2O	97.4
d_6-Norgestrel	74.7	Tetracycline (TC)	94.9
d_6-Thiabendazole	92.6	Triclocarban	99.2
Diltiazem	60.6	Ranitidine	54.2
Sarafloxacin	138	Mestranol	102
Sulfamethizole	76.6	Lomefloxacin	458
Digoxigenin	97.2	Estrone	98.3
Norfloxacin	128	Virginiamycin	147
Trimethoprim	95.5	Digoxin	106
Ormetoprim	120	Diphenhydramine	103
Ciprofloxacin	119	Diphenhydramine	124
17 alpha-Estradiol	136	Lincomycin	58.8
Cotinine	113	Oxacillin	120
Warfarin	113	Androsterone	95.7
Estrone	121	Diltiazem	104
Virginiamycin	57.1	Oxytetracyclin (OTC)	105
d_4-17 alpha-Ethinyl-Estradiol	78.9	Oxytetracyclin (OTC)	85.5
Sulfamethizole	81.5	Penicillin G	130
Albuterol	114	Oxytetracyclin (OTC)	88.2
Diltiazem	66.2	**Phase 4 OPRs**	
d_4-17 alpha-Ethinyl-Estradiol	90.3	Digoxin	80.9
Albuterol	110	Sulfadimethoxine	100
Chlortetracycline (CTC)	99.2	Demeclocycline	99.3
Equilenin	129	Anhydrotetracycline (ATC)	120
4-Epitetracycline (ETC)	113	17 alpha-Ethinyl-Estradiol	120
Ibuprofen	104	Sulfachloropyridazine	106
4-Epioxytetracycline (EOTC)	125	Norfloxacin	133
d_3-Albuterol	105	Estrone	111
Anhydrochlortetracycline (ACTC)	81.6	Oxacillin	100
Testosterone	93.5	Cefotaxime	130
Penicillin G	99.1	Clarithromycin	120
Progesterone	127	Oxytetracyclin (OTC)	92.8
1,7-Dimethylxanthine	122	Caffeine	75.9
$^{13}C_2$-Erythromycin-H_2O	65.4	Chlortetracycline (CTC)	110
Caffeine	83.6	4-Epianhydrotetracycline (EATC)	98
4-Epichlortetracycline (ECTC)	80.7	Sulfamethoxazole	107
4-Epianhydrotetracycline (EATC)	74.8	Trimethoprim	104
d_9-Progesterone	57.2	Sarafloxacin	130
Enrofloxacin	110	Oxolinic Acid	112
		Doxycycline	88.2

Note: Phase 1 and 2 are shown together as they were performed simultaneously.

APPENDIX 2. Data for Assorted Miscellaneous PPCPs

Biosolids 4ºC

Biosolids -20ºC

POTW Effluent 4ºC

POTW Effluent -20ºC

Biosolids 4ºC

Biosolids -20ºC

POTW Effluent 4ºC

POTW Effluent -20ºC
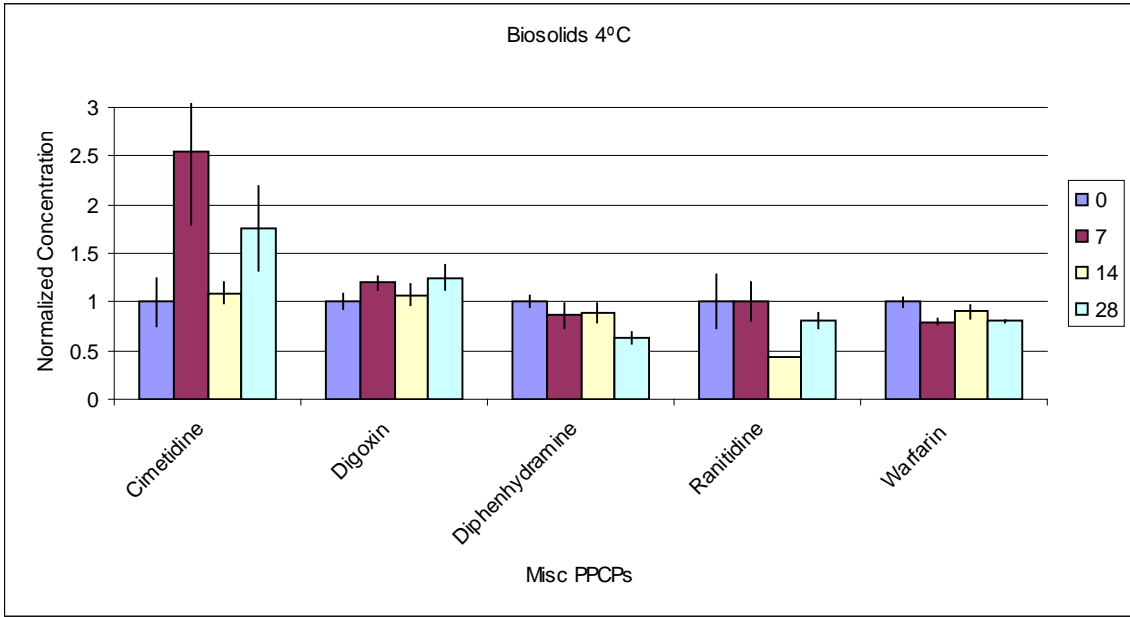
Data for "best combination samples"

POTW Effluent, 0.5 mg/L CL2, Ascorbic Acid, 4ºC

POTW Effluent, 0.5 mg/L CL2, Ascorbic Acid, 4ºC

POTW Effluent, 0.5 mg/L CL2, Ascorbic Acid, 4ºC

B Lactam & Other Antibiotics

POTW Effluent, 0.5 mg/L CL2, Ascorbic Acid, 4ºC

Macrolide Antibiotics

POTW Effluent, 0.5 mg/L CL2, Ascorbic Acid, 4ºC

POTW Effluent, 0.5 mg/L CL2, 4ºC

POTW Effluent, 0.5 mg/L CL2, Ascorbic Acid, 4ºC