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Analysis of Nucleosides in Municipal Wastewater by Large-Volume Liquid Chromatography Tandem Mass Spectrometry

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Abstract: Nucleosides are components of both DNA and RNA, and contain either a ribose (RNA) or 2deoxyribose (DNA) sugar and a purine or pyrimidine base. In addition to DNA and RNA turnover, modified nucleosides found in urine have been correlated to a diminished health status associated with AIDS, cancers, oxidative stress and age. Nucleosides found in municipal wastewater influent are potentially useful markers of community health status, and as of now, remain uninvestigated. A method was developed to quantify nucleosides in municipal wastewater using large-volume injection, liquid chromatography, and mass spectrometry. Method accuracy ranged from 92 to 139% when quantified by using isotopically labeled internal standards. Precision ranged from 6.1 to 19% of the relative standard deviation. The method's utility was demonstrated by the analysis of twenty-four hour composite wastewater influent samples that were collected over a week to investigate community nucleoside excretion. Nucleosides originating from RNA were more abundant that DNA over the study period, with total loads of nucleosides ranging from 2 to 25 kg/day. Given this relatively high amount of nucleosides found over the study period they present an attractive analyte for the investigation of community health.

INTRODUCTION

Municipal wastewater contains community scale information [1-3]. There have been numerous methods developed for the quantification of illicit drugs [4-6], personal care products [7, 8], and pharmaceuticals [9, 10] in municipal wastewater influent and effluent. Endogenous compounds such as steroids have also been investigated in municipal wastewater [11]. The concentrations of these substances are converted to mass loads by the multiplication of wastewater volume in order to account for dilution [12-14]. Community drug use, which is an important indication of community health has been investigated with the use of influent loads [6, 12]. Prescription pharmaceuticals also have some potential as indicators of community health with the use of prescription records, dose estimation and pharmacokinetic data when compared to loads. Possible prescription drug loads that could be used to determine the overall health status of a community could include anti-cancer, anti-viral drugs or metabolites. The usefulness of prescription drug loads may be limited due to potentially sparse use throughout the community.

Nucleosides are components of both DNA and RNA, and contain a purine or pyrimidine base and either a 2deoxyribose (DNA) or a ribose (RNA) sugar. Nucleosides are damaged in oxidation or alkylating reactions that produce modified nucleosides which remain incorporated into DNA, repaired or ultimately excreted in urine [15, 16]. Nucleoside modification is typically the result of reactions that modify the base. Alkylating electrophiles commonly form adducts at N^7, N^3, and O^6 guanine, and at N^3 and N^1 of adenine [17]. Electrophilic free radicals (i.e.

superoxide anion, hydroperoxyl radical, hydrogen peroxide, and hydroxyl radical) attack sites of rich electron density of pyrimidine (guanine and adenine) and purine (cytosine, uracil and thymine) bases and form oxidative adducts [15]. The generation of these free radicals can be either be from endogenous and or exogenous sources such as mitochondria, inflammatory cells, redox cycling compounds (e.g. diphenols, quinones, nitroaromatics) and metals [17]. The modified nucleoside 8-hydroxyguanosine has been studied in urine [18, 19] and organ tissue [20] as a marker for aging. The urinary concentrations of modified and un-modified nucleosides have been used as markers of health status, within small groups of individuals [21, 22]. The excretion profile of modified and unmodified nucleosides have be observed to be a function of age [23, 24], oxidative stress [25, 26], environment, cancer [21, 27, 28], lifestyle [29], pregnancy [30] and increased exposure to UV radiation [31]. Given the variability of modifications, there are a number of modified nucleosides that have not been investigated due to the lack of analytical standards. 8-hydroxydeoxyguanosine (8OHdG) has been mentioned as a possible marker of community health in wastewater but never fully investigated [3]. While there is no “ideally” health community, an investigation of nucleoside loads in municipal wastewater influent could potentially be compared to more traditional community (i.e. public) health data such as cancer occurrence, community levels of HIV, and environmental data that could impact health, such as air quality or proximity to sources of pollution and or radiation. Thus, nucleoside loads have the potential to be complementary to these traditional data.

The objective of this study is to develop a method using large-volume injection, liquid chromatography, mass spectrometry to quantify nucleosides and modified nucleosides in municipal wastewater influent. Large-volume injection liquid chromatography and mass spectrometry has been used for the determination of contaminants and metabolites in urine [32], as well as illicit drugs [6, 33], and steroids [11], in municipal wastewater influent. Large-volume injection was chosen to minimize sample preparation, and to maximize sensitivity needed for detection and quantification. It is known that hydrophilic analytes such as nucleosides are difficult to separate on C8 and C18 columns, and therefore alternative approaches have been developed [34-36]. In order to retain and separate nucleosides and modified nucleosides a polar-modified column employed. Nine nucleosides were chosen to a proof of concept and demonstrate method applicability. Twenty-four hour composite influent samples were obtained and analyzed in order to demonstrate the feasibility of the developed method as a step to achieve the overall goal of investigating community health via wastewater analyses.

EXPERIMENTAL

Chemicals and Materials. Adenosine (A), 2'-deoxyadenosine (2dA), guanosine (G), 2'-deoxyguanosine (2dG), cytidine (C), 2'-deoxycytidine (2dC), uridine (U) , N^2-methylguanosine (N2-MG), 7-methlyguanosine (7-MG), and HPLC grade ammonium acetate (>99%) where purchased from Sigma-Aldrich (St. Louis, MO, USA). Internal standards of [$^{13}C_5$] adenosine (AC5), [$^{13}C_5$] guanosine (GC5) and [$^{13}C_5$] cytidine (CC5) were purchased from Toronto Research Chemicals (North

York, ON, CAN). LC-MS optima™ grade methanol was purchased from Fisher Scientific (Waltham, MA, USA). Ultra pure water was obtained via a Miili Q advantage 10 (EMD Millipore, Billerica, MA, USA) equipped with a Q guard T2 purification cartridge, quantum TEX polishing cartridge and millipak express 0.22 µm filter.

Liquid Chromatography. Large volume injection liquid chromatography (LVI-LC) was performed using a Shimadzu liquid Chromatograph (Kyoto, JPN) consisting of two LC-20AD pumps coupled with a Phenomenex (Torrance, CA, USA) model DG-4400 on-line degasser, CBM-20A control unit, SIL-20AC autosampler equipped with a large volume injection kit (part # 228-45405-94) along with a climate controlled sample tray, and a CTO-20A column heater equipped with a two way switching valve (part # 228-45013-94). Mobile phase (A) consisted of 10mM ammonium acetate at pH 5.3, and mobile phase (B) was 100% methanol. Sample volumes of 1 mL were injected onto a 10 x 4.0 mm 5 µm RESTEK (Bellefonte, PA, USA) Ultra aqueous C18 guard column coupled with a 150 x 4.6 mm 5 µm analytical Ultra aqueous C18 column.

Mass Spectrometry. Detection was performed using an AB SCIEX (Framingham, MA, USA) model 3200 Q TRAP equipped with a Turbo V™ ion source with an electrospray ionization probe operated in positive ion mode. Mass spectrometric parameters (Table 1) were controlled (along with the LC) using Analyst version 1.5.1. Ion source temperature was set to 375ºC, ionization potential set to 5 kV, nebulization gas was set to 20 arbitrary units, desolvation

gas was set to 45 arbitrary units, curtain gas was set to 25 arbitrary units and CAD gas set to high.

Wastewater flow. Flow data was recorded using a Foxboro 9300 series flow meter (Houston, TX, USA), which is operated and maintained by the municipal wastewater treatment plant. All flow data was recorded by the minute for the duration of the study. Precipitation data was collected via daily climate reports published by the National Oceanic and Atmospheric Administration for Lawrence, Kansas (http://www.nws.noaa.gov/climate/index.php?wfo=top).

Sample Collection. Composite (twenty-four hour) wastewater samples were collected using an in-line sampling device installed within the municipal wastewater treatment plant after influent screening. Sample collection was started on the morning of September 30, 2014 (Monday) and ended on October 5, 2014 (Saturday). Samples were collected in a volume-dependent manner, with one sub-sample being collected after every 6.9×10^4 L of influent. 50 mL aliquots of composite samples were collected at 4ºC in HDPE centrifuge tubes, transported directly to the lab, and immediately frozen at -20ºC until analysis.

Standard and Sample Preparation. Standard calibration and quality control solutions were prepared for analysis at concentrations ranging from 3×10^3 to 2×10^5 ng/L using 10 mM ammonium acetate spiked with the internal standard mix to a final concentration of 5×10^3 ng/L. All samples were prepared by thawing to room temperature and then centrifugation at 4×10^3 g for 30 min using a VWR clinical 100 centrifuge (Radnor, PA, USA) in 15 mL tubes. 1425 µL of sample was then transferred into a 1.5 mL autosampler vial, spiked with 75 µL's of

internal standard mix (AC5, GC5, CC5) to a final concentration of 5 x 10^3 ng/L and then vortexed.

Quantification and Identification. All analytes were infused into the mass spectrometer at a concentration ~1 mg/L made up in 10 mM ammonium acetate and methanol 1:1 (v/v) using an analytical syringe and integrated syringe pump to optimize mass spectrometric parameters. MRM transitions that were the most abundant were chosen for quantification (Table 1). Analyte responses were normalized to internal standard responses and all linear analyte calibration regression lines had a coefficient of determination (R^2) of >0.98.

Quality control. Blanks were made up of 10mM ammonium acetate, and quality control samples were calibration standard solutions. Blanks and quality control samples were analyzed following eight wastewater samples. Quality control samples made up 25% of the total sample sequence, which was completed in one day. Rejection criteria of the quality control samples were +/- 15% of the initial calculated concentrations at the beginning of the run sequence.

Standard Addition, Accuracy, Precision. The use of a solvent-based calibration curve was determined appropriate for quantification of wastewater samples by using standard addition. Internal standards were deemed appropriate for quantification based on if there was a statistically significant difference (95%CI) between the solvent-based calibration derived value of the initial concentration in the sample and quantification using a calibration curve within the matrix using a 95% CI of the slope. The initial concentration of the sample was determined in the matrix by extrapolating the linear regression to the

x intercept by using the additional concentrations. Wastewater samples were initially analyzed along with five different concentrations to extrapolate to the x intercept by calibration in the wastewater matrix. The accuracy of the method was determined by analyzing an over-spiked sample 8 times (Table 2).

Limits of detection, quantification and ion suppression. Limits of detection (LODs) were determined by spiking concentrations of internal standard into a wastewater sample and analyzing four times to determine the lowest concentration that yielded a S/N (peak to peak) ≥ 3. LOQs were defined as the concentration of the lowest standard with a S/N ≥ 10. The percent of ion suppression was determined subtracting the ratio of the average area counts of internal standard peaks (n=8) in 10 mM ammonium acetate, by the average area counts of internal standard in wastewater (n=9) and multiplying by 100 (Equation 1). The method detection limit (MDL), and method quantification limit (MQL) was calculated by multiplying the LOD (ng/L) or LOQ (ng/L) by 100 and dividing the product by the percent of the sample recovery (100%) multiplied by the concentration factor of one.

Analyte Stability During Collection and Storage. Analyte stability during sample collection was determined by collecting 500 mL of fresh wastewater influent and storing an aliquot at 4ºC over 24 h, and another aliquot at 24ºC (room temperature) for three hours. Samples at 4ºC were collected initially and at 12, and 24 h and directly frozen. Samples held at 24ºC were taken initially and after three hours and then directly frozen. Samples were then analyzed in quadruplicate during one analytical sequence.

RESULTS AND DISCUSSION

Large volume injection liquid chromatography. The Liquid chromatograph flow path configuration was optimized to minimize gradient delay and to speed up column equilibration, effectively decreasing total analysis time (Figure 1). A two-way switching valve was installed inside the column heater and between the solvent mixer and autosampler in order to bypass the autosampler after the sample volume was injected onto the column. The design of the autosampler is such that the sample is at the head of the injection needle and directly injected onto the column. After sample injection the initial mobile phase composition of 0% B flowed at 0.4 mL/min through the autosampler and column to waste. At 5 min after sample injection, mobile phase was diverted away from the autosampler and directly through the column using the switching valve located inside of the column heater. When using a 1 mL injection volume, a switch in the flow path away from the autosampler was done 5 min after injection so that the injection loop containing the sample was flushed with two volumes (2 mL) to insure no carryover. The %B mobile phase was then linearly increased to 80% starting at 5 and ending at 12 min. Given the time when the front of the sample reaches the column (2.5 min) and then completely passed through the column (6.25 min using a flow rate of 0.4 mL/min, ~1.5 mL void volume and 1 mL sample volume) the valve leading to the mass spectrometer ion source was set to divert flow after injection until 10.3 min in order to keep salts and unretained compounds from fouling the instrument. At 10.3 min the mass spectrometer divert valve diverted mobile phase from inject (waste) to load (into ESI probe).

The mobile phase was then held at 80% B until 13.5 min and then linearly ramped to 0% B at 13.75 min. The mass spectrometer divert valve switched from load back to inject at 15 min. The flow rate was then increased to 0.6 ml/min at 15.10 min and held until 19.50 min when the switching valve diverted flow back to the autosampler, which was done in order to speed up column equilibration. The earliest analyte, cytidine (Figure 2), has an elution time of approximately 1 min after the divert valve switch, which is 6 min after sample injection.

Method validation. The accuracy which was determined by calculating the percent difference between quantification using a solvent based calibration curve and calibrating in the matrix by standard addition were 100 +/- 20% except for 2dC (100 +/- 39%) (Table 2). Precision was also determined using the same set of samples and ranged from 6.05 %RSD in U to 18.59 %RSD in 7-MG (Table 2). Calibration standards were linear from 100 to $2x10^5$ ng/L. LOD was observed to be 50 ng/L for AC5 and CC5, while GC5 was 100 ng/L. The LOQ was determined to be 100 ng/L for AC5 and CC5, and 1×10^3 ng/L for GC5. The LOQ of for analysis was 3×10^3 ng/L, which was the concentration of the lowest standard. Linear ranges for all calibration curves used to analyze samples were from $3x10^3$ to $2x10^5$ ng/L for all analytes. The MDL and MQL are 5% higher than the LOD and LOQ respectively due to the total recovery of the sample (100%) and a concentration factor of 0.95 (1425 µL of sample with the addition of 75 µL of IS). The percent of ion suppression was calculated to be 31.97 +/- 12.67 (95%CI, Equation 1).

Samples collected and stored at 4ºC generally displayed a slight increase in concentration. 2dG concentrations were significantly (95%CI) higher within the 24 h sample, while A displayed significantly (95%CI) lower concentrations for the 12 h and 24 h samples. The samples stored at 22ºC resulted in a significant (95%CI) increase in 2dG between initial sample and after 3 h, while over the same time a decrease in A was observed.

Method demonstration. Twenty-four hour composite wastewater samples were collected starting on the morning of September 30, 2014 (Monday) and ending on the morning of October 5, 2014 (Saturday). The calculated concentration (ng/L) of every nucleoside was above the LOD and LOQ except for N^{2}-MG and 7-MG which were below the LOD (Figure 3). The volume of wastewater entering the treatment plant was multiplied by the concentrations of all analytes to calculate loads (mg) (Figure 4). The loads of nucleosides containing ribose, were significantly higher than nucleosides containing 2deoxyribose on every day sampled (Figure 4). The total uncertainty of calculated loads were calculated using the sum of the analytical uncertainty which is analyte dependent, the uncertainty associated with the flow meter (0.25%) and estimated sampling uncertainty of 5% (Equation 2). This would indicate that there are more nucleosides originating from RNA than DNA, which makes sense given that the turnover of DNA is slower than RNA [28]. Wastewater influent flow displayed diurnal variation on four out of the six days (Figure 4), with the other two days (October 2 to 3) appearing to have been influenced by rain events (Figure 4). During October 2 and October 3 there seems to be a significant amount of

infiltration of stormwater into the system given that this is not a combined (stormwater and wastewater) sewer system. The loads of nucleosides are lowest on October 1 when there was no precipitation (Wednesday) and increase until sampling is complete on October 4 (Saturday).

In order to estimate the average mass of A, U, C and N2-MG (which are ribose containing nucleosides) excreted in a day, published concentrations of nucleosides in urine [21] were multiplied by average estimated daily volume of urine (1.1 L) [37], then multiplied by a population estimate for Lawrence [38], Kansas USA (93,742). This estimate ranged from 0.2 to 45, with a mean of 8 kg/day for the sum of A, U, C and N^2-MG [21, 39]. These values are similar to the loads of A, U, C found during the entirety of this study, which range from 1.2 to 16, with an average of 8.4 kg (Table 3). The highest loads (25 kg, Table 3) were calculated on October 4 (Saturday) and could potentially be an indication of commuters staying within the city on their workday off, thus the total amount of nucleosides may represent possible population markers. Although the usefulness of nucleosides (especially nucleosides 2deoxyribose nucleosides) as population markers are plausible, an investigation into there full potential is beyond the scope of this study.

Nucleosides are components of all living organisms and some non-living organisms (i.e. viruses), thus the total loads of nucleosides in wastewater could be influenced by unused food [40] disposal, and biofilm [41] (i.e. bacteria). Direct disposal is a possible interference for the interpretation of loads of illicit drugs, pharmaceuticals, as well as other potential analytes that are not directly excreted

as a metabolite originating from human use. Nucleosides in this study were observed to be relatively stable in wastewater at 4ºC and room temperature without the influence of biofilm. Given these factors future investigations related to comparing the loads of nucleosides between communities may have to take nucleoside transformation and or addition via biofilm into consideration.

Conclusions. The goal of this study was to develop an analytical method to quantify nucleosides in municipal wastewater. The method developed in this study was applied to analyze 24 h composite samples, which were used to verify the presence and to quantify nucleosides in wastewater and to investigate their stability. This method offers an insight into the total amounts of selected nucleosides found in municipal wastewater influent. Thus, the method developed here is a complimentary tool for the future investigation of community health. For further analyses and interpretation it may be beneficial to employ non-targeted detection to obtain a profile of excreted nucleosides which could be used to detect nucleosides for which standards would be expensive or impossible to obtain. Ultimately analytical methods such as the one developed in this study need to provide data of any proposed health marker in wastewater, which will lead to investigations and correlations of these markers to known health stressors and human health endpoints.

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Table 1. Mass spectrometric parameters for MRM scans (collision cell exit potential of 2V for all analytes) Transitions used for quantification are labeled with an *.

Identifier	Precursor ion (m/z)	Product ion (m/z)	Dwell time (msec)	Declustering potential (V)	Collision energy (V)
A-1*	268.3	119.2	125	35	50
A-2	268.3	136.1	125	35	30
2dA-1*	252.2	136.1	125	35	35
2dA-2	252.2	119.2	125	35	75
AC5-1*	273.5	136.1	125	35	27
AC5-2	273.5	119.2	125	35	61
G-1*	284.0	135.2	125	75	50
G-2	284.0	152.3	125	35	30
2dG-1*	268.4	152.3	125	35	20
2dG-2	268.4	135.2	125	35	60
GC5-1*	289.4	152.5	125	35	35
GC5-2	289.4	135.5	125	35	45
C-1*	244.2	112.0	125	15	35
C-2	244.2	95.2	125	15	65
2dC-1*	228.5	95.2	125	15	65
2dC-2	228.5	112.3	125	15	35
CC5-1*	248.9	112.0	125	15	35
CC5-2	248.9	95.2	125	15	65
U-1*	245.1	113.2	125	15	35
U-2	245.1	70.4	125	20	50
N^2-MG-1*	298.3	149.2	125	75	50
N^2-MG-2	298.3	110.4	125	75	50
7-MG-1*	299.4	149.2	125	75	50
7-MG-2	299.4	124.3	125	75	50

Table 2. Accuracy as determined as the percent difference between solvent based calibration curve quantification and standard addition, and precision determined from multiple analysis of one wastewater sample.

Identifier	Accuracy (mean % difference n=8)	Precision (%RSD n=8)
A	108	6.7
2dA	99	8.1
G	92	15
2dG	116	10
C	103	6.1
2dC	139	11
U	98	6.1
N2-MG	102	12
7-MG	115	19

Table 3. Daily loads of all nucleosides collected during study period.

Date	A	2dA	G	2dG	C	2dC	U	Sum (kg)
29-Sep	4.1	0.37	5.7	0.69	2.4	0.23	2.8	16
30-Sep	4.1	0.37	5.5	0.65	2.1	0.34	2.3	15
1-Oct	0.60	0.08	0.54	0.09	0.22	0	0.44	2
2-Oct	2.6	0.27	2	0.36	1.3	0.15	1.7	8
3-Oct	4.7	0.30	4.7	0.46	2.2	0.18	3.0	15
4-Oct	9.7	0.40	7.4	0.79	3	0.36	3.5	25

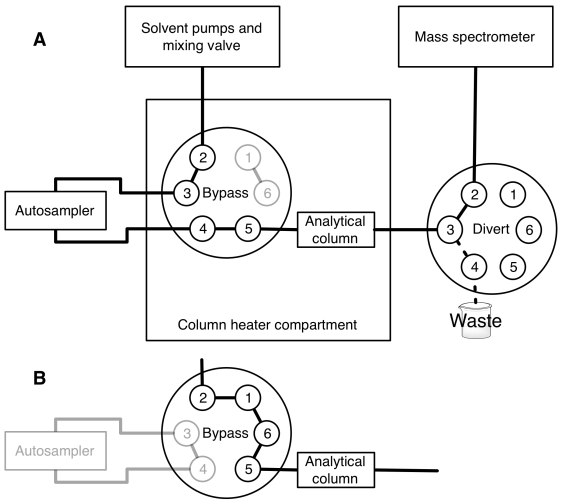

Figure 1. Schematic diagram showing the flow path while the sample is being loaded onto the column from 0-5 min (A), and when the flow path is routed around the autosampler 5-19.5 min (B). Faded areas of the "bypass" valve indicate ports of the valve not being used at each respective time. The "divert" valve was set to send flow into the mass spectrometer ionization source from 10.3-15 min.

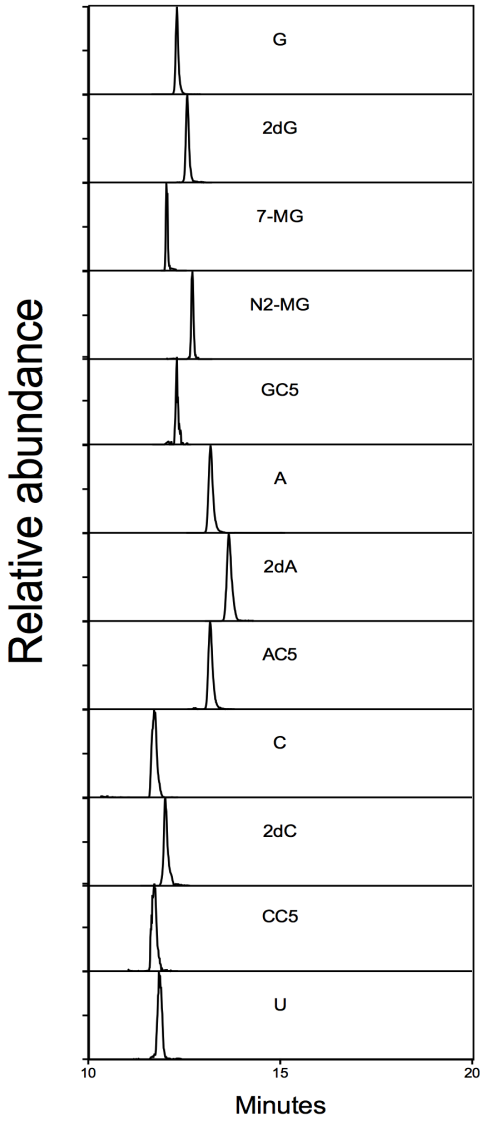

Figure 2. Example of typical wastewater sample chromatogram showing relative abundance (0-100%) of guanosine (G), 2deoxyguanosine (2dG), 7methylguanosine (7-MG), N2methylguanosine (N^2-MG), guanosine C5 (GC5), adenosine (A), 2deoxyadenosine (2dA), adenosine C5 (AC5), cytidine (C), 2deoxycytidine (2dC), cytidine C5 (CC5), uridine (U).

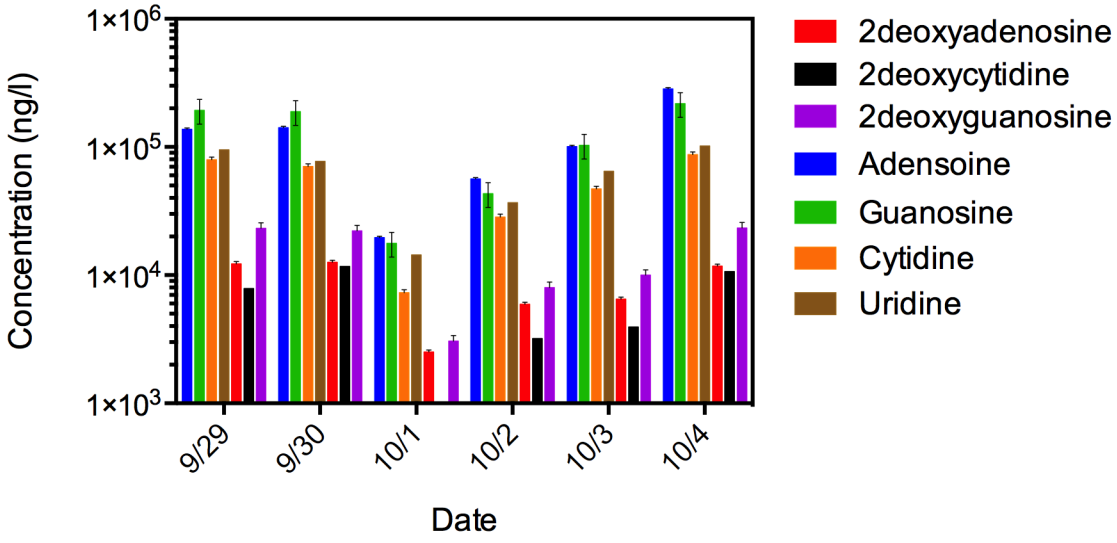

Figure 3. Bar graph showing the concentration of nucleosides in wastewater influent samples from September 29 to October 4, 2014

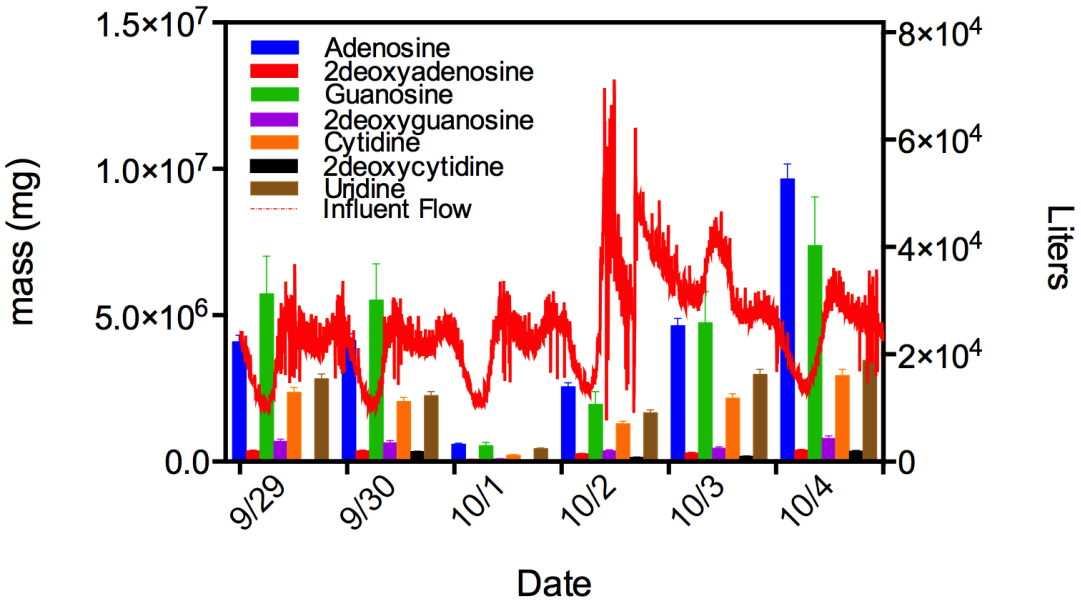

Figure 4. Loads (mg) of nucleosides and influent flow (L) from September 29 through October 4, 2014, error bars represent total uncertainty which is the sum of analytical, flow meter, and sampling uncertainty (%RSD). Total daily rainfall was 0.25, 22, 43, and 1 mm starting on September 30th and ending on October 3rd.

Equation 1. Calculation of ion suppression (%) in wastewater compared to 10 mM ammonium acetate buffer.

$$Ion\ suppression\ \% = 1 - \frac{IS\ peak\ area\ in\ wastewater}{IS\ peak\ area\ in\ buffer} \times 100$$

Equation 2. Calculation of total uncertainty from analysis, sampling, and flow. [12]

$$Total\ uncertainty = \sqrt{(RSD\ analytical)^2 + (RSD\ sampling)^2 + (RSD\ flow)^2}$$

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