

## <sup>1</sup>H qNMR and Chemometric Analyses of Urban Wastewater

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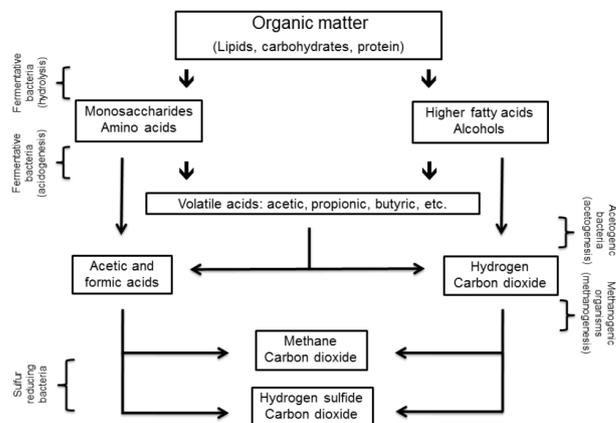
The industrial development, urbanization and agriculture play a major role in the degradation of the global environmental. Thus, the wastewater treatments need to be monitored continuously to ensure efficient operation. This manuscript presents an application of <sup>1</sup>H nuclear magnetic resonance (NMR) associated with chemometric and quantitative analyses to study the wastewater before and after the sewage treatment plant (STP). The concentration of compounds related to organic matter degradation ranged with treatment and seasonality. Anomalous discharges and the influence of stormwater on the sewage composition were further identified. All the variations indicated that the employed procedure might be useful to enhance the effectiveness of STPs, plan prevention actions for equipment protection and preserve the environment.

**Keywords:** wastewater composition, <sup>1</sup>H qNMR, chemometrics

### Introduction

The goals of economic and social development must be adjusted with the principles of sustainability to overcome the cost of pollution. Population growth and environmental constraints have induced a rising demand for water for several uses. Besides, aquatic pollution is one of the main concerns of the world today since the hydric resources are in significant decline, demanding fast recovery for subsequent reuse. Nowadays, the industrial and domestic sewage must be treated by the sewage treatment plants (STPs) to avoid the spread of diseases and harmful situations, to prevent ground water contamination, to preserve aquatic life, and to maintain the aquatic resources for the future uses.<sup>1,2</sup>

The organic matter (OM) from wastewater is a complex mixture. To promote contaminant elimination in order to protect receiving water, several steps are employed in the STPs.<sup>3</sup> In general, the primary stage aims to remove the solid material present while the second step comprises aerobic and/or anaerobic treatment. This stage is one of the most important in sewage treatment since it promotes the OM degradation (Figure 1) at the upflow anaerobic



**Figure 1.** Representation of OM degradation by the different microorganisms.

sludge blanket (UASB) reactors and the efficiency of its degradation depends on STP operating conditions.<sup>4-6</sup>

The accumulation of compounds, such as formic, acetic, propionic and butyric acid that arises from incomplete metabolism of the microorganisms may occur due to non-ideal conditions for the microorganism development or limitations in capacity and performance of the UASB. Thus, these compounds might be used as chemical markers to characterize the efficiency of the treatment process, seasonality, and abnormal discharges. The tertiary

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treatment involves filtration (nanofiltration and reverse osmosis) and advanced oxidation schemes as ozone light.<sup>7</sup> Therefore, information regarding sewage composition, quantification and monitoring of the organic compounds are indispensable. These data may assist in the optimization of the performance of the STPs as well as design parameters in order to maintain stable and favorable conditions for achieving efficiency of the treatment of wastewater.<sup>8,9</sup>

The study of organic pollutants in environmental samples is largely carried out by chromatographic techniques coupled with specific detection schemes.<sup>10</sup> However, methodologies as nuclear magnetic resonance (NMR) are widely used in the study of complex matrices, because they are non-destructive and provide qualitative and quantitative information (by applying correct protocols) about the sample composition with little pretreatment.<sup>11,12</sup> In addition, recent advances in NMR such as cryogenic probes, new pulse sequences, and software packages for pattern matching of both 1D and 2D NMR spectra allow overcoming several obstacles.<sup>13</sup> Nevertheless, due to highly complex NMR datasets and the inherent similarity, applications of chemometric methods to complement the analytical methodologies are essential.<sup>14-16</sup>

The present work aims to draw a profile of organic compounds in untreated and treated wastewater from an STP of São Carlos-SP, Brazil. The contributions of domestic and industrial discharges in the common wastewater system of the city and seasonal variations were investigated by qualitative and quantitative <sup>1</sup>H NMR analysis coupled to multivariate statistical analysis to monitor the sewage treatment operations.

## Experimental

### Wastewater samples

The STP of São Carlos-SP provided the samples that were collected before (untreated wastewater, UW) and after the treatment (treated wastewater, TW). The samples were collected weekly (at every hour for 24 h) in a specific day of the week over the course of one year (June 2011 to June 2012). The hourly samples were then mixed to obtain the representative samples of the day/week. The pH was ca. 7.5 for all the wastewater samples, for this reason all the compounds identified in this study are reported based on their neutral form.

### NMR data

Immediately after the collection, the UW and TW samples were filtered through filter paper (80 g) and Teflon

filter (0.45 μm) to remove the fine particulates. Then, 20 mL of the filtrated were transferred to vials and dried in a vacuum centrifuge (SpeedVac Concentrator, Labconco™) at 10,000 rpm. The dried samples were redissolved in 600 μL of D<sub>2</sub>O (99.9%) containing 0.014% of sodium-3-trimethylsilylpropionate-2,2,3,3-*d*<sub>4</sub> (TMSP-*d*<sub>4</sub>, 98%) from stock solution and transferred into 5 mm NMR tubes. The NMR experiments were performed on a Bruker Avance™ III 600 spectrometer operating at 14.1 T, equipped with a <sup>1</sup>H-<sup>13</sup>C/<sup>15</sup>N 5 mm TCI cryoprobe™ and z gradient coil. One-dimensional <sup>1</sup>H NMR spectra were acquired at 298 K with the Bruker zgpgpr pulse sequence (for water suppression at 4.71). Typically, 128 scans (free induction decays, FIDs) were collected into 260k data points using an 8.4 μs pulse width (90° pulse angle), spectral width of 30.0 ppm, acquisition time of 7.0 s and relaxation delay of 15.0 s. The spectra were referenced to the TMSP-*d*<sub>4</sub> resonance (0.0 ppm). The <sup>1</sup>H NMR spectra were acquired in quintuplicate for quantification and chemometric analyses. The <sup>1</sup>H NMR spectra were processed by applying an exponential multiplication of the FIDs by a factor of 0.3 Hz before Fourier transform. Phase correction was performed manually for each spectrum and the baseline correction was applied over the entire spectral range. The automatic mode was used for signal integration choosing the same width for each compound.

The identification of the constituents within the matrices was performed through 2D NMR such as gradient correlation spectroscopy (g-COSY), gradient heteronuclear single quantum coherence (g-HSQC), and gradient heteronuclear multiple bond correlation (g-HMBC) (NMR data acquisition and processing are in the Supplementary Information) and the assessments using a Bruker Biofluid Reference Compound Database (v 2.0.3) with the pattern matching performed by the Amix™ (version 3.9.3, Bruker BioSpin) as well as supplementary existing open access databases and literature reports.<sup>17</sup>

The quantification of the compounds that did not exhibit overlapping resonances was performed by the Eretic2™ method (TopSpin™ 3.1). This technique is based on pulse length based concentration determination (PULCON) that arises from the principle of reciprocity and the NMR signals strengths are correlated with a reference sample.<sup>18</sup> The Eretic2 method is beginning to replace the use of external radio frequency (rf) signal in Eretic since it is not suitable due to electronic instability and requires special acquisition and processing.<sup>19</sup>

The combined uncertainty of the method was estimated from the errors of balance and pipette, TMSP-*d*<sub>4</sub> purity (98%), and the standard deviation from quintuplicate of <sup>1</sup>H NMR analysis.

## Chemometric analysis

Chemometric analyses were performed using a quintuplicate of 49 UW and 48 TW samples. The  $^1\text{H}$  NMR spectra were utilized as input data for Amix<sup>TM</sup> program for principal component analysis (PCA) to create an overview and show trends, groupings and outliers in the data. Each spectrum was divided into 0.04 ppm wide buckets, using simple rectangular bucketing with integration mode by sum of intensities and scale to biggest bucket. The spectra were divided into 195 buckets between the chemical shift of 0.70 and 8.50 ppm for full spectra analyses and into 48 buckets between 6.50 to 8.40 ppm for analysis of olefinic and aromatic region. The area around the non-deuterated water was excluded of the bucketing process. Previous to the PCA analyses, the bucket tables were pre-processed by two different methods: mean-centered, with the mean value of each column subtracted from individual elements; and autoscaling that is mean-centered data divided by the square root of the standard deviation resulting in zero mean to each column and unit variance. Thus, PCA analyses were carried out after the mean-centered processing since this pretreatment provided better difference between UW and TW samples. In general, the first five principal components (PCs) explain 98% of initial variation. However the extractions of relevant information from chemical data were obtained at the PC1 and PC2.

## Results and Discussion

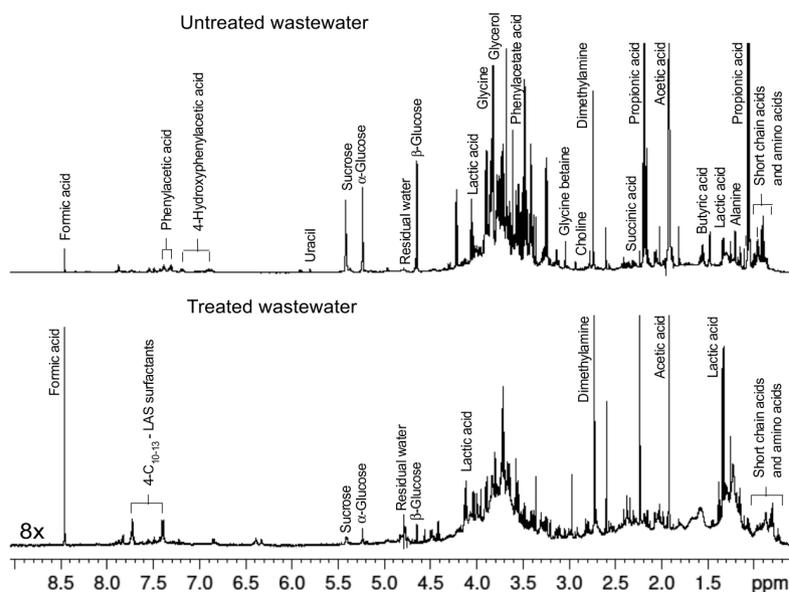
The  $^1\text{H}$  NMR spectra showed that the UW samples comprise a high level of aliphatic structures, mainly alkyl

chains (Figure 2) (structures, chemical shifts and coupling constants are in the Supplementary Information).<sup>20-22</sup> The STP resulted in a sharp decrease in the OM content and changes in wastewater composition.

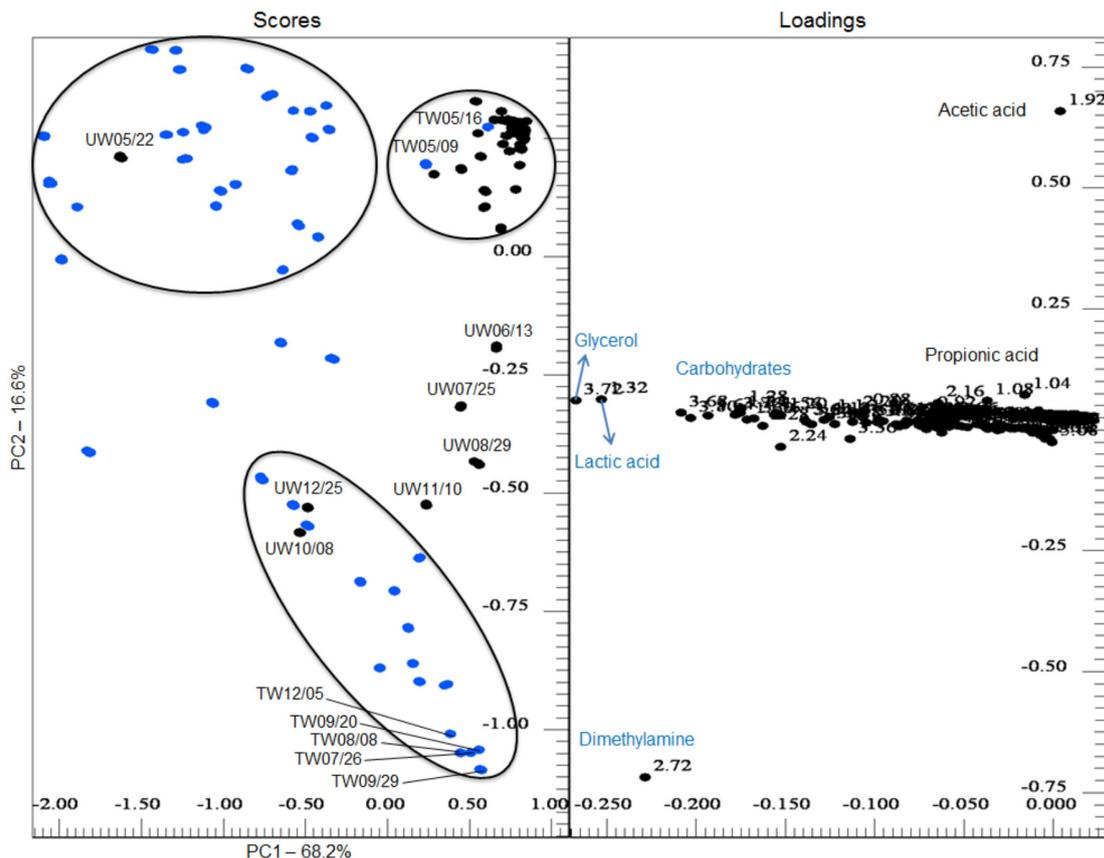
The application of PCA using entire spectra (Figure 3), in general resulted in the separation of UW (black color, ●) and TW samples (blue color, ●) as expected since the treatment must remove the major organic compounds leading to the reduction in intensity of  $^1\text{H}$  NMR signals (Figure 2). Therefore, PC1 vs. PC2 scores plot (Figure 2, left) clearly shows grouping of the samples into three distinct clusters with 85.4% of the total variance: one with most of the UW samples; and the TW samples divided into two main groups according to changes in STP conditions.

The PC1 is associated primarily with the activity of the acidogenic and acetogenic bacteria which convert carbohydrates, proteins, and fats into simple OM compounds as sugars, amino and fatty acids.<sup>23</sup> Most UW samples and two TW samples at positive scores of PC1 and PC2 show high concentration of complex OM compounds while TW samples at negative scores of PC1 showed less complex OM amount. In addition, the TW samples at negative scores of PC1 presented high concentration of lactic acid ( $\delta$  1.32). This compound arises from carbohydrate fermentation showing that the biological process is occurring. However, the presence of compounds such as lactic acid and glycerol ( $\delta$  3.72) might indicate the incomplete OM degradation at the UASB reactor.<sup>24</sup>

The acetic acid ( $\delta$  1.92) and the slight influence of propionic acid ( $\delta$  1.04-1.08 and 2.16) allocated the UW group to positive scores of PC1 and PC2. These compounds are present in UW due to degradation of complex OM



**Figure 2.** Representative  $^1\text{H}$  NMR spectra of untreated and treated wastewater.



**Figure 3.** PC1 and PC2 scores (left) and loadings (right) coordinate system for the UW (black) and the TW (blue) samples.

previous to STP entrance. The TW samples located at positive scores of PC1 and negative scores of PC2 (especially the 07/25/11, 08/08/11, 09/20/11, 09/29/11, and 12/05/11) presented the highest content of dimethylamine (DMA) ( $\delta$  2.72). This compound is the most abundant aliphatic amine from urine and also arises from degradation of proteins and amino acids. Its presence shows that the DMA removal might not occur during conventional wastewater treatment.<sup>25-27</sup> The DMA and nitrogen species that are sources of DMA may produce nitrosamine, a highly mutagenic compound with carcinogenic activity to humans.<sup>28,29</sup> Thus, actions to reduce the DMA content in effluent of the STP must be regarded.

For UW samples located outside of their group (10/08/11, 12/25/11, 11/10/11, 07/25/11, and 05/22/12) a decrease of the concentration of acetic and propionic acids was observed (Figure 3). This occurs due to overflows caused by stormwater entering into the sewer system in rain periods when clogging of the catchment of rainwater, piping defects, and illegal connections ensues. This additional water increases the sewage flow above the limits of STP, decreasing the residence time of wastewater in the UASB reactors, and also compromises the entire STP operation.

The PCA regarding only UW samples with 66.6% of the total variance in the first two principal components (Figure 4) highlights some trends of separations: September 2011 to April 2012 (black, ●) associated with high amount of acetic ( $\delta$  1.90-1.94) and propionic ( $\delta$  1.06 and 2.18) acids from OM degradation in hot seasons (which increase the bacterial activity);<sup>30</sup> May to August 2011, May and June 2012 (green, ●) associated with relatively low amount of these organic acids in the cold season; and samples influenced by stormwater (blue, ●) associated with low amount of OM compounds. In addition, positive values of PC2 are associated with high amount of urea and dimethylamine, mainly for the sample collected on 08/29. This unusual sewer composition might indicate an anomalous discharge in the wastewater catchment system. Thus, the protocol applied for UW may be used as a new approach for the STP monitoring.

PCA analysis was performed only for TW samples (Figure 5) with 79.4% of the total variance in the first two principal components. The carbohydrates and highest dimethylamine contents allocate the samples in blue (●) at the positive scores of PC2 and the highest concentration of the acetic and lactic acids allocate samples in black (●) at negative scores of PC2, showing dissimilar discharges on the Monjolinho River.

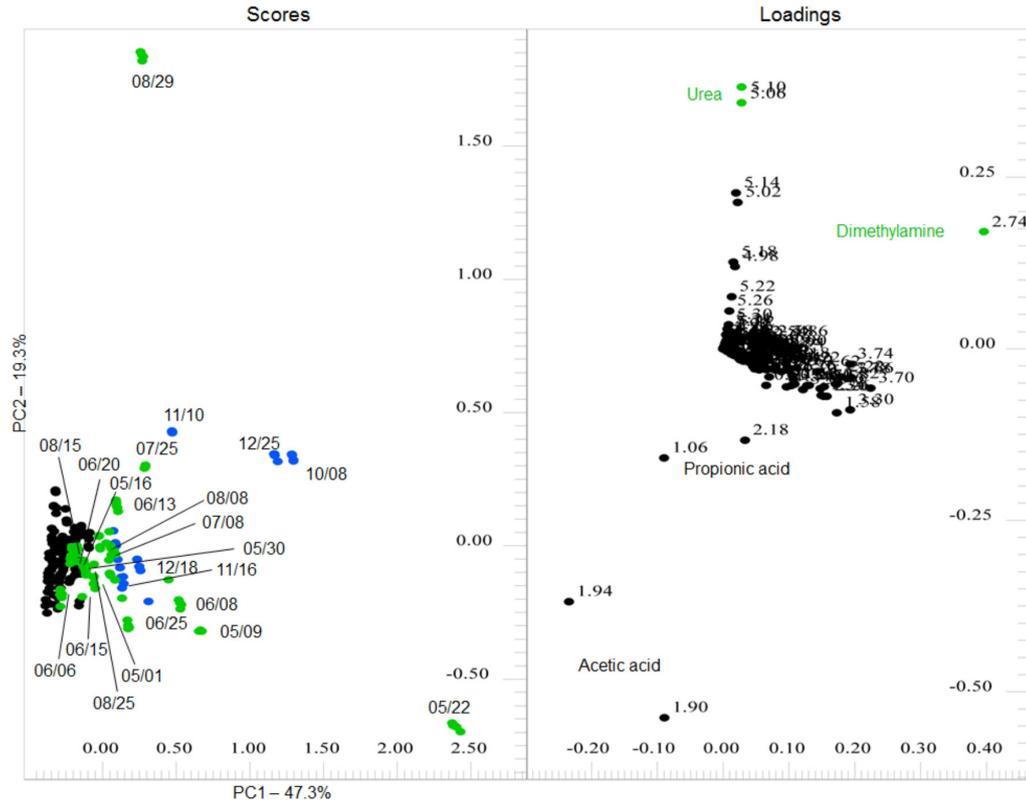


Figure 4. PC1 and PC2 scores and loadings system for UW samples.

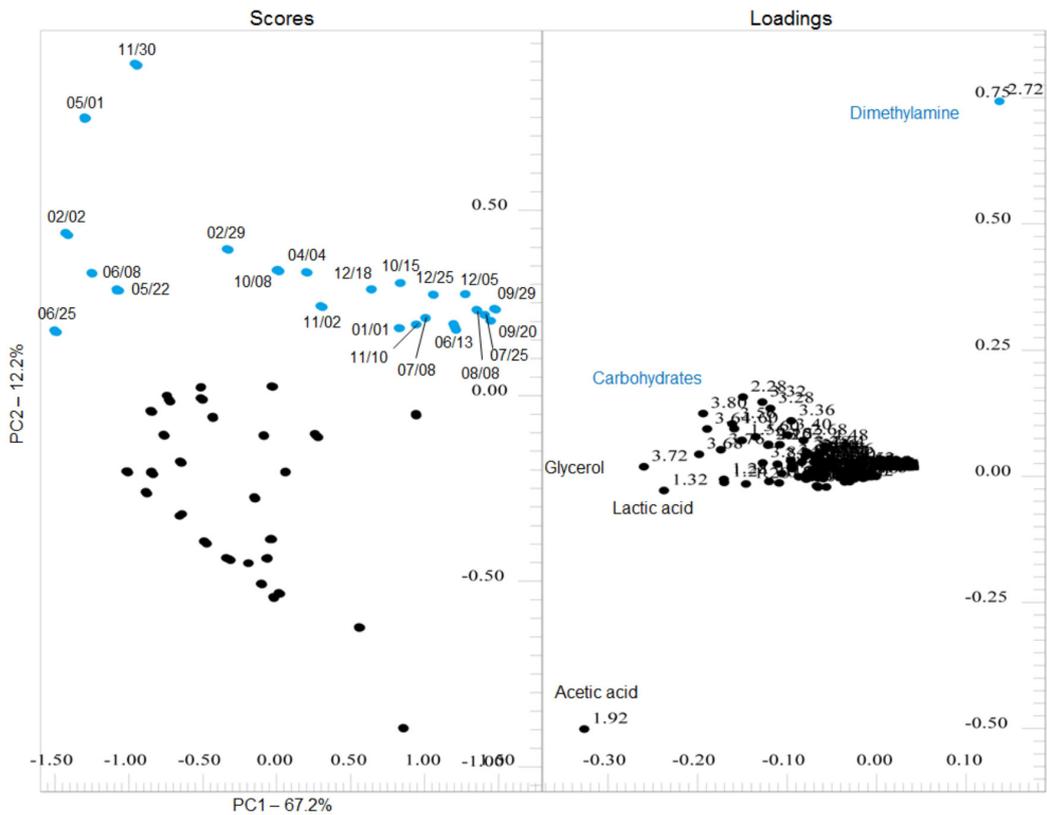


Figure 5. PC1 and PC2 scores and loadings system for TW samples. The blue color represents the highest dimethylamine and carbohydrate contents at the specified dates; and black the highest concentration of the acetic and lactic acids.

The presence of these compounds (Figure 5) in the effluents is a problem because they are nutrient sources for microorganism and phytoplankton growth in the receptor river, which may lead to eutrophication resulting in significant changes to the structure and function of aquatic ecosystems.<sup>31</sup>

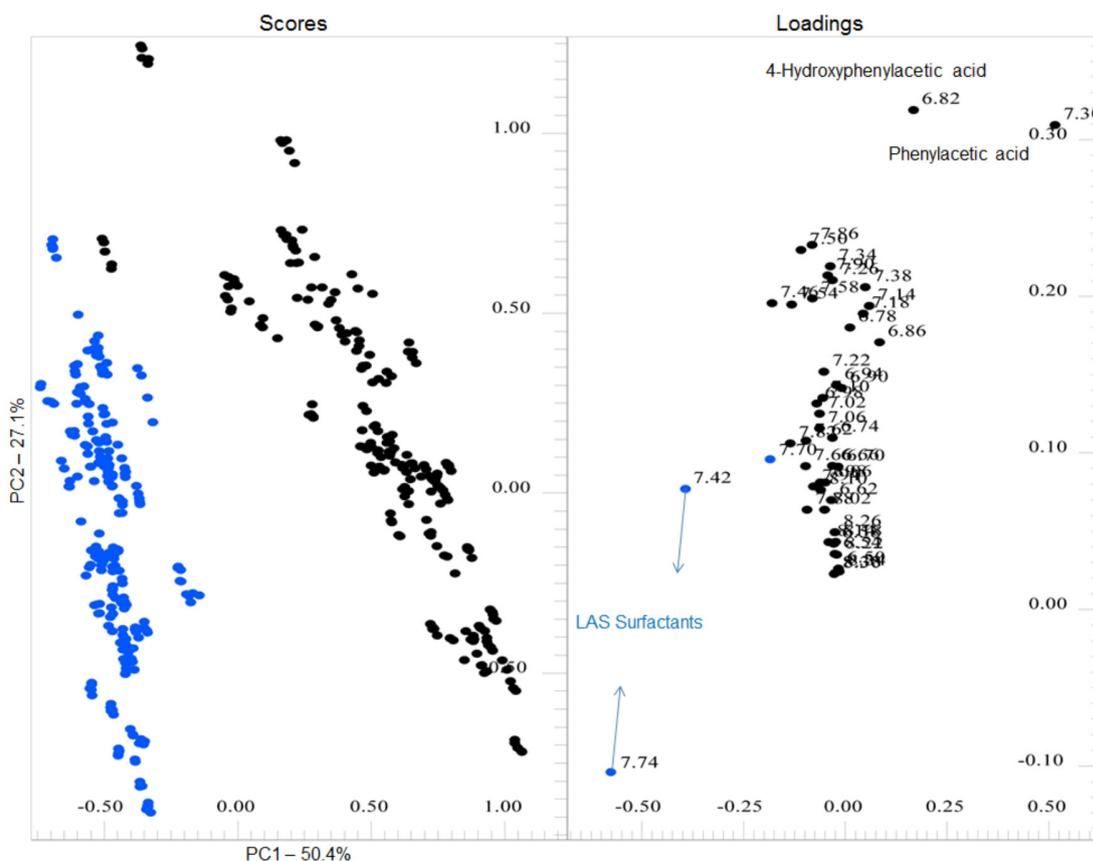
Considering only the olefinic and aromatic region the PCA was applied with UW samples in black (●) and TW samples in blue (●) (Figure 6).

The separation of two groups (with 77.5% of the total variance in the first two principal components) arises due to presence of 4-hydroxyphenylacetic ( $\delta$  6.82) and phenylacetic acids ( $\delta$  7.30, precursor of non-steroidal drugs) in UW samples (●), and the 4-C<sub>10-13</sub>-linear alkylbenzene sulfonate (LAS) surfactants ( $\delta$  7.42 and 7.74) in TW samples (●). As a result, the PCA showed the efficient removal of the 4-hydroxyphenylacetic and phenylacetic acids by STP. However, the LAS surfactants remain in the effluent. The recalcitrance occurred due to the type of biological treatment employed by STP, which is only anaerobic and does not degrade most LAS surfactants.<sup>32</sup>

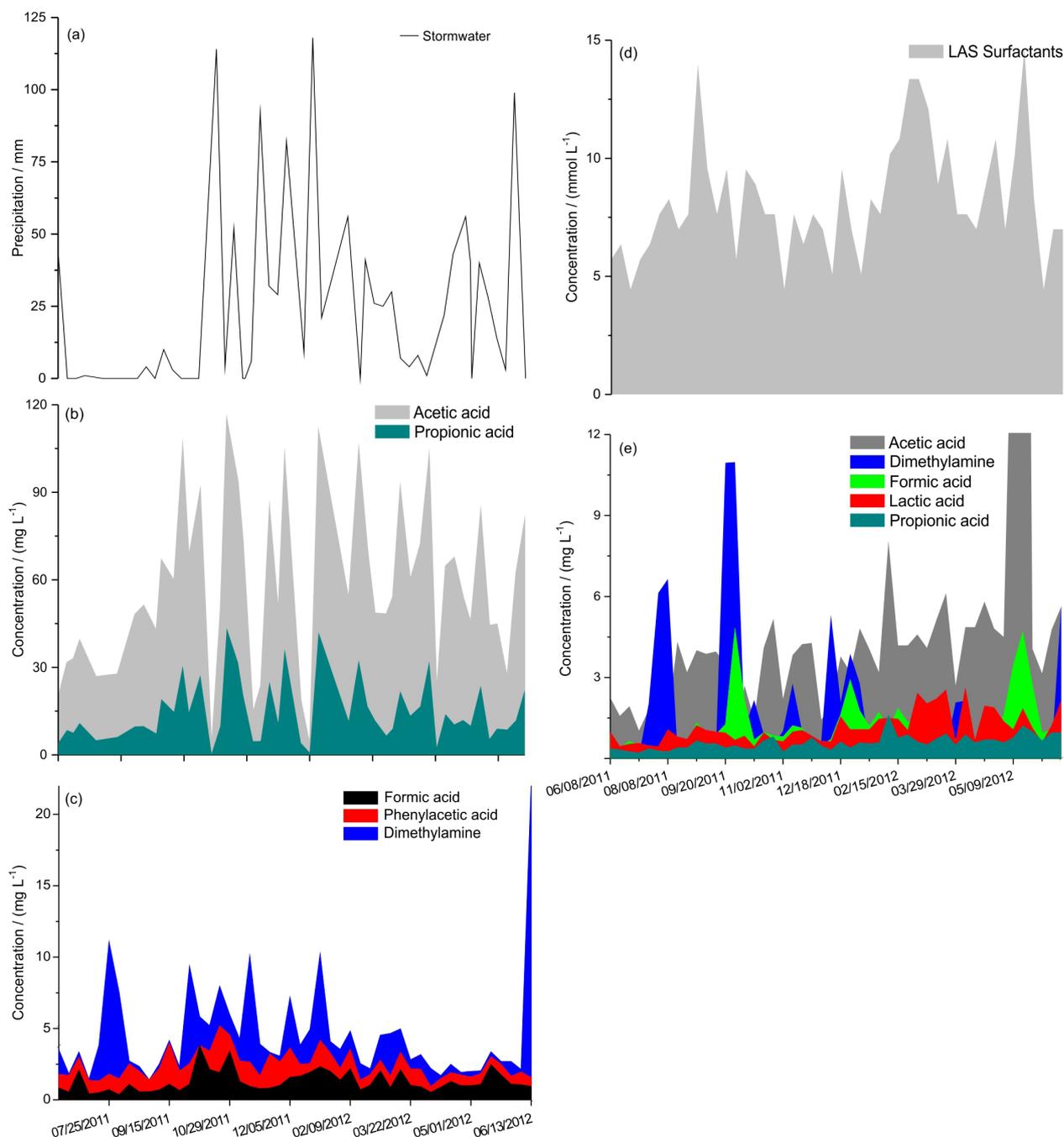
The compounds that had a high variation in concentration and that did not exhibit overlapping resonances were quantified. Figure 7 shows the quantification of the selected

compounds from UW (b and c) and TW (d and e) and the precipitation graph (a) from São Carlos city during the year of the sampling.

The acetic and propionic acids in UW samples (Figure 7b) were the major emitted species with maximum concentrations of 115.4 and 43.4 mg L<sup>-1</sup>, respectively. The variations in the concentrations of formic and phenylacetic acids and dimethylamine in UW (Figure 7c) might represent different disposals in common wastewater system of São Carlos during the year. The Figure 7d only shows the variation in the concentration of the anionic LAS surfactants discharged in Monjolinho River. The dimethylamine was the major emitted species in TW (Figure 7e), mainly on 07/25, 08/08, 09/20, 09/29, and 12/05, exhibiting maximum concentration of 11.0 mg L<sup>-1</sup>. Two TW samples in May 2012 presented the highest concentration of acetic acid (19.6 and 56.4 mg L<sup>-1</sup>) which grouped with UW samples in chemometric analysis (Figure 3). The graph of precipitation (Figure 7a) clearly shows the influence of stormwater in the concentration of acetic and propionic acids (Figure 7b) on 10/08/11, 11/10/11, 11/16/11, 12/25/11, and 04/04/12. The quantification results corroborated the chemometric analyses.



**Figure 6.** PC1 and PC2 scores and loadings coordinate system of olefinic and aromatic region for the UW (black) and the TW (blue) samples.



**Figure 7.** (a) Precipitation graph from São Carlos-SP;<sup>a</sup> (b) and (c) concentrations ( $\text{mg L}^{-1}$ ) in UW; (d) and (e) concentrations ( $\text{mmol L}^{-1}$  and  $\text{mg L}^{-1}$ ) in TW. <sup>a</sup>The data were obtained from reference 33.

## Conclusions

The  $^1\text{H}$  NMR combined with chemometrics was a valuable tool to provide comprehensive information on the wastewater. Seasonal variations and abnormal discharges of organic compounds in the sewage indicated that this protocol might be applied to monitoring the STP in order to know the processes involved in the complex system of surface water pollution. Monitoring of general pollution by

STPs showed that the concentration level may be used for searching of pollution source, planning of prevention action to equipment protection and environment preservation.

## Supplementary Information

Supplementary data (NMR data for compounds identified in wastewater) are available free of charge at <http://jbcbsbq.org.br> as PDF file.

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