Assessment of organic micropollutants occurrence in treated wastewater using heat shock protein 47 stress responses in Chinese hamster ovary cells and GC/MS-based non-target screening

Selma Etteieb, Atsushi Kawachi, Junkyu Han, Foued Elayni, Jamila Tarhouni and Hiroko Isoda

ABSTRACT

Combining bioassays and analytical chemistry screening is a powerful approach to assess organic micropollutants which are the main contributors to toxic potential in complex mixtures of treated wastewater (TWW). The aim of this study was to perform a comprehensive toxicity assessment of treated effluents using stress response bioassays and then to assess the occurrence of the organic micropollutants which were responsible for this biological response using gas chromatography coupled with a mass spectrometry detector (GC/MS). Results showed that TWW samples induced significant stress response on Chinese hamster ovary cells, stably transfected with heat shock protein 47 promoter, at 0.1%, 1%, 5% and 10% concentrations. The organic chemical compounds responsible for stress response potential were identified at different percentage values using non-target chemical screening. Of the compounds detected in TWW1 and TWW4, 55.09% and 74.5% respectively, fell within the class of aliphatic hydrocarbons. Aliphatic hydrocarbons were also present in TWW3 at 26.46% whereas 11.96% corresponded to 6-acetyl-1,1,2,4,4,7-hexamethyltetralin and 16.08% to triethoxysilane. Moreover, 76.73% of TWW2 was recorded as decamethylcyclopentasiloxane (D5) and 17.44% as n-hexadecanoic acid.

Key words | gas chromatography/mass spectrometry, HSP 47 assay, organic micropollutants, stress response, treated wastewater

INTRODUCTION

Organic micropollutants involve emerging contaminants which are considered as any synthetic or naturally occurring chemical that is not commonly monitored in the environment but has the potential to impact environmental ecosystems, human health and safety (Kuster et al. 2008; Deblonde et al. 2011). However, conventional sewage treatment plant processes are inefficient at eliminating organic contaminants such as pharmaceuticals and personal care products, surfactants and their degradation products, plasticizers, pesticides, insect repellents and flame retardants from treated effluent (Narita et al. 2007). Moreover, these persistent organic compounds released with the treated sewage effluents into recipient water bodies enter the environment and pose a risk to animal and human health as they are biologically active and bioaccumulating (Grover & Kaur 1999; Smital et al. 2011; Düsman et al. 2012). Previous studies have assessed chemical and bacterial pollution related to wastewater discharge. However, these traditional methods underestimate the occurrence of potentially toxic chemicals and do not consider the chemical mixture present in the water. Moreover, chemical analysis cannot predict harmful effects to the environment and human health (Smital et al. 2011). Therefore, the potential risk of chemical mixtures present can be evaluated using...
bioassay systems with living cells that give a global response to the pool of potential toxic micropollutants present in the water (Smital et al. 2014). In fact, the main advantage of bioassays is their ability to detect the combined toxicity of mixtures of known and unknown compounds with the same toxic mode of action without chemical analysis (Zegura et al. 2009). A range of toxicity bioassays have been developed to establish the toxicity level of wastewater against different organisms (Abu Ngozi & Ezeugwu 2008). Stress response bioassays were applied in this study using Chinese hamster ovary (CHO) cells stably transfected with heat shock protein (HSP) 47 promoter. In fact, HSPs are a distinctive class of molecules that protect cells against a wide range of injuries (Bierkens 2000; Malaspina & Silva-Zacarín 2006; McKenna 2009; Fontanetti et al. 2011). The production of stress proteins is induced as a result of the reaction of cells with a stressor such as heat, a chemical substance or a heavy metal to protect against cytotoxic conditions through their molecular chaperoning activity, maintaining cytoskeleton stability, and assisting in cell signaling (Welch 1987; Taguchi & Razzaque 2007). Stress gene expression has proved promising to evaluate the level of toxicity of chemicals based on a direct correlation between the level of stress and the amount of HSPs synthesized (Schlesinger 1986; Fujita 1999; Gupta et al. 2010). The role of HSPs as biomarkers of chemical stress has been recognized by many researchers (Mukhopadhyay et al. 2003; Mahmood et al. 2014). Sorensen et al. (2005) confirmed the importance of HSPs in resistance towards a range of stresses including insecticides and heavy metals. Increased levels of HSP 70 particularly could be used as a very sensitive biomarker in HeLa cells and juvenile rainbow trouts exposed to different classes of environmental pollutants such as metals in water (Muller et al. 1995; Williams et al. 1996; Alt-Aissa et al. 2000). In addition, Porte et al. (2001) confirmed that HSP 70 could be used as a biomarker in Mytilus galloprovincialis against polyaromatic hydrocarbon (PAH) toxicity. HSP 70 was also considered as a biomarker of exposure to heavy metals (Ni, Cr, and Cu) and trace organic pollutants (PAH and organochlorine pesticides (aldrin, DDT and its metabolites DDD and DDE)). Guiziani et al. (2012) also supported HSP to act as biomarkers of environmental stress.

Furthermore, to identify the occurrence of organic micropollutants responsible for ecotoxicity in water samples, bioassay results should be complemented by analytical chemical analysis. Progress in instrumental analytical chemistry and robust extraction techniques have made it possible to detect more compounds and at lower concentrations (Kotowska et al. 2012). For instance, gas chromatography coupled with a mass spectrometry detector (GC/MS) has been proven to analyze organic trace compounds while characterizing unknown compounds as well as their naturally occurring metabolites and breakdown products in environmental samples (Shareef et al. 2008). However, analytical chemistry methods are usually limited to pre-selected compounds like pesticides, pharmaceuticals, and industrial chemicals, whereas emerging contaminants are unknown. As an alternative, a method of non-target screening could reveal priority pollutants as well as emerging ones, a wide group of chemicals which includes but is not limited to synthetic fragrances, antiseptics, antioxidants, and insect repellents (Shaikh et al. 2014). Within this context, the aim of this study was to assess the stress response effect of the occurrence of organic micropollutants, including emerging contaminants, in treated wastewater (TWW) using a combined approach of applied bioassay and analytical chemistry screening of water matrices. The toxic potentials of TWW effluents from an activated sludge process were assessed by HSP bioassay, and the analysis was followed by identification of the organic micropollutants responsible for this effect with a detailed non-target screening using GC/MS.

MATERIALS AND METHODS

Sample collection

A total of four effluents from wastewater treatment plants (WWTPs), discharging on a daily basis into the Medjerda river in northern Tunisia, which is used for irrigation and drinking purposes, were sampled using sterile glass bottles in August 2013 (Figure 1). These WWTPs receive municipal wastewater to which is applied activated sludge as a biological treatment process. The characteristics of the four sampled WWTPs are summarized in Table 1. All samples (TWW1, TWW2, TWW3 and TWW4) were placed in a cooler, brought to the laboratory and analyzed the same day for chemical identification. Water samples were immediately filter-sterilized using a 0.45 μm filter (Millipore, MA, USA) and stored at −80 °C until use for bioassay analyses.

Heat shock protein 47 assay

CHO cells stably transfected with (+) or without (−) an HSP 47 promoter were provided by S. Yokota (Kaneka, Osaka, Japan) and used for this experiment. HSP assay is used to
determine the stress response of HSP 47 promoter transfected cells by measuring the enzymatic activity of β-galactosidase. When introduced into a chromosome, the HSP 47 plasmid can express β-galactosidase efficiently during stress induction. Experimental CHO cells were transformed by inserting the β-galactosidase gene downstream of the HSP 47 promoter while control CHO cells had the β-galactosidase gene under the control of the SV40 pA promoter. Isoda et al. (2003) developed this system for detecting trace amounts of environmental pollutants and natural toxins.

HSP 47-transformed cells were plated onto 96-well plates at initial concentrations of $1 \times 10^4$ cells per well in 100 µL of F12 medium (Gibco®, Invitrogen, Tokyo, Japan) supplemented with 10% fetal bovine serum (Sigma), 0.1% of G418 (Gibco) and 0.2% kanamycin solution (Sigma). The cells were allowed to attach for 48 h before removing the medium and adding 100 µL of TWW samples diluted with medium, followed by incubation for 3 hours in a 5% CO$_2$ incubator at 37 °C. The water sample was diluted to 0.1, 1, 5 and 10% of total volume of the medium (100 µL). The medium was then carefully removed and the cells washed twice with phosphate-buffered saline solution (0.8% NaCl, 0.22% Na$_2$HPO$_4$7H$_2$O, 0.02% KCl, 0.02% KH$_2$PO$_4$). Fifty microlitres of lysis buffer (Promega) was then added and the plates incubated for 30 min at room temperature. Twenty microlitres of cell lysate was transferred to a black plate, to which 100 µL of substrate solution (10 mmol/L NaH$_2$PO$_4$2H$_2$O, 100 mmol/L NaCl, 1% BSA, 0.005%
NaN₃, 1 mmol/L MgCl₂·6H₂O, 1% 4-methylumbelliferyl-β galactose (MUG), pH 7.0) was added in order to trigger the conversion of MUG into galactose and methylumbelliferyl. After allowing the reaction to occur in the dark for 30 min at room temperature, 60 μL of reaction stop buffer (1 mol/L glycine-NaOH, pH 10.5) was added and the fluorescence at 365 nm excitation/450 nm emission was then determined using a multidetection microplate reader.

In the evaluation, the relative value of HSP production was calculated by the following equation:

\[
\text{The relative stress response} = 100 \times \frac{\text{sample HSP}(+) - \text{sample HSP}(-)}{\text{control HSP}(+) - \text{control HSP}(-)}
\] (1)

Statistical analysis

All experiments were independently repeated three times and in each experiment six replicates were considered per plate for all sample doses. Mean values were calculated; statistical analysis of the results was done using Student’s t-test to determine the significance of the results versus control. For a statistically significant difference, the rejection/acceptance of the null hypothesis was taken into account at a risk of <5% and 1%, respectively. Data are shown as mean ± standard deviation; p-values <0.05 are considered ‘statistically significant’ (*) and those <0.01 are considered ‘statistically highly significant’ (**).

Water sample extraction

Chemical analysis was done using liquid-liquid phase extraction followed by GC/MS analysis.

In the liquid–liquid extraction phase, a 50 mL volume of dichloromethane was added to 1 L of filtered water before adding 50 mL of n-hexane, then shaken vigorously for half an hour and allowed to settle. After complete separation, the organic and aqueous phases were separated in a 1,000 mL conical flask from which the organic phase was recovered and filtered. The extracted organic phase was combined and dried by passing through a glass funnel containing anhydrous sodium sulfate. The organic fraction was concentrated to approximately 2 mL on a rotary evaporator and transferred into a vial suitable for GC/MS measurement.

GC/MS non-target analysis

Analytical determination using gas chromatography analysis was done using a Clarus 680 gas chromatograph (PerkinElmer, Inc., USA) along with an MS Clarus 600 T. This device was equipped with an RTX-5MS column (5% diphenyl; 95% dimethyl polysiloxane) 30 m long × 0.25 mm, coated with 0.25 μm thick film. The carrier gas was helium used at a flow rate of 1 mL/min. The temperature of the injector operating in splitless mode was held constant at 150 °C. The oven column temperature was programmed starting at 45 °C, increasing to 150 °C at a rate of 10 °C/min, and was thus maintained for 30 min. The ionization source temperature was 280 °C with ionization energy of 70 eV. The transfer line between GC and MS was kept at 280 °C. For analyte confirmation, mass spectra records during analyses were compared to the spectra in the NIST MS database.

RESULTS AND DISCUSSION

HSP 47 stress responses in CHO cells exposed to TWW effluents

Results of the HSP 47 assay showed that stress response in CHO cells was significant to very significant (t-test, p < 0.05 and p < 0.01) in presence of the tested TWW samples (Figure 2). Results demonstrated that TWW samples induced significant stress response at 0.1%, 1%, 5% and 10% concentrations. The maximum stress response in TWW 1 and TWW 2 reached a relative value of almost 142% while it reached relative values of 159% and 189% for TWW 3 and TWW 4, respectively.

The enhanced expression of HSPs can be detected in response to many kinds of stressors, including chemical pollutants. In fact, elevated stress protein values indicate proteotoxic conditions, since HSPs play an essential role in protein integrity maintenance and preventing aggregation. Decreasing cellular HSP amounts, however, can be interpreted as signs of a very intense stress response resulting in heading towards physiological breakdown and destruction of the organism (Vincze et al. 2014). Thus, the HSP 47 assay has proven its effectiveness as a highly sensitive system that can be used for studying the effect of trace contaminants such as organic pollutants and biotoxins (Narita et al. 2007; Funamizu et al. 2008; Talorete et al. 2008; Ben Fredj et al. 2010; Guizani et al. 2012). Previous studies confirmed that the secondary effluent showed high...
levels of stress response, as biological treatment generates toxic compounds. Indeed, a previous work applying bioassay using CHO cells with an HSP 47 promoter to the effluent of the WWTPs in Sapporo declared a statistically significant HSP production (Funamizu et al. 2011). This implied that the effluent contained organic compounds which stress the CHO cells. Moreover, Guizani et al. (2012) found that although the organic matter initially found in wastewater decreased during the treatment process, HSP response of the effluent was found to be higher than that of the influent. Narita et al. (2007) confirmed that the toxicity of the effluent was more intensive than the influent, as return flows from sludge treatment facilities and the organic matter released from activated sludge bacteria during their decay process contributed to the increase in toxicity in the secondary effluent.

Poor wastewater management practices adopted in Tunisia generate many micropollutants which are persistent in the environment and are not readily biodegraded (Wepener et al. 2011). These micropollutants have been detected in TWW effluents due to their physico-chemical properties and partial resistance to biotransformation (Stumpf et al. 1996; Cargouet et al. 2004). In fact, the conventional treatment systems like activated sludge technique applied in this case remove only biodegradable chemicals, microbial agents, and suspended particulate matter but has many limitations for removal of other toxic micropollutants (Mahomed et al. 2012). Additionally, various categories of compounds detected in the TWW are probable candidates for the observed toxicity depending on the plant’s capacity to reduce total organic carbon concentration, chemical oxygen demand and biological oxygen demand (Limam et al. 2007). Moreover, the treatment efficiency depends on many factors such as sludge retention time, biomass concentration, temperature, pH value and dominant class of micropollutants (Ciria et al. 2008). Furthermore, even low levels of individual chemicals and combinations of chemicals may pose a potential health risk to humans, animals and aquatic life. Thus, the stress response effect of our TWW samples on mammalian cells

![Figure 2](https://iwaponline.com/wst/article-pdf/74/10/2407/456911/wst074102407.pdf)

| The relative stress response of CHO cells incubated with TWW samples measured in HSP 47 assay. The results are expressed as percentage relative to control (CTR: non-treated cells), and represent the means ± standard deviation (\(n = 3\)). *\(P < 0.05\), **\(P < 0.01\).
 urged us to identify the organic substances which may contribute to toxic potentials, using non-target chemical analysis ensured by GC/MS screening.

**Chemical identification of organic micropollutant occurrence in TWW effluents**

The chemical composition of the TWW samples in terms of the various organic compounds detected in percentage values is presented in Table 2. These results showed that 55.09% and 74.5% of organic compounds detected in TWW1 and TWW4, respectively, fall within the class of aliphatic hydrocarbons. TWW3 showed predominantly aliphatic hydrocarbons (26.46%), followed by 11.96% 6-acetyl-1,2,4,4,7-hexamethyldotriacontane (AHTN) and 16.08% triethoxysilane. In TWW2, the highest percentage values of 76.73% and 17.44% were recorded for decamethylcyclopentasiloxane (D5) and n-hexadecanoic acid, respectively.

Thus, these organic contaminants are responsible for the stress response effect detected by the HSP 47 bioassay. Indeed, aliphatic hydrocarbons, AHTN, triethoxysilane, D5, and n-hexadecanoic acid contribute the most to toxic potentials of treated effluents. These toxic chemical substances have mainly industrial origins and they are persistent in TWW, which poses a major risk to aquatic organisms and human health. The toxicity of aliphatic hydrocarbons, AHTN, triethoxysilane, D5, and n-hexadecanoic acid was confirmed by previous findings. First, aliphatic hydrocarbons (alkanes), which can originate from anthropogenic or natural resources, are one of the earlier molecular markers in identifying hydrocarbon pollution and its sources (Bouzid et al. 2012; Fagbote & Olanipekun 2013). Wang et al. (2007) confirmed that long-chain alkane as the main components of wastewater would usually contribute greatly to toxicity. In fact, the toxicity of alkane tends to increase with increasing numbers of carbon in the molecule (Hamilton 1977). Thus, any simple alkane present in the source oil would contribute to acute toxicity (Donlan et al. 2005). In addition, the main factors determining the toxicity of long-chain aliphatic compounds is their concentration in aqueous solution coupled with the ability of the organism to metabolize such materials (Gill & Ratledge 1972). Furthermore, alkane with carbon chain lengths ranging from C8–C40 can result in toxicity to plants and/or soil invertebrates and pose risks to humans and wildlife through direct contact with these soils or soil organisms (Liu et al. 2015). Second, the toxicity of AHTN, which is the second largest volume product of the fragrance materials known as polycyclic musks and usually found in treated effluents, was discussed by previous studies (Wormuth et al. 2005). In fact, it is not readily biodegradable and is lipophilic and therefore likely to accumulate in sewage sludge and also in aquatic fauna. A previous *in vitro* cytogenetic assay in CHO cells in the presence of metabolic activation showed structural aberrations observed with AHTN. Moreover, *in vivo* experiments in rats showed that a single high dose produced acute hepatic damage characterized by single cell necrosis, inflammation, swelling of liver parenchymal cells, the presence of cytoplasmic condensations in the hepatocytes, disorganization of the rough endoplasmic reticulum and mitochondria, and focal cytolysis (HERA 2004). Furthermore, AHTN has been found in human adipose tissue and in mothers’ milk, suggesting regular and extensive exposure of consumers. Third, the toxicity of triethoxysilane, which is an organic silicon substance used in the production of silicone polymers or silicone resins (Vandenbergh et al. 1991), was suggested to be similar to the toxicity of silanes. In fact, silane showed mainly nephrotoxicity. Mice subjected to silane acute exposure (2,500, 5,000, and 10,000 ppm) developed acute tubular necrosis, and tubulo-interstitial nephritis was observed in mice which survived the 2 week observation period (Nakashima et al. 1998). Then, n-Hexadecanoic acid is a fatty acid (saturated) used in foodstuffs to add texture to processed foods and to produce soap, cosmetics, and release agents (Aparna et al. 2012). The toxicity of n-hexadecanoic acid was discussed by Luo et al. (2012), declaring that high levels of palmitic acid induce cell death. Moreover, a study of the dose response of HepG2 cells to palmitic acid treatment identified the optimal concentrations of palmitic acid which lead to minimal cytotoxicity. In fact, palmitic acid increased lactate dehydrogenase release and caspase activity at a concentration of 200 μM, and it increased cytotoxicity at concentrations greater than 300 μM. Previous studies have investigated the toxic mechanism following the accumulation of this saturated fatty acid in human Chang liver cells. After a 24-h exposure to palmitic acid, cell viability decreased with an ATP reduction and G2/M phase arrest (Park et al. 2014). Finally, given the persistence of D5, which is used as an intermediate for producing other chemicals (silicone polymers), and in personal care products, household products, and industrial/institutional cleaning (Brooke et al. 2009), its toxicity was discussed previously. In fact, findings from subacute and subchronic inhalation studies in rats have shown evidence of effects on the liver causing weight changes and hepatocellular hypertrophy. Effects on the uterus were also observed: mainly increased incidence of endometrial adenocarcinoma, endometrial adenoma, and
Table 2 | Chemical identification of organic compounds in TWW

<table>
<thead>
<tr>
<th>Peak retention time (min)</th>
<th>Chemical component</th>
<th>Component, [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>TWW 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.61</td>
<td>Tetratetracontane C_{44}H_{90}</td>
<td>6.86</td>
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<tr>
<td>11.36</td>
<td>Tetratetracontane, 1,54-dibromo-C_{54}H_{108}Br_{2}</td>
<td>5.08</td>
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<tr>
<td>11.97</td>
<td>Hexacosane C_{26}H_{54}</td>
<td>8.44</td>
</tr>
<tr>
<td>12.61</td>
<td>Tetraatriacontane C_{34}H_{70}</td>
<td>10.73</td>
</tr>
<tr>
<td>13.24</td>
<td>Pentatriacontane C_{35}H_{72}</td>
<td>11.51</td>
</tr>
<tr>
<td>13.88</td>
<td>Dodecan, 1-fluoro-C_{12}H_{25}F</td>
<td>9.16</td>
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<tr>
<td>14.52</td>
<td>Hexatriacontane C_{36}H_{74}</td>
<td>9.14</td>
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<tr>
<td>15.16</td>
<td>Pentacontane C_{36}H_{102}</td>
<td>8.41</td>
</tr>
<tr>
<td>15.81</td>
<td>Sulfurous acid, butyl octadecyl ester C_{22}H_{46}O_{3}S</td>
<td>7.55</td>
</tr>
<tr>
<td>16.46</td>
<td>Methyl 2-hydroxyicosanoate C_{22}H_{42}O_{2}</td>
<td>6.36</td>
</tr>
<tr>
<td>17.11</td>
<td>3-Methyl-2-(2-oxopropyl) furan C_{16}H_{10}O_{2}</td>
<td>5.09</td>
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<td>17.75</td>
<td>1-Hexyl-2-nitrocyclohexane C_{21}H_{23}NO_{2}</td>
<td>4.57</td>
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<td>18.01</td>
<td>Tricyclo[20.8.0.0(7,16)]triacontane, 1(22), 7 (16)-diepoxy C_{30}H_{52}O_{2}</td>
<td>7.10</td>
</tr>
<tr>
<td>TWW 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.53</td>
<td>Cyclopentasiloxane, decamethyl C_{10}H_{30}O_{5}Si_{5}</td>
<td>76.73</td>
</tr>
<tr>
<td>12.22</td>
<td>Benzeneacetic acid, ALPHA-cyano-,ethyl ester C_{11}H_{11}NO_{2}</td>
<td>2.56</td>
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<tr>
<td>13.75</td>
<td>Indolizine, 3-methyl-C_{6}H_{8}N</td>
<td>3.26</td>
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<tr>
<td>21.57</td>
<td>N-hexadecanoic acid C_{16}H_{32}O_{2}</td>
<td>17.45</td>
</tr>
<tr>
<td>TWW 3</td>
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<td></td>
</tr>
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<td>9.53</td>
<td>Cyclopentasiloxane, decamethyl C_{10}H_{30}O_{5}Si_{5}</td>
<td>8.30</td>
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<td>Cyclohexasiloxane, dodecamethyl C_{12}H_{30}O_{5}Si_{6}</td>
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<tr>
<td>17.91</td>
<td>2,6,10-dodecatrien-1-ol,7,11-trimethyl-9-(phenylsulfonfyl)-, (E,E)-C_{21}H_{36}O_{2}S</td>
<td>9.32</td>
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<tr>
<td>18.16</td>
<td>Dotriacontane C_{32}H_{66}</td>
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<td>19.59</td>
<td>Triaccontane C_{30}H_{62}</td>
<td>6.31</td>
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<td>19.72</td>
<td>2-Ethylhexyl salicylate C_{15}H_{22}O_{3}</td>
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<td>19.77</td>
<td>3-Isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris (trimethylsiloxy) tetrasiloxane C_{18}H_{52}O_{3}Si_{5}</td>
<td>2.72</td>
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<tr>
<td>20.38</td>
<td>6-Acetyl-1,1,2,4,4,4,7-hexamethyltetralin C_{18}H_{26}O</td>
<td>11.96</td>
</tr>
<tr>
<td>20.50</td>
<td>Triethoxysilane C_{8}H_{16}O_{5}Si</td>
<td>16.08</td>
</tr>
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<td>20.64</td>
<td>3-Cyclohexene-1-ethanol, ALPHA-ethenyl-ALPHA, 3-dimethy1-6-(1-methylethylidene)-</td>
<td>4.78</td>
</tr>
<tr>
<td>TWW 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.53</td>
<td>Cyclopentasiloxane, decamethyl C_{10}H_{30}O_{5}Si_{5}</td>
<td>4.04</td>
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<tr>
<td>12.52</td>
<td>Cyclohexasiloxane, dodecamethyl C_{12}H_{30}O_{5}Si_{6}</td>
<td>2.90</td>
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<tr>
<td>16.78</td>
<td>Hentriacontane C_{35}H_{64}</td>
<td>4.69</td>
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<td>17.45</td>
<td>Nonane, 2,2,4,6,8,8-heptamethyl-C_{16}H_{34}</td>
<td>4.90</td>
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<td>Dotriacontane C_{32}H_{66}</td>
<td>5.21</td>
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<td>5.97</td>
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<tr>
<td>19.48</td>
<td>Pentacosane C_{25}H_{52}</td>
<td>8.80</td>
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(continued)
adenomatous polyps in several animals. Effects also on respiratory tract/lungs were identified such as inflammatory responses (OARS 2015).

CONCLUSIONS

This study combines both biological and chemical approaches to better characterize sewage effluent composition and assess its environmental impacts. The current study revealed significant stress response on mammalian cells, which was related to the occurrence of organic micropollutants in treated effluents. This stress potential of treated effluents, revealed by the applied bioassays, was linked to causative chemicals identified by non-target chemical analysis using GC/MS. Hence, the most important compounds responsible for the toxic potential of treated effluents were aliphatic hydrocarbons, AHTN, triethoxysilane, D5 and n-hexadecanoic acid. Conventional treatment processes are not designed to remove these organic contaminants, presenting the risk of high contamination load to river water designated for irrigation and drinking purposes. These compounds are toxic at very low concentrations and have the potential for bioaccumulation and biomagnification in the food chain, which impacts human health. Thus, the continued discharge of TWW will only lead to environmentally critical contamination of surface water resources. Consequently, managers should be urged to explore the alternatives for removing hydrocarbons and other organic micropollutants by introducing sophisticated water purification technologies aimed at protecting recipient water bodies from further degradation.

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